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par

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The Mechanism of Action of Polerovirus P0 in RNA Silencing Suppression

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Abstract

The poleroviruses are an agronomically important genus of plant viruses which can infect a wide range of hosts. Their genome is a single-stranded plus-sense RNA. The 5'-terminal ORF encodes the 29 kDa protein P0, a strong suppressor of Post-Transcriptional Gene Silencing (PTGS), an important antiviral defense system in plants. I have investigated the mechanism of action of P0 in suppression of RNA silencing. A yeast two-hybrid screen of an *Arabidopsis thaliana* cDNA library identified two closely related Arabidopsis SKP1-like proteins (ASK) as a cellular partner for P0. ASK is a component of the SCF class of E3 ubiquitin ligases involved in the protein ubiquitination and degradation pathway. A conserved F-box like-motif was identified near the N-terminus of P0, suggesting that P0 is a viral-coded F-box protein. F-box proteins are the components of the SCF complex that specifically recognize target proteins. The targets are then usually polyubiquitinated as a marker for proteolysis by the 26S proteasome. P0 mutated in the F-box motif did not interact with ASK and conferred low pathogenicity to the virus in plants. *Nicotiana benthamiana* in which SKP1 levels were knocked down by virus-induced gene silencing were resistant to polerovirus infection. The F-box motif was also essential for silencing suppression activity of P0 in an agroinfiltration assay. Transgenic Arabidopsis expressing P0 under control of an inducible promoter showed abnormal phenotypes. A subset of miRNAs in the induced P0 plants accumulated less abundantly than in non-induced plants and miRNA-targeted endogenous transcripts were upregulated, indicating that P0 interferes with the miRNA pathway. P0 also suppressed IR-PTGS at a step downstream of Dicer (DCL) activity, suggesting that ARGONAUTE1 (AGO1), the slicer protein in the RNA-induced silencing complex (RISC) might be the target of P0. Indeed, P0 specifically provoked AGO1 degradation in both transient expression experiments and in crossed P0-FlagAGO1 Arabidopsis. A physical interaction between P0 and AGO1 was demonstrated both *in vitro* and *in planta*, favoring the hypothesis that AGO1 is the direct target of P0. Our data support a model in which P0 acts as an F-box protein, recruiting the post-translational modification system to overcome the post-transcriptional gene silencing system. In this model P0 interacts with SKP to constitute a SCF^{P0} complex which presumably addresses AGO1 for ubiquitination and degradation by 26S proteasome. This would be the first example of a suppressor of RNA silencing that acts in an SCF complex to promote degradation of an essential component of the silencing pathway, thereby, inhibiting the plant antiviral defense.

In Persian (Farsi)

مکانیسم عمل پروتئین P0 متعلق به Polerovirus در بازدارندگی از RNA Silencing

چکیده: ویروسهای متعلق به جنس Polerovirus دامنه وسیعی از گیاهان دارای اهمیت اقتصادی را آلوده می کنند. ژنوم آنها از یک RNA تک رشته ای مثبت تشکیل شده و ORF انتهای '5 آن پروتئینی بنام P0 به وزن 29kDa را کد میکند که یک بازدارنده قوی از فرایند Gene Silencing است. این فرایند نقش دفاعی در برابر ویروسها را در گیاهان به عهده دارد. برای پی بردن به مکانسم عمل P0 در بازدارندگی از این سیستم دفاعی میزبان، از روش جستجوی شریک سلولی بکمک دوبل هیبرید مخمر در بانک cDNA گیاه مدل، *Arabidopsis thaliana*، استفاده شد و دو پروتئین مشابه هم بنام ASK (Arabidopsis SKP1-like) کشف گردید. ASK جزئی از تیپ SCF از مجموعه آنزیمهای E3 یوبی کویتین لیگاز است که در سیستم یوبی کویتینه کردن و تجزیه پروتئینها توسط پروتئازوم بکار میروند. F-box پروتئین جزء دیگری از SCF است که به ASK می چسبد و نقش شناسایی پروتئین هدف را برای یوبی کویتینه کردن به عهده دارد. F-box پروتئینها دارای یک توالی اسید آمینه ثابت در انتهای آمینه خود هستند. بررسی توالی P0 چندین Polerovirus نشان داد که چنین توالی در تمام P0 ها حفاظت شده است نشانگر اینکه P0 میتواند یک پروتئین F-box باشد. P0 موتانت در توالی ثابت F-box نتوانست با ASK تعامل کند و چنین موتاسیون در ویروس باعث کاهش چشمگیر بیماریزایی ویروس در گیاه گردید. گیاهان *Nicotiana benthamiana* که ژن SKP آنها توسط Virus Induced Gene Silencing خاموش شده بود به آلودگی توسط Polerovirusها مقاوم بودند. آزمایشات دیگر نشان داد که توالی F-box در P0ها برای بازدارندگی از RNA Silencing ضروری است. گیاهان Arabidopsis ترانسژنیک بیان کننده P0 تحت کنترل پروموتور قابل القاء فنوتیپ غیرطبیعی نشان دادند. تجمع تعدادی از miRNAها در گیاهان P0 القاء شده نسبت به القاء نشده کمتر بود و تعدادی از mRNAهایی که توسط miRNAها کنترل میشوند در گیاهان القاء شده تجمع چشمگیری یافتند حاکی از اینکه P0 علاوه بر مسیر siRNA، مسیر miRNAها را هم در گیاه مختل میکند. P0 همچنین توانست Gene Silencing ناشی از ساختار Inverted-Repeat را هم در مرحله ای پایینتر از عمل Dicer مختل نماید نشانگر اینکه پروتئینی از کمپلکس RISC (RNA-Induced Silencing Complex) در سیستم RNA Silencing توسط P0 مورد هدف قرار میگیرد. آزمایشات بعدی با استفاده از بیان همزمان P0 با ARGONAUTE1 (AGO1) این فرضیه را ثابت کرد و پروتئین ARGONAUTE1 در حضور P0 تجزیه گردید. در ادامه، تعامل مستقیم بین P0 و AGO1 توسط متدهای *in vitro* و *in planta* ثابت گردید. در مجموع، نتایج منتهی به ارائه مدلی برای مکانیسم عمل P0 شد. در این مدل P0 یک پروتئین F-box است که توسط ویروس در گیاه بیان میشود و با تعامل با ASK یک کمپلکس SCF^{P0} تشکیل میدهد و پروتئین AGO1 را که یک عضو ضروری در فرایند Gene Silencing است احتمالا از طریق یوبی کویتینه کردن تجزیه می نماید. این اولین گزارش از یک پروتئین بازدارنده از Gene Silencing است که در کمپلکس SCF عمل میکند و باعث تجزیه شدن عضوی ضروری در سیستم Gene Silencing میشود و بدین ترتیب دفاع میزبان را شکسته و زمینه را برای آلودگی ویروسی فراهم مینماید.

Etude du Mode d'action de la Protéine P0 des Polerovirus dans la suppression du RNA Silencing

Résumé: Les polerovirus appartiennent à une famille de phytovirus (Luteoviridae) capables d'infecter de nombreuses plantes d'intérêt agronomique. Leur génome est composé d'un RNA simple brin de polarité positive dont l'ORF, situé à l'extrémité 5', code pour la protéine P0 de 29 kDa, un suppresseur fort de Post-Transcriptional Gene Silencing (PTGS). Ce phénomène d'extinction de gènes est un moyen de défense antiviral chez les plantes. Afin d'étudier le mécanisme d'action de la protéine P0, nous avons criblé une banque cDNA d'Arabidopsis thaliana en système double hybride de levure. Deux protéines de la famille des protéines SKP1-like d'Arabidopsis (ASK) ont été identifiées comme partenaires cellulaires de la protéine P0. Les protéines ASK font partie des complexes SCF, un type d'E3 ubiquitine ligases impliquées dans la voie d'ubiquitination et de dégradation par le protéasome 26S. Ces complexes renferment également une protéine à F-box qui se lie à la protéine ASK par son domaine F-box et dont le rôle est la reconnaissance spécifique des protéines à dégrader. Un motif F-box a été identifié dans la partie N-terminale de la protéine P0. Une mutation ponctuelle dans ce motif entraîne la perte d'interaction avec les protéines ASK ainsi que la perte d'activité de suppression de silencing de la protéine P0. Introduite dans le génome viral, cette mutation confère une baisse importante de la pathogénicité du virus. Par ailleurs, des plantes de Nicotiana benthamiana, dans lesquelles l'expression du gène SKP1 a été diminuée par la technique du Gene Silencing induite par un virus (VIGS), se sont avérées résistantes à l'infection par les polerovirus. Une deuxième approche du mode d'action de la protéine P0 résulte de la transformation d'A. thaliana par le gène codant pour P0 sous le contrôle d'un promoteur inductible. En condition d'induction, ces plantes présentent un phénotype anormal rappelant certains mutants touchés dans le développement. L'analyse de l'expression des mRNAs endogènes, cibles de miRNAs a montré que certains sont surabondants dans les plantes induites, suggérant que P0 pourrait interférer avec la voie des miRNA. Par ailleurs nous avons montré que la protéine P0 est capable de supprimer le PTGS de type «Inverted-Repeat», indiquant qu'elle agirait à une étape située en aval de l'activité de Dicer (DCL). Parmi les protéines candidates cibles de P0 figure

ARGONAUTE1 (AGO1), une protéine essentielle du complexe RISC (RNA-Induced Silencing Complex). Nous avons pu montrer que l'expression de P0 conduit à la dégradation spécifique de la protéine AGO1 aussi bien en condition d'expression transitoire que dans les plantes d'Arabidopsis transformées «P0-FlagAGO1». De plus, une interaction physique entre P0 et AGO1 a été démontrée in vitro et in planta, favorisant l'hypothèse qu'AGO1 est bien la cible directe de P0. Nos données soutiennent un modèle dans lequel P0 agirait comme une protéine à F-box, recrutant la voie de modification post-traductionnelle pour inhiber le système de Gene Silencing post-transcriptionnel. Dans ce modèle, la P0 interagirait avec la protéine SKP pour constituer un complexe SCFP0 conduisant la protéine AGO1 vers l'ubiquitination et la dégradation par le proteasome 26S. C'est le premier exemple de suppresseur de silencing capable d'intégrer un complexe SCF pour induire la dégradation d'un composant essentiel de la voie du gene silencing, et de ce fait, inhiber la défense antivirale de la plante hôte.

Introduction

Les Polerovirus sont des phytovirus répandus à travers le monde entier qui occasionnent des dégâts considérables sur des cultures très variées telles que les céréales, la betterave sucrière, la pomme de terre, ou les cucurbitacées. Ils constituent l'un des trois genres de la famille des *Luteoviridae* caractérisés par une infection limitée aux tissus du phloème et une transmission obligatoire par puceron. Les particules sont de symétrie icosaédrique et le génome est constitué d'un RNA simple brin (~6 Kb) de polarité positive contenant six cadres ouverts de lecture. Les phases de lecture (ou open reading frame, ORF) 0, 1 et 2 sont traduites à partir du RNA génomique et les ORFs situés dans la partie 3' du génome (ORFs 3, 4 et 5) sont exprimés à partir d'un RNA sub-génomique. Les ORF 1 et 2 codent pour les sous-unités de la replicase. La protéine de capside est codée par l'ORF3 et la protéine P4 (ORF4) présente les propriétés d'une protéine du mouvement. La protéine P5 est traduite par un mécanisme de translecture du codon stop de l'ORF3. Associée aux particules virales, elle est essentielle à la transmission du virus par puceron et participe au mouvement et à l'accumulation efficace du virus dans la plante. Enfin, l'ORF0 qui est situé à l'extrémité 5' du RNA assure la synthèse de la protéine P0. Cette protéine a été caractérisée comme

suppresseur de post-transcriptional gene silencing (PTGS). Indétectable dans les plantes infectées, il semble que son expression soit fortement régulée par le virus. Par ailleurs l'absence de synthèse de la protéine P0 aboutit à une forte diminution de l'accumulation des RNA viraux, ce qui suggère l'importance de la protéine P0 pour l'infection virale.

L'extinction de gènes ou «gene silencing» est un mécanisme universel chez les métazoaires basé sur la reconnaissance d'un RNA double brin et qui conduit à sa dégradation en RNA de 21-24 nucléotides (siRNA), ainsi qu'à celle de tous les RNA homologues.

Il existe trois voies majeures d'extinction de gènes chez les plantes : (1) la voie des siRNA cytoplasmiques comprenant le PTGS dont le rôle est essentiellement antiviral, (2) la voie des miRNA destinée à la régulation de messagers endogènes codant pour des facteurs de transcription impliqués principalement dans le développement et (3) la voie nucléaire associée à la méthylation de l'ADN et l'inhibition de la transcription et dont le rôle est de protéger le génome contre les DNA parasites tels que les transposons. La molécule clé qui initie le RNA silencing est une molécule d'ARN double brin qui est reconnue par une enzyme de type RNAse III appelée Dicer (ou DCL pour Dicer-like) et qui est clivée en petits RNA de 21-24 nucléotides, les siRNA. Il existe 4 gènes DCL chez *Arabidopsis thaliana*. Les siRNA sont ensuite transférés à un complexe multiprotéique dénommé RISC (RNA-induced gene silencing) où ils vont assurer la reconnaissance spécifique du RNA homologue à dégrader. Une des sous-unités essentielles du complexe RISC est la protéine ARGONAUTE1 (AGO1) dont la programmation est dépendante de sa capacité à fixer les siRNA et les miRNA. L'activité ribonucléase attribuée au complexe RISC a été localisée par des études biochimiques et cristallographiques au niveau du domaine PIWI situé dans la partie C-terminale de la protéine AGO de drosophile. Cette activité RNAse H encore appelée «Slicer» a ensuite été caractérisée chez la souris et plus récemment chez AGO1 d'*A. thaliana* capable de recruter spécifiquement les miRNA et certains siRNA. Les protéines AGO possèdent un deuxième domaine caractéristique appelé PAZ (dans la partie N-terminale) nécessaire à l'incorporation des petits RNA.

Pour faire face à ce système de défense, les virus codent pour des protéines qui peuvent inhiber le PTGS induit par leur hôte. De nombreuses protéines dites «suppresseur de gene silencing» ont été identifiées à ce jour chez les virus de plante mais elles ne présentent

aucune homologie de séquence ou de structure. Au début de ma thèse, seul le mode d'action de la protéine P19 du *Tomato bushy stunt tombusvirus* avait été élucidé: la protéine P19 est capable de fixer et de séquestrer les siRNA inhibant ainsi le fonctionnement du complexe RISC. Depuis, le mode d'action d'autres protéines commence à être compris: par exemple la protéine HCPro des *potyvirus* ou la protéine P21 des *closterovirus* sont également capables de lier les siRNA, les caractéristiques d'interaction étant spécifiques à chaque protéine. La protéine P38 du *Turnip crinkle carmovirus* interfère avec l'enzyme DCL4. Tout dernièrement, il a été montré que la protéine 2b du *Cucumber mosaic virus* est capable d'inhiber l'activité de la protéine AGO1 en interagissant directement avec elle.

Résultats:

1. La protéine P0 s'associe aux protéines ASK1/2 dans un complexe E3 ubiquitine-ligase.

L'objectif de ma thèse était de comprendre le mécanisme d'action de la P0 dans la suppression du gene silencing. Dans ce but, nous avons privilégié une première approche au moyen d'un crible d'une banque cDNA d'*A. thaliana* en système double hybride de levure, en utilisant comme appât les protéines P0 de deux Polerovirus, *Beet western yellows virus* (BWYV) et *Cucurbit aphid-borne yellows virus* (CABYV). Ainsi nous avons identifié les protéines ASK1 et ASK2 (Arabidopsis SKP1-related protein) qui sont des orthologues de la protéine SKP1 de levure intervenant dans les complexes E3 ubiquitine ligase de type SCF (SKP-Culline-F-box). Ces complexes participent à l'ubiquitination de protéines destinées à la dégradation par le protéasome 26S dans le système de régulation post-traductionnelle des protéines. L'interaction entre les protéines P0 et ASK1 (ou ASK2) a été confirmée par deux approches : *in vitro* celle du GST pull-down et *in planta* celle du BiFC (Bi-molecular fluorescence complementation). Dans le complexe SCF, les protéines ASK ont un rôle d'adaptateur entre la protéine d'échafaudage Culline1 et une protéine à domaine F-box. Chez *A. thaliana*, plus de 700 gènes codant pour des protéines à F-box ont été identifiés, chacune étant responsable de la dégradation spécifique d'une protéine cible. L'alignement de séquence des protéines P0 de plusieurs polerovirus montre qu'elles possèdent toutes un

domaine F-box dans leur partie N-terminale avec un motif caractéristique (LPxxI/L). En introduisant des mutations ponctuelles dans ce motif, les protéines P0 du CABYV et du BWYV perdent leur capacité à interagir avec les protéines ASK dans la levure. Cette perte d'interaction conduit également à la perte de l'activité de suppression de gene silencing en système ectopique d'agro-infiltration sur *Nicotiana benthamiana*, ainsi qu'à la diminution du pouvoir pathogène dans un contexte viral hétérologue PVX (*Potato X potexvirus*). Transposée dans le génome viral, cette mutation est responsable de la forte diminution d'accumulation de virus. Afin de démontrer l'importance de l'interaction P0-ASK dans l'infectivité des Polerovirus, nous avons utilisé une approche VIGS (Virus Induced Gene Silencing) pour inhiber l'expression des gènes *SKP* de *N. benthamiana* (orthologues des gènes *ASK*). Au préalable, nous avons vérifié en système double hybride de levure l'existence de l'interaction entre les protéines $P0^{CA}$ et $P0^{BW}$ et NbSKP. Le vecteur viral choisi a été le virus X de la pomme de terre (PVX). Les plantes *N. benthamiana* ont été inoculées dans un premier temps avec le PVX ou un PVX recombinant PVX-SKP de manière à induire l'extinction du gène SKP. Ces plantes ont ensuite été sur-inoculées par des pucerons virulifères chargés en BWYV. Les plantes PVX-SKP qui présentent une forte diminution du taux de la protéine SKP sont résistantes à l'infection par le BWYV, alors que les plantes témoins PVX restent sensibles au virus. Cette expérience démontre que l'interaction P0-SKP est indispensable au développement de l'infection virale.

En jouant le rôle d'une protéine à F-box dans un complexe E3 ubiquitine-ligase, la protéine P0 des Polerovirus pourrait changer la destinée d'un facteur cellulaire essentiel du PTGS en l'adressant vers le protéasome. Cette stratégie de détournement de la voie d'ubiquitination bien connue chez les virus animaux constitue ici un premier exemple chez les plantes.

Ces résultats sont détaillés dans le chapitre 1 relatant la première publication ainsi que certaines expériences complémentaires non publiées.

Pazhouhandeh M., Dieterle M., Marrocco K., Lechner E., Berry B., Brault V., Hemmer O., Kretsch T., Richards K.E., Genschik P. & Ziegler-Graff V. (2006). F-box-like domain in the Polerovirus protein P0 is required for silencing suppressor function. PNAS, 103(6):1994-1999.

2. La protéine ciblée par P0 est la protéine ARGONAUTE 1.

Afin d'identifier l'étape du PTGS entravée par la protéine P0, nous avons dans un premier temps analysé l'accumulation des petits RNA produits par DCL par une approche d'expression ectopique des protéines dans N. benthamiana. Par une 2ème approche, j'ai caractérisé des plantes exprimant la protéine P0, ce qui m'a amené à proposer la protéine ARGONAUTE1 (AGO1) comme candidat cible de la protéine P0.

L'activité suppresseur de silencing de la protéine P0 a été démontrée pour la première fois dans des conditions de PTGS induit par une construction GFP en orientation sense (S-PTGS). Afin d'étudier l'activité de la protéine P0 sur le PTGS induit par une construction inversement répétée (Inverted Repeat-PTGS), nous avons co-infiltré des plantes *N. benthamiana* sauvages avec les constructions codant pour les protéines P0, GFP (sens) et une construction produisant une « tige-boucle » à partir du mRNA de la GFP appelé GFFG. L'observation de la restauration de la fluorescence ainsi que l'ananlyse des siRNA produits, nous a permis de constater que la protéine P0 supprime aussi le PTGS de type IR. L'accumulation des siRNA primaires n'étant pas affectée par la présence de la protéine P0, contrairement à l'effet de la protéine P38 du TCV connue pour inhiber l'activité DCL, nous avons pu conclure que P0 bloque une étape en aval de DCL.

Afin d'aborder le mécanisme d'action de la protéine P0 dans un contexte *in vivo*, nous avons transformé des plantes d'Arabidopsis par le gène codant pour la protéine P0. Son expression constitutive sous la dépendance du promoteur 35S du CaMV étant létale pour le développement précoce des plantes, nous avons choisi de l'exprimer à partir d'un promoteur inductible par l'oestradiol (XVE). Le traitement chimique des plantules XVE-P0^{BW} conduit après une semaine à l'apparition de déformations foliaires rappelant le phénotype de plantes exprimant d'autres protéines suppresseurs forts tels que les protéines HCPro des *potyvirus* ou P15 du *Peanut Clump Pecluvirus*, à savoir des feuilles enroulées et dentelées présentant une forte déformation spatiale. On peut également noter certaines malformations au niveau de la hampe florale, comme la disparition de la phyllotaxie et des tiges courbées et fasciées. Ces phénotypes pleiotropiques rappellent également les altérations présentées par certains mutants touchés dans des gènes impliqués dans le développement et dont la régulation post-transcriptionnelle implique les miRNA. Une approche par RT-PCR quantitative a montré que les plantes XVE-P0 présentent une forte accumulation de plusieurs de ces mRNAs

endogènes ciblés par les microRNA. Par contre, au niveau des miRNA, seuls certains présentent une faible diminution, les autres demeurant constants. Il est intéressant de noter que l'ensemble des modifications observées au niveau des mRNA et miRNA suivent parfaitement celles mesurées pour un mutant hypomorphe d'AGO1, le mutant *ago1-11*. Ces observations suggèrent donc une dérégulation de la dégradation des mRNA par la voie des microRNA. Un des facteurs essentiel et commun à la cascade du PTGS et à la voie des microRNA qui agit en aval d'activité de DCL, est la protéine ARGONAUTE1 (AGO1). Cette protéine était donc le candidat idéal pour être la cible de la protéine P0.

Afin de confirmer cette hypothèse, les protéines P0 et AGO1 ont été co-exprimées en système transitoire ectopique *in planta*. On observe la dégradation spécifique de la protéine AGO1 en présence de la protéine P0. De plus, la disparition est dépendante du motif F-box de la protéine P0, suggérant que l'effet est dépendant du complexe E3 ubiquitine ligase. Par ailleurs, les plantes XVE-P0 ont été croisées avec des plantes transformées avec une version étiquetée (FLAG) d'AGO1 (dont l'expression est contrôlée par le propre promoteur d'*AGO1*). Il apparaît que l'induction du gène P0 conduit à la déstabilisation de la protéine AGO1, étayant l'hypothèse que la protéine AGO1 est bien la cible de la protéine P0.

Afin de démontrer que l'interaction entre les protéines AGO1 et P0 est de type direct, j'ai tout d'abord tenté l'approche par double hybride dans la levure, malheureusement sans succès. J'ai ensuite mis au point une technique de co-sédimentation. Il s'avère que seule la protéine AGO1 produite dans un système eucaryotique (par traduction en réticulocytes de lapin ou directement extraite des plantes transgéniques FLAG-AGO1), est capable d'interagir avec la protéine P0, qu'elle ait été traduite en réticulocytes de lapin ou produite à partir de bactéries sous forme de GST-P0. Enfin, par une approche de complémentation de BiFC, nous avons confirmé *in planta* l'existence de l'interaction directe entre les protéines P0 et AGO1.

L'ensemble de ces résultats sont regroupés dans le deuxième chapitre ainsi que dans la publication N° 2:

Bortolamiol D., Pazhouhandeh M., Marrocco K., Genschik P. & Ziegler-Graff V. (2007). The Polerovirus F-box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Current Biology, acceptée le 20 juillet 2007.

Le chapitre n°2 présente également des résultats non publiés, comme les essais d'infection de mutants d'arabidopsis de la voie du gene silencing. Ceux-ci n'ont pu être exploités en raison de la redondance de fonction des différents gènes DCL, RDR et AGO. Les premiers résultats de l'analyse des plantes issues de croisement entre les plantes XVE-P0 et une lignée indicatrice silencée pour le gène de la GFP sont également rapportés. J'ai pu constater que l'expression de la protéine P0 conduit à la réapparition de la fluorescence dans les tissus vasculaires foliaires et racinaires. Par ailleurs, j'ai caractérisé des plantes d'arabidopsis transformées par le gène P0 placé sous le contrôle d'un promoteur spécifique aux cellules compagnes (AtSUC2). Ces plantes présentent un phénotype sévère d'enroulement des feuilles comparable à celui des plantes trasgéniques exprimant d'autres suppresseurs forts viraux tels que HCPro et P15. Croisées avec la lignée indicatrice silencée pour le gène de la GFP, on observe la restauration de la fluorescence dans les tissus vasculaires.

Enfin, la dernière partie du chapitre est consacrée au début d'une étude fonctionnelle des protéines $P0^{CA}$ et $P0^{BW}$. Les mutants ponctuels dans la partie C-terminale ont été analysés pour leur interaction avec les protéines ASK ainsi que leur propriétés de suppression du gène silencing.

Conclusion

L'ensemble de ces résultats nous a permis de mettre en évidence un nouveau mécanisme de suppression de gène silencing dans lequel la protéine P0 joue le rôle d'une protéine à F-box, en ciblant la protéine AGO1 pour l'adresser vers la voie de dégradation par le protéasome, inhibant de ce fait le processus de défense de la plante. La protéine P0 permet donc de tisser un premier lien entre deux mécanismes importants de la régulation cellulaire : l'interférence à RNA et la régulation post-traductionnelle des protéines par le protéasome. Cette étude nous a également permis d'éclaircir une étape essentielle de la biologie des polerovirus.

Dans un avenir proche, il s'agira tout d'abord de confirmer l'intervention de l'ubiquitination dans la dégradation de la protéine AGO1 par la protéine P0, en inhibant par exemple le processus par un inhibiteur du protéasome et en mettant en évidence la modification d'AGO1 par l'ubiquitine.

Récemment, le mode d'action de la protéine 2b du CMV, un autre suppresseur de gene silencing, a été caractérisé. Tout comme la protéine P0 des polerovirus, elle agit au niveau d'AGO1 mais selon un mécanisme différent. La protéine 2b bloque l'action d'AGO1 en inhibant son activité de dégradation des RNA. Plus précisément, elle interagit avec une région du domaine PAZ et une partie du domaine PIWI. La caractérisation moléculaire de l'interaction entre les protéines P0 et AGO1 reste à étudier.

A. thaliana code pour une famille de dix protéines ARGONAUTE. Les homologies de séquence entre les domaines PAZ et PIWI des dix membres de cette famille suggèrent que la protéine P0 puisse également reconnaître les autres protéines AGO. On peut noter que les mutants simples *ago1* nuls d'Arabidopsis sont viables (mais stériles), alors que l'expression constitutive de P0 est létale. Quant aux doubles mutants *ago1ago10* homozygotes, ils sont embryon-letaux. Ces observations laissent présumer que la protéine P0 pourrait cibler plusieurs protéines AGO, hypothèse qu'il faudra tester *in vivo*.

Classiquement, les protéines à domaine F-box caractérisées à ce jour, interagissent avec leurs cibles via leur région C-terminale. Ni les recherches *in silico* de domaines d'interaction entre protéines, ni les premiers résultats de mutagénèse ne nous ont permis d'identifier le domaine de P0 impliqué dans l'interaction avec AGO1. Ces études vont être poursuivies. Par ailleurs, on peut noter que de nombreuses protéines cibles nécessitent une/des modifications post-traductionnelles pour être reconnues par la protéine à F-box. Il sera intéressant à l'avenir d'étudier ces modifications potentielles en relation avec la déstabilisation d'AGO1.

En conclusion, malgré de fortes présomptions concernant l'implication du protéasome dans la dégradation de la protéine AGO1 par P0, nous ne pouvons pas éliminer la possibilité que P0 reconnaisse un autre facteur qui serait dégradé par la voie ubiquitine-dépendante. Ce

facteur intermédiaire pourrait jouer un rôle de stabilisateur d'AGO1, qui entraînerait la disparition dégradation d'AGO1 lors de sa propre dégradation.

Enfin, en croisant les plantes exprimant P0 avec des plantes indicatrices transformées par un gène rapporteur GFP silencé, nous avons constaté un profil de restauration de la fluorescence associé au phloème. Ces observations ont été réalisées avec des plantes exprimant la protéine P0 théoriquement dans toutes les cellules (plantes XVE-P0) ou uniquement dans le phloème (plantes Suc-P0). Placé dans le contexte de la restriction des polerovirus au phloème, ces résultats suggèrent l'existence de facteurs cellulaires spécifiques au phloème dont l'interaction avec la protéine P0 conditionnerait son activité de suppresseur de silencing. Cette question sera abordée par le biais des plantes transgéniques P0 croisées avec d'autres plantes mutantes ou transformées avec d'autres gènes rapporteurs ou encore avec un crible génétique d'une banque cDNA de cellules compagnes.

Remerciements

En tout premier lieu, je remercie chaleureusement Véronique Ziegler-Graff pour m'avoir accueilli dans son équipe et pour m'avoir guidé et conseillé au cours de ces trois années. Un Grand merci au "Triple Chief Block": Gérard Jonard, Hubert Guilley (ma référence pour toutes les question pratiques) et plus particulièrement, Kenneth Richards pour m'avoir accompagné pas à pas tout au long de cette thèse, pour répondre à toutes mes questions et pour corriger attentivement ma thèse.

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Abbreviations

Virus Acronyms

ACMV	African cassava mosaic Begomovirus
BChV	Beet Chlorosis Polerovirus
BCTV	Beet curly top Curtovirus
BMYV	Beet mild yellowing Polerovirus
BWYV	Beet western yellows Polerovirus
BYDV	Barley yellow dwarf Luteovirus
BYV	Beet yellows Closterovirus
CABYV	Cucurbit aphid-borne yellows Polerovirus
CaLCuV	Cabbage Leaf Curl Geminivirus
CaMV	Cauliflower mosaic Caulimovirus
CIRV	Carnation italian ringspot virus
CMV	Cucumber mosaic Cucumovirus
CtRLV	Carrot red leaf Polerovirus
CTV	Citrus tristeza Closterovirus
CYDV	Cereal Yellow Dwarf virus
CymRSV	Cymbidium ringspot Tombusvirus
EBV	Epstein-Barr virus
FHV	Flock house Virus
LMV	Lettuce mosaic Potyvirus
MYMV	Mungbean yellow mosaic Begomovirus
ORMV	Oilseed Rape Mosaic Tobamovirus
PCV	Peanut clump Pecluvirus
PEMV-1	Pea enation mosaic Enamovirus-1
PEMV-2	Pea enation mosaic Umbravirus-2
PFV-1	Primate Foamy Retrovirus type-1
PLRV	Potato Leafroll Polerovirus
PSTVd	Potato spindle tuber viroid
PVX	Potato X Potexvirus
PVY	Potato Y Potyvirus
RCNMV	Red clover necrotic mosaic Dianthovirus
RHBV	Rice Hoja Blanca Tenuivirus
SbDV	Soybean dwarf virus
ScYLV	Sugarcane yellow leaf Polerovirus
SeMV	Sesbania mosaic virus
TBSV	Tomato bushy stunt Tombusvirus
TCV	Turnip crinkle Carmovirus
TEV	Tobacco Etch Potyvirus
TGMV	Tomato golden mosaic Begomovirus
TMV	Tobacco Mosaic Tobamovirus
ToMV	Tomato mosaic Tobamovirus
TRV	Tobacco rattle Tobravirus
TuMV	Turnip mosaic Potyvirus
TuYV	Turnip yellows Polerovirus
TYLCV	Tomato yellow leaf curl Begomovirus
TYMV	Turnip Yellow Mosaic Tymovirus

Abbreviations

A. thaliana	*Arabidopsis thaliana*
A. tumefaciens	*Agrobacterium tumefaciens*
AAP	acquisition access period
AD	activation domain
ADK	Adenosine Kinase
AGO	ARGONAUTE
APC	Anaphase-Promoting Complex
APS	Ammonium Persulfate
ARF	Auxin response factor
ASG	accessory salivary gland
ASK	Arabidopsis S-phase Kinase
*ASK*1	Arabidopsis *SKP1*-like genes
ATP	adenosine triphosphate
BD	binding domain
BiFC	Bimolecular Fluorescent Complementation
bp	Base-paired
BSA	bovine serum albumin
BTB	Broad-complex, Tramtrack, Bric-a-Brac
C. elegans	*Caenorhabditis elegans)*
C. quinoa	C*enopodium quinoa*
CAND1	Cullin-Associated and NeddylationDissociated1
ca-siRNA	cis-acting siRNA
cDNA	Complementary DNA
CHS	Chalcone synthase
CMT3	Chromo Methyltransferase
COI1	Coronatine Insensitive1
Col	Colombia
CP	coat protein
CP	core protease
Cullin	CUL/CDC53
D. melanogaster	Drosophila melanogaster
D-box	Destruction box
DCL	Dicer-like
DDB1	Damaged DNA-Binding1
DDM1	Decrease in DNA Methylation
DET1	De-Etiolated 1
dip	Day(s) post infection/ infiltration/induction
DMSO	Dimethylsulfoxide
DRB/dsRBD	double-strand RNA binding domains
DRM	Domains Rearranged Methyltransferase
dsRNA	double-stranded RNA
DTT	dithiothreitol
DUB	Deubiquitinating Enzyme
E6AP	E6-associated protein
ECR1	E1-Conjugating enzyme-Related1
EDTA	ethylene diamine tetra-acetate
EGTA	ethylene glycol tetra-acetate
ELISA	Enzyme linked immunosorbent assay
EMS	Ethyl methyl sulfate
Exp5	Exportin-5
GFP	Green fluorescent protein
GST	Glutathione S-Transferase
GUS	β-Glucoronidase
HA	Hemagglutinin
HCPro	Helper component proteinase
hc-siRNA	heterochromatin siRNA
HECT	Homology to E6AP C Terminus
HEN1	HUA Enhancer1
HEPES	N-2 hydroxyethyl piperazine acid N'-2ethane sulfate
HMV	high molecular weight
HR	hypersensitive reaction
HRP	horse radish peroxidase
HST	HASTY
HUB	Homologous to Ubiquitin

HYL1	Hyponastic Leaves1
IAP	inoculation access period
ICTV	International Committee on Taxonomy of Viruses
IPTG	Isopropyl-β-D-thiogalactopyranoside
IR	inverted-repeat
kb	kilo base
kDa	kilo dalton
LB	Luria Bertoni
L*er*	Landesberge *erecta*
LMP	low melting point
LP	latent period
LRR	leucine-rich repeats
LTR	long terminal repeat
MET1	methyltransferase1
miRNA	MicroRNA
MP	movement protein
MS	Murashige and Skoog
N. benthamiana	*Nicotiana benthamiana*
N. clevelandii	*Nicotiana clevelandii*
nat *cis*-acting siRNA	natural antisense transcript *cis*-acting siRNA
NBS	nucleotide-binding site
NLS	nuclear localization signals
nt	nucleotide
OAS	origin of assembly sequence
ODC	Ornithine Decaroxylase
ORF	Open reading frame
P. syringae	*Pseudomonas syringae*
P35S	35S promoter
P5CDH	pyrroline-5-carboxylate dehydrogenase
PAMP	pathogen-associated molecular patterns
PAZ	Piwi, Argonaute, and Zwille/Pinhead
pBin	Binary vector
P-bodies	Processing bodies
P-bodies	processing-bodies
PBS	Phosphate buffer saline
PCR	polymerase chain reaction
PEG	poly ethylene glycol
PNH/ZLL	PINHEAD/ZWILLE
PNK	T4-Polynucleotide Kinase
PPR	pentatricopeptide repeat proteins
PTGS	post-transcriptional gene silencing
PVDF	polyvinylidene fluoride
PVP	Polyvinyl pyrrolidone
QDE-1	*Neurospora crassa* RDR
Q-RT-PCR	Quantitative Reverse transcription PCR
ra-siRNA	repeat-associated siRNA
RBX	Ring-Box protein
RdDM	RNA directed DNA methylation
RDR	RNA-dependent RNA polymerase (in Arabidopsis)
RdRP	RNA dependent RNA polymerase (in viruses)
RING	Real Interesting New Gene
RISC	RNA induced silencing complex
RITS	RNA-induced transcriptional silencing
RNA pol	RNA polymerase
RNAi	RNA interference
RNP	ribonucleoprotein
RP	regulatory particle
rpm	Rotation per minute
rRNA	Ribosomal RNA
RT	readthrough
RT	Reverse transcriptase
RT	Room temperature
RTD	readthrough domain
RUB1	Related to Ubiquitin1
RUP	Ub/proteasome-dependent processing
SAP	Shrimp Alkaline phosphatase
SAR	systemic acquired resistance

SCF	SKP1, Cullin/CDC53, F-box protein complex
SDE	Silencing DEfective
SDS	Sodium dodecyl sulfate
SE	Serrate
sgRNA	Subgenomic RNA
SGS	Suppressor of Gene Silencing
SGT1	Suppressor of G2 allele of SKP1
siRNA	short interfering RNA
SKP	S-phase Kinase-associated protein
SON1	Suppressor of Nim1
S-PTGS	sense-PTGS
SSC	Salt sodium citrate
ssDNA	Single strand DNA
ssRNA	Single strand RNA
SUC	Sucrose symporter promoter
SUL	sulphur
SUMO	Small Ubiquitin-related Modifier
T35S	35S terminator
Taq	*Thermus aquaticus*
tasiRNA	*trans*-acting siRNA
TBE	Tris-borate EDTA
T-DNA	Transfer-DNA
TEM	transmission electron microscopy
TEMED	NNNN'-tetramethyl ethylene diamine
TGS	transcription gene silencing
Ti	Tumor inducing
TIR	terminal inverted repeat
TPR	Tetratricopeptide repeats
TrAP	Transcriptional Activator Protein
Tris	Tri(hydroxyl)aminomethane
tRNA	Transfer RNA
Ub	ubiquitin
UBC	Ub-carrier protein
U-Box	UFD2 homology protein
UBP	Ub-specific proteases
UFO	Unusual Floral Organs
UPL	Ub Protein Ligase
URM	Ubiquitin-related Modifier
UTR	untranslated region
UV	ultraviolet
VIGS	Virus-induced gene silencing
VPg	Viral protein linked genome
WEL	Werner Exonuclease-Like
WEX	Werner Exonuclease
XVE	LexA,VP16,Estrogene

Introduction

Part 1: Polerovirus

Part 2: RNA Silencing

Part 3: Viral Suppressor of Silencing

Table 1: Luteoviridae family: its genera and their species (ICTVdB Management, 2006).

Genus	Virus species
Luteovirus	***Barley yellow dwarf virus* – PAV (BYDV-PAV)** (*Rhopalosiphum* ***p****adi* and *Macrosiphum* ***av****enae*) *Barley yellow dwarf virus* - MAV [*Sitobion* (*Macrosiphum*) *avenae*] *Barley yellow dwarf virus* - RGV (Rice Giallume virus)
Polerovirus	***Potato leaf roll virus* (PLRV)** *Beet western yellows virus* (BWYV) *Cucurbit aphid-borne yellows virus* (CABYV) *Cereal yellow dwarf virus* - RPV (*Rhopalosiphum padi*) (CYDV-RPV) *Cereal yellow dwarf virus - RPS* (CYDV-RPS) *Beet mild yellowing virus* (BMYV) *Beet chlorosis virus* (BChV) (proposed to be in this genus by Hauser *et al*., 2002) *Turnip yellows virus* (TuYV) *Sugarcane yellow leaf virus* (SCYLV) *Carrot red leaf virus* (CtRLV)
Isolates that were found similar to known viruses:	Tobacco yellow top virus (synonym of PLRV) Capsicum yellows virus (synonym of PLRV) Potato phloem necrosis virus (synonym of PLRV) Tomato yellow top virus (synonym PLRV) Solanum yellows virus (synonym PLRV) Malva yellows virus (synonym of BWYV) Turnip mild yellows virus (synonym of BWYV) Pea leaf roll virus (synonym of BWYV) Radish yellow virus (synonym of BWYV)
Enamovirus	***Pea enation mosaic virus - 1* (PEMV-1)**
Unassigned Viruses in this Family	Barley yellow dwarf virus - SGV (*Schizaphis graminum*) Barley yellow dwarf virus - RMV (*Rhopalosiphum maidis*) Barley yellow dwarf virus - GPV (*S. graminum* and *R. padi*) Barley yellow dwarf virus - ORV (Oat Red-leaf Virus) Bean leafroll virus (BLRV) Chickpea chlorotic stunt virus (CpCSV) Groundnut rosette assistor virus (GRAV) Indonesian soybean dwarf virus (ISDV) Soybean dwarf virus (SbDV) (between Luteovirus and Polerovirus without ORF0 and 6) (Terauchi *et al*., 2001) Sweet potato leaf speckling virus (SPLSV) Tobacco necrotic dwarf virus (TNDV) Brassica yellowing virus (BrYV) (isolate of BWYV on brassicaceae that does not infect beet) (Hauser *et al*., 2000) Ryegrass chlorotic streak virus (synonym of BYDV) Legume yellows virus (synonym) Michigan alfalfa virus (synonym) Strawberry mild yellow edge associated virus (isolates) Subterranean clover red leaf virus (isolates)

Introduction Part 1: **Polerovirus**

1. Early record and classification

The poleroviruses represent an important genus of plant viruses that can infect a wide range of hosts, causing important losses in agriculture. The first records of symptoms attributable to a polerovirus are probably those of "potato curl" in Europe in the second half of the 18th century. Because of the economic importance of potato, the first polerovirus that attracted the attention of virologists was *Potato Leafroll Polerovirus* (PLRV). The yellowed leaves of sugar beet with economically important yield losses led to the discovery of two other polerovirus, *Beet Western Yellows Polerovirus* (BWYV) and *Beet Mild Yellowing Polerovirus* (BMYV) (Watson, 1952). Since then, other polerovirus were discovered from a wide variety of hosts and the genus is now recognized as one of the most ecologically successful and economically important taxa of plant viruses (Smith and Barker, 1999).

The viruses that we now refer to as poleroviruses were first classified in a subgroup in the *Luteovirus* genus based on serological relationships, physiochemical properties of the virus particle and biological properties such as tissue localization and vector relations. However, as it became available, nucleotide sequence information has greatly affected taxonomic thinking on this group. In 1999, the seventh report of the International Committee on Taxonomy of Viruses (ICTV), used genome organization to define three distinct genera in a new *Luteoviridae* family: *Luteovirus* (*Luteus*: in Latin means yellow) with the type species *Barley yellow dwarf virus* - PAV (BYDV-PAV), *Polerovirus* with the type species PLRV, and *Enamovirus* with the type species *Pea enation mosaic virus-1* (PEMV-1) (Mayo, 1999; D'Arcy and Domier, 2000). For the members of each genus and phylogeny relationships see Table 1 and Figure 1. There are also several viruses that are apparent members of the family but that have not yet been formally classified in genera.

2. Hosts and viral symptom

Luteoviruses such as BYDV exclusively infect plant species in the grass family, *Poaceae*, including weeds and cereal crops such as barley, maize, oats, rye, rice, and wheat. Polerovirus-susceptible host species are found in the Family *Amaranthaceae, Brasicaceae* (*Cruciferae*), *Portulacaceae, Cucurbitaceae and Solanaceae.* Susceptible host species for enamoviruses are found in the Family *Chenopodiaceae, Leguminosae-Papilionoideae* and *Solanaceae* (ICTVdB Management, 2006). Symptoms of *Luteoviridae* infection consist typically of rolling, yellowing or reddening of leaves (Figure 2), stunting of growth and reduced yield in grain. Phloem necrosis in PLRV-infected potato is also observed.

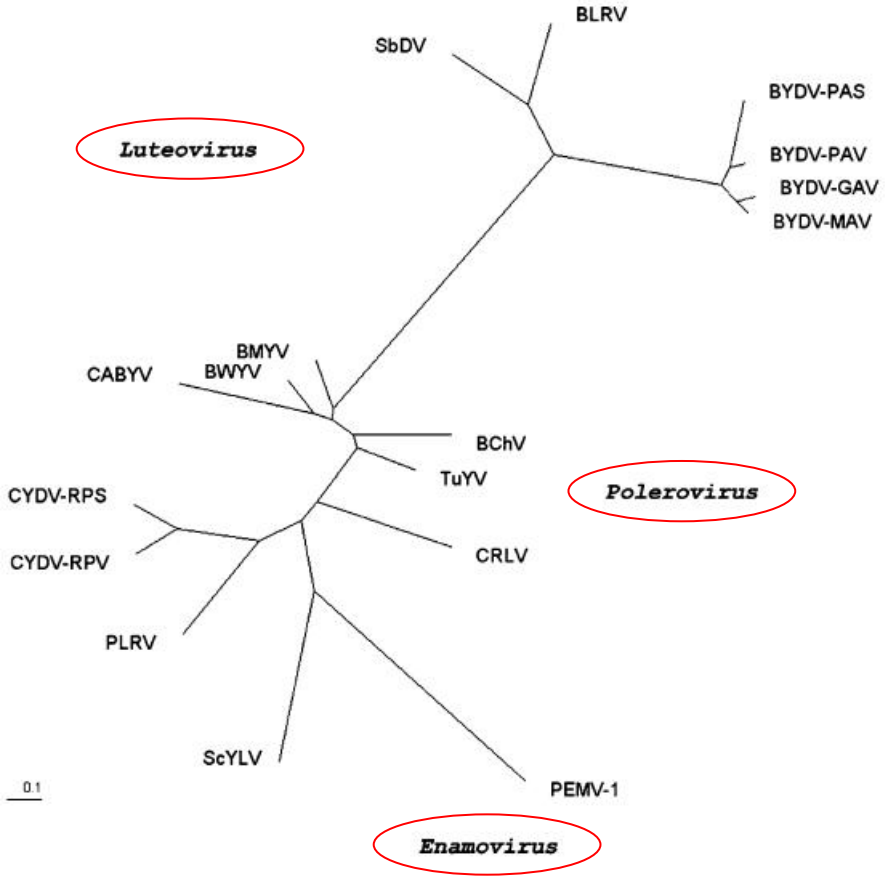


Figure 1: Unrooted phylogenetic tree for the complete genome sequences of luteoviridae species (Maximum Likelihood method). There are three branches corresponding to the three genera. The scale bar indicates the branch length (Huang *et al.*, 2005).

Table 2: Aphid vectors and susceptible hosts of BWYV, CABYV and PLRV (ICTVdB Management, 2006).

virus	Aphid species that transmit the virus	Susceptible economically important hosts
BWYV	*Myzus persicae,* *Aphis craccivora* *Aphis gossypii* *Acyrthosiphon (Aulacorthum) solan,* *Brachycaudus helichrys* *Brevicoryne brassicae* *Macrosiphum euphorbiae* *Myzus (Sciamyzus) ascalonicus* *Myzus ornatus* *Myzus (Phorodon) humuli*	*Beta vulgaris* (sugar beet), *Spinacia oleracea* (spinach), *Helianthus annuus, Lactuca sativa* (lettuce), *Brassica napus, B. campestris, B. nigra, B. oleracea, Raphanus sativus* (radish), *Crambe abyssinica, Citrullus lanatus, Cucumis sativus* (cucumber), *Cucurbita pepo, Cicer arietinum, Glycine max, Pisum sativum, Trifolium subterraneum, Vicia faba, Phlox drummondii, Capsicum annuum, Lycopersicon esculentum.*
CABYV	*Myzus persicae, Aphis gossypii*	Cucurbitaceae
PLRV	*Myzus persicae* *Macrosiphum euphorbiae*	*Solanum tuberosum* (potato) *Lycopersicon esculentum* (tomato)

3. Viruses studied

In the course of my research, I have worked with the three following poleroviruses: BWYV, *Cucurbit aphid-borne yellows virus* (CABYV) and, to a much lesser extent, PLRV. Basic information concerning the diseases provoked by these viruses is provided below.

3-1. BWYV. BWYV is a widespread and economically important plant virus with a host range including more than 150 species in 23 dicotyledonous families. The susceptible economically important species are listed in Table 2. This virus was first reported by Duffus (1960) in North America, then was found in Europe, Asia and Africa. The most obvious symptom, as in many polerovirus-infected plants, is leaf yellowing (Figure 2), although the leaves often also develop a crinkled appearance and undergo changes in their characteristics (due to elevated starch content). BWYV was initially named *Radish yellows virus* but was renamed because of its importance in sugar beet (Hauser *et al*., 2000). The term "beet polerovirus" refers to sugar beet-pathogenic poleroviruses such as BWYV and BMYV. Several synonyms of BWYV are: beet mild yellowing virus, malva yellows virus, pea leaf roll virus, New Zealand radish yellows virus and turnip mild yellows virus.

The virus used in my studies is the isolate BWYV-FL1, which was originally isolated from lettuce (Lot and Maury-Chovelon, 1985). BWLV-FL1 will generally be referred to from now on as BWYV unless a distinction with other BWYV isolates is important. The complete genome sequence (5641 nt) of BWYV-FL1 was determined by Veidt *et al.* (1988) and can be found under EMBL GenBank accession numbers X13063. It should be noted that, in terms of host range, BWYV-FL1 is atypical compared to other BWYV-like strains and species isolated from sugar beet and rape (Hauser *et al*., 2000). In particular, the cloned BWYV-FL1 does not efficiently infect sugar beet (Hauser *et al*, 2002). The principal natural vector is *Myzus persicae*. Other aphid species that transmit BWYV are listed in Table 2.

3-2. CABYV. CABYV was first reported by Lecoq *et al*. (1992) following the appearance of an unusual obligately aphid-transmitted disease of cucurbits in France. The complete sequence of the genomic RNA (5669 nt) was determined by Guilley *et al.* (1994) and is listed under EMBL GenBank accession number X76931. This virus is widespread in cucurbits (almost all *Cucurbitaceace*) throughout the world and has economic importance. The most characteristic visual symptom is yellowing of leaves (Figure 2). Both *Myzus persicae* and *Aphis gossypii* transmit efficiently CABYV.

3-3. PLRV. This virus is distributed world-wide and infects economically important crops such as potato and tomato. The symptoms on infected potato plants include yellowing or reddening of leaves, which can also roll and become erect (Figure 2). Plants grown from infected tubers are stunted and vein necrosis is observed in susceptible varieties. Yield losses of 10-95% have been reported for potato infected by PLRV

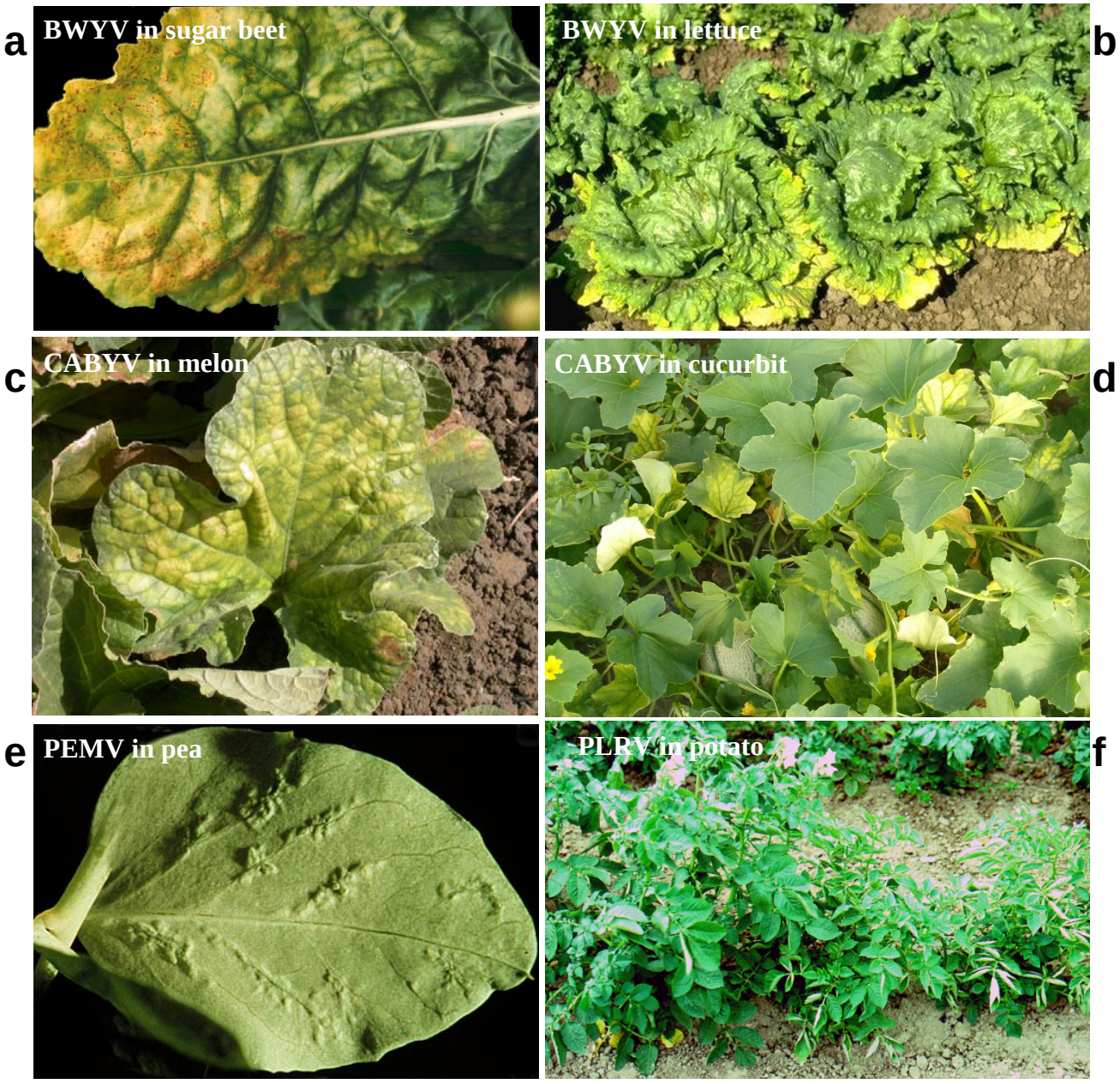


Figure 2: The polerovirus symptoms on economically field crops. BWYV in sugar beet (a) and in lettuce (b). CABYV in melon (c) and in cucurbit (d). PEMV in pea (e). PLRV in potato (f).

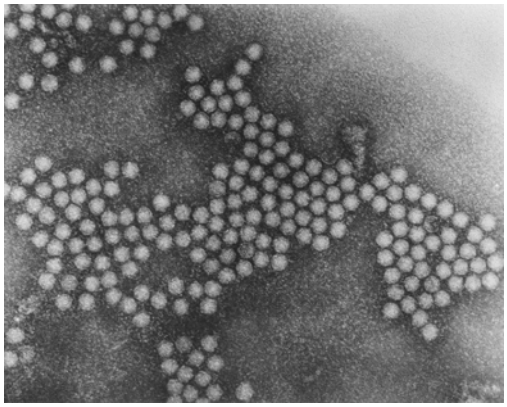


Figure 3: Icosahedrical particles of virions in the *Luteoviridae* family (25 nm diameter, CABYV virions, Catherine Reinbold, INRA Colmar)

(Watson and Wilson, 1956). Although PLRV, like other poleroviruses, is not seed-transmitted, potato and other plants with vegetative propagation can transmit the virus easily from year to year via tubers, thus necessitating control procedures. PLRV was first reported by Quanjer *et al.* (1916, see Peters, 1967). Potato phloem necrosis virus, Tobacco yellow top virus, Tomato yellow top virus and Capsicum yellows virus are PLRV synonyms. The complete PLRV genome sequence (5882 nt) was first determined by Mayo *et al*. (1989) and Van der Wilk *et al*. (1989) and are listed under EMBL GenBank accession number D00530 and X14600, respectively. *Myzus persicae* is the most efficient and important natural vector for PLRV. *Macrosiphum euphorbiae* also transmits the virus, but less effectively.

4. General characteristics of the *Luteoviridae*

Defining characteristics of the *Luteoviridae* family are obligatory transmission by aphids, yellowing symptoms in their hosts and the 25 nm diameter isometric (icosahedral) unenveloped particles (Figure 3) containing a single-stranded linear RNA molecule of positive polarity (about 6 Kb) with 6 ORFs (Figure 4) and at least one subgenomic RNA for expression of 3'-terminal ORFs. The RNA 3' terminus has neither a poly(A) tail nor a tRNA-like structure. Virions contain 28% nucleic acid. They are restricted to the phloem tissue of host plants and strictly transmitted by aphids in a persistent, circulative, and non-propagative manner. They show vector specificity in transmission and are not transmitted by mechanical inoculation, seeds and pollen (Smith and Barker, 1999; ICTVdB Management, 2006). Distinctions among the genera include the following:

Luteoviruses are characterized by uncapped RNA, lack of a viral genome-linked protein (VPg) at the RNA 5' end and the absence of ORF0. During translation, the ribosome performs a –1 frameshift so as to fuse the ORF2-encoded amino acid sequence to that of ORF1 just upstream of the stop codon of ORF1 (only 8 to 13 nt overlap). The length of the non-coding sequence between ORF2 and ORF3 is about 100 nucleotides. ORFs 3-5 are expressed from a subgenomic RNA. A small ORF6 (~150 nt) is present near the 3' extremity and is expressed from a second subgenomic RNA. ORF6 plays a role in virus replication (see Figure 4). In ***Poleroviruses*** and ***Enamovirus*** the 5' end of the viral RNA is covalently bound to a VPg and there is an ORF0 near the 5' extremity of their genomes. A –1 frameshift from ORF1 to ORF2 also occurs upstream of the stop codon of ORF1. The central non-coding region is about 200 nucleotides in length (see Figure 4). Unlike the poleroviruses, ***Enamovirus*** does not contain an equivalent to ORF4 and ORF5 is shorter (900 nt) than ORF5 of polerovirus (1500 nt). There is no evidence that PEMV-1 can spread in plants. Its infectivity is dependent on co-infection by an *umbravirus* (PEMV-2) which does not contain coat protein. In co-infection the PEMV-1/PEMV-2 complex can be transmitted mechanically as well as by aphids and can invade mesophyll tissues in infected plants.

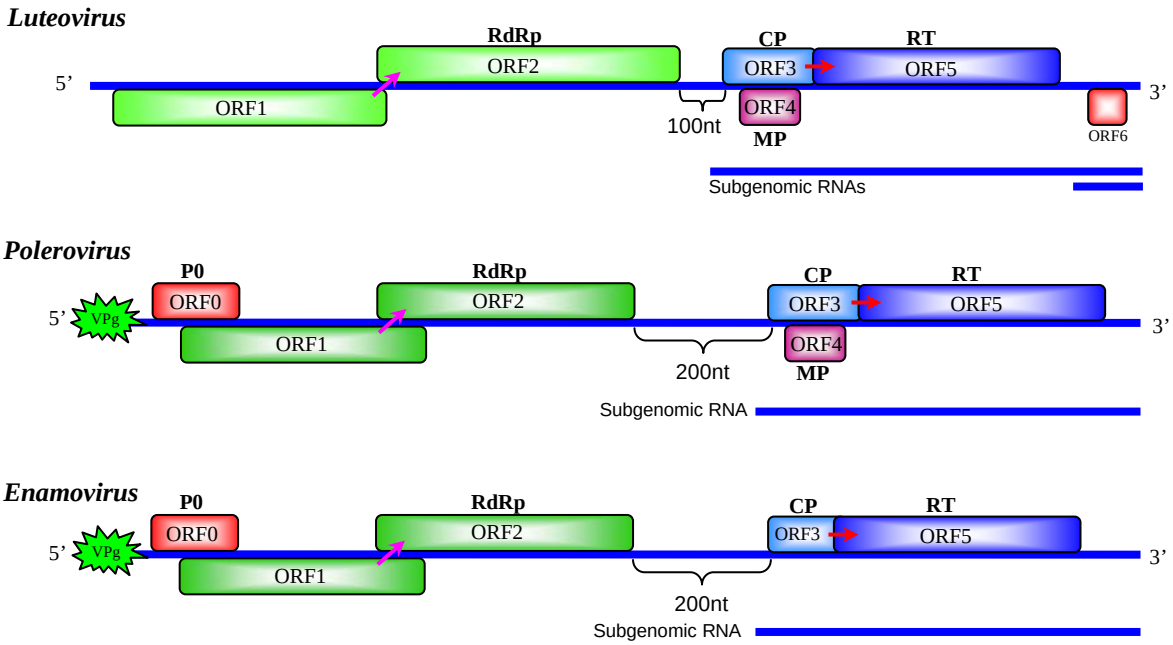


Figure 4: Genomic organization of genera in *Luteoviridae* family (not to scale).

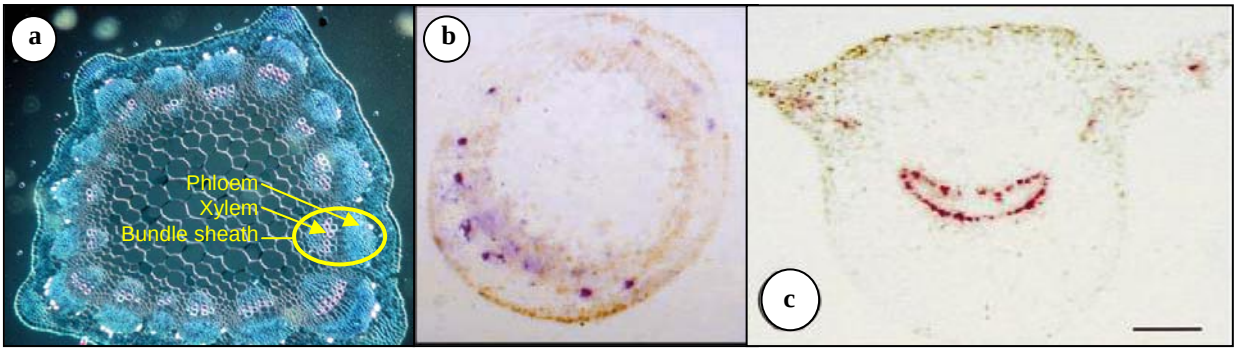


Figure 5: Restriction of polerovirus to phloem tissues. (a) stem cross-section of Rannuncluus demonstrating typical bundle sheaths. (b) stem cross-section of BMYV-infected *N. benthamiana* plant (Dirk and Edgar, 2006) and (c) Petiole cross-section of BWYV-infected *N. clevelandii* plant (Mutterer *et al.*, 1999). virions are stained in (b and c) by immunoassay.

Evolution of genera. Viruses in the Family *Luteoviridae* have replication proteins (product of ORF1 + ORF2) which are sufficiently similar to those in other genera to suggest evolutionary relationships. The putative polymerases of viruses in the Genus *Luteovirus* resemble those of members of the genus *Carmovirus* (*Tombusviridae* family). In contrast, polymerases of viruses in the Genera *Polerovirus* and *Enamovirus* resemble those of viruses in the Genus *Sobemovirus*. These polymerase types are thought to be very distant in evolutionary terms and it has been suggested that the origin of these genomes was recombination between ancestral genomes containing the coat protein genes characteristic of the Family *Luteoviridae* and genomes containing either of the two polymerase types (Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999; ICTVdB Management, 2006).

5. Histo- and Cyto-pathological studies

Plant vascular tissues are composed of xylem and phloem within a bundle sheath (Figure 5a). The phloem conducts the products of photosynthesis from the places where they are manufactured (sources) to the places where they are consumed or stored (sinks). In contrast to xylem, phloem tissues are living. They contain sieve tube elements with perforated end walls (sieve plates). This allows cytoplasmic connections between vertically-ranged cells. The sieve tube elements lose their nuclei at maturity and are filled with a complex proteinaceous material called P-proteins. In losing their nuclei, sieve tube elements lose most of the machinery permitting molecular control. Nucleated cells adjacent to sieve tube elements are thought to take over the control of cellular functions within these phloem transport cells. These nucleated cells are of two types: companion cells and phloem parenchyma cells. The sieve tube elements, the companion cells and parenchyma cells are linked via plasmodesmata. The plasmodesmata which connect sieve elements to companion cells have larger size exclusion limit than other plasmodesmata in plants (Oparka and Turgeon 1999). The companion cells typically have much smaller vacuoles than parenchyma cells and contain a higher density of ribosomes.

Poleroviruses are introduced into the vascular tissue by their aphid vectors and their infection is generally confined to the companion cells and phloem parenchyma cells (Figure 5b and c). Cytopathological studies on plants infected with PLRV, BWYV and BYDV have identified virus particles in mature sieve elements, companion cells and phloem parenchyma cells, but not in other cell types (Jensen, 1969; Esau and Hoefert, 1972b; Gill and Chong, 1975; Shepardson *et al*., 1980). The phloem limitation of Polerovirus is not due to their inability to replicate in other cells. Thus, it was shown for PLRV (Barker and Harrison, 1982) and BWYV (Veidt *et al*., 1992) that virus can replicate in inoculated mesophyll protoplasts. Replication of PLRV in phloem tissues as well as in mesophyll cells of transgenic potato plants constitutively expressing PLRV full length cDNA has been demonstrated. These plants displayed symptoms typical of PLRV infection (Schmitz *et al*., 1997; Prufer *et al*., 1997; Franco-Lara *et al*., 1999). The mechanism by which poleroviruses are restricted to phloem tissues remains to be explained.

Different localizations of virus particles at the subcellular level have been reported. Nuclear accumulation of CYDV-RPV has been described (Gill and Chong, 1975) and accumulation in virus-induced vesicles close to but not in the nucleus has been reported for PLRV (Shepardson *et al*., 1980). Particles of PEMV-1 were detected within the nucleus as well as in the cytoplasm of protoplasts (Demler *et al*., 1994) and it has been suggested that BWYV particles assemble in the nucleus (Esau and Hoefert, 1972b). Coat protein of PLRV is observed in the cytoplasm as well as in the nucleus, particularly the nucleolus. Virus particles were not seen in the nuclear compartment, however, suggesting that the capsid protein accumulates in them in a form other than virus particles (Haupt *et al*., 2005). The diverse and even contradictory nature of these observations is evidence as to how much remains to be learned about the subcellular behavior of the poleroviruses.

6. Agroinoculation and the other means of inoculation

Certain of the characteristics of the *Luteoviridae* have made them an attractive model for fundamental research on topics such as virus trafficking in the phloem, virus-vector interactions, virus genome expression strategies and RNA-induced gene silencing. Many of these studies, however, only became possible with the development of agroinoculation as a means of overcoming our inability to transmit *Luteoviridae* by mechanical inoculation. Agroinoculation takes advantage of the ability of *Agrobacterium tumefaciens* harboring a Ti plasmid to efficiently transfer T-DNA to plant cells in the vicinity of a wound (Zambryski *et al*., 1984). A viral genome sequence inserted into the T-DNA can be carried along and, in appropriate circumstances, initiate an infection. The first successful agroinoculation experiments were carried out with Cauliflower mosaic virus (CaMV), a virus with a circular DNA genome. The infectious construct contained a full copy of the genome plus a partial copy in tandem that permitted homologous recombination and genome escape from the T-DNA (Grimsley *et al*., 1986).

Application of agroinoculation to RNA viruses with a linear genome requires addition of a promoter, generally the CaMV 35S promoter, upstream of the viral cDNA. Sometimes a transcription termination sequence and /or a ribozyme sequence is positioned at the cDNA 3' terminus but this does not appear to be strictly necessary (Leiser *et al.,* 1992). Once in the plant cell, the viral cDNA can be transcribed and the resulting transcripts can move from the nucleus to the cytoplasm and undergo translation. Production of viral RNA dependent RNA polymerase (RdRP) then permits the autonomous replication of the viral RNA. *A. tumefaciens* has a wide range of host plants. This technique has been extensively applied to studies of poleroviruses molecular biology. Unfortunately, however, *A. tumefaciens* does not effectively transform monocotyledonous plants so agroinoculation of these plants with BYDV is not easily achieved.

It has also been reported that poleroviruses are transmissible by biolistic inoculation (Hoffmann *et al*., 2001). Biolistic inoculation with purified virus and viral RNA extracts of PLRV resulted in 30–50% systemically infected *Nicotiana occidentalis* plants and produced 15–30% infection of *N. clevelandii* plants. Particle bombardment was also used successfully to infect *N. clevelandii* plants with *in vitro* RNA

transcripts of BWYV (Hoffmann *et al*., 2001). In protoplast experiments, *in vitro* viral transcripts are easily and routinely used for inoculation of protoplasts by electroporation (Veidt *et al*., 1992).

7. Gene Expression Mechanisms

Although they have a genome of less than 6 Kb, the Luteoviridae infection cycle involves a panoply of expression strategies. These include:

a. translation frameshift between overlapping ORFs (ORF1-ORF2)

b. leaky scanning by ribosomes to translate an ORF downstream of a start codon (ORF1 and ORF4)

c. production of subgenomic mRNA to express downstream ORFs (ORF3, 4, 5 and 6)

d. translational readthrough of a termination codon to express a downstream ORF (the ORF5 polypeptide) as a fusion protein

e. proteolysis of a precursor protein to produce several proteins from one ORF (e.g., the VPg from the ORF1 gene product)

In the following sections, I will mostly focus on research carried out on members of the polerovirus genus although many of the conclusions reached also apply to the luteoviruses and enamoviruses.

8. Polerovirus genome structure and translation strategies

The prototype polerovirus genome is composed of a single-strand positive-sense RNA of 5.5 to 6 kb. A VPg is bound to its 5' end. Coding sequences are in two blocks separated by a 200 nucleotide non-coding sequence (Figure 4). The 5' block consists of three ORFs (0, 1 and 2). In order to maintain consistency with the names of ORFs in the *Luteoviridae* family, the 5'-proximal ORF in the poleroviruses, for which there is no counterpart in the luteoviruses, is referred to as ORF0. ORF0, ORF1 and ORF2 are expressed by translation of genomic RNA in a cap-independent manner. The first AUG codon of the genome is the initiation codon of ORF0. Leaky scanning of this codon allows some ribosomes to bypass it and initiate at the AUG of ORF1. There is extensive overlapping between the first three ORFs. ORF2 is expressed when ribosomes undergo a -1 frameshift from ORF1 to ORF2 to produce a fusion protein. The site of frameshift has a consensus sequence XXXYYYZ, where X is any base, Y is A or U and Z represents any base except G. This sequence (for example in BWYV: GGGAAAC) is followed five or six bases downstream by a structured region, known as a pseudoknot (Miller *et al*., 1995) (Figure 6).

The 3' block consists of three ORFs (3, 4 and 5) that are expressed by translation of a subgenomic RNA (2.3 Kb) (see Figure 4). The 5'-terminal sequence of the subgenomic RNA is identical to the 5'-terminal sequence of the genomic RNA and the first 8 nt (ACAAAAGA) are conserved in poleroviruses; they presumably represent a conserved replication recognition sequence. In the subgenomic RNA, ORF3 codes for coat protein and ORF4 which is embedded in ORF3 but in another reading frame, codes for a

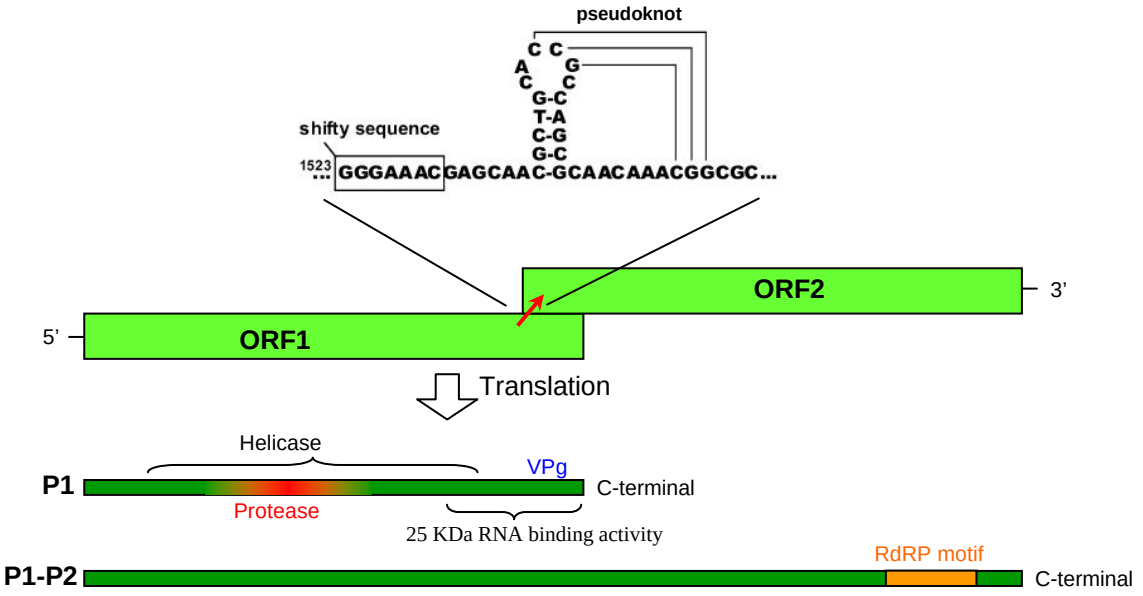


Figure 6: The expression of P1 and fusion P1-P2 proteins in polerovirus. The site of frameshift from ORF1 to ORF2 in BWYV is indicated (↗). Consensus shifty sequence is followed six bases downstream by pseudoknot.

Table 3: The proteins coded by polerovirus (BWYV), their size (Veidt *et al*., 1988) and functions.

Protein	Molecular weight	Function
P0	29 kDa	Suppressor of silencing
P1	66 kDa	VPg and Protease (Replicase?)
P1-P2	115 kDa (P2: 67 kDa)	Viral RNA polymerase (Replicase)
P3	22.5 kDa	Coat protein, indispensable for long distance movement and systemic infection of virus
P4	19 (17-21) kDa	Short distance movement protein
P3-P5 (RT)	75 kDa	Role in aphid transmission, accumulation of virus and efficient long distance movement

putative movement protein by a leaky scanning mechanism. It has been reported for PLRV (Tacke *et al*., 1990) and in *Barley yellow dwarf Luteovirus-PAV* (BYDV-PAV) (Dinesh-Kumar and Miller, 1993) that initiation at the second AUG (for P4) is more frequent than at first AUG (for P3). This may be because the AUG of ORF1 (compared with ORF0) and of ORF4 (compared with ORF3) have more favorable contexts for initiation (Miller *et al*., 1995). ORF5 is translated by in-frame readthrough of the ORF3 stop codon. Readthrough of this leaky stop codon (UAG) requires the presence of CCXXXX repeats downstream as well as a sequence located about 750 nt downstream of the stop codon (Brown *et al*., 1996; Bruyere *et al.*, 1997). The resulting ~75 kDa ORF3-5 fusion protein is generally referred to as readthrough (RT) protein (Mayo and Ziegler-Graff, 1996; Smith and Barker, 1999).

9. Function of proteins

The different virus-coded proteins will be referred to by the prefix "P" followed by the number of the corresponding ORF. Note, however, that the amino acid sequences corresponding to P2 and P5 are not expressed as independent proteins but as the fusion proteins P1-P2 and P3-P5, respectively (see Table 3 for molecular weights). Basic knowledge concerning P1-P2, P3, P4 and P3-P5 are briefly presented below. A full description of P0, the polerovirus protein which is of particular concern for my work, will be presented in Chapter 1.

9-1. P1 and P2 will be considered together because P2 is expressed by a ribosomal frameshift to make the P1-P2 fusion protein. P1 and P1-P2 are essential for virus replication. The P2 polypeptide is highly conserved (about 57% identity in poleroviruses) and contains an amino acid sequence motif ($GXXXTXXXNX_{25\text{-}40}GDD$) near the C-terminus that is conserved in all known RNA-dependent RNA polymerases (Miller *et al*., 1995; Mayo and Ziegler-Graff, 1996). In protoplasts infection experiments frameshift mutations in ORFs 1 and 2 were lethal, providing direct evidence that P1-P2 is required for replication (Reutenauer *et al*., 1993). P1 is a polyprotein which undergoes proteolytic cleavage. It contains a domain characteristic of a chymotrypsin-like serine protease (Sadowy *et al*., 2001a) (Figure 6). A 25 kDa protein (containing the VPg) that originates by proteolytic processing from the C-terminal region of P1 is detected readily in PLRV-infected plants and has RNA binding activity (Prufer *et al*., 1999). The protease domain is upstream of the VPg (Figure 6) (Kamer and Argos, 1984; Habili and Symons, 1989; van der Wilk *et al*., 1997b). There is a protein sequence characteristic of a helicase in P1 that overlaps with the N-terminal residues and cleavage site of the VPg (Habili and Symons, 1989).

9-2. P3 is the major coat protein (CP) and is required for infection of whole plants, efficient systemic spread and long-distance movement within the phloem (Ziegler-Graff *et al*., 1996). P3 is conserved in poleroviruses (50-90% identity) and recapitulates polerovirus phylogeny based on the complete

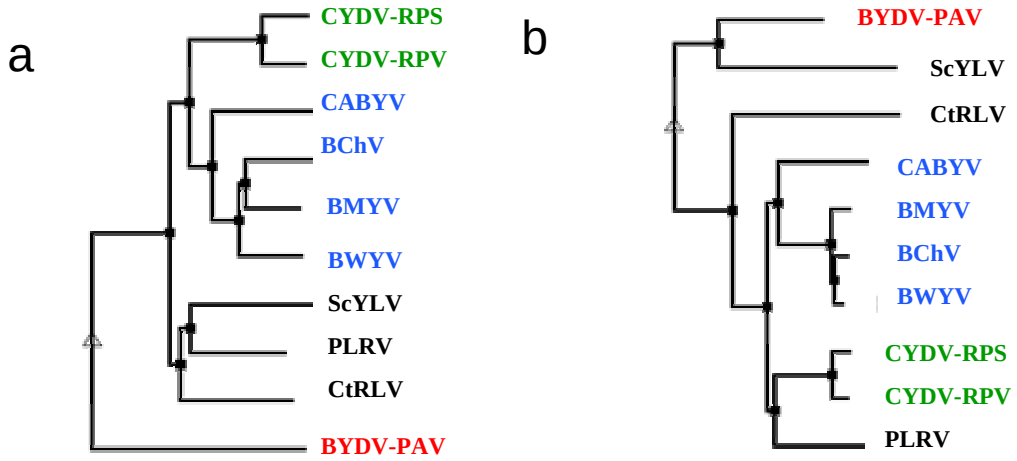


Figure 7: (a) The phylogenetic tree and distance between polerovirus based on complete nucleic acid sequence is correlated with (b) the phylogenetic tree and distance between polerovirus based on amino acid sequence of CP. BYDV-PAV, a luteovirus, was added for comparison.

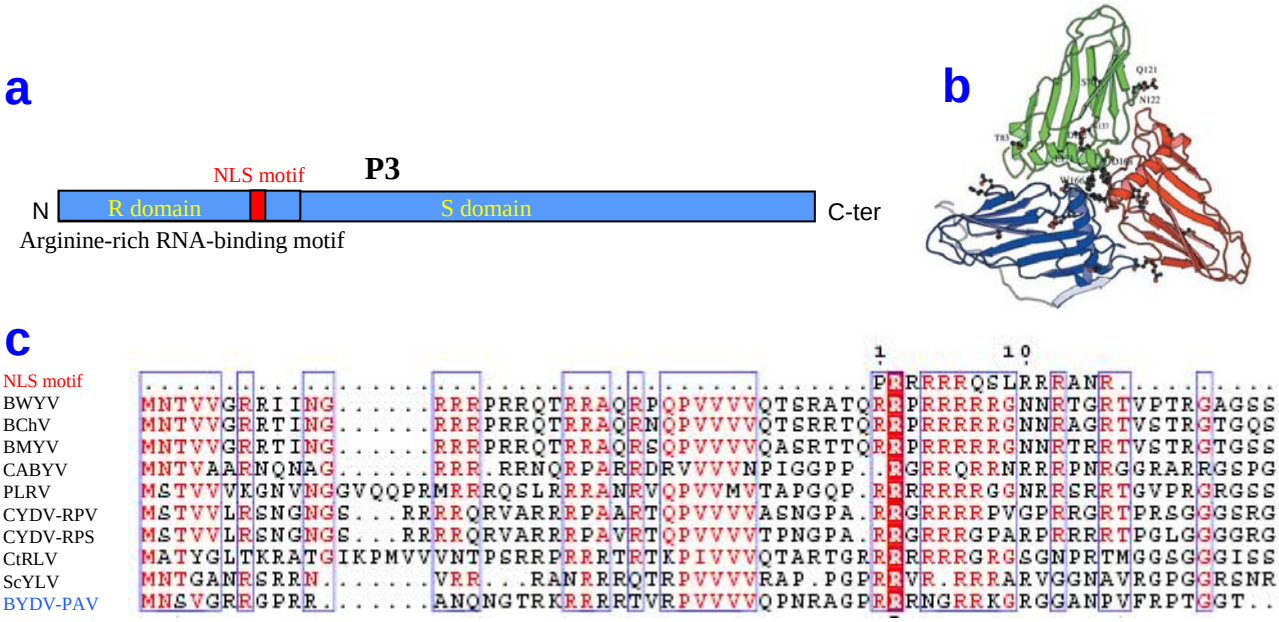


Figure 8: (a) The domains of polerovirus CP. (b) The asymmetric trimer of BWYV P3 subunits in a viral capsid model (Brault *et al.*, 2003) indicating the acidic residues at the center of the trimer. (c) The remarkable identity between CP of polerovirus and luteovirus (only the N-terminal region is aligned on http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). The Arginine (R) rich domain and NLS motif are shown.

nucleotide sequence (Figure 7) (Mayo and Ziegler-Graff, 1996). Polerovirus capsids are thought to be assembled from approximately 180 CP subunits according to $T = 3$ symmetry (Waterhouse *et al.,* 1988). A variable but minor number of P5 subunits are likely to be incorporated into the virion via their CP moiety (Brault *et al*., 1995; Lee *et al*., 2005). A crystallographic structure is not yet available for the capsid of any member of the *Luteoviridae* family, but studies of other small icosahedral plus-strand RNA viruses have revealed that the CPs often contain several characteristic domains: An N-terminal R domain (Arginine-rich domain, see Figure 8) that localizes in the inner part of the capsid and is believed to interact with the RNA; Such Arginine-rich RNA-binding motifs are found in many plant and non-plant viral CP (Rao, 2006); The central S domain (shell) forms the bulk of the protective capsid structure on the capsid surface; In some cases, an outwardly projecting C-terminal domain also exists. The S domain exhibits a barrel of two β-sheets, each sheet consisting of four strands that form a so-called jelly roll structure (Harrison *et al.,* 1978). Attempts have been made to produce a three-dimensional structure model of PLRV CP (Terradot *et al*., 2001; Lee *et al*., 2005) and BWYV CP (Brault *et al*., 2003) by homology with known viral structure (*Southern cowpea mosaic Sobemovirus* and *Tomato bushy stunt Tombusvirus* for PLRV and *Southern bean mosaic Sobemovirus* for BWYV). These models were partially confirmed by some functional mutational analysis.

There are two acidic domains in P3 which are highly conserved among all *Luteoviridae.* Based on the PLRV model, these two domains would be brought into close proximity at the center of a CP trimer when the virion is assembled (Figure 8b). Mutagenesis of PLRV CP revealed that the acidic domains are biologically important regions involved in CP subunit interactions, plant-virus interactions, and aphid-virus recognition (Lee *et al*., 2005).

Analysis of CP amino acid sequence of different luteoviruses showed the presence of the conserved putative nuclear localization signals (NLS) motif (PRRRRRQSLRRRANR) in the N-terminal region coincident with the Arginine-rich RNA-binding motif (Figure 8a and c) (Haupt *et al*., 2005). This motif is responsible for the preferential targeting to the nucleolus of PLRV CP.

It has been reported that CP can encapsidate RNAs other than own viral RNA (Creamer and Falk, 1990; Wen and Lister, 1991). When the PLRV CP was expressed in insect cells, virus-like particles that encapsidate random cellular RNAs were formed although they were rather unstable (Gildow *et al.,* 2000b). Poleroviruses also encapsidate umbravirus RNA and satellite or satellite-like RNAs (Taliansky *et al*., 2000; Lee *et al*., 2002). See Table 4 for a list of important experiments which have done on CP.

9-3. P4 and Virus Movement

P4 is thought to be a virus movement protein (MP) based on its biochemical properties and subcellular localization. ORF4 is present and highly conserved (42-90% identity) in poleroviruses and luteoviruses but is not found in enamoviruses. It has been shown that P4 of PLRV has affinity for single-stranded nucleic acids in a sequence non-specific manner (Tacke *et al*., 1991). By analyzing deleted forms of P4,

Table 4: Important experiments on CP

Experiment	Result	Reference
Infection of *Chenopodium quinoa* protoplasts with **BWYV** knockout mutant of CP or of P3-P5	Protoplast infection but fourfold reduction in accumulation of progeny viral RNA	Reutenauer *et al*., 1993
Infection of *C. quinoa* and Agroinoculation of melon and cucumber with **CABYV** knockout mutant of P3 or substitution with PLRV CP	lower level (twofold) of viral RNA accumulation (unstability of the nonencapsidated progeny RNA)	Prufer et al., 1995
Agroinoculation of *N. clevelandii* with **BWYV** knockout mutant of CP	No viral infection and very low level of progeny viral RNA near the inoculation site (the CP is indispensable for long-distance movement)	Ziegler-Graff *et al*., 1996
Infection of *C. quinoa* protoplasts and Agroinoculation of *N. clevelandii* with **BWYV** point mutants in the S-domain of CP (Encapsidation-defective mutants)	No protection of viral RNA from RNase, no systemic infection of whole plant and no transmission by aphid (virion formation is essential for virus movement)	Brault *et al*., 2003
Agroinoculation of *N. clevelandii, N. benthamiana and solanum tuberosum* with **PLRV** point mutants in CP (Alanine substitution)	Unstable virus particle, no systemic infection and reduced aphid transmissibility (virions play key roles in phloem-limited virus movement and transmission by the aphid)	Lee *et al*., 2005
Agroinfiltration of *N. benthamiana* with constructs expressing GFP fused to CP and P3-P5 and the mutants in the CP NLS motif	Localization of CP and P3-P5 in the cytoplasm, nucleus and nucleolus. Virus particles were not seen in the two last structures. The NLS motif is indispensable for nucleolar localization. P3-P5 lost its nucleolar localization in the presence of replicating **PLRV**.	Haupt *et al*., 2005

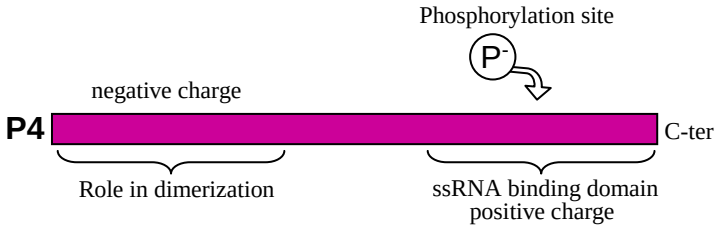


Figure 9: Functional domains of P4 protein of PLRV.

the binding domain was found to be located near the C-terminal part of protein (Figure 9). The sequence has a net positive charge and contains β-sheet regions (Mayo and Ziegler-Graff, 1996). Nucleic acid binding has been reported for MPs of other viruses, including *Tobacco Mosaic Tobamovirus* (TMV, see Table 5), *Cucumber Mosaic Cucumovirus* (CMV), and *Alfalfa Mosaic Alfamovirus* (AMV) (reviewed by Smith and Barker, 1999). Another property of TMV MP is the existence of an MP-MP interaction (Citovsky *et al*., 1993). It has been reported that PLRV P4 can form homodimers and mutational analysis indicated that the N-terminus is responsible for dimerization of this protein (Tacke *et al*., 1993) (Figure 9). Finally, localization of P4 to plasmodesmata in phloem tissues has also been shown (Schmitz *et al*., 1997).

In PLRV-infected plants and P4-expressing transgenic plants, P4 was reported to undergo phosphorylation and this post translational modification did not inhibit the nucleic acid binding activity of protein. Indeed, P4 is predominantly present in a phosphorylated form in plants (Tacke *et al*., 1993). This finding parallels the situation with TMV MP, which is also post-translationally phosphorylated. PLRV MP is phosphorylated near its C-terminus by a membrane-associated protein kinase and phosphorylation takes place in membranous structures, possibly at the deltoid plasmodesmata (Sokolova *et al*., 1997). Phosphorylation may control P4 localization to plasmodesmata or binding to viral RNA. Introduction of phosphate moieties in the C-terminal region of P4 (which harbors RNA binding activity) would lower the net positive charge of this region and could lead to dissociation of the MP-RNA complex.

Normally, viruses employ two types of movement in plants. First occurs "horizontal" cell-to-cell movement via plasmodesmata that will spread the infection from the initial infection site in the epidermis into mesophyll cells, then bundle sheath cells, and then phloem parenchyma and companion cells. Once the infection has attained the nucleate phloem cells it can enter the sieve elements via plasmodesmata. Viral particles can then undergo "vertical" long-distance movement along with photoassimilates to distant parts of the plant where cell-to-cell movement can occur again, but in the reverse direction. Because of the polerovirus restriction to phloem tissues, their movement by phloem-associated long-distance movement will obviously represent an important pathway in virus dispersal in the plant. Nevertheless, the evidence suggests that cell-to-cell movement may occur between nucleate cells of the phloem tissues and that this movement is important for whole plant infection, at least in certain hosts (Mutterer *et al*., 1999).

Ziegler-Graff *et al*. (1996) observed that BWYV carrying a knockout mutation of P4 accumulated to high levels in certain host plants such as *N. benthamiana* and *N. clevelandii* although symptom onset was delayed and initial virus titers were lower than in infections with wild-type virus. The virus in these plants could be aphid-transmitted to other host plants, including *N. benthamiana*, *N. clevelandii* and *Physalis floridiana*, but certain other hosts, notably lettuce, were resistant to such infection (V. Brault, personal communication). Experiments with PLRV P4 mutants (one knockout mutant and another with expression of P4 missing the first four amino acids) showed that they were able to replicate and accumulate in agroinoculated leaves of potato and *Physalis floridana*, but that they were unable to move into vascular

Table 5: Some characteristics of the 30 kDa MP of TMV, the first identified and the best known viral MP (Heinlein and Epel, 2004; Ashby *et al*., 2006) in comparison with PLRV P4.

MP of TMV	P4 of PLRV
interacts with itself and forms homodimers	also
binds nucleic acids *in vitro.*	also
is phosphorylated by cellular kinases.	also
binds directly to microtubules and actin microfilaments and uses actin filaments to move viral RNA	not determined
interacts with the plasmodesmata, the cortical endoplasmic reticulum, the cytoskeleton, and peripheral punctuate structures.	Interacts with the plasmodesmata
modifies the size exclusion limit of plasmodesmata.	not determined

Table 6: Important experiments on P4

Experiment	Result	Reference
Infection of *C. quinoa* protoplasts with **BWYV** knockout mutant of P4	Viral RNA was replicated and encapsidated (P4 plays no essential role in single cell infection)	Reutenauer *et al*., 1993
Agroinoculation of *N. clevelandii* with **BWYV** knockout mutant of P4	Viral infection occurred but with delay. The mutant virus titers were lower than in wild type. Mutant virus could be aphid-transmitted to other hosts.	Ziegler-Graff *et al*., 1996
Subcellular localization of **PLRV** P4 In infected potato plants	P4 was immunolocalized in phloem cells (associated with chloroplast, mitochondria, virus-induced vesicles and plasmodesmata connecting companion cells to sieve tubes)	Schmitz *et al*., 1997
In transgenic potato plants with full-length cDNA of PLRV	Same localization in phloem cells as well as in mesophyll cells but not to plasmodesmata in mesophyll cells	
In P4 transgenic potato plants	P4 was localized to plasmodesmata in vascular tissue and their connecting mesophyll cells but never observed on organelles	
Expression of **BYDV** P4-GFP fusion protein in insect cells	Interaction of P4 with nuclear envelope and protrusions from its surface	Liu *et al*., 2005

tissues and initiate a systemic infection in these plants. In contrast, these mutants were able to spread systemically from inoculated leaves in *N. benthamiana* and *N. clevelandii*, although the efficiency of infection was reduced relative to wild-type PLRV. Examination of virus distribution in *N. benthamiana* plants using tissue immunoblotting techniques revealed that the wild-type PLRV and P4 mutants followed a similar movement pathway out of the inoculated leaves. The P4 mutants infected fewer phloem-associated cells and they were slower than wild-type virus in moving out of the inoculated tissue (Lee *et al*., 2002). Therefore the evidence suggests that PLRV P4 is a host-dependent movement protein. See Table 6 for a summary of important experiments which have performed on P4.

9-4. P5, also known as the readthrough domain (**RTD**), is expressed as a P3-P5 fusion *in planta* by translational readthrough of the P3 termination codon and is a minor coat protein, probably anchored into the capsid structure via the N-terminal P3 moiety (Brault *et al*., 1995) (Figure 10). During virus purification the ~75 kDa P3-P5 (which will be referred to as RT protein) undergoes C-terminal truncation to produce a protein of ~55 kDa, and this is the form present in purified virus preparations. The RTD consists of several subdomains. A Proline-rich sequence encoding 7 to 13 alternating Proline residues is located just downstream of the CP stop codon, this is followed by a region of about 200 amino acid residues with considerable sequence similarity throughout the *Luteoviridae*. The C-terminal half of the RTD, on the other hand, is divergent (Guilley *et al*., 1994). The C-terminal non-conserved half of the RTD is dispensable for whole-plant infection and aphid transmission (Brault *et al*., 1995; Bruyere *et al*., 1997). The conserved N-terminal of the RTD, on the other hand contains motifs which are important for aphid transmission of the virus (Brault *et al*., 1995; Chay *et al*., 1996; Bruyere *et al*., 1997), efficient accumulation of the virus in whole plants (Chay *et al*., 1996; Bruyere *et al*., 1997; Mutterer *et al*., 1999) (presumably by facilitating virus movement and efficient suppression of major coat protein translation-termination (Brown *et al.,* 1996; Bruyère *et al*., 1997). See Table 7 for more detail.

9-4-1. Role of P5 Protein in Aphid Transmission. As noted above, the conserved N-terminal subdomain of P5 contains sequence motifs essential for aphid transmission. Here I will summarize the published results concerning the role of the RTD in aphid transmission. For simplicity, I will refer to all viruses in the *Luteoviridae* as luteoviruses.

9-4-2. Transmission by Aphid

Obligate transmission by aphids (Homoptera, Aphididae) is one of the major distinguishing features of Luteoviridae. Aphids play an important role in horizontal transmission (plant to plant) of luteoviruses because of lack of mechanical and seed transmission. Interestingly, the yellowing symptoms characteristic of luteovirus infections act as an attractant for aphids. Moreover, increased nutritional

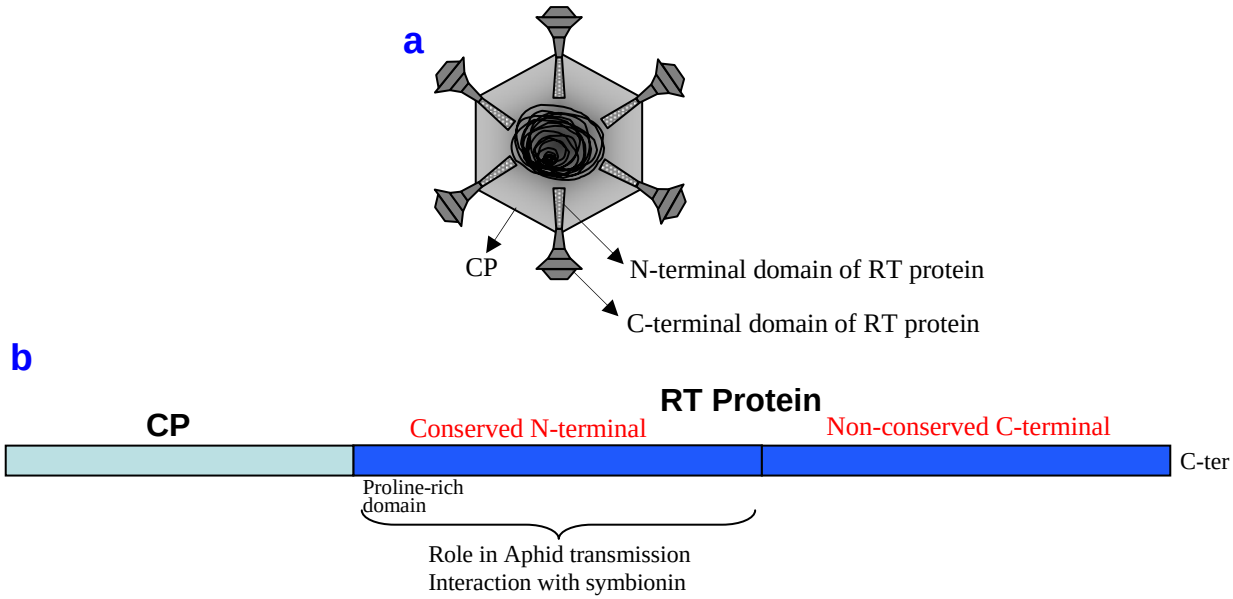


Figure 10: (a) Polerovirus virion composition. (b) Readthrough (RT) protein

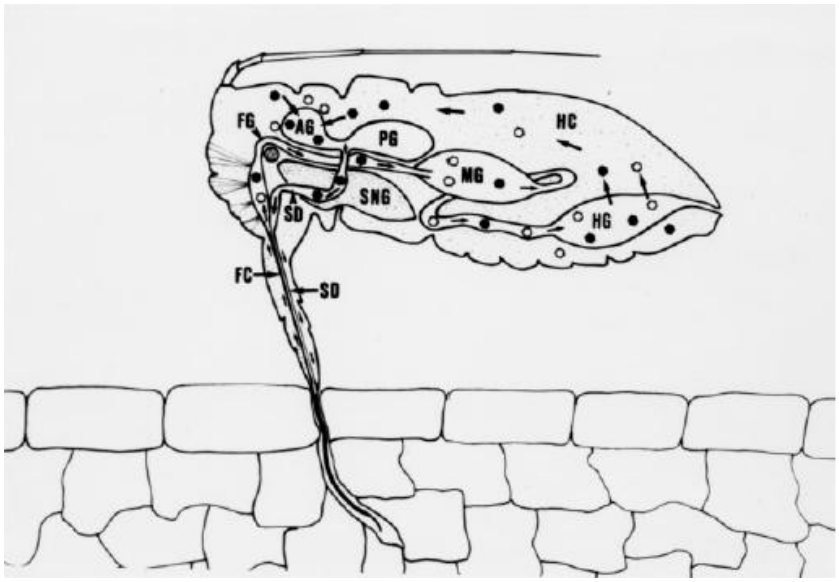


Figure 11: Aphid transmission of Polerovirus. Anatomy of aphid body demonstrating different organs implicated in transmission of Polerovirus. Foregut (FG), midgut (MG), hindgut (HG), hemocoel (HC), accessory salivary gland (AG), principal salivary gland (PG), salivary duct (SD), food canal (FC) and suboesophageal nerve ganglion (SNG) (adapted from Smith and Barker, 1999).

quality of sap for aphids and increased fecundity and longevity of aphids on virus-infected plants have been reported (Herrbach, 1999). Aphid transmission is highly specific, meaning that one virus is effectively transmitted by only one or few aphid species. This specificity argues for the existence of a recognition phenomenon during transmission. Aphid transmission of luteoviruses is a circulative, non-propagative type of transmission, characterized by the circulation of virions throughout the aphids' bodies suspended in the hemolymph and by the lack of multiplication in vector tissues. Transmission is persistent because, after acquisition, infectious virus persists in the aphid and may be transmitted over a period of weeks. Virus cannot be transmitted congenitally to the progeny of the vector. The limitation of luteoviruses to phloem tissues does not permit virus acquisition during short test probes made by aphids into epidermal cells.

Luteovirus acquisition/transmission is divided into four processes: virus ingestion into the alimentary canal, acquisition of virus into hemocoel, retention in the hemolymph and transfer to a salivary gland and then into plants (Figure 11). A minimal 15 minute (normally 1h) acquisition access period (AAP) and 30 minute inoculation access period (IAP) are required for aphids to achieve 50% transmission efficiency although the exact times may vary with aphid species and virus isolate. The latent period (LP) refers to the period between virus ingestion and the time at which the aphid can successfully transmit the virus. A minimal latent period for efficient transmission is at least 24 hours.

9-4-3. Receptor-mediated endocytosis/exocytosis mechanism of virus transport in the aphid body

Ingestion of virus begins with penetration of aphid stylet into phloem tissues and feeding. The virus particles pass into the intestine where transport of virions across the intestinal epithelial cell barrier into the hemocoel can occur. Intestine epithelial cells transport the virions into hemolymph in coated pits and vesicles through basal lamina by an endocytosis/exocytosis mechanism. This transport mechanism is believed to provide opportunities for virus recognition by epithelial cells that is probably a basis for vector specificity. The same receptor-mediated endocytosis/exocytosis process operates in the reverse direction at epithelial cells of the accessory salivary gland (ASG). This transport supplies a second site for vector-specific recognition for virus.

Successful recognition of the virus at the intestinal epithelia is a first prerequisite for virus acquisition although this barrier appears to less selective than the ASG barrier (Gray and Gildow, 2003). Some luteoviruses, including BYDV, CYDV and Soybean dwarf virus (SbDV), have evolved to utilize the hindgut as the specific site for acquisition whereas others (PLRV and BWYV) use the midgut and CABYV uses both sites for transport (Gildow *et al.*, 2000a,b; Brault *et al.*, 2005). Mutagenesis analyses have identified amino acid sequence motifs in both P3 and the P5 (conserved region) that are probably recognized by the receptor in aphid body. Aphid feeding experiments and experiments in which mutant virus is injected into hemolymph have shown that certain of the critical motifs act at the level of the intestinal barriers while others act at the ASG.

Table 7: Some important experiments on RTD

Experiment	Result	Reference
infection of *C. quinoa* protoplasts with BWYV deletion mutant in RTD but not in P3	Mutant multiplied efficiently and formed viral particles. (RTD is dispensable for formation of viral particle.)	Reutenauer *et al*., 1993
agroinoculation of *N. clevelandii* with BWYV RTD deletion mutant	Mutant was infective but lower accumulation (tenfold) of mutant virus in both inoculated and non-inoculated leaves (by ELISA) (RTD may play a role in accumulation, stability or systemic movement of virus); mutant was not transmitted by aphid	Brault *et al*., 1995
infection of *C. quinoa* with CABYV mutant in which BWYV RTD was substituted	Low level (four fold) of viral RNA accumulation (heterologous readthrough protein may slightly impair virion assembly or the resulting virions are somewhat less stable.)	Prufer et al., 1995
infection of *C. quinoa* protoplasts and Agroinoculation of *N. clevelandii* with BWYV RTD deletion mutants	Mutant with no RTD replicated to very low levels (ten fold) (RTD is essential for accumulation or systemic movement of virus in plant); mutant was not transmitted by aphid	Bruyere *et al*., 1997
with BWYV RT mutants in conserved N-terminal subdomain	Accumulated to very low levels (tenfold) (role in stable incorporation of RT protein into virion); mutants did not induce symptoms and were not transmitted by aphid	
with BWYV RT mutants in non-conserved C-terminal subdomain	Only small decline in virus accumulation. Mutants did not develop symptoms in plants and were aphid transmissible	
agroinoculation of *N. clevelandii* with BWYV RTD deletion mutant	Dramatically reduction in number of infection sites in systemically infected leaves (RTD intervenes in virus movement to increase new infection loci)	Mutterer *et al*., 1999
expression of BWYV 3'-proximal genes from *Bombyx mori* nuclear polyhedrosis virus (BmNPV) vector in BmN cells and *B. mori* larvae	Detection of P3, P4 but not P3-P5 when expressed all together from a vector. Virus particle formed when P3 alone or P3 plus P3-P5 expressed but particle were not detected when P3-P5 was expressed alone (P3-P5 can not form viral particles alone and P3 can encapsidate subgenomic RNA or other cellular RNAs)	Tian *et al*., 1995
Yeast two hybrid screen of a cDNA bank of *Arabidopsis thaliana* to identify cellular proteins which interact with RTD of BWYV and CABYV.	The microtubule motor protein (Kinesin) interacts with C-terminal subdomain of RT. A Kinase protein (Casein Kinase II, CK2) interacts with P3 as well as with C-terminal subdomain of RT. (CK2 has a role in movement of Potato virus A)	Boissinot *et al*., 2007

Virus recombinants obtained by exchanging the RTD sequences of BWYV and CABYV, two viruses which are different in their vector specificities, showed that the recombinant viruses are capable of replicating in *C. quinoa* protoplasts and in whole plants (*Montia perfoliata* which are hosts for both viruses) and to incorporate into virions. The hybrid viruses, however, showed a reduced accumulation in protoplasts and whole plants compared to the wild-type viruses. Aphid transmission experiments using infected plants or purified virions and *Aphis gossypii* (vector only for CABYV) and *Macrosiphum euphorbiae* (vector only for BWYV) revealed that vector specificity is driven by the RTD, presumably motifs in the conserved subdomain. Localization of hybrid virions in aphids by transmission electron microscopy (TEM) revealed that gut tropism is also determined by the RT protein (Brault *et al*., 2005). This conclusion is in agreement with studies on SbDV strains differentially transmitted by *Aulacorthum solani* or *Acyrthosiphon pisum.* Sequence comparison among the strains provided circumstantial evidence that vector specificity correlates with the N-terminal half of the P5 protein (Gildow *et al*., 2000a; Terauchi *et al*., 2003).

Acquired virus particles persist for several weeks in the aphid hemolymph. It is known that **symbionin**, a GroEL homologue protein produced by the primary endosymbiont bacteria (genus *Buchnera*) of the aphid, is abundantly present in the hemolymph. This association has a nutritional basis and is required for both bacterium and aphid. Isolated bacteria cannot be cultured and bacteria-free aphids grow poorly and produce few offspring. Symbionin-like proteins are found in most aphid taxa. Interestingly, the virions of several poleroviruses, (BWYV, PLRV and Bean leaf roll virus, BLRV) as well as BYDV and PEMV displayed affinity for native symbionin from their aphid vectors. *In vitro* assays showed that BWYV mutants in the conserved subdomain (N-terminal) of P5 protein lost their ability to bind to *Buchnera* symbionin. These mutants were not transmissible by aphids after oral acquisition or after direct injection of the purified virus into the hemolymph. Mutants targeting the non-conserved C-terminal subdomain behaved as did wild-type BWYV (Bruyere *et al*., 1997). Therefore, it appears that the conserved N-terminal subdomain of P5 protein is sufficient for the interaction with *Buchnera* symbionin. Concordantly, direct injection of BWYV carring mutations in the N-terminal subdomain of the P5 protein into aphid hemolymph showed significantly less persistence of virions and rapid degradation in hemolymph (van den Heuvel *et al*., 1997).

In vivo binding of symbionin to virus has not been strictly confirmed. Nevertheless, these findings have led to the hypothesis that symbionin may protect the virus from targeting by the aphid immune system or function as a chaperonin which could modify the structure of the capsid and facilitate virus movement into the ASG. When aphids were treated with antibiotic, their ability to transmit virus was significantly reduced and the amount of virus detected in the aphid was diminished (van den Heuvel *et al*., 1994). These observations suggest that luteoviruses associate with symbionin in the hemolymph to inhibit or retard their breakdown (Herrbach, 1999).

Introduction Part 2: RNA Silencing

There are numerous different types of interaction between plants and microorganisms which have evolved over the time. One of the most interesting is pathological parasitism in which a microorganism diverts the resources of a plant to its own multiplication, accompanied by deleterious effects on the infected plant. Thus situation is evidently different from that in which both the microorganism and plant benefit (symbiosis).

If a pathogenic microorganism cannot infect a plant species, the plant species is described as a **non-host.** Failure to infect a non-host species is usually due to basal defenses, which include physical barriers to infection such as the cell wall, waxy cuticle and bark, as well as the production of various antimicrobial compounds. If a microorganism can infect and replicate in a plant species, the plant is referred to as a **host** for this microorganism. A **compatible** interaction occurs when a pathogen infects and replicates (**virulent** pathogen) in the host (**susceptible**). An **Incompatible** interaction refers to a relationship between a pathogen that can enter but not replicate (**avirulent**) in the host plant (**resistant**).

A. Protein-mediated resistance or innate immunity

To defend themselves, plants have resistance **(*R*) genes** that confer resistance to specific pathogens. Most *R* genes encode proteins that contain a nucleotide-binding site (NBS) and leucine-rich repeats (LRR). The pathogen molecule that specifically elicits R-protein-mediated responses is an avirulence (**Avr**) determinant, known also as an elicitor. In plant-virus interactions, any protein component of a virus can function as the specific Avr determinant to elicit resistance. In a compatible plant-pathogen interaction the elicitor escapes recognition by an R protein, leading to development of disease. In an incompatible interaction, the elicitor is recognized by an R protein, triggering a cascade of defense reactions. **Innate immunity** is a host rapid defense mechanism in which recognition of a pathogen can occur upon binding to a specific cytoplasmic receptor or to a Toll-like receptor (TLR). These TLRs recognize conserved patterns of proteins, lipoproteins, double-stranded RNA (dsRNA), or unmethylated C and G residues in DNA. These characteristics features are often referred to as pathogen-associated molecular patterns (**PAMP**).

The first line of defense in most *R*-gene-mediated resistance responses is the hypersensitive reaction (**HR**). The HR includes programmed cell death (**PCD**), which occurs in cells at the site of infection and manifests itself as discrete necrotic lesions. The virus is usually confined to the lesion and to the cells immediately surrounding it and fails to spread from lesions into adjacent healthy tissues. The second line of *R*-gene mediated resistance is systemic acquired resistance (**SAR**). Once, the pathogen enters a site in the plant, alarm signals spread throughout the plant and distant tissues become resistant to varaeities of

pathogens. Among known systemic signals for SAR are lipid-derived compounds, hormones, such as jasmonic acid (JA), ethylene, salicylic acid (SA), reactive oxygen species (ROS) and nitric oxide (NO). Interestingly, SAR is durable and can last for several weeks. The mechanism(s) by which SAR produces resistance are complex and are not fully understood. One response involves the increased expression of several genes, named pathogenesis-related (**PR**) genes that encode antimicrobial compounds. Also, transient changes in ion fluxes occur upon infection and are believed to activate several kinase cascades. These cascades induce expression of defense-related genes, including several transcription factors (reviewed by Soosaar *et al*., 2005).

The **guard hypothesis**, originally proposed by Van der Biezen and Jones (1998), postulates that R proteins (guards) are constitutively associated with host cellular proteins (guardees) that are required by pathogens for establishment of infection. Upon infection, the pathogen causes modifications to the guardee that are detected by the guard. Any modification of the guardee could result in detection of the pathogen and activation of the guard to initiate a signaling cascade. Importantly, resistance mediated by the guard protein function does not necessitate a direct interaction between the R and Avr protein. One R protein could recognize the presence of multiple Avr proteins through either a single or multiple guardees. There are approximately 200 *R* gene-like sequences in the 125 Mb *A. thaliana* genome that could confer resistance to thousands of pathogens (reviewed in Soosaar *et al*., 2005).

B. RNA-mediated resistance or RNA silencing

RNA silencing can be considered as a type of immune system that operates at the nucleic acid level. Indeed, there may well prove to be overlaps between nucleic acid-mediated and protein-mediated resistance. RNA silencing is a general term used to describe **post-transcriptional gene silencing (PTGS)** in plants, quelling in fungi, and **RNA interference** (**RNAi**) in animals. It is an RNA surveillance system that is conserved among eukaryotes, and that acts as a natural defense mechanism against invasive nucleic acids, such as viruses. Undoubtedly, in many cases both the protein- and RNA- mediated resistance mechanisms can work together to establish resistance against viruses. RNA silencing also plays an essential role in plant and animal development by providing a gene expression control system that is mediated by RNA degradation, translational inhibition or chromatin modification. Control of movement of transposable elements at the transcriptional and post-transcriptional level in plants and animals is another function of RNA silencing.

PTGS was first discovered unexpectedly in transgenic Petunia as a loss of expression of both a transgene and the homologous endogenous gene. Attempts to overexpress Chalcone synthase (CHS) in pigmented petunia petals so as to reinforce anthocyanin pigment production by introducing a chimeric petunia CHS gene, resulted in complete blockage of anthocyanin synthesis in the petals (Napoli *et al*., 1990). At that time, it was not clear how the introduced gene silenced the homologue endogenous gene but further

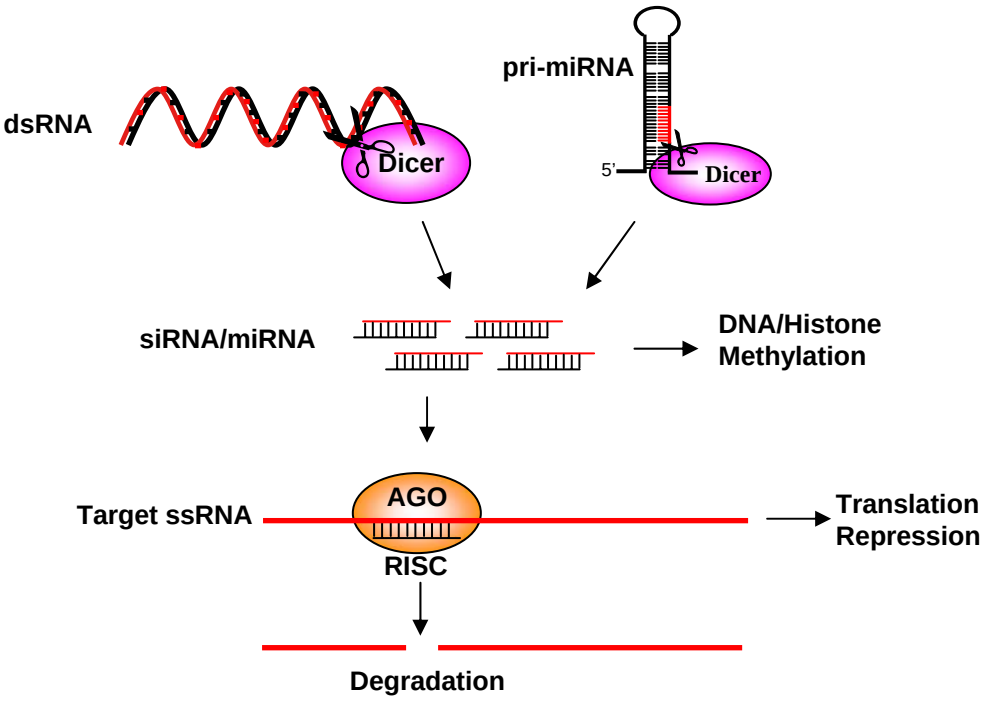


Figure 1: Basic pathway of RNA silencing

research on similar phenomena in other systems ultimately revealed the mechanism. Now we know that there are several partially overlapping but distinct RNA silencing pathways in plants and animals. Commonly, silencing in all these pathways is triggered by **dsRNA** that may vary in length and origin. These dsRNAs are processed into small RNA duplexes of 21 to 25 nucleotides in length known as short interfering RNA (**siRNA**) which guide for the cleavage or translational repression of complementary single-stranded RNAs, such as messenger RNAs or viral genomic RNAs (Figure 1). I will first introduce briefly the different RNA silencing pathways in plants (generally discovered in *Arabidopsis thaliana*) and then I will discuss the essential components of the pathways (reviewed by Baulcombe 2004; Meister and Tuschl, 2004; Voinnet, 2005a; Vaucheret 2006; Brodersen and Voinnet, 2006; Jones-Rhoades *et al*., 2006).

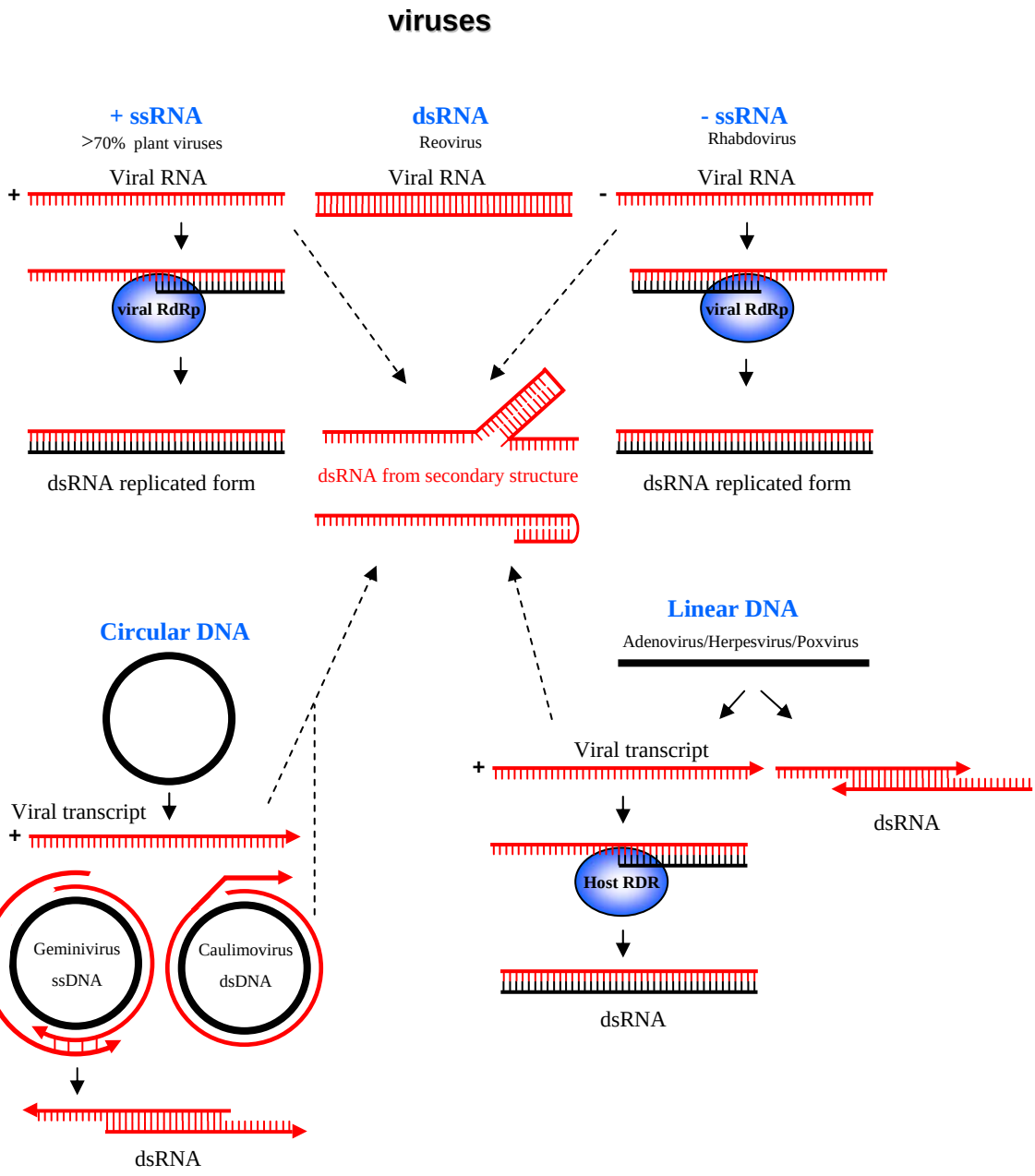


Figure 2: Viruses with different genome structures produce dsRNA by different mechanisms.

1. Different RNA silencing pathways

1-1. siRNA/Antiviral pathway

1-1-1. Antiviral defense: Early evidence that indicated an antiviral role for RNA silencing came from molecular analyses of transgenic plants following infection with a potyvirus from which the transgene was derived. The infection of these plants displayed symptoms initially but later recovered and became resistant to subsequent infection with the homologous virus. Recovery and establishment of the virus-resistant state were correlated with silencing of transgene (Lindbo and Dougherty, 2005). **Virus-induced gene silencing (VIGS)** supported the evidence that a virus can trigger RNA silencing in plant. When the infecting virus with either an RNA or DNA genome was engineered to express an endogenous gene, it triggered silencing against the viral target and also against the endogenous gene (Ruiz *et al*., 1998). Furthermore, it was reported that viral RNAs are targeted for silencing (English *et al*., 1996; Covery *et al*., 1997) and that the infection of plants by RNA viruses results in the accumulation of viral siRNAs (Hamilton and Baulcombe, 1999), demonstrating that viruses are both inducers and targets of RNA silencing in plants.

The idea that RNA silencing is an antiviral mechanism in plants is further supported by experiments showing that Arabidopsis carrying mutations in essential genes implicated in RNA silencing displayed enhanced susceptibility to virus infection (will be referenced in details). Further support has come from the discovery that viruses encode proteins which can suppress RNA silencing (Brigneti *et al*., 1998). Therefore, the siRNA pathway of RNA silencing is generally believed to be a natural antiviral defense mechanism in plants (Brigneti *et al*., 1998; reviewed by Li and Ding, 2006).

Replication of RNA viruses produces double-stranded hybrids of positive and negative stranded copies of genomic RNA, called replicative forms. These dsRNA, or partial regions of secondary structure of the single-stranded viral RNA, could serve as the dsRNA PAMP (Figure 2). In the case of plant DNA viruses (such as Geminivirus), the dsRNA may be formed by annealing of overlapping complementary transcripts because of bi-directional transcription (Shivaprasad *et al*., 2005) (Figure 2).

1-1-2. S-PTGS and IR-PTGS: Elements of the antiviral silencing pathway are also important in silencing of a transgene. The dsRNA trigger can be derived from inverted-repeat (IR) transcripts that anneal by base pairing and form dsRNA (IR-PTGS) or from single-stranded RNA [single copy transgene, sense-PTGS (S-PTGS)] that is transcribed to high levels and is copied into a duplex by a host RNA-dependent RNA polymerase (**RDR**) (Figure 3). In Arabidopsis, RDR6 recognizes and use as templates certain transgene transcripts with yet to be identified aberrant features, possibly including absence of the 5' cap (Gazzani *et al*., 2004) or 3' polyA. The limiting levels of cofactors could be a reason for production of aberrant mRNAs when high levels of transgene expression occur (reviewed by Baulcombe, 2004; Brodersen and Voinnet, 2006).

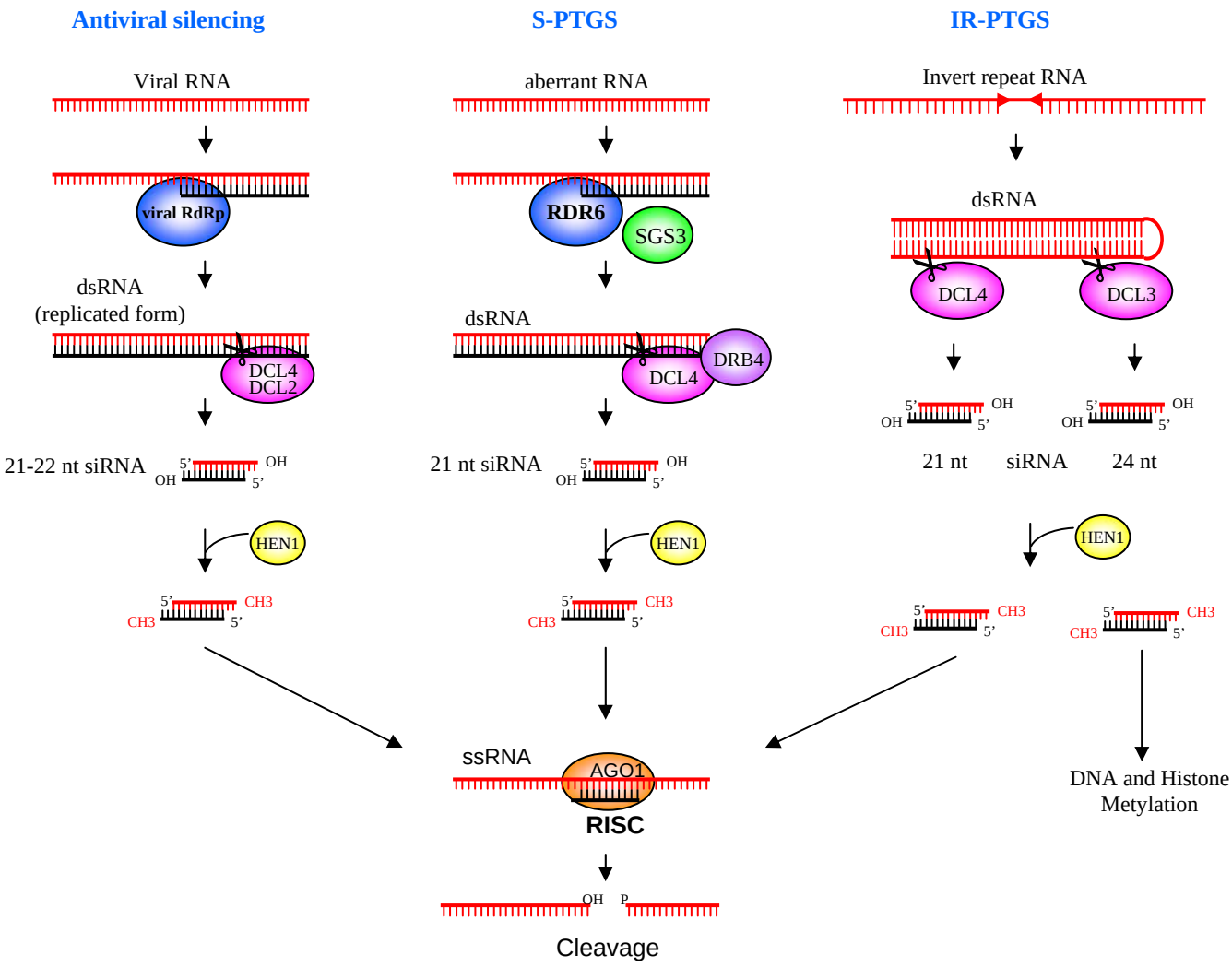


Figure 3: General antiviral silencing, S-PTGS and IR-PTGS pathways

1-1-3. The siRNA Pathway: Once introduced or produced into the cytoplasm dsRNA is diced by an RNaseIII-like protein known as **Dicer** into siRNAs. The family of RNaseIII nucleases cleaves dsRNAs, generally leaving 2nt overhangs at the 3' ends. There are four Dicer like (**DCL**) in Arabidopsis that are probably specialized to cleave dsRNA of different origins but some redundancy has also been reported (Xie *et al*., 2005b; Deleris *et al*., 2006). For instance, DCL4 and DCL2 are implicated in antiviral defense (Deleris *et al*., 2006; Bouche *et al*., 2006; Fusaro *et al*., 2006). DCL2, DCL4 and DCL3 are used in IR-PTGS and DCL4 in S-PTGS (Dunoyer *et al*., 2005; Fusaro *et al*., 2006). The resulting siRNA vary in size depending on the DCL that cleaves the dsRNA. DCL1 and DCL4 produce 21nt siRNA whereas DCL2 generates 22nt and DCL3 produces 24nt siRNA (Xie *et al*., 2005b; Dunoyer *et al*., 2005; Gasciolli *et al*., 2005) (Figure 3). The siRNAs are methylated at 3' termini by **HEN1** (HUA Enhancer1, a small RNA-specific methyltransferase) (Boutet *et al*., 2003). Methylation probably protects siRNAs from degradation and polyuridylation. The two strands of these siRNAs are then separated. One or the other of the two strands is recruited as the guide RNA in a so-called RNA induced silencing complex (**RISC**) to target and cleave the single-stranded RNA that bear a sequence of perfect or near-perfect complementarity. Cleavage is performed by an RNA-binding and Slicer protein, **ARGONAUTE1** (**AGO1**), which cuts the target RNA at the position between the 10 and 11thnt of the siRNA (Elbashir *et al*., 2001; reviewed by Bartel, 2004). In Arabidopsis, there are 10 members of *AGO* family, and *AGO1* is involved in the different RNA silencing pathways (Fagard *et al*., 2000; Morel *et al*., 2002; Baumberger and Baulcombe, 2005; Zhang *et al*., 2006; Csorba et al., 2007).

In addition to RDR, DCL and AGO1, the siRNA pathway in Arabidopsis requires a protein of unknown function, **SGS3** (Suppressor of Gene Silencing3, an RNA stabilizer) (Mourrain *et al*., 2000; Peragine *et al*., 2004) as well as **WEX** (Werner Exonuclease, an RNaseD exonuclease) (Glazov *et al*., 2003) whose role in the pathway is not clear (reviewed in Brodersen and Voinnet, 2006). Because transposon-, virus-, and transgene-derived siRNAs target the degradation of RNAs from which they derive, they are called *cis*-acting siRNAs, and the silencing phenomenon that relies on their action is referred to as **autosilencing** (Bartel, 2004). *Trans*-acting silencing of host mRNAs is also possible (Figure 4) and it is also now clear that viral siRNAs not only degrade viral genome/transcript but also promote the cleavage of host transcripts (Moissiard *et al*., 2007).

1-1-4. Source of siRNA in viral RNA: Sequencing of the cloned siRNAs from *N. benthamiana* infected with *Cymbidium ringspot Tombusvirus* (CymRSV) indicated that 80% of viral siRNAs were derived from the positive-strand viral RNA. More over the siRNA sequences have a nonrandom distribution along the length of the viral genome, suggesting that there are hot spots for virus-derived siRNA generation (Molnar *et al*., 2005). It has also been demonstrated that the leader sequence of 35S RNA of CaMV (Caulimovirus, Pararetrovirus, with dsDNA genome) is a major source of siRNAs and that they down-regulate a large number of Arabidopsis transcripts (Moissiard and Voinnet, 2006). These experiments, together with the detection of viroid-derived siRNAs and animal DNA virus-derived small RNAs suggest

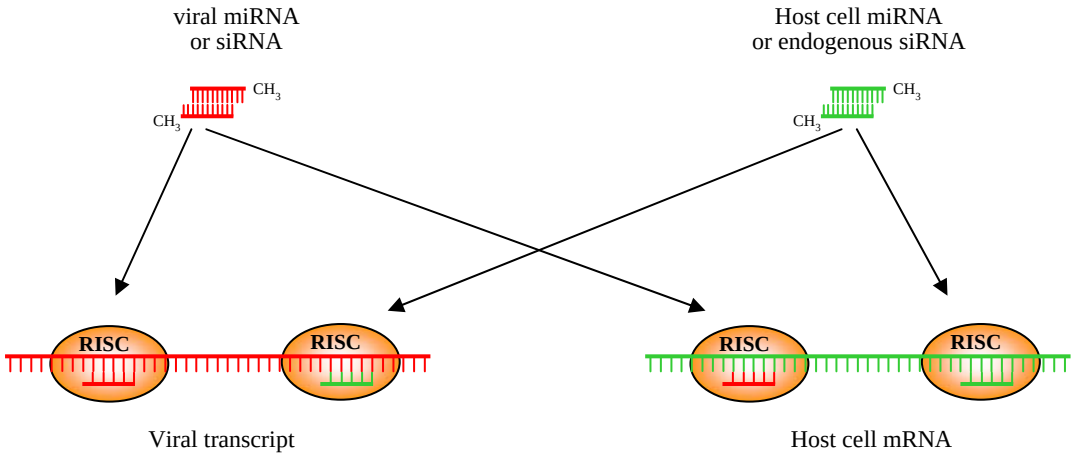


Figure 4: The silencing interactions between host and virus reveal an important aspect of RNA silencing: sequence-specific recognition. Host cell miRNA or endogenous siRNA can target both host mRNA and viral transcript. Inversely, viral derived miRNA or siRNA can target its own transcript as well as host mRNA.

that infections by viruses and viroids could down-regulate the host mRNAs by RNA interference (Figure 4).

1-1-5. Non-silencing viroid: Viroids largely escape RNA degradation by the small RNA pathway despite a high degree of base pairing throughout their circular RNA genome. It has been reported that *Potato spindle tuber viroid* (PSTVd) RNA produces siRNAs that accumulate in the cytoplasm and mediate symptomatology, probably by targeting plant mRNAs. Apparently, however, the tight viroid RNA structure allows them to be largely resistant to siRNA-directed degradation (Denti *et al*., 2004; Wang *et al*., 2004).

1-1-6. Role of siRNA in resistance: As noted above, RNA silencing directed against viruses is probably a more general strategy against pathogens in plants. Recently, induction of a plant endogenous siRNA by the bacterial pathogen *Pseudomonas syringae* has been reported. This siRNA contributes to race-specific resistance (Katiyar-Agarwal *et al*., 2006).

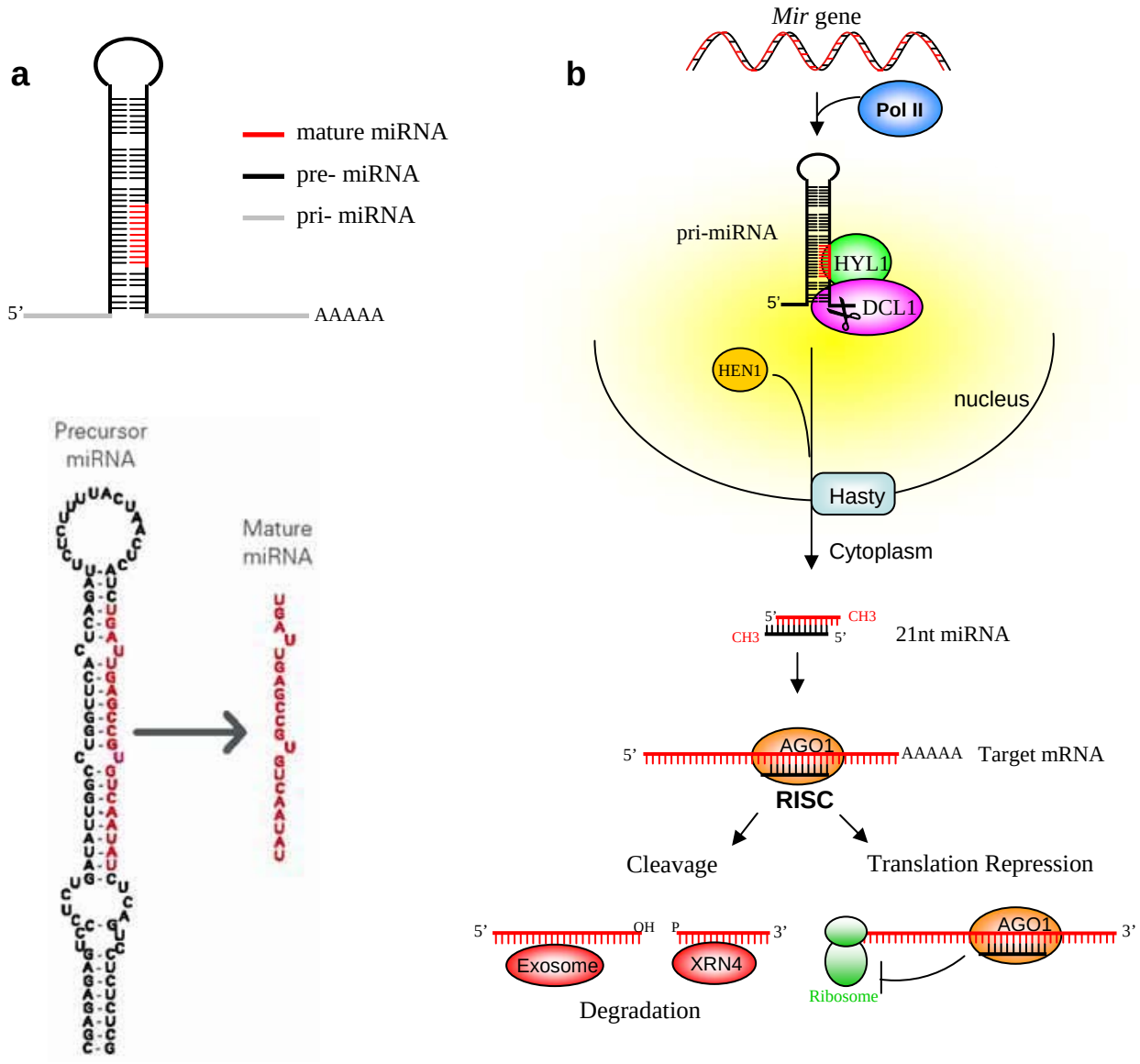


Figure 5: (a) Schematic structure of pri-miRNA. An examples of plant miRNA in their pre-miRNA secondary structure. (b) General miRNA pathway.

1-2. miRNA pathway

1-2-1. *MIR* genes: A second important pathway mediated by small RNAs is the silencing of endogenous messenger RNAs by miRNAs. The miRNAs negatively regulate gene expression in plants and animals and this regulation is a crucial phenomenon for development. The first discovered miRNAs were from the *lin-4* gene, a gene that controls the timing of *Caenorhabditis elegans* larval development (Lee *et al*., 1993). miRNAs are short 21–24nt endogenously expressed RNAs, potentially processed from one arm of a stem loop precursor (Figure 5) by DCL cleavage. They are generally conserved in evolution (Bartel, 2004). Plant and animal miRNA pathways are fundamentally similar. The miRNA precursor RNA from the *MIR* gene is transcribed by RNA polymerase II (Xie *et al*., 2005a). Most *MIR* genes are placed in non-coding regions or within introns. They are independent transcription units and their expression show tissue specificity or even cell-type specificity in agreement with a role in patterning and maintenance of differentiated cell states (Bartel and Bartel, 2003; Parizotto *et al*., 2004). Currently, ~100 Arabidopsis *MIR* genes classified into 25 distinct families have been identified based on evolutionary conservation (Xie *et al*., 2005a) but many more are thought to exist that may be genus- or even species-specific.

1-2-2. miRNA processing pathway: Animal *MIR* gene transcripts (**pri-miRNA**) are processed in two steps that take place in the nucleus and cytoplasm first by **Drosha** (RNaseIII endonuclease) to liberate a 60-70nt stem loop intermediate precursor (**pre-miRNA**) and then by **DCL1** to liberate the miRNA. In plants, miRNAs are thought to be produced in one step by **DCL1** and this is most likely to occur in the nucleus (Park *et al*., 2002; Papp *et al*., 2003; Kurihara and Watanabe, 2004; Bartel, 2004) (Figure 5). In the animal miRNA pathway, both Drosha and Dicer are assisted in the cleavage processes by specific dsRNA-binding proteins such as Pasha and Loquacious (Du and Zamore 2005). In the plant miRNA pathway, DCL1 requires and interacts with **HYL1** (Hyponastic Leaves1, a dsRNA-binding protein, **DRB1**) to make both cuts within the miRNA precursor (pri- and pre-miRNA) so as to liberate a miRNA/miRNA* duplex (Han *et al*., 2004; Vazquez *et al*., 2004a; Kurihara *et al*., 2006). The **miRNA*** is the complementary strand to miRNA that is less stable and is also called the passenger strand (Lau *et al*., 2001).

Like siRNAs, miRNA duplexes are 5' phosphorylated and have 2nt 3' overhangs (Vazquez, 2006). The mature miRNA is methylated at its 3' extremity by HEN1 (Yu *et al*., 2005). Like DCL1, HYL1 and HEN1 are found in the nucleus, suggesting that miRNA processing is essentially nuclear and may be carried out in Dicing body complexes (Fang and Spector, 2007). Dicing bodies are nuclear bodies in which a series of proteins involved in the miRNA pathway are co-localized. In animals, Exportin-5 (**Exp5**) regulates the transport of pre-miRNAs from the nucleus to the cytoplasm and in Arabidopsis, **HASTY** (HST) probably provides a related function for miRNA transport (Park *et al*., 2005). Once in the cytoplasm, the miRNAs are charged to RISC and their miRNA strand (but not the miRNA* strand which appear to be degraded) is used as a guide for recognition of any complementary ssRNA in cytoplasm. Biochemical and genetic studies revealed that AGO1 is implicated in plant miRNA RISC (Qi *et al*., 2005;

Baumberger and Baulcombe, 2005). Plant miRNAs typically are more perfectly paired than are animal miRNAs to their target RNA. They mainly bind to unique complementary sites within the coding sequence or even in the 5'UTR (untranslated region) of their target mRNAs and guide their cleavage (Olsen and Ambros, 1999; Jones-Rhoades *et al.,* 2002; Jones-Rhoades and Bartel, 2004). The 5' fragment generated by cleavage is believed to be degraded by the **Exosome**, whereas the 3' fragment is digested by the 5'-3' exonuclease **XRN4** that degrades uncapped mRNAs (Souret *et al*., 2004). Animal miRNAs normally target the 3'UTR with less perfect complementarity and use a translation repression mechanism to inactivate their target. The translational repression mechanism is not fully understood but there is evidence that RISC mediates sequestration of target transcripts away from the translational machinery into cytoplasmic foci termed P (processing)-bodies (Rossi *et al*., 2005). Another proposed scenario is that translational repression is the result of binding of RISC to multiple target sequences within an mRNA so as to interfere with ribosome movement along the transcript (Tolia and Joshua-Tor, 2007). The stalled ribosomes might be the signal that the complex should be transported to the P-bodies.

1-2-3. miRNA targets: In Arabidopsis the miRNAs targets which have been identified computationally and validated experimentally mostly correspond to mRNAs for transcription factors and other proteins involved in developmental regulation. The mRNAs encoding proteins associated with ubiquitin mediated protein degradation are also potential miRNA targets (Llave *et al.*, 2002; Jones-Rhoades and Bartel, 2004). Most plant miRNA have perfect or near perfect complementarity with their targets (Schwab *et al*., 2005, 2006). Therefore they affect only a small number of targets while animal miRNAs usually have hundereds of targets with limited complementarity. Lim *et al.* (2005) showed that delivering a cell-type specific miRNA into human cells down regulated about 100 mRNAs.

Most plant miRNAs exhibit up to four or less mismatches with their targets and these mismatches are usually located in the 3' region of the miRNA (Mallory *et al*., 2004; Parizotto *et al.,* 2004; Schwab *et al*., 2005). Indeed, experiments with mutated targets show that mismatches between positions 3 and 11 of the miRNAs result in poor cleavage, whereas mismatches at the 3' end had a slighter effect (Mallory *et al*., 2004). Although, as noted above, most plant miRNAs that have been studied promote degradation of their target transcript, miR172 is known to repress translation of the floral regulator *AP2* mRNA (Aukerman and Sakai, 2003; Chen, 2004) but also guide mRNA cleavage (Schwab *et al*., 2005). It will be interesting to determine if other plant miRNAs have secondary targets which are subject to translation inhibition rather than degradation.

1-2-4. Virus and miRNA pathway: there is now evidence that viruses can use the host miRNA pathway to their profit. Several mammalian DNA viruses encode their own miRNAs. Viral-encoded miRNAs can act both in *cis*, to ensure accurate expression of the viral genome, and in *trans*, to modify the expression of host transcripts (Figure 4). Epstein-Barr virus (EBV), a 172 kb dsDNA human Gammaherpesvirus, produces nuclear-localized transcripts with partially dsRNA structures that are processed into miRNAs which target viral transcripts (to trigger a change of stage in the virus infection cycle) as well as many

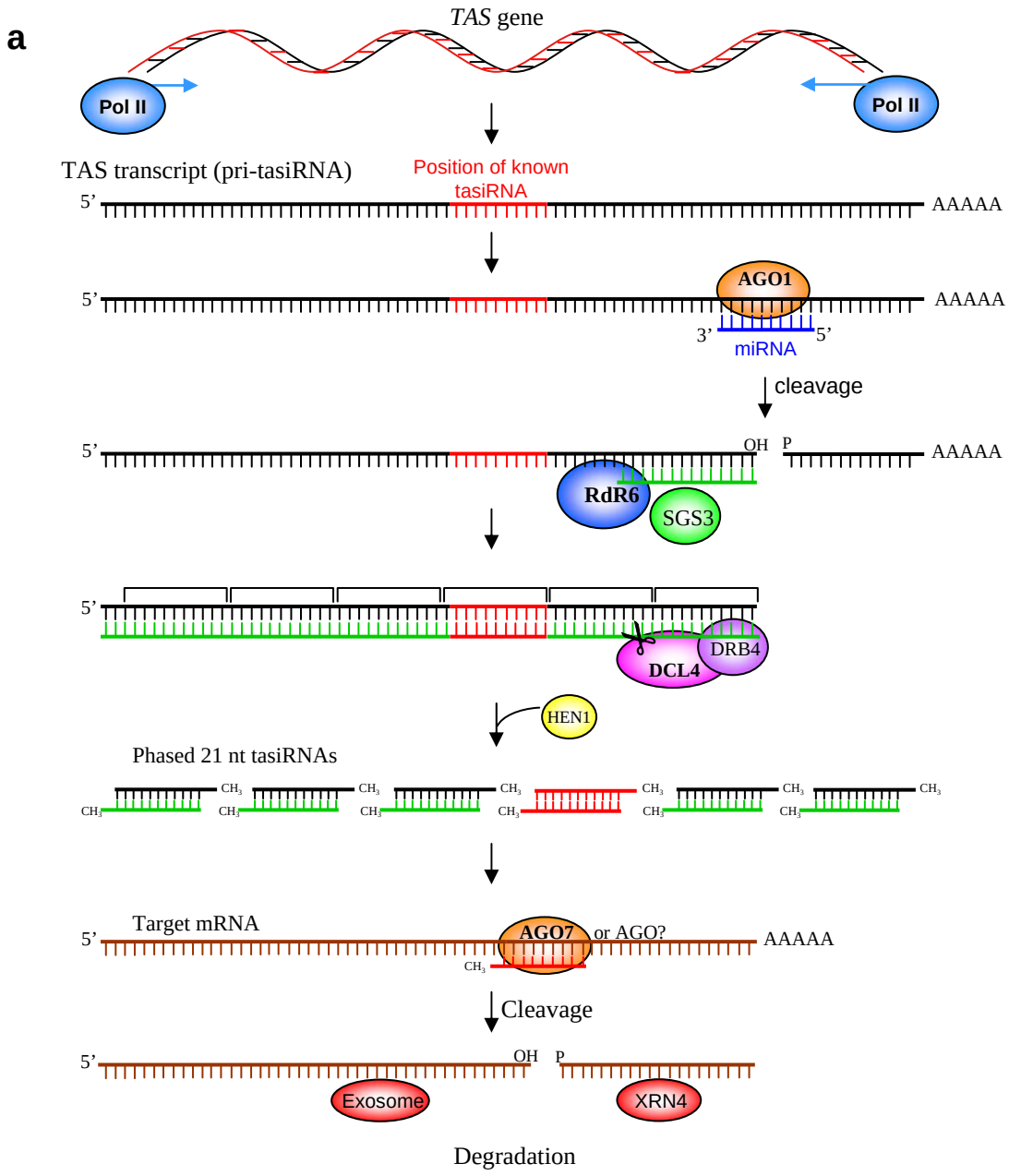


Figure 6: (a) General tasiRNA pathway. (b) tasiRNA position and miRNA cleavage site on the sequence of pri-tasiRNA from the TAS3 locus (Allen *et al.*, 2005).

cellular mRNAs in human cells (Pfeffer. *et al.,* 2004). So far, a total of 74 miRNAs which are mainly encoded by human Herpesviruses have been identified (reviewed by Li and Ding, 2005; Pfeffer and Voinnet, 2006).

On the other hand, it has also been shown that the miRNA pathway in mammalian cells can play an antiviral role. Primate miR32 targets mRNAs of Primate Foamy Retrovirus type-1 (PFV-1) (Lecellier *et al*., 2005). In plants, induction of a plant miRNA by flagellin of the bacterial pathogen *P. syringae* has been reported. Perception of a flagellin-derived peptide induces synthesis of miR393 which down regulates mRNAs implicated in auxin signaling so as to reduce auxin levels and consequently restrict bacterial growth (Navarro *et al*., 2006). This is the first example of a role for the plant miRNA pathway in nature resistance to a bacterium at the step of perception of a PAMP molecule. The miRNA pathway, like the siRNA pathway, can also be used to produce virus-resistance transgenic plants. It has been reported that transgenic expression of artificial miRNA targeting viral sequences can confer resistance to the corresponding virus (Niu *et al.,* 2006).

1-3. tasiRNA pathway

Another class of endogenous small RNA is ***trans*-acting siRNA** (**tasiRNA**) that derives from ***TAS* genes**. Several *TAS* loci have been identified in Arabidopsis (Allen *et al*., 2005) (Table 1). The tasiRNA pathway provides a second cellular regulation system for development. *TAS* genes transcribe long primary RNAs that are not predicted to encode proteins and seem to function by serving as the precursors (pri-tasiRNA) for tasiRNA production (Figure 6). Pri-tasiRNAs contain a binding site for a specific miRNA (Table 1) that guides RISC-mediated cleavage at a defined point, so the production of tasiRNAs requires a functional miRNA pathway (Figure 6b). The cleavage products seem to be protected against degradation by **SGS3** (Yoshikawa *et al*., 2005). **RDR6** is believed to transform one of the two single-stranded TAS transcript cleavage products into dsRNA (Figure 6a) since null *rdr6* and *sgs3* mutants lack tasiRNAs (Peragine *et al*., 2004; Vazquez *et al*., 2004b; Allen *et al*., 2005; Adenot *et al*., 2006; Howell *et al*., 2007).

The miRNA-mediated cleavage of pri-tasiRNA is required for dsRNA formation by RDR6 and subsequent production of phased 21nt tasiRNAs by **DCL4** (Gasciolli *et al*., 2005; Xie *et al*., 2005b; Yoshikawa *et al*., 2005), which associates with the dsRNA binding protein **DRB4** (Nakazawa *et al*., 2007). DRB4 is required for TAS3 tasiRNA production (Adenot *et al*., 2006; Nakazawa *et al*., 2007) as reduced accumulation of the TAS1 and TAS3 tasiRNA and over-accumulation of their targets were detected in both *drb4* and *dcl4* mutants. DCL4 cleavage starts from the miRNA-cleaved end of the TAS precursors to generate clusters of tasiRNAs. Active tasiRNAs are accurately phased with respect to the miRNA-guided cleavage site (Allen *et al*., 2005).

tasiRNA biogenesis differs from other pathways in that both strands of the tasiRNA locus are transcribed. It has been reported that the accumulation of tasiR-ARF requires RDR6 and DCL1 (Williams *et al*.,

Table 1: TAS loci, related tasiRNAs and their targets in Arabidopsis (Allen *et al*., 2005; Williams *et al*., 2005; Yoshikawa *et al*., 2005; Rajagopalan *et al*., 2006; Howell *et al*., 2007).

TAS genes	Locus	Related miRNA	Resulted tasiRNAs	targets
TAS1	TAS1a: At2g27400 TAS1b: At1g50055 TAS1c: At2g39675	miR173	tasiR255	four mRNAs with unknown function and PPR
TAS2	At2g39680 (antisense)	miR173	tasiR255 tasi1511	PPR
TAS3	TAS3a: At3g17185 TAS3b: At5g49615 TAS3c: At5g57735	miR390	tasiR2141 tasiR-ARF	ARF2, 3 and 4
TAS4	Between At3g25790 and At3g25800	miR828	tasiR81(-)	MYB75, 90 and 113

2005). Like miRNAs and siRNAs, tasiRNAs are also methylated by HEN1. The 5' half of tasiRNAs and miRNAs show a high level of complementarity with their endogenous target mRNAs. tasiRNAs regulate the expression of their target mRNAs by guiding mRNA cleavage. Interestingly, different members of the same gene family can be targeted by either miRNAs or tasiRNAs. The *TAS1* family is composed of three genes that encode a closely related set of tasiRNAs (one of them known as tasiR255) that target four mRNAs encoding proteins of unknown function. *TAS2*-derived tasiRNAs (like tasiR1511) regulate a set of mRNAs encoding pentatricopeptide repeat proteins (PPR). The *TAS3* locus specifies tasiRNAs that target several Auxin response factors (ARFs), including ARF2, ARF3 and ARF4 (Peragine *et al*., 2004; Vazquez *et al*., 2004b; Allen *et al*., 2005; Williams *et al*., 2005) (Table 1).

RDR6, SGS3 and DCL4 are required for biogenesis of tasiRNA. Knock out mutant in Arabidopsis, *rdr6*, *sgs3*, *dcl4* have in common an accelerated **juvenile-to-adult phase transition** (Peragine *et al*., 2004; Xie *et al*., 2005b) indicating that tasiRNAs could regulate the corresponding mRNAs governing this trait. Recently Fahlgren *et al*. (2006) showed that the juvenile to adult phase transition is normally suppressed by TAS3-tasiRNAs through negative regulation of ARF3 mRNA. Therefore, in mutants, transcripts of ARF3 cannot be regulated by tasiRNA and juvenile to adult transition is accelerated. tasiRNAs deriving from the (-) strand (which is complementary to the primary TAS RNA) have the potential to target and regulate the primary TAS RNA (Vazquez *et al*., 2004b). **AGO1** seems to be involved in TAS1 and TAS2-mediated regulation, whereas **AGO7** (Hunter *et al*., 2003; Howell *et al*., 2007) is involved in TAS3-mediated regulation. a*go7* mutants display also a shortened juvenile-to-adult phase transition. In animals, tasiRNAs have not been reported (reviewed by Brodersen and Voinnet, 2006; Vaucheret, 2006).

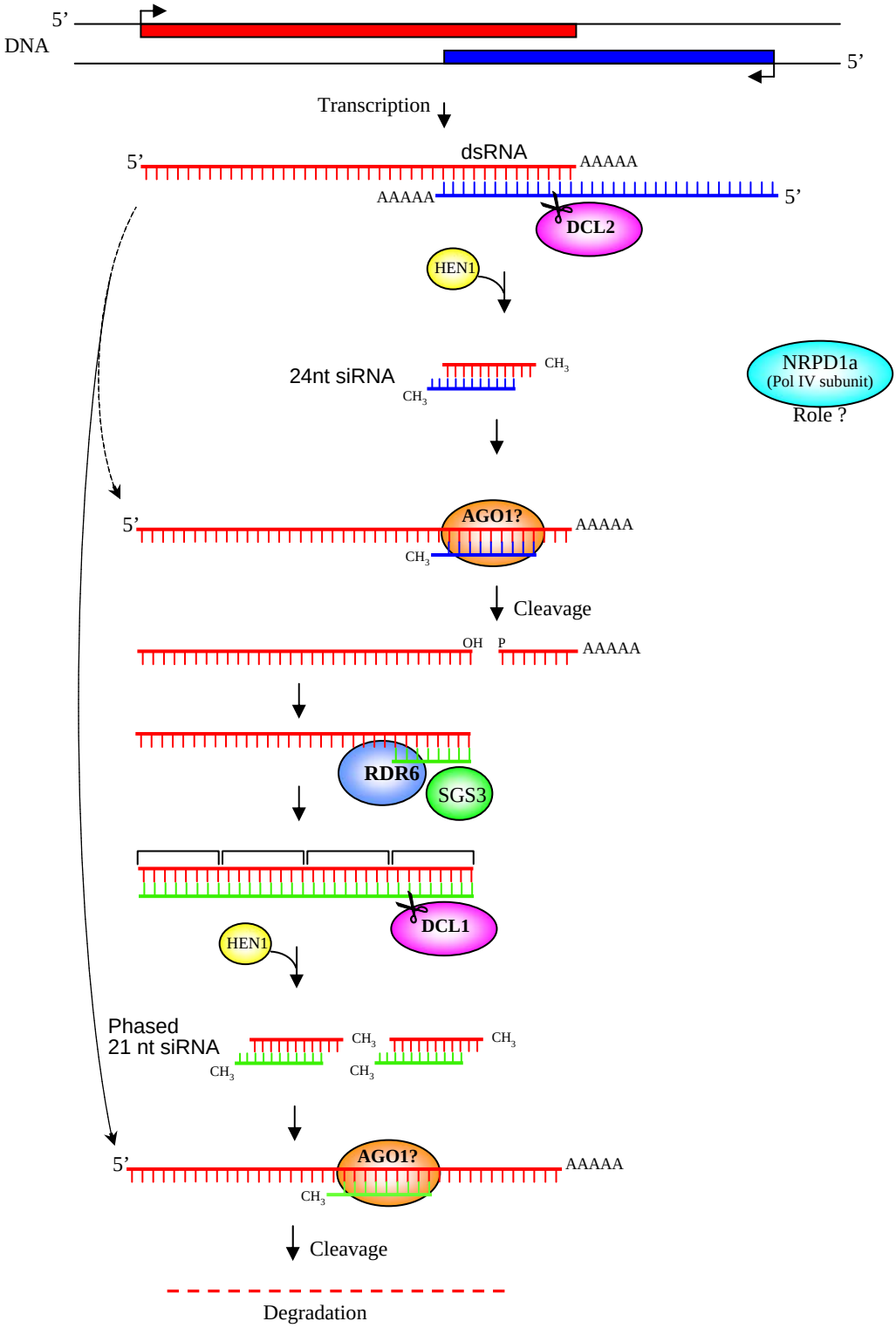


Figure 7: General nat-siRNA pathway.

1-4. nat *cis*-acting siRNAs

Another class of endogenous regulatory small RNA which are only expressed under particular conditions of growth has been discovered recently and named **natural antisense transcript *cis*-acting siRNA** (**nat *cis*-acting siRNA**). In the only known example of this pathway (Wang *et al*., 2005), expression of a pair of neighboring genes on opposite DNA strands (*cis*-antisense genes), SRO5 (At5g62520 with unknown function) and pyrroline-5-carboxylate dehydrogenase (P5CDH, At5g62530), upon salt stress, gives rise to a single 24nt siRNA species from the overlapping region of their transcripts by the action of DCL2 (Figure 7). Cleavage of the P5CDH transcript by the 24nt nat-siRNA sets the phase for accurate production of further 21nt P5CDH nat-siRNAs by DCL1. These nat-siRNAs down-regulate the expression of P5CDH by causing mRNA cleavage. Down-regulation of P5CDH leads to proline accumulation, which is important for salt tolerance. This novel pathway involves **DCL2, DCL1, RDR6, SGS3** and the atypical DNA dependent RNA polymerase-like subunit **NRPD1a** (a subunit of Pol IV). The finding that 4–20% of the genes in many eukaryotes show cis-antisense overlapping organization raises the possibility that the nat-siRNA pathway could be a major mechanism for gene expression regulation (Borsani *et al*., 2005).

1-5. DNA methylation pathway and chromatin silencing

1-5-1. Euchromatin and Heterochromatin: Eukaryotic chromosomes are composed of two general types of chromatin domains: (1) euchromatin, which is gene-rich chromatin that is less condensed and more accessible to factors involved in transcription, and (2) heterochromatin, which is highly condensed and composed of repeated (thousands of times) DNA sequences with few or no accessible coding regions. Most heterochromatin is found near centromeres and telomeres (Figure 8). Heterochromatin is characterized by methylated cytosines and specific methylated lysine residues in histones and these modifications are important for chromatin remodeling, condensation and control of numerous genetic processes in the cell, including replication, transcription, DNA repair, recombination and gene transposition (Lippman and Martienssen, 2004; Grant-Downton and Dickinson, 2005; Grewal and Jia, 2007).

1-5-2. Transposable elements: These are DNA sequences that have the capacity to move from place to place within a genome. They have been divided into two classes. Class I are **retrotransposons** that amplify their copy number through reverse transcription of an RNA intermediate. They have direct **long terminal repeats** (**LTR**s). Class II transposons have **terminal inverted repeats** (**TIR**s) ranging in size from 11 to several hundred base pairs. Within this class, one or more element encodes a transposase protein that has the potential to interact with TIRs to excise the elements and integrate them into other regions of the genome. Both classes can move around plant genomes, altering the function and structure

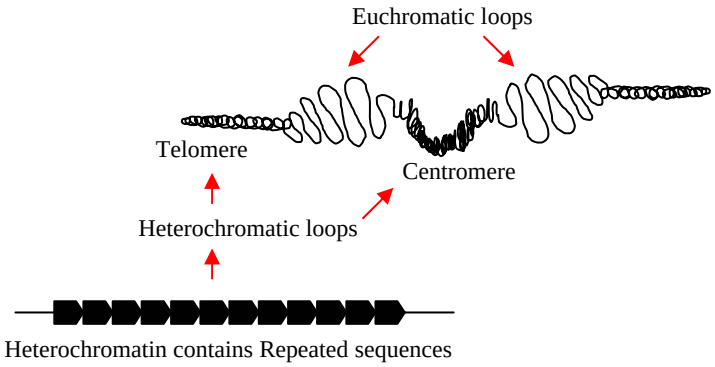


Figure 8: Chromosome structure, hetero- and eu-chromatin.

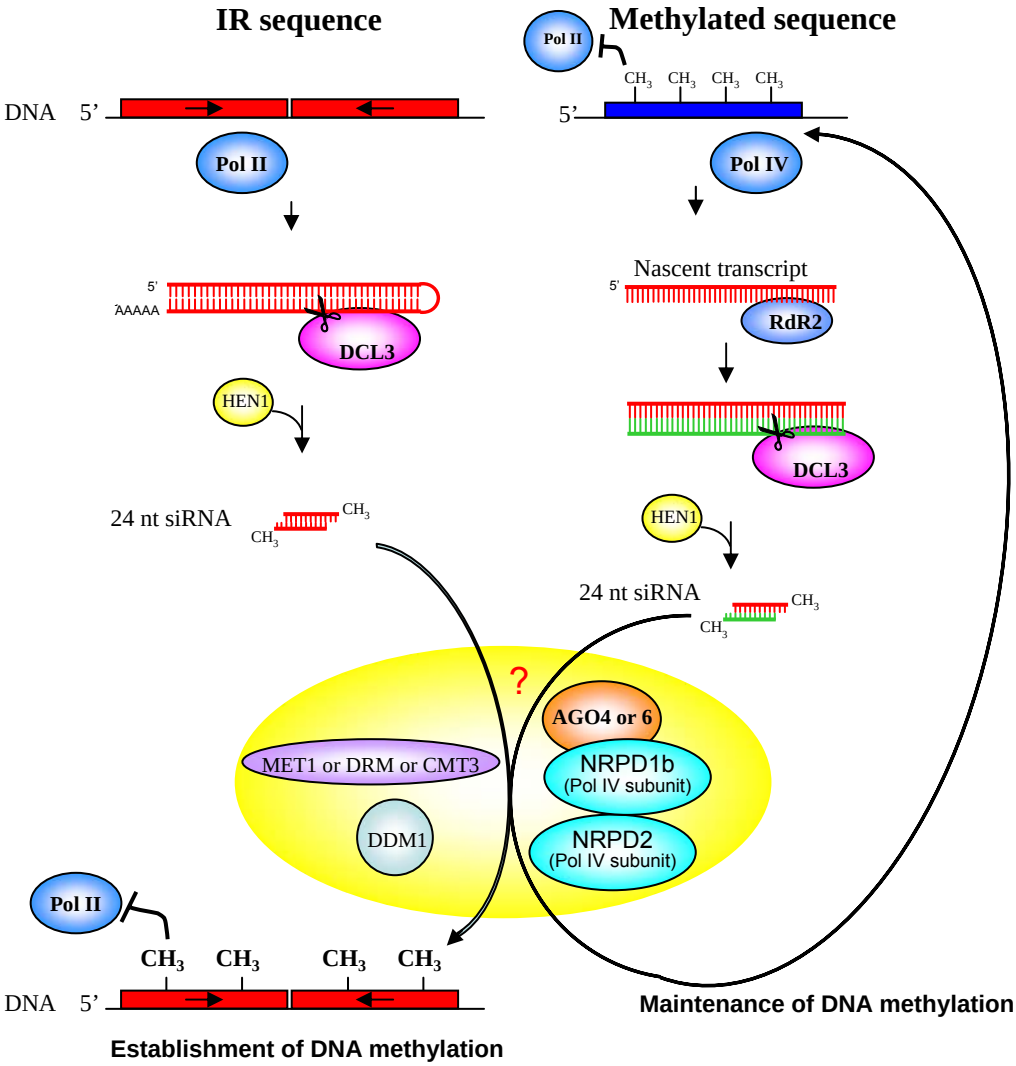


Figure 9: General DNA methylation pathway

of genes, and thus accelerating genomic evolution. However, they are by their nature also parasitic mutagenic agents that have the potential to perturb a genome (Kumar and Bennetzen, 1999; Waterhouse *et al*., 2001). DNA methylation has clear roles in taming transposons and in maintenance of genome integrity in plants, because loss of methylation in mutants reactivates transposon activity (Zilberman *et al*., 2003; Xie *et al*., 2004). Thus taken together, DNA methylation has two essential roles, defending the genome against transposons and regulating gene expression in plant.

1-5-3. DNA methylation pathway: The small RNA-mediated silencing pathway in which RNA causes methylation of DNA is called **RNA-directed DNA methylation** (**RdDM**). The first evidence for this type of silencing came from experiment with viroid-infected tobacco plants. When the RNA of a viroid was integrated as a transgene into the tobacco genome, cDNA copies of the viroid became methylated only during replication of the homogenous inoculated viroid (Wassenegger *et al*., 1994). These results indicated that the presence of the replicating viroid RNA could induce methylation of homologous DNA sequences. This type of silencing depends on the presence of dsRNA like other pathways. Thus, endogenous, sense transgene- or invert repeat transgene-derived siRNAs guide DNA methylation in homologous DNA sequences (Aufsatz *et al*., 2002). RdDM typically involves methylation of both CG and non-CG sequences and the consequence of C methylation can be the occasional transition of C to T that is heritable. The siRNA-directed DNA methylation in plants is also linked to histone methylation (Soppe *et al*., 2002; Zilberman *et al*., 2003; reviewed by Grant-Downton and Dickinson, 2005; Kanazawa *et al*., 2007).

The siRNAs corresponding to several endogenous silent loci, including retrotransposons, 5S rDNA and centromeric repeats are referred to as **repeat-associated siRNAs** (**ra-siRNAs**), **cis-acting siRNAs** (**ca-siRNA**s) or **heterochromatin siRNAs** (**hc-siRNA**s). In plants, ra-siRNAs are the most abundant siRNA class; they are predominantly 24nt in size and methylated by **HEN1**. They originate from the transcripts which are converted into long dsRNAs by **RDR2**, which are then processed by **DCL3** into 24nt siRNAs (Xie *et al*., 2004; Li *et al*., 2005; Kasschau *et al*., 2007) (Figure 9) although partially redundant function of DCL2, DCL3 and DCL4 in this pathway have recently been reported (Henderson *et al*., 2006). ra-siRNA accumulation also requires a plant-specific putative DNA dependent RNA polymerase (containing subunits **NRPD1a** and **NRPD2**), termed **Pol IV** (Herr *et al*., 2005). The effector complex implicated in this pathway, different from the RISC involved in siRNA and miRNA directed cleavage, is referred to as the **RNA-induced transcriptional silencing** (**RITS**) complex because the resulting small RNAs silence target sequences at the transcriptional level (Finnegan and Matzke 2003; Verdel *et al*., 2004). RITS contains at least **AGO4** (Zilberman *et al*., 2003), although it has recently been reported that **AGO6** also has a role in the accumulation of specific chromatin-related siRNAs, in DNA methylation and in transcriptional gene silencing and that is partly redundant with AGO4 (Zheng *et al*., 2007).

The mechanism of methylation directed by ra-siRNA and how an siRNA interacts with its homologous genomic DNA is unknown. The nascent transcripts and/or the DNA itself are possible targets of ra-

siRNAs. There is an hypothesis that siRNAs interact directly with DNA and Pol IV could facilitate this by transiently unwinding the DNA double helix by moving along the DNA with associated helicases (Brodersen and Voinnet, 2006).

In addition to silencing elements, methylation requires other components such as methyltransferases. There are three known **methyltransferases** that add methyl groups to cytosine. **MET1** (methyltransferase1) maintains CG methylation by methylating newly synthesized DNA and is required to maintain methylation patterns at repetitive and single copy sequences. CNG (where N is any nucleotide) methylation is maintained by **CMT3** (Chromo Methyltransferase) and, to a lesser extent, by **DRMs** (Domains Rearranged Methyltransferase). DRMs are responsible for establishing methylation in all sequences. Mutations in RNA silencing pathway genes prevent establishment of methylation by DRMs. In addition to methyltransferases, the **DDM1** (Decrease in DNA Methylation, a chromatin remodeling helicase) is a major factor for maintaining methylation in both CG and non-CG contexts (reviewed by Bender, 2004; Chan *et al*., 2005; Gehring and Henikoff, 2007).

Silencing by DNA methylation is a type of transcription gene silencing (**TGS**). In this form of silencing, the promoter and sometimes the coding region are densely methylated. Methylation, or methylation-associated chromatin remodeling of promoter sequences is thought to prevent binding of factors necessary for transcription. Patterns of DNA methylation are inherited and maintained across generations in plants but demethylation by a **DNA glycosylase** is required for the expression of some genes in specific tissues. Changes in DNA methylation that arise somatically during the plant life cycle have the possibility to be propagated (Gehring and Henikoff, 2007). Finally in addition to action by siRNA, there is a simple example demonstrating that miRNA target genes are specifically methylated downstream of the miRNA complementary site and that DNA methylation occurs in *cis* and depends on the ability of the miRNA to bind to the transcribed RNAs (Bao *et al.,* 2004).

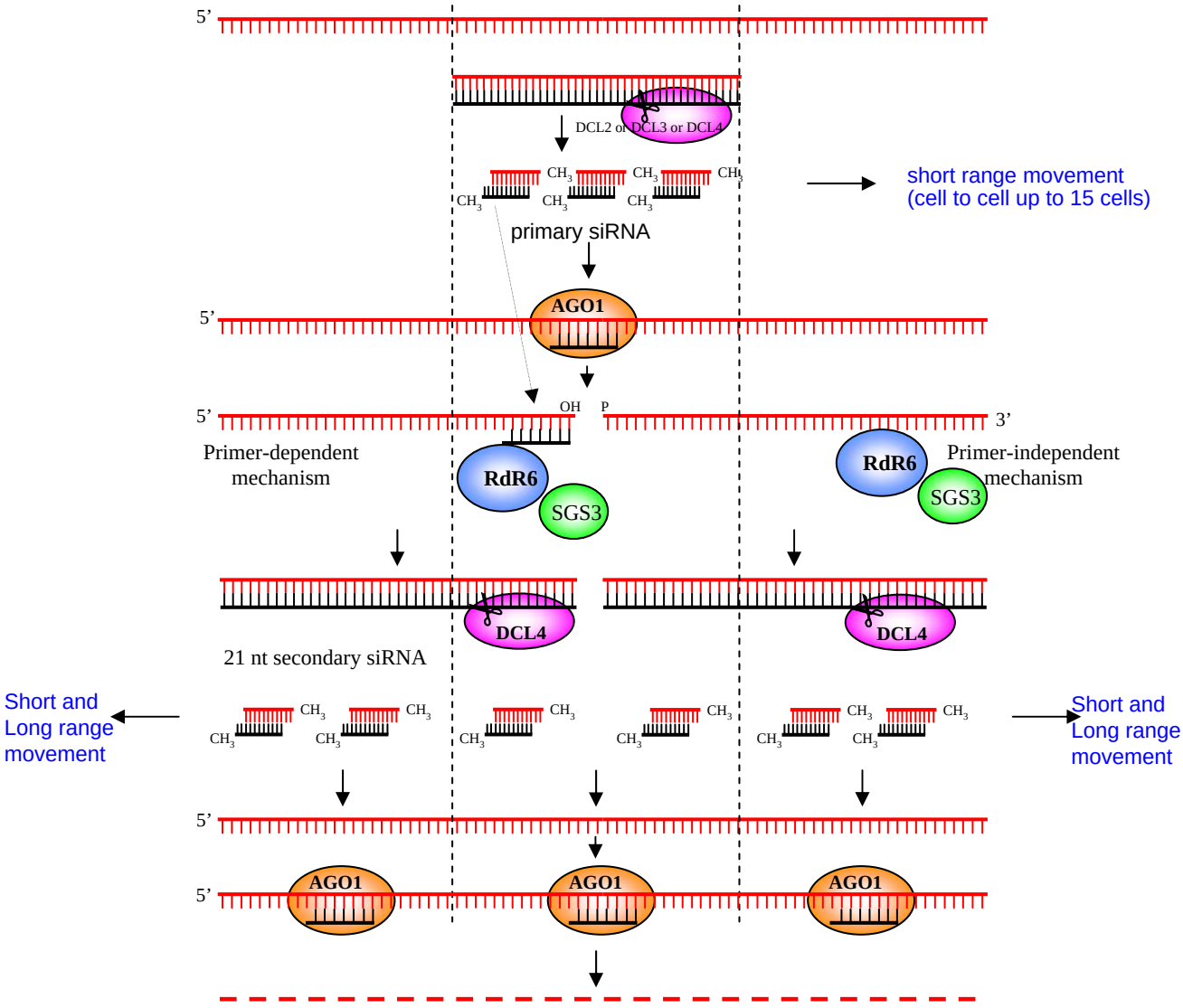


Figure 10: Transitivity and secondary siRNA production.

2. Initiation, amplification and spread of silencing signal

Like systemic acquired resistance (SAR) mediated by *R* gene, locally triggered RNA silencing can produce systemic silencing by dispersion of a specific signal cell-to-cell via plasmodesmata and at long distance through the vascular tissues (Voinnet *et al*., 1998). Evidently, the spread of the silencing signal is an important component of the plant defense response against ongoing virus infections, especially for protection of the shoot apical meristem (Schwach *et al*., 2005). The 21nt siRNAs produced by DCL4 are the signal active at the cell-to-cell level (Dunoyer *et al*., 2005). The RDRs are apparently key factors for amplification of these signals since non cell autonomous silencing of viruses and transgenes involves RDR6 in Arabidopsis (Dalmay *et al*., 2000; Vaistij *et al*., 2002; Himber *et al.,* 2003; Parizotto *et al*., 2004).

2-1. Mechanism of production of secondary siRNAs: Primary siRNAs produced by the initial action of DCL on a dsRNA have two functions: as guide and as primer. First, they incorporate into RISC to guide sequence-specific cleavage of homologous RNA (Figure 10). The resulting cleavage products are perceived as aberrant RNAs and promote further production of dsRNA by **RDR6**. It can be considered two mechanisms for function of RDR6: the primer-dependent and the primer-independent mechanisms (Figure 10). The first mechanism is important for production of dsRNA from a single-stranded 5' cleavage product (Moissiard *et al*., 2007). The antisense strand of this primary siRNAs anneals by base pairing to a target ssRNA and serve as a primer for primer-dependant production of more dsRNA by RDR6 (Sijen *et al*., 2001). The resulting dsRNA then is cleaved by **DCL4**, and gives rise to 21nt secondary siRNAs. The secondary siRNAs may also be produced in a primer-independent manner from 3' cleavage product because this fragment is not capped and is perceived as an aberrant RNA by RDR6. Thus, the cleavage of target RNA by ARGONAUTE is a starting point for the production of dsRNA by RDR6 for secondary siRNA production in the siRNA, tasiRNA and nat-siRNA pathways (reviewed by Brodersen and Voinnet, 2006; Vaucheret, 2006; Baulcombe, 2007).

2-2. Transitivity: In Arabidopsis, the synthesis of secondary siRNAs (or amplification of the signal) requires the activities of **RDR6** and **SDE3** (RNA-helicase) (Himber *et al*., 2003). The secondary siRNAs are derived not only from the initiator region but also from adjacent regions (on both the 5’ and the 3’ side) of the initial target sequence (Sijen *et al*., 2001; Vaistij *et al*., 2002). Therefore, a primary siRNA molecule could generate many dsRNAs which would then trigger silencing of even more target molecules. The transition from production of primary siRNAs (corresponding to a specific sequence of a targeted RNA) to secondary siRNAs (targeting regions outside the initial target sequence) is called **transitivity**. The result of transitivity can be methylation of a target DNA as well as cleavage of its transcript (Vaistij *et al*., 2002). It should be emphasized that transitivity as an siRNA amplification mechanism is important in virus defense because it allows defense system to keep pace with the replicating viral RNAs. Similarly, in genome defense, this amplification step would ensure that just a few

molecules of transposon RNA could activate the chromatin silencing pathway sufficiently to suppress all copies of a transposable element (reviewed in Baulcombe, 2004, 2007).

Systemic RNAi has also been discovered in nematodes but secondary siRNAs in *C. elegans* differ from those in plants. They appear to result from unprimed dsRNA synthesis by RdRP, are only found upstream of the initial dsRNA trigger, are only of antisense polarity and are bound by a different set of ARGONAUTE proteins (Sijen *et al*., 2007).

2-3. Mobility of the silencing signal: Mobility of siRNA is a crucial characteristic of an antiviral defense system. A mobile silencing signal could move either with or ahead of the virus to silence the viral RNA before, or at the same time, as the virus moves into a new cell. There are two signaling mechanisms in plants: the short range (up to 15 cells) and the longer range. As noted above, in short range signaling, 21nt siRNAs produced by DCL4 are the mobile signals because *dcl4* mutants fail to exhibit short distance spreading of silencing triggered by an inverted-repeat construct driven by the phloem-specific promoter (SUC-SUL) to silence the endogenous SUL mRNAs. This type of signaling is RDR6-independent. The long-range silencing signals may be secondary 21nt siRNAs generated by RDR6-mediated amplification. Their synthesis would require the presence of siRNA homologous transcripts in recipient cells to produce dsRNA to be template of subsequent DCL4 dicing (Himber *et al*., 2003; Dunoyer *et al*., 2005). In addition to secondary siRNAs, the primary 21nt siRNA can move to 10-15 adjacent cells independently of the presence of homologous transcripts in those cells (Himber *et al*., 2003; reviewed by Voinnet, 2005b).

2-4. Mobility of miRNA: *In situ* hybridizations experiments suggest that most miRNAs are not mobile (Kidner and Martienssen, 2004) or at least that they do not accumulate at distances far from their site of production, although there could be limited mobility within a tissue. However, some miRNAs have been cloned from vascular tissues, suggesting that they could move within certain parts of the plant (Yoo *et al*., 2004). Movement of miRNAs could contribute to the gradients of gene expression that are often required for patterning. The study of miR171 revealed cell-autonomous expression and activity of this miRNA (Parizotto *et al*., 2004) and studies of miR165 and miR166, two negative regulators of leaf polarity, showed a gradual distribution in leaf primordia (Kidner and Martienssen, 2004; reviewed by Voinnet, 2005b).

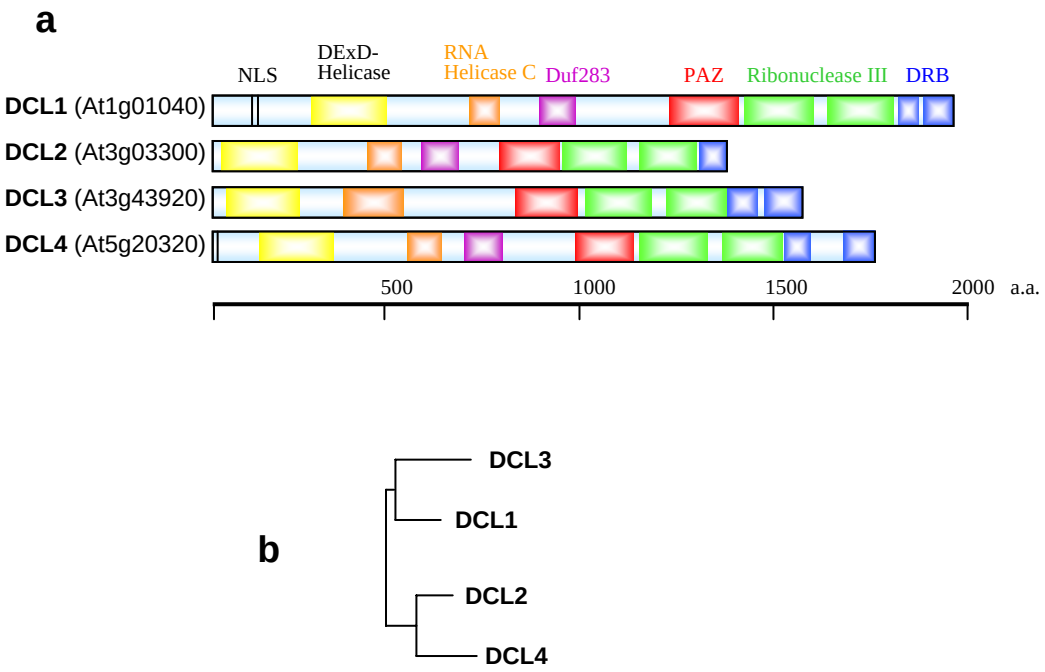


Figure 11: (a) schematic diagram of the four Arabidopsis DCLs and position of different domains (adapted from Vazquez, 2006). (b) phylogenetical tree of the four DCLs based on amino acid (a.a.) sequence (analyzed in http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

3. Essential components of RNA silencing

3-1. Dicers

Dicer proteins are members of the RNaseIII protein family that catalyze the cleavage of dsRNA to generate small RNA duplexes. Human, mice, nematode and yeast (*Schizosaccharomyces pombe*) each possess only one Dicer gene that is invoved in all pathways. Insects (*Drosophila melanogaster*) and fungi (*Neurospora. crassa* and *Magnaporthe oryzae*) possess two Dicer like proteins (DCLs) (Tomari and Zamore, 2005; Catalanotto *et al*., 2004), while plants have even more DCL genes: *Arabidopsis thaliana* four, poplar (*Populus trichocarpa*) five and rice (*Oryza sativa*) six (Gasciolli *et al*., 2005; Margisa *et al*., 2006). In Arabidopsis, the DCL1 gene was identified under different names (*SIN1*, *SUS1* and *CAF*) in genetic screens for mutants with abnormal embryo, ovule and flower development (Schauer *et al*., 2002). Because of amino acid sequence similarity to the *D. melanogaster* Dicer and *Caenorhabditis elegans* DCR1 proteins it was renamed as **AtDCL1**. The other members of the gene family were then identified by sequence homology followed by analysis of the properties of mutants (Schauer *et al*., 2002; Xie *et al*., 2004; Gasciolli *et al*., 2005). It is worth noting, however, that there are also six non-DCL RNAseIII enzymes in the Arabidopsis genome (Bouche *et al*., 2006).

3-3-1. Protein Structure and conserved domains: Structurally speaking, RNaseIII enzymes fall into three classes (Hammond, 2005). Class I enzymes are found in bacteria and yeast and contain a single RNaseIII domain joined to a dsRNA-binding domain (dsRBD/DRB domain). Class II and III enzymes contain two RNaseIII catalytic domains. Class III enzymes are characterized by one or more helicase domains and a PAZ (PIWI/ARGONAUTE/ZWILLE) domain, which is also present in ARGONAUTE proteins. Dicers belong to class III. As many as six domains are present in animal, fungal and plant Dicer proteins although many individual proteins lack one or more of them (Figure 11). These six domains are the DExD-Helicase, RNA Helicase-C, Duf283 (unknown function), PAZ (PIWI/ARGONAUTE/ZWILLE), two Ribonuclease-III motifs and one or two dsRNA-binding (DRB) domains at the C-terminus (Schauer *et al*., 2002; reviewed by Margisa *et al*., 2006).

PAZ is a dsRNA binding domain and binds to the 2nt 3' overhang of dsRNA termini (Ma *et al*., 2004). This interaction positions the two catalytic residues of each RNaseIII domain at a distance of ~20nt from each free end of the dsRNA. Each catalytic residue independently cleaves each of the two strands. DCL products have 5′-monophosphates and 2′,3′-hydroxy termini (Zhang *et al*., 2004).

A crystallographic study performed on a lower eukaryote (*Giardia intestinalis*, Protista) confirmed that the distance between the PAZ and RNaseIII domains of Dicer determines the length of siRNA (MacRae *et al*., 2006). Possibly, the distinct lengths of the siRNA produced by the various Arabidopsis DCLs are a consequence of differences in PAZ-RNaseIII distances in the different enzymes. It is also possible however, that siRNA size is determined not only by the DCL but also by the partners of DCL proteins,

Table 2: Arabidopsis Dicer family, their function and produced siRNA size.

A. thaliana **Dicers**	**small RNA size**	**Implicated in pathway of**	**Ref.**
DCL1 (At1g01040)	21 nt	miRNA (can produce siRNA)	(Park et al., 2002) (Bouche et al., 2006)
DCL2 (At3g03300)	24 nt and 22 nt	antiviral siRNA (minor effect) nat-siRNA tasiRNA (minor effect) Antagonistic effect on miRNA	(Xie et al, 2004) (Deleris et al., 2006)(Bouche et al., 2006) (Fusaro et al., 2006) (Borsani et al., 2005) (Xie et al., 2005b) (Gasciolli et al., 2005) (Bouche et al., 2006)
DCL3 (At3g43920)	24 nt	DNA methylation (casiRNA or hc-siRNA) IR-PTGS tasiRNA (minor effect)	(Xie et al, 2004) (Fusaro et al., 2006) (Gasciolli et al., 2005)
DCL4 (At5g20320)	21 nt	antiviral siRNA (major effect) tasiRNA (major effect) S-PTGS IR-PTGS	(Bouche et al., 2006) (Deleris et al., 2006) (Fusaro et al., 2006) (Xie et al., 2005b) (Gasciolli et al., 2005) (Deleris et al., 2006) (Dunoyer et al., 2005) (Dunoyer et al., 2005)(Deleris et al., 2006)(Fusaro et al., 2006)

particularly HYL1 and related DRB proteins. Interestingly, the dsRNA binding domain not only binds to dsRNA but also functions as a protein-protein interaction domain. In particular, it has been shown that DRB of DCL1, DCL3 and DCL4 can bind to members of the DRB family (Hiraguri *et al*., 2005).

3-3-2. Subcellular localization: DCL1, which is expressed throughout the plant (Jacobsen *et al*., 1999), accumulates mainly in the nucleus where probably it ensures the processing of miRNA in so called ‘‘Dicing bodies’’ (Papp *et al*., 2003; Fang and Spector, 2007). DCL2, DCL3 and DCL4 also were detected in the nucleus. (Xie *et al*., 2004; Hiraguri *et al*., 2005). One or more DCLs must, however, also function in the cytoplasm for dicing of viral RNAs; the presence of DCL(s) in the cytoplasm is also supported by the capacity of wheat-germ extract to process long dsRNA (Tang *et al*., 2003).

3-3-3. *dcl* mutants: An Arabidopsis *dcl1* null mutant is embryo lethal (McElver *et al*., 2001). A number of viable hypomorphic *dcl1* mutants have been characterized (Schauer *et al*., 2002). Knockout *dcl2* and *dcl3* mutants developed normally, whereas a *dcl4* null mutant had leaves with downward curled margins similar to *rdr6* phenotype (Xie, *et al*., 2004; Gasciolli *et al*., 2005). All combinations of *dcl2, 3* and *4* mutations were viable and fertile but the *dcl4* mutants in every combination showed developmental defects associated with defective tasiRNA production (Henderson *et al*., 2006).

3-3-4. Function and redundancy: Function of the four DCLs and the size of the small RNAs they produce are recapitulated in Table 2. DCL1 produce most miRNA (Park *et al*., 2002; Bartel, 2004), DCL2 in addition to acting in the viral silencing pathway could be involved in the production of nat-siRNAs (Borsani *et al*., 2005), DCL3 is required for the DNA methylation pathway (Xie, *et al*., 2004) and DCL4 is implicated in tasiRNA biogenesis (Xie *et al*., 2004; Gasciolli *et al*., 2005) and antiviral defense (Deleris *et al*., 2006).

A recent study showed that both the 21nt and 22nt siRNA products of DCL4 and DCL2 respectively are implicated in the antiviral defense. Indeed, DCL2 can act as a DCL4 substitute when DCL4 is genetically removed or inhibited although DCL2 appears to be less effective than DCL4. When both DCL4 and DCL2 were inactive in mutants, plants were hypersusceptible to virus [*Tobacco rattle Tobravirus* (TRV) or *Turnip crinkle Carmovirus* (TCV)] and viral siRNAs produced by DCL3 and DCL1 were not effective for antiviral activity (Deleris *et al*., 2006). Similar results have been obtained with CMV and *Turnip mosaic Potyvirus* (TuMV) infections (Fusaro *et al*., 2006). The major Dicer generating viral siRNAs from *Oilseed rape mosaic Tobamovirus* (ORMV) is reported to be DCL4 dependent, and in its absence, DCL2 takes over this role (Blevins *et al*., 2006). When both DCL4 and DCL2 are absent, DCL3 generates 24nt viral siRNAs. Work with two DNA viruses, *Cabbage Leaf Curl Geminivirus* (CaLCuV, ssDNA) and CaMV (*Pararetrovirus*, dsDNA), showed involvement of all four DCLs in viral siRNA biogenesis (Blevins *et al*., 2006; Moissiard and Voinnet, 2006). DCL3 appears to be of particular importance consistent with another report that DCL3 produces a geminivirus-derived siRNAs (Akbergenov *et al*., 2006), but even DCL1 has a role as well, at least in facilitating the process. Inversely, Red clover necrotic mosaic Dianthovirus (RCNMV), an RNA virus, requires DCL1 for its replication (Takeda *et al*., 2005).

The hierarchical behavior of DCLs with respect to virus-derived dsRNAs is similar to that observed with hairpin constructs (Fusaro *et al*., 2006) and tasiRNA precursors. RDR6-dependent tasiRNA biogenesis involves preferentially the activity of DCL4, but both DCL3 and DCL2 gain access to precursors in the absence of DCL4. In parallel, RDR2-dependent heterochromatic siRNAs (such as ra-siRNA02) are produced primarily by DCL3. However, DCL4 and DCL2 produce RDR2-dependent siRNAs in the absence of DCL3 (Xie *et al*., 2004; Gasciolli *et al*., 2005). DCL4 also produces siRNAs from sense or inverted-repeat transgenes (Dunoyer *et al*., 2005). On the other hand, the embryo-lethality of *dcl1* null mutants suggests that no other DCL can efficiently process stem-loop miRNA precursors and that only DCL1 has this specialized function. **miR162** directs the cleavage of DCL1 mRNA and **miR838** directs the cleavage of DCL1 pre-mRNA (targets intron) indicating that these miRNAs feedback regulate the activity of the miRNA pathway (Xie *et al*., 2003; Rajagopalan *et al*., 2006). DCL2 partially antagonizes the production of miRNAs by DCL1 (Bouche *et al*., 2006) probably by competing for a limiting factor (such as a DRB).

3-2. DRBs

In Arabidopsis, five double-strand RNA binding domains (DRB/dsRBD) have been implicated in silencing pathways (Qi and Hannon, 2005). In animals, interaction with the DRB protein **Pasha** (DGCR8) is required for proper processing of pri-miRNAs to pre-miRNAs by Drosha (Landthaler *et al*., 2004). Similarly, interaction with DRB partners is required for Dicer function. As discussed earlier, in Arabidopsis, interaction of DCL1 with **HYL1** (Hyponastic Leaves1/DRB1, At1g09700) is required for the efficient and precise processing of pri-miRNA during plant miRNA biogenesis in Dicing bodies in the nucleus (Han *et al*., 2004; Vazquez *et al*., 2004a; Hiraguri *et al*., 2005; Kurihara *et al*., 2006; Fang and Spector, 2007). *HYL1* mRNA accumulates in all tissues and organs (Lu and Fedoroff**,** 2000).

3-2-1. Protein structure: The Arabidopsis HYL1 protein has two double-strand RNA binding domains (dsRBD1 and dsRBD2) in its N-terminal half and it preferentially binds dsRNA *in vitro* (Lu and Fedoroff, 2000). The dsRBD1 of HYL1 is essential for dsRNA binding *in vitro*. HYL1 also has a nuclear localization sequence (NLS) and a putative protein-protein interaction domain to which dsRBD2 contributes. This domain is probably implicated in the interaction of HYL1 with DCL1 and also with AGO1, interactions which have been detected by Bimolecular Fluorescent Complementation (BiFC) (Fang and Spector, 2007). Interaction of HYL1 with both DCL1 and AGO1 suggests that HYL1 might act as a bridge between the two important components of silencing. Such a complex between DCL1 and AGO1 has not yet been identified in Arabidopsis, whereas in *D. melanogaster, C. elegans* and human, an interaction between the equivalent of AGO1 and DCL1 has been reported (reviewed by Sontheimer, 2005).

3-2-2. *hyl1* mutants: *hyl1* null mutants exhibit reduced miRNA levels and an increase in uncleaved target mRNAs levels, resulting in developmental defects such as leaf hyponasty, delayed flowering, altered root gravitropic response, altered responses to several hormones and abnormality in adaxial/abaxial polarity (Lu and Fedoroff, 2000; Vazquez *et al*., 2004a; Yu *et al*., 2005). The transformation of a *hyl1* null mutant with a series of HYL1 deletion constructs showed that the N-terminal region containing the dsRBD1 and dsRBD2 domains completely rescued the mutant phenotype, triggering the accumulation of miRNAs and resulting in reduced mRNA levels of the targeted genes. Therefore, the N-terminal dsRBDs of HYL1 are the most important domains for processing miRNA precursors and the generation of mature miRNA (Wu *et al*., 2007).

Transgene silencing occurs in the *hyl1* mutant, suggesting that HYL1 has specialized functions in the plant miRNA pathway (Vazquez *et al*., 2004a) presumably HYL1 cooperates exclusively with DCL1 and other DRB proteins cooperate with the other DCLs. Indeed it has been shown that DRB4 interacts *in vitro* and *in vivo* with DCL4 (Hiraguri *et al*., 2005; Nakazawa *et al*., 2007) and that together they have a key role in tasiRNA biogenesis. It is reported that *drb4* mutants, which exhibit leaf morphology defects, lack TAS3 but not TAS1 and TAS2 tasiRNAs (Adenot *et al*., 2006). Reduced accumulation of the TAS1 and TAS3 tasiRNA and over accumulation of their targets in both *drb4* and *dcl4* mutants (Nakazawa *et al*., 2007) are consistent with the hypothesis that DRB4 functions with DCL4 in biogenesis of at least some tasiRNA.

Redundancy: Although HYL1 helps to position the precise cleavage site, DCL1 still cleaves pri-miRNA precursors in *hyl1* mutants, suggesting that other DRB proteins can compensate for HYL1 deficiency. Redundancy in DRB–DCL interactions is also supported by immunoblot analysis and pull-down assays showing that DCL1 interacts strongly with HYL1 but weakly with DRB2 and DRB5. For DCL3, weak interactions with HYL1, DRB2 and DRB5 were also reported (Hiraguri *et al*., 2005). This redundancy between DRB proteins can explain the viability of the *hyl1* null mutant and could even be important in the reported functional redundancy between DCL proteins (reviewed by Vazquez, 2006).

3-3. RDRs

Six Arabidopsis RDRs (RNA-dependent RNA polymerases) have been identified by homology to the tomato RDR (LeRDR1) and the *Neurospora crassa* RDR (QDE-1) both of which have proven RNA-dependent RNA polymerase activity. RDRs are required for the silencing pathways in nematodes, fungi and plants (Baulcombe, 2004). They share a sequence motif that is distantly related to the catalytic domain of DNA-dependent RNA polymerases (Iyer *et al.*, 2003). The RDRs are enzymes that use ssRNA as templates to synthesize dsRNA. A mutation in the GDD motif, which is essential for the catalytic activity of all characterized RDRs, abolished transgene silencing (Mourrain *et al*., 2000).

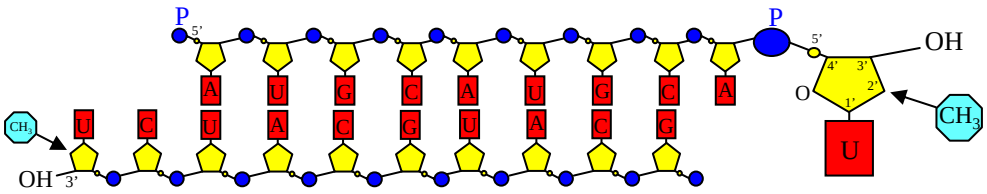


Figure 12: Schematic molecular structure of a duplex siRNA or miRNA demonstrating 3' two nucleotides overhang and the position of methyl group at 3' end (the real number of nucleotide is 21 to 24).

RDR1 (At1g14790) appears to be important for resistance to virus infection because the protein is induced by virus infection and also by salicylic acid treatment. In addition *rdr1* loss-of-function mutants exhibit increased accumulation of PVX and TMV RNAs (Xie *et al*., 2001, Yu *et al*., 2003) but the mechanism remains unknown.

RDR2 (At4g11130) is implicated in the methylation of histones and silencing of certain repetitive DNAs. It is expressed in inflorescences but not leaves, appears to have a role in the timing of flowering, and is required for de novo methylation. (Xie *et al*., 2004, Chan *et al*., 2004; Kasschau *et al*., 2007).

RDR6 (At3g49500, also known as *SDE1/SGS2*) are required in the S-PTGS but not the IR-PTGS pathway (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Peragine, *et al*., 2004). RDR6 is a key factor in the tasiRNA pathway because tasiRNAs were undetectable in *rdr6* mutants, which show developmental abnormalities such as leaf curling (Beclin *et al*., 2002; Vazquez *et al*., 2004b and Peragine *et al*., 2004; Adenot *et al*., 2006). RDR6 is also responsible for transitivity in transgene silencing (Vaistij *et al*., 2002) and is required for systemic movement of antiviral silencing signals (Dunoyer *et al*., 2005). The RDR6 homologue in *N. benthamiana* is involved in defense against systemic spreading of PVX and in exclusion of the virus from the apical growing point (Schwach *et al*., 2005). Different viruses behave differently with respect to RDR6. For example, *rdr6* mutants were hypersusceptible to CMV and PVX but not to TMV, TCV, TuMV and TRV (Dalmay *et al*., 2000; Mourrain *et al*., 2000; Xie *et al*., 2001; Deleris *et al*., 2006). It has been reported that *RDR1*, *RDR2*, and *RDR6* are not required for miRNA pathway (Xie *et al*., 2004; Vazquez *et al*., 2004b). It has been reported that N. benthamiana plants with reduced expression of NbRDR6 were more susceptible to all viruses tested and that this effect was more pronounced at higher growth temperatures (Qu et al., 2005).

3-4. HEN1

In plants, the biogenesis of miRNAs and all types of siRNAs involves methylation. Arabidopsis miRNAs and siRNAs carry a methyl group on the ribose of the 3' terminal nucleotide. Methylation of the small RNAs requires the protein HEN1 (HUA ENHANCER1, At4g20910) (Boutet *et al*., 2003; Yu *et al*., 2005) which was first identified as a gene that plays a role in the flower development (Chen *et al*., 2002) before its role in accumulation of miRNA was reported (Park *et al*., 2002). This protein has no known motif but its C-terminus shows 40-50% similarity to predicted proteins from human, *D. melanogaster*, *C. elegans* and *S. pombe* (Park *et al*., 2002).

HEN1 places the methyl group on the ribose 2' hydroxy moiety of the 3' terminal nucleotide (Figure 12) (Yang *et al*., 2006). HEN1 was shown to act *in vitro* on miRNA/miRNA* duplexes but not with DNA duplexes, single-stranded miRNA, pre-miRNA or tRNA (Li *et al*., 2005). It was reported that both the ribose 2'OH and 3'OH moieties of the 3' terminal nucleotide in the miRNA/miRNA* duplexes are required for the activity of HEN1 (Yu *et al*., 2005). HEN1 is localized in both the nucleus and the

cytoplasm suggesting that methylation of miRNA and siRNA can occur in both compartments (Fang and Spector, 2007).

***hen1* mutants:** *hen1* mutants exhibit pleiotropic phenotypes such as reduced leaf size and plant height, altered leaf shape and increased number of florescences, suggesting that HEN1 plays multiple roles in plant development as well as in organ identity specification in the flower (Chen *et al*., 2002), all presumably mediated by perturbation of miRNA function. It has also been reported that *hen1* mutants are defective in siRNA and tasiRNA silencing, in addition to miRNA biogenesis (Park *et al*., 2002; Boutet *et al*., 2003; Vazquez *et al*., 2004a). Mutants either display low small RNA accumulation and/or the apparent size of miRNAs is increased by one or more nucleotides. Small RNAs in the *hen1* background lack methylation and have additional nucleotides, primarily uridines, on their 3' ends, suggesting that one function of small RNA methylation is to protect the 3' ends of the small RNAs from uridination (Li *et al*., 2005). An *in vitro* RISC activity assay revealed that methylation of siRNA did not have an impact on RISC activity or on affinity of the small RNAs with AGO1 (Qi, *et al*., 2005). Finally, a *hen1* mutant was reported to be hypersensitive to CMV infection (Boutet *et al*., 2003).

3-5. Serrate

Serrate (*SE*), which encodes a zinc finger protein, is distinguished in Arabidopsis by mutants with leaf developmental defects (Prigge and Wagner, 2001). The *serrate-1* (*se-1*) mutant shows a highly pleiotropic phenotype, which overlaps with the phenotypes of mutants defective in miRNA accumulation such as *hyl1*, *dcl1* and *ago1*. Among several characterized mutant alleles of Serrate, one is embryo-lethal, another (*se-1*) causes only a partial defect and two other show more severe defects in leaf development. Microarray analysis shows upregulation of many genes known to be the targets of miRNAs in *se-1* plants. The levels of several miRNAs and tasiRNAs were reduced in *se-1*, suggesting that SE is another basic component of the miRNA-processing complex in plants. It has been shown that SE is not required for S-PTGS. SE interacts with HYL1 in the yeast two-hybrid system and is localized in the nucleus like HYL1. It affects the processing of pri-miRNA to miRNA. Taken together, these observations suggest that SE and HYL1 probably act with DCL1 in processing pri-miRNAs in miRNA biogenesis (Lobbes *et al*., 2006; Yang *et al*., 2006).

3-6. SDE family

At least four loci exist in the Arabidopsis SDE (Silencing DEfective) family. SDE1 is identical to the RDR6 described above.

SDE3 (At1g05460) was identified by a forward genetic screen through its loss of PTGS phenotype in transgene silencing (Dalmay *et al*., 2000). In plant carrying the *sde1* mutation, there is complete loss of transgene silencing whereas in the *sde3* mutant, silencing was reduced in leaves and flowers but was strong in hypocotyls and cotyledons. The *sde3* mutant was hyper-susceptible to infection with CMV but not with TRV and TCV. The product of SDE3 is similar to previously identified RNA helicases involved in RNA silencing in mouse, Drosophila and humans (Dalmay *et al*., 2001) although it lacks some important motifs. The data indicate that SDE3 is a regulator of PTGS rather than an essential factor. It has been suggested that SDE3 might have a role in the systemic spread of silencing and its maintenance (Willmann, 2001).

SDE5 (At3g15390) is also required for transgene silencing and the production of tasiRNAs. Mutation in SDE5 also results in hyper-susceptibility to CMV but not to TuMV. However, like RDR6 (*SDE1*), SDE5 is not involved in IR-PTGS, in the biogenesis of miRNAs or in silencing by the 24nt siRNAs produced by DCL3. SDE5 is, however, important in the tasiRNA pathway because tasiRNAs from three TAS loci were reduced in *sde5* mutants. It has been proposed that SDE5 acts together with RDR6 in generating dsRNA from specific ssRNAs. The sequence of SDE5 has similarity with TAP, a human mRNA export factor, and it is suggested that SDE5 could have a role in the transport of ssRNA molecules that will be recruited by RDR6 (Hernandez-Pinzon *et al*., 2007).

3-7. SGS3

SGS3 (Suppressor of Gene Silencing 3, At5g23570), which was identified through a screen for PTGS defective mutant in Arabidopsis, is an RNA stabilizer but its exact role in the different small RNA pathways is not clear. It is thought that SGS3 stabilizes the ssRNA cleavage products which serve as template for RDR6 (SGS2) function and dsRNA production (Mourrain *et al*., 2000; Peragine *et al*., 2004) in the tasiRNA pathway. After miRNA cleavage, the products would be protected against degradation by SGS3 consistent with the findings that a null *sgs3* mutant lacks TAS1, TAS2, and TAS3 tasiRNAs (Adenot *et al*., 2006). Abnormal leaf development such as downward-curled leaf margins is the phenotype of *sgs3* mutant, as also observed in *dcl4*, *rdr6*, and *ago7* mutants. Hypomorphic *sgs3* mutants (like the hypomorphic *rdr6* mutants) are defective in PTGS but not in leaf development and both null and hypomorphic mutants are hypersusceptible to CMV infection (Adenot *et al*., 2006).

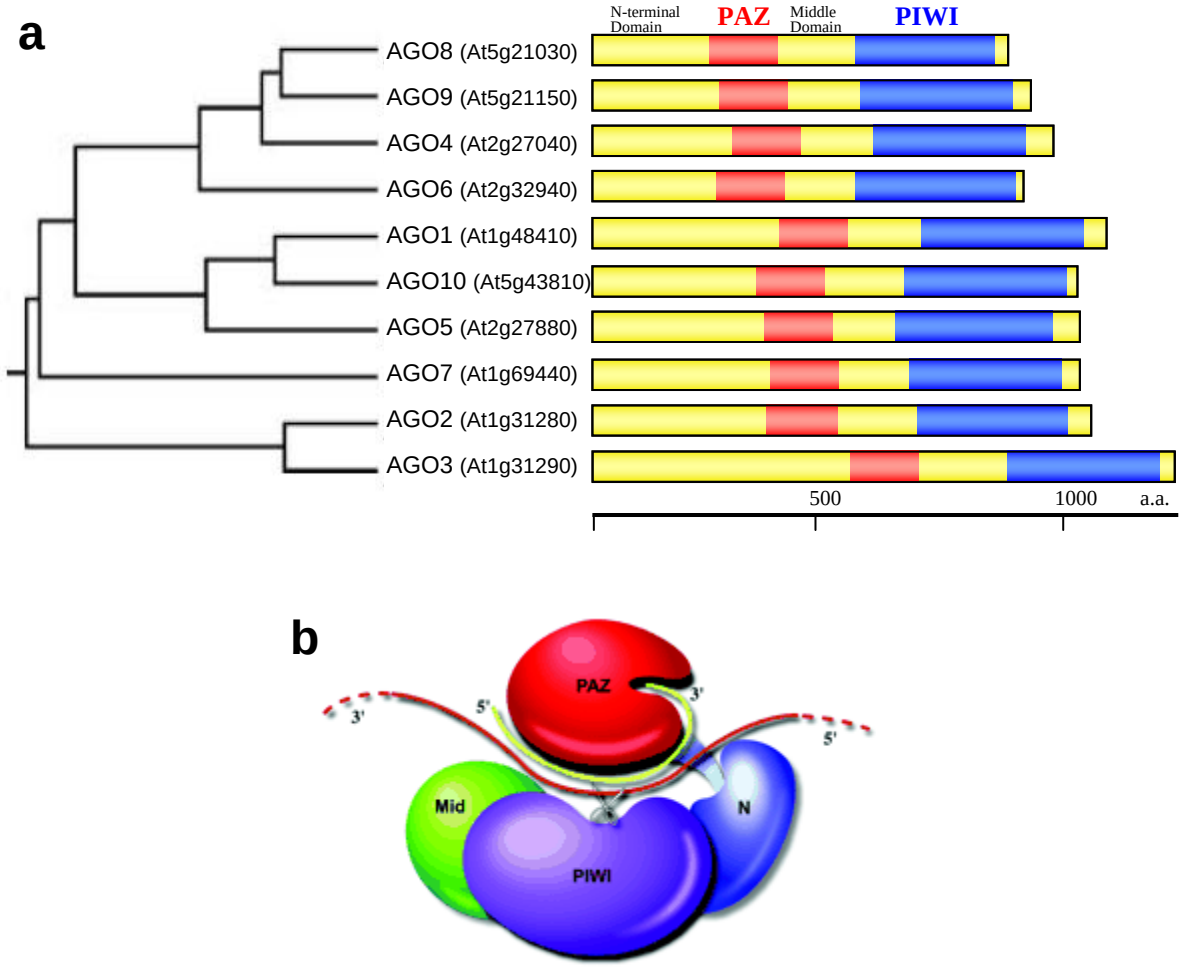


Figure 13: (a) Phylogenetical tree of the ten Arabidopsis ARGONAUTES based on amino acid sequence (Zheng *et al.*, 2007) and their schematic diagram of conserved domains (adapted from Vazquez, 2006). (b) Schematic spatial structure of ARGONAUTE and model for siRNA-guided mRNA cleavage, based on crystal structure of archaebacterium *Pyrococcus furiosus* ARGONAUTE (Song *et al.*, 2004).

3-8. AGO family

The ARGONAUTE (AGO) family in Arabidopsis contains ten conserved members (Figure 13) (Fagard *et al*., 2000; Carmell *et al*., 2002). The importance of the AGO family is established by the fact that AGO proteins have been implicated as effectors in all pathways of RNA silencing and that they are indispensable for development. AGO proteins, also called PPD proteins because they have PAZ (PIWI, ARGONAUTE, AND ZWILLE/PINHEAD) and PIWI domains (Figure 13) highly conserved between different kingdoms. AGO proteins in different organisms have similar characteristics: (1) they are essential components of RISC complexes; (2) they bind small RNAs such as siRNA and miRNA by means of the PAZ domain; and (3) the PIWI domain has an RNaseH-type motif that "slices" a bound target RNA (Baulcombe, 2004, 2005).

3-8-1. Structure and Slicer activity: ARGONAUTES are composed of four domains: the N-terminal, the PAZ, the middle and the PIWI domains (Song *et al*., 2006). The crystal structure of an archaebacterial *Pyrococcus furiosus* (Song *et al*., 2004) AGO homologue revealed a crescent-shaped base made up of the N-terminal, middle, and PIWI domains. The PAZ domain is positioned above the base (Figure 13b). The architecture of the molecule and the placement of the PAZ and PIWI domains define a groove for substrate binding. The PAZ domain is an oligonucleotide binding domain that has a high affinity for the 3' end of ssRNA and duplex siRNA-like ends (with 2nt 3' overhangs). PAZ has a reduced affinity for a duplex with a mononucleotide 3' overhang and reduced or undetectable affinity for a blunt-ended duplex (Song *et al*., 2003; Yan *et al*., 2003). The middle domain has structural homology to the sugar binding domain of lac repressor. The inner surface of the groove is lined with positive charges suitable for interaction with the negatively charged phosphate backbone and the 2'OH moieties of RNA, implicating the groove for substrate binding. The PIWI domain is similar to ribonuclease H, with a C-terminal conserved Aspartate-Aspartate-Glutamate motif (DDE), which is the most conserved region in AGOs. RNaseH enzymes cleave ssRNA guided by the DNA strand in an RNA/DNA hybrid. Similarly, AGOs specialize in RNA cleavage, guided by the siRNA strand in a siRNA-target RNA duplex. In addition, RNaseH enzymes produce products with 3'OH and 5'phosphate groups, in agreement with the properties of products of mRNA cleavage by RISC (Martinez and Tuschl, 2004). A requirement for Mg^{2+} for activity of the AGO PIWI slicer is also conserved for RNAseH (Schwarz *et al*., 2004). These shared attributes between RNaseH and AGO evidently support the idea that the PIWI domain could function similarly during RNA silencing. Mutation analyses of the PIWI DDE motif have confirmed this hypothesis. In Arabidopsis, all ten AGO family members have either a DDH or DDD motif (Histidine may substitute functionally for Aspartic acid), which suggests that all could be active slicers (reviewed by Sontheimer, 2005; Vazquez, 2006; Parker and Barford, 2006; Tolia and Joshua-Tor, 2007).

3-8-2. Arabidopsis AGO1

AGO1 was first identified as a mutant that affected general plant architecture. Strong *ago1* mutants exhibit numerous phenotypic abnormalities such as radialized leaves and abnormal infertile flowers with filamentous structures. The corresponding gene was named ARGONAUTE for the squid tentacle-like appearance of the leaves of mutant plants (Bohmert *et al*., 1998; Lynn *et al*., 1999). Then through a genetic screen Fagard *et al*. (2000) identified *ago1* mutants deficient in S-PTGS. Because *ago1* knockout mutants are sterile, hypomorphic *ago1* mutants that are fertile but defective in PTGS and hypersusceptible to virus infection (CMV) were characterized (Morel *et al*., 2002; Vaucheret *et al*., 2004)**.** Furthers phenotypical observations showed that AGO1 is required for stem cell function, organ polarity and floral meristem identity (Kidner and Martienssen, 2005). *ago1* null mutants, like *dcl1*, *hyl1* and *hen1* mutants, exhibit reduced levels of miRNAs and increased levels of the corresponding uncleaved target mRNAs. In hypomorphic *ago1* mutants Vaucheret *et al*. (2004) reported that uncleaved target mRNA accumulation occurred without a substantial change in miRNA accumulation.

3-8-2-1. Slicer activity of AGO1: The predicted slicer activity of Arabidopsis AGO1 was confirmed by showing that the *in vitro* cleavage of a miRNA target RNA depended on the slicer motif in the PIWI domain. This domain constitutes therefore a predicted RNase catalytic center similar to that described in human AGO2 (Baumberger and Baulcombe, 2005; Qi *et al*., 2005). Cleavage of miRNA target mRNAs was reduced but not abolished in an *ago1* null mutant, indicating that redundant slicer activity exists in Arabidopsis (Ronemus *et al*., 2006).

3-8-2-2. Interaction with miRNA and siRNA: AGO1 protein can directly associate with the miRNA and siRNA. Analysis of miRNA levels in *ago1* mutants has revealed that most miRNAs were unstable in *ago1* null mutants, pointing to a stabilization role of AGO1 (Vaucheret *et al*., 2004). Then, it has shown that purified AGO1 is associated with miRNAs, endogenous tasiRNAs, and transgene-derived siRNAs (either from an IR or sense GFP transgene) but not the 24nt siRNAs produced by DCL3 (siRNA02 and siRNA1003) that are involved in chromatin silencing. Theses observations suggest that miRNA-mediated silencing and transgene silencing both employ AGO1 but that DCL3-mediated processes do not. (Baumberger and Baulcombe, 2005; Qi *et al*., 2005). Baumberger and Baulcombe were unable to detect CMV, TCV or crucifer TMV siRNAs associated with AGO1. More recently, Zhang *et al.* (2006) detected viral siRNAs, derived from CMV or from *Turnip yellow mosaic Tymovirus* (TYMV), in Flag-AGO1 immunoprecipitates recovered from virus-infected Flag-AGO1 plants. Furthermore, *ago1* mutants were hypersensitive to viral infection (Morel *et al*., 2002; Zhang *et al*, 2006) clearly suggesting implication of AGO1 in antiviral pathway. Taken together, its Slicer activity and its interaction with miRNA and siRNA indicate that AGO1 is the cleavage-competent of Arabidopsis RISC.

3-8-2-3. AGO1 homeostasis: *AGO1* and AGO2 are the only members of the AGO family that are known to be regulated by a miRNA, AGO1 by **miR168** (Jones-Rhoades *et al*., 2002) and AGO2 by **miR403** (Allen *et al*., 2005). miR168, like other miRNAs is stabilized by interaction with AGO1 protein.

Moreover, the fact that miR168 also directs the cleavage of AGO1 mRNA indicates that miR168 is involved in a negative feedback loop that regulates the activity of the miRNA pathway. Expression of a miR168-resistant AGO1 gene in Arabidopsis results in developmental defects, suggesting that over-production of AGO1 protein as well as AGO1 deficiency causes defects in the miRNA pathway. These developmental defects were accompanied by accumulation of miRNA-target transcripts like that observed in *ago1* mutants (Vaucheret *et al*., 2004). The large excess of AGO1 protein probably interferes with the function of RISC or sequesters miRNAs or other RISC components resulting in a decrease in miRNA accumulation and an increase in miRNA targets. Therefore, both the absence and the over expression of AGO1 protein results in similar developmental defects and plants have to regulate tightly the synthesis of this protein.

3-8-2-4. Expression and localization of AGO1 in Arabidopsis: AGO1 is expressed throughout the plant at all stages of development (Bohmert *et al*., 1998). Interestingly, the *AGO1* and *MIR168* genes are co-regulated transcriptionally. Thus, the expression of a *GUS* reporter gene under control of the *AGO1* or *MIR168a* promoters revealed that *AGO1* and *MIR168* genes have an identical expression pattern (measured by *GUS* reporter gene under control of *AGO1* or *MIR168* promoters) and are expressed in all tissues and organs, especially in the shoot and root apical meristems, which are undergoing active development. Expression also was detectable in vascular tissues of leaves and roots (Vaucheret *et al*., 2006). In terms of its subcellular localization, AGO1 was observed in both the nucleus and the cytoplasm (Fang and Spector, 2007).

3-8-3. AGO4

AGO4, which is implicated in the small RNA-mediated DNA methylation pathway, was first identified because its mutation resulted in partial suppression of epigenetic silencing at the Superman locus (Zilberman *et al*., 2003). This was followed by reports that *AGO4* is required for maintenance of DNA methylation at several endogenous loci. Mutation in *AGO4* substantially reduces the maintenance of DNA methylation triggered by IR transgenes, but *ago4* loss-of-function does not block the initiation of DNA methylation by IR. The *ago4* mutations strongly decrease non-CG methylation but only slightly affect CG methylation (Zilberman *et al*., 2004).

Maintenance of the heterochromatic state involves Pol IV function followed by siRNA production and assembly of silencing complex containing AGO4 and NRPD1b (Pontes *et al*., 2006). AGO4 interacts with the C-terminal domain of NRPD1b and localizes to distinct bodies in the nucleolus (Li *et al*., 2006; Pontes *et al*., 2006). It is proposed that AGO4 can function at target loci through two distinct and separable mechanisms. First, AGO4 can recruit DNA methylation components in a manner independent of its catalytic activity. Second, through the catalytic activity of AGO4, secondary siRNAs are generated to reinforce silencing by methylation (Qi *et al*., 2006).

3-8-4. AGO6

It has been reported that AGO6 has a role in the accumulation of specific chromatin-related siRNAs, in DNA methylation and transcriptional gene silencing but does not have a substantial effect on production of miRNA and of siRNA from inverted repeat transgenes. Its function is partly redundant with AGO4. The *ago6* mutation reduces the accumulation of heterochromatin-related siRNAs from transgene and endogenous loci. Analysis of cytosine methylation at several endogenous loci revealed that the levels of CNG methylation and asymmetric methylation are lower in both the *ago6* and *ago4* single mutants relative to the wild type and the levels are even the lower in the *ago6ago4* double mutant (Zheng *et al*., 2007). AGO6 protein is mainly localized in nuclei, although low amounts are also present in the cytoplasm. AGO6 is strongly expressed in roots and cotyledons, very weakly in young leaves and is not detectable in floral tissues (Zheng *et al*., 2007).

3-8-5. AGO7

AGO7 or ZIPPY (ZIP) functions in the regulation of developmental timing of the juvenile-to-adult transition and is needed for the TAS3 tasiRNAs pathway. This gene was initially identified in a screen for mutations that cause a premature transition to the adult state. AGO7 plays little or no role in S-PTGS (Hunter *et al.*, 2003, 2006; Adenot *et al*., 2006). Fahlgren *et al*. (2006) showed that the juvenile-to-adult phase transition is normally suppressed by TAS3-tasiRNAs, in an AGO7-dependent manner, through negative regulation of ARF3 mRNA. Therefore, in *ago7* mutants, ARF3 transcript levels are not down-regulated by tasiRNA and the juvenile-to-adult transition is accelerated. Destabilization of TAS3-tasiRNAs has also been observed in *ago7* mutants (Fahlgren *et al*., 2006; Adenot *et al*., 2006).

3-8-6. AGO10

AGO10 or PINHEAD/ZWILLE (PNH/ZLL) plays a critical role in maintaining undifferentiated stem cells in the shoot apical meristem, but was found not to participate in PTGS (Lynn *et al*., 1999). Defects in meristem formation and abnormal embryos in *pnh* mutants have been observed. Particularly, high levels of expression of AGO10 were detected in the developing vascular system (Moussian *et al*., 1998; Lynn *et al*., 1999; Morel *et al*., 2002). The functional roles of other AGO proteins in Arabidopsis remain to be determined.

3-8-7. Redundancy

It is likely that the diversification of RNA silencing mechanisms in Arabidopsis is linked to the multiplicity of members of the AGO and DCL families. As observed for the DCLs, functional redundancy probably also occurs in the AGO family. Up to now, among the ten AGO proteins only AGO1 has been unambiguously implicated in the miRNA and PTGS pathways. Because *dcl1* null mutants lacking this essential enzyme for miRNA biogenesis are not viable, it might be expected that plants lacking a functional AGO1 are not viable either. However, *ago1* null mutants are viable, although they exhibit dramatic developmental defects. This viability is probably a consequence of partial functional

complementation by other AGO proteins, in particular AGO10 which has 75% similarity with AGO1 and has a pattern of expression like AGO1 (Lynn *et al*., 1999). Lynn *et al*., (1999) reported that plants homozygous for an *ago10* null mutation and heterozygous for an *ago1* null mutation exhibited developmental defects stronger than those of single homozygous *ago10* mutants, whereas plants homozygous for an *ago1* null mutation and heterozygous for an *ago10* null mutation showed developmental defects stronger than those of single homozygous *ago1* mutants. In addition, plants homozygous for both *ago1* null and *ago10* null mutations died at the embryo stage, indicating the important roles of these two proteins in plant development. As mentioned above, AGO1 and AGO10 are both required for stem cell function and miRNA-guided determination of organ polarity (Kidner and Martienssen, 2004, 2005).

There is also evidence that more than one AGO protein is associated with tasiRNA-guided cleavage. As mentioned above, the targets of tasiRNA-ARF accumulate in *ago7* mutant, suggesting that AGO7 has slicer activity for this particular target but that another AGO protein(s) provides the slicer functions associated with tasiRNAs derived from the other TAS loci (reviewed by Vazquez, 2006).

Introduction Part 3: **Viral Suppressor of Silencing**

Not surprisingly, the development of RNA silencing as an antiviral defense system has resulted in viruses evolving various strategies to overcome this defense strategy. The mechanisms include:

a. Evasion of RNA silencing. Some viruses localize and replicate in subcellular sites that are not exposed to the RNA silencing machinery. For example, replication of *Brome mosaic Bromovirus* occurs in membrane-bound vesicles, keeping viral RNAs away from host ribonucleases (Schwartz *et al*., 2002). Similarly, the chloroplastic replication of *Avsunviroidae* probably protects them from silencing (Tabler *et al*., 2004).

b. Protection of the viral genome from silencing. Viroids have apparemtly evolved an RNA secondary structure that is resistant to silencing. Although their genomes are substrates for Dicer, viroid sequences are inaccessible to the RISC (Wang *et al*., 2004). Protection of viral genomes might also result from their association with proteins. For example, encapsidation protects viral genomes from silencing (Angell and Baulcombe, 1997). Finally, an umbraviral protein (product of ORF3) interacts with viral RNA to form cytoplasmic filamentous ribonucleoprotein (RNP) complexes that protects the viral RNA (as well as being implicated in movement of virus has been reported (Taliansky *et al*., 2003).

c. Overwhelming silencing. It is believed that some viruses may replicate and spread at such high rates than the defensive capacity of the RNA silencing machinery at the cellular and/or systemic levels can simply not keep up.

d. Silencing suppression. The majority of viruses have evolved suppressor proteins of silencing which are encoded by the genomes of both RNA and DNA viruses. These proteins evolved independently in different virus groups and are structurally diverse and have no sequence similarity. Some viruses are also suspected to recruit a host suppressor protein to their own benefit (reviewed by Voinnet, 2005a). The first discovered viral silencing suppressor was the potyvirus Helper Component Proteinase (**HCPro**). Preliminary experiments about the synergism in co-infections with potyviruses and potexviruses led to the identification of HCPro as the synergism determinant (Pruss *et al*., 1997) and it was proposed that HCPro suppresses a host defense system. Based on the finding that PVX induces RNA silencing in hosts (Ruiz *et al*., 1998), the possibility emerged that HCPro could act as a suppressor of RNA silencing. This hypothesis was subsequently verified independently and, in the course of these experiments, the 2b protein of *Cucumber mosaic Cucumovirus* (CMV) was also identified as a silencing suppressor (Brigneti *et al*., 1998; Anandalakshmi *et al*., 1998; Kasschau and Carrington, 1998). A key to the identification of new silencing suppressors came from the realization that HCPro and 2b had been previously characterized as pathogenicity determinants that are not strictly required for viral replication but are needed for efficient accumulation of virus in plant (Brigneti *et al*., 1998). Re-investigation of

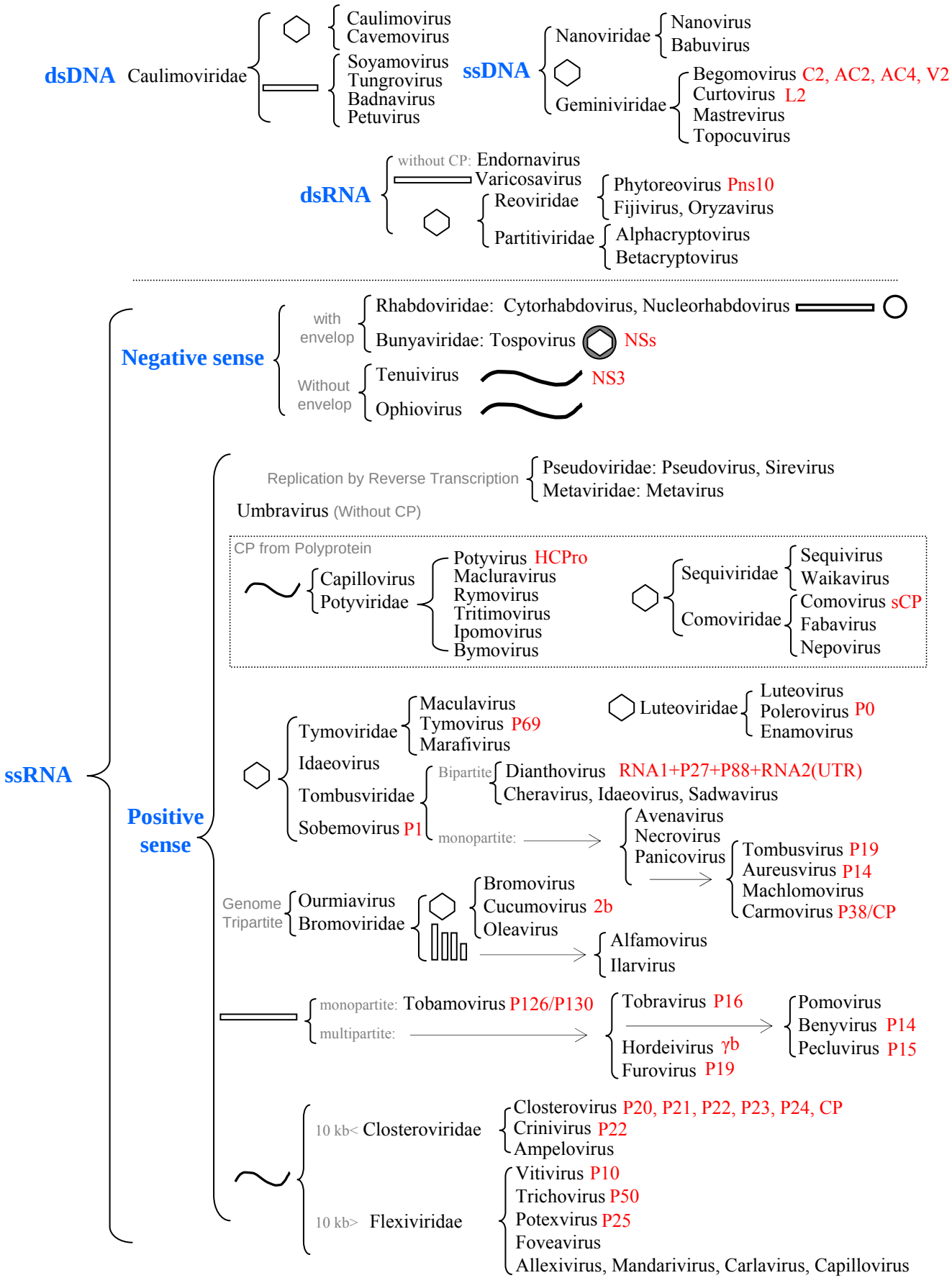


Figure 1: Classification of Plant viruses (according to VIII[th] report of ICTV) showing known silencing suppressors (in red).

pathogenicity determinants from diverse viruses revealed that many are indeed silencing suppressors (Voinnet *et al*., 1999) and since then, by using diverse methods, many plant viruses have been shown to encode one or more suppressors of silencing (Figure 1).

1. Methods for identification of suppressor proteins

Different methods have been used to identify plant viral suppressors of silencing (reviewed by Moissiard and Voinnet, 2004; Li and Ding, 2006). These methods are reported below:

1-1. Agrobacterium-mediated transient suppression assay (Patch Test): In this transient assay, the candidate gene and a construct that triggers RNA silencing of a stably integrated reporter transgene (such as GFP) are cloned separately in binary vectors and then transferred to Agrobacterium. Then Agrobacterium cultures are co-infiltrated in the transgenic reporter (GFP) plant. If the candidate protein is not a silencing suppressor, the reporter transgene will be silenced. If the candidate protein, however, is a suppressor of silencing, the transgene degradation will be impaired and the reporter will be expressed. Coinfiltration is the most popular assay used in the identification of suppressor proteins because it is simple and fast.

1-2. Reversal of a silenced transgenic reporter gene: This system involves a cross between a silenced transgenic plant (such as a constitutively GFP-silenced plant) and a second transgenic plant expressing a candidate silencing suppressor protein. Alternatively, the candidate protein can be expressed from a heterologous viral vector inoculated on the silenced transgenic plant. If the candidate protein is a suppressor of silencing, the reporter gene (such as GFP) may be expressed.

1-3. Heterologous complementation of a suppressor protein: In this system the gene for the candidate silencing suppressor protein is cloned in the place of a known suppressor of silencing in its viral context. If the candidate protein complements functionally the suppressor, it is considered as a silencing suppressor protein.

1-4. Expression of suppressor by another virus resulting in enhanced symptom: In this method, the candidate protein is expressed from a harmless viral vector and if the candidate protein is a suppressor of silencing a dramatic increase in symptoms usually appears. The favorite viruses used in these assays are PVX and TRV.

1-5. Grafting: Grafting experiments allow identification of suppressors that block spread of silencing signals and systemic silencing. This assay uses a transgenic plant that carries a silenced reporter transgene (for example, GFP plus a GFP hairpin) which can silence a reporter gene (GFP) in the scion. Plants stably expressing a candidate protein are crossed with the silenced reporter transgenic plant. Whether or not expression of the candidate protein suppresses spread of the silencing signal can be determined by grafting reporter scions onto rootstocks made from the progeny of crossed plants.

Table 1: Animal viral silencing suppressor proteins: (reviewed by Li and Ding, 2006)

Virus	Genome	Suppressor	Ref
Poxvirus: vaccinia virus	dsDNA	E3L	(Li et al., 2004)
Adenovirus	dsDNA	VA1 RNA	(Lu and cullen, 2004)
Reovirus	dsRNA	σ3	(Lichner et al., 2003) (Yue and Shatkin, 1997)
Orthomyxovirus: Influenza virus A, B and C	-sRNA	NS1	(Li et al., 2002, 2004) (Delgadillo et al., 2004) (Bucher et al., 2004)
Orthobunyavirus:La Crosse virus	-sRNA	NS1	(Soldan et al., 2005)
Lentivirus: HIV-1	retrovirus	Tat	(Bennasser et al., 2005)
Spumavirus: Primate foamy virus type 1(PFV-1)	retrovirus	Tas	(Lecellier et al., 2005)
Nodavirus: Flock house virus, Nodamura virus, Striped jack nervous necrosis virus, Greasy grouper nervous necrosis virus	+sRNA	B2	(Li et al., 2002, 2004) (Fenner et al., 2006) (Iwamoto et al., 2005)

Table 2: Several suppressors of silencing and their effects on the miRNA pathway

Suppressor of silencing	Accumulation of miRNA	Accumulation of miRNA*	Upregulation of miRNA-targeted mRNAs	Coimmunoprecipitation with miRNA and siRNA
P19	+	+	+	+
HcPro	+	+	+	-
P21	+	+	+	+
P69	+		-	
P15	-	-	+	
2b	+	+	+	
P38	-	-	-	
P25	-	-	-	

Ref: Chapman *et al*., 2004; Chen *et al*., 2004; Dunoyer *et al*., 2004; Zhang *et al*, 2006; Li and Ding, 2006

Table 3: Suppressors of silencing which have been shown to bind dsRNA (Merai *et al*., 2006)

Virus	VSP	dsRNA binding selectivity
Turnip crinkle Carmovirus (TCV)	P38	without size selection
Pothos latent Aureusvirus (PoLV)	P14	without size selection
Peanut clump Pecluvirus (PCV)	P15	size-specific (21 nt)
Barley stripe mosaic Hordeivirus (BSMV)	γb	size-specific
Beet yellows Closterovirus (BYV)	P21	size-specific
Tobacco etch Potyvirus (TEV)	HcPro	size-specific
Tomato bushy stunt Tombusvirus (TBSV)	P19	size-specific (21 nt)
Flock house virus (FHV)	B2	without size selection

2. Modes of action of suppressor proteins

The known viral suppressor proteins are diverse in sequence and structure, probably as a consequence of independent events of co-evolution between different viruses and hosts. They act via different mechanisms at different stages of silencing pathway (Voinnet *et al*., 1999; Voinnet, 2005a). Several known suppressors suppress RNA silencing in both animal and plant cells, regardless of the host origin of the virus (Dunoyer *et al*., 2004; Lakatos *et al*., 2004, 2006). Silencing suppressor proteins have also been reported for animal viruses (Table 1). Among them, B2 of FHV, NS1 of influenza virus, E3L of vaccinia virus, and Tas of PFV-1 efficiently suppress RNA silencing in plant systems. Among plant viruses, P19 of TBSV, HCPro of TEV, P21 of BYV, P38 of TCV, P15 of PCV and NS3 of RHBV have silencing suppression activities in animal cell cultures, suggesting that RNA silencing is a defense mechanism conserved in both animals and plants, and that these suppressor proteins target important conserved components of the silencing pathway. Several studies have shown that transgenic expression of silencing suppressors can alter the accumulation and/or functioning of miRNAs (Table 2) leading to developmental abnormalities related to the loss of function of miRNAs. Indeed, some of the symptoms resulting from virus infection are probably the consequence of perturbation of the miRNA pathway. This finding emphasizes the overlap between the siRNA and miRNA pathways and represents evidence that suppressors direct the common component between the two pathways.

The molecular mechanism of suppression and modes of action of different suppressors of silencing has been the subject of much research in the last few years. It has been suggested that dsRNA binding is a general plant viral silencing suppression strategy because many diverse silencing suppressor proteins (recapitulated in Table 3) show affinity to dsRNA (Merai *et al*., 2006; Lakatos *et al*., 2006). However, recent findings show that other modes of action have been developed by virus like the 2b of CMV, P38 of TCV and AC2 of Begomoviruses.

Here I summarize what we have learned about mechanisms of silencing suppression up to this time (see also Table 4 and Figure 5). The mechanisms of action of plant silencing suppressor proteins can be classified as follows:

a. suppression by dsRNA binding activity (P19, P21, HCPro and P15)
b. suppression by inhibiting DCL function (P38)
c. suppression by inhibiting AGO1 activity (2b)
d. suppression by transactivation of host genes (AC2)
e. other examples of silencing suppression of plant viral proteins (P25, NS3, combination of proteins and RNAs)

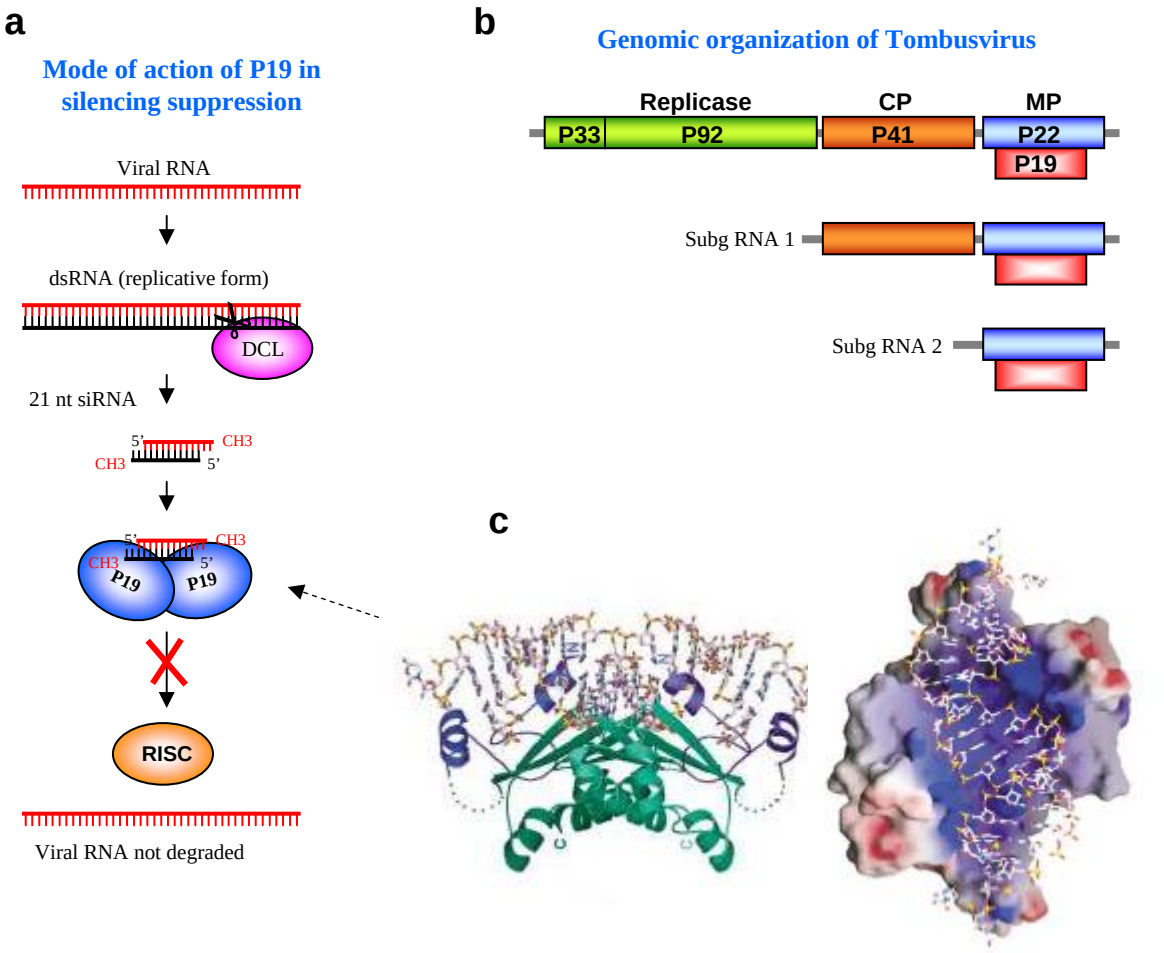


Figure 2: (a) The mechanism of action of P19 in suppression of silencing by binding and sequestering siRNA so as to prevent them from being loaded to RISC. (b) Genomic organization of Tombusvirus ~4.8 kb positive-sense ssRNA. P19 is expressed from subgenomic 2 by leaky scanning of the first AUG. (c) crystal structure of P19 dimer shows binding to 21 nt siRNA (Vargason *et al*., 2003).

3. Suppression by dsRNA binding activity

3-1. Tombusvirus encoded P19

P19 from a number of Tombusviruses have been studied including *Tomato bushy stunt virus* (TBSV), *Cymbidium ringspot virus* (CymRSV) and *Carnation italian ringspot virus* (CIRV). The role of P19 as a silencing suppressor was first suggested by its role as a symptom determinant (Dalmay *et al*., 1993). Indeed, P19 is required in a host-dependent manner for viral infection (Scholthof *et al*., 1995b), is an elicitor of the hypersensitive response (HR) on *N. tabacum*, and induces a systemic necrosis in *N. benthamiana* (Scholthof *et al*., 1995a). Later it was shown directly in a Patch Test that P19 suppresses RNA silencing (Voinnet *et al*., 1999). Gel mobility shift assays showed that the P19 protein of CymRSV binds to chemically synthesized 21nt dsRNA with 2nt 3′ overhanging ends, a characteristic of siRNAs (Silhavy *et al*., 2002). P19 could not reverse silencing once it had been established but did prevent its onset in upper tissues (Qiu *et al*., 2002), suggesting that P19 impairs the systemic movement of the silencing signal. P19 expressing plants showed developmental abnormalities, increased miRNA and miRNA* accumulation and upregulation of miRNA-targeted mRNAs (Dunoyer *et al*., 2004; Chapman *et al*., 2004). Indeed, it has been shown that P19 co-precipitate with miRNA duplex.

P19 is a cytosolic protein and is exclusively present as a dimer (reviewed by Scholthof, 2006). The crystal structure of the CIRV P19 protein (Vargason *et al*., 2003; Ye *et al*., 2003) revealed that protein dimerization is required for binding to 21nt siRNA. On the other hand, the 2nt, 3' overhangs of the siRNA are not necessary for high-affinity binding, but binding is enhanced with 5' phosphate group (Figure 2c). The length of the duplex region of the siRNA is critical for high-affinity binding. In the structure of the complex, p19 forms a homodimer that binds one face of the RNA duplex through interactions with a phosphate backbone and 2' hydroxyl groups in a non-sequence specific manner. In addition, critical tryptophan residues projecting from symmetrically positioned helices were found to cap both ends of the 19 bp duplex, providing a caliper-like mechanism for measuring duplex length. The affinity dramatically decreases if the dsRNA segment is either shorter or longer than 19 bp. P19 also has no affinity for ssRNA or dsDNA (Lakatos *et al*., 2004). Therefore, P19 functions by specifically binding and sequestering siRNAs from being incorporated into an active RISC (Figure 2a). Recently it was confirmed that P19 is a dsRNA-binding protein that interacts physically with siRNA duplexes *in vitro* as well as *in vivo*; P19 inhibits initiation of RISC assembly but it cannot interfere with preassembled RISC activity (Lakatos *et al*., 2006).

P19 not only suppresses silencing in viral hosts but also in Arabidopsis, which is not a host for TBSV (Dunoyer *et al*., 2004; Chapman *et al*. 2004), insect cells (Li *et al*., 2004), human cells (Dunoyer *et al*., 2004; Lecellier *et al*., 2005), *C. elegans* (Lu *et al*., 2005), and even in a *Drosophila*-derived *in vitro* system (Lakatos *et al*., 2004).

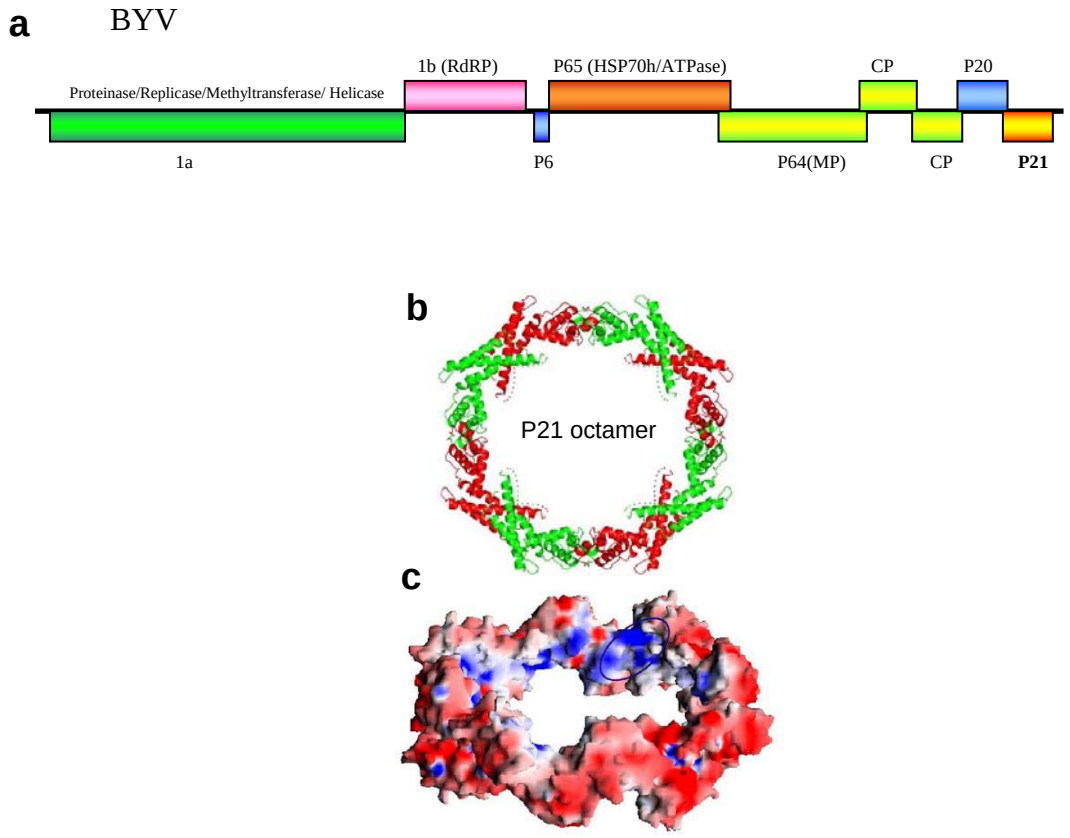


Figure 3: (a) genome organization of Beet Yellow Closterovirus ~20 kb positive-sense ssRNA. (b) Schematic representation of P21 crystal structure. P21 octamer creates a ring. (c) The inner surface of the ring contains positive charge (blue), which could bind to RNA. Red color presents negative charge on the outside of the ring (Ye and Patel, 2005).

In situ hybridization experiments revealed that expression of P19 allowed the virus to exit the vascular bundles and invade the surrounding tissues and beyond in systemically infected leaves (Havelda *et al*., 2003). siRNA sequestering by P19 may prevent the viral 21nt siRNAs from entering the vasculature in the inoculated leaves and/or exiting the vasculature in the first systemically infected leaves (reviewed by Li and Ding, 2006). Interestingly, P19 specifically interacts with members of a multigene-family known as ALY proteins. These proteins are RNA-processing factors that serve as transcriptional cofactors and are involved in subsequent RNA transport. ALY proteins accumulate predominantly in the nucleus, but upon TBSV infection or heterologous expression of P19 they are relocalized to the cytosol (Uhrig *et al*., 2004; Park *et al*., 2004). However, there seems to be no correlation between this interaction and silencing suppression.

3-2. Closterovirus encoded suppressors

Beet yellows Closterovirus (BYV), encodes P21 which is the only one among its nine encoded proteins that suppresses RNA silencing in an IR-PTGS assay (Reed *et al*., 2003) (Figure 3a). Conversely, the related *Citrus tristeza Closterovirus* (CTV) encodes at least three suppressors, P20, P23 and CP (P25) (Lu *et al*., 2004), P23 being a homolog of BYV P21. A gel mobility shift assay showed that P21 binds siRNA and miRNA duplexes *in vitro* and in transgenic plants, P21 was found to co-immnuoprecipitate with miRNAs and siRNAs (Chapman *et al*., 2004; Merai *et al*., 2006; Lakatos *et al*., 2006; Yu *et al*., 2006). P21-expressing plants exhibited developmental abnormalities, miRNA and miRNA* over-accumulation and miRNA-targeted mRNA upregulation (Chapman *et al*., 2004). The crystal structure (Ye and Patel, 2005) showed that P21 forms an octameric ring (Figure 3 b) whose inner surface might be involved in RNA binding (Figure 3c). It has also been shown that P21, unlike P19, has no strict binding specificity for siRNA duplexes. However, recent evidence indicates that P21, like P19, is a dsRNA-binding protein that interacts physically with siRNA duplexes *in vivo* as well as *in vitro*. It inhibits initiation of RISC assembly by siRNA sequestration but cannot impair preassembled RISC activity (Lakatos *et al*., 2006). It was also reported that in transgenic plants expressing P21, P19 and HCPro, the levels of unmethylated miRNA and miRNA* were more elevated than in wild-type plants, suggesting that these proteins interfere with methylation of miRNAs (Yu *et al*., 2006). The suppressors may compete with HEN1 for miRNA/miRNA* duplex substrates and sequestration by the suppressors could impede HEN1 from interacting with the duplexes or otherwise prevent HEN1 access to the 2' OH of the 3' terminal nucleotide.

3-3. Potyvirus encoded HCPro

The RNA genome of potyviruses (figure 4a) is translated into a polyprotein that is further processed by three virus-encoded proteinases (Carrington *et al*., 1990). One of these proteinases, the helper component proteinase (HCPro), is a multifunctional protein that acts as a strictly *cis* acting proteinase for its self-

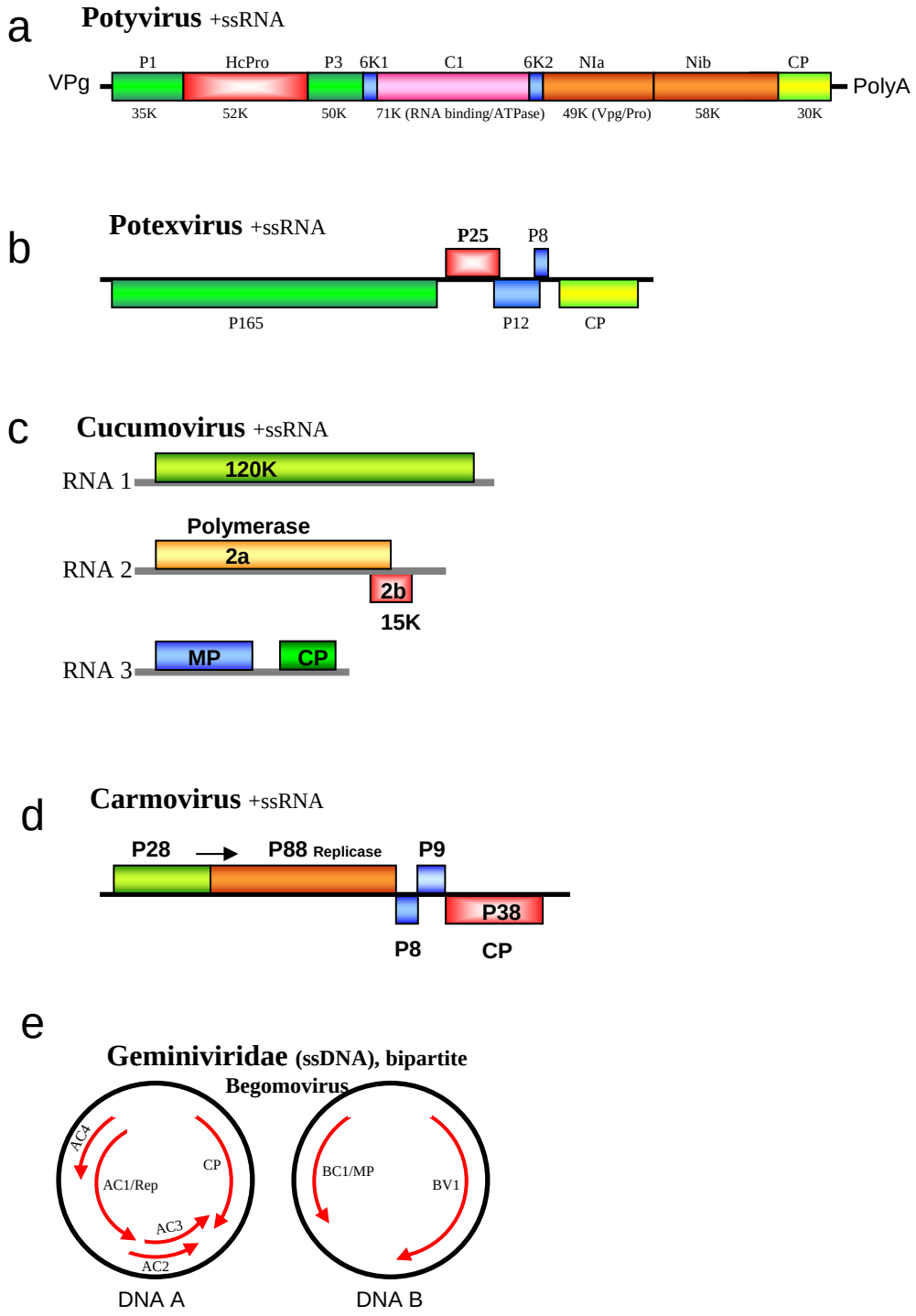


Figure 4: Genome organization of a Potyvirus (a), Potexvirus (b), Cucumovirus (c), Carmovirus (d) and Begomovirus (e).

cleavage from the polyprotein precursor (Carrington and Herndon, 1992). It is also involved in a number of infectious processes as aphid transmission, cell-to-cell and long distance movement (Maia *et al*., 1996; Syller, 2006). Initial work showed that HCPro enhances the replication of many unrelated viruses (Pruss *et al*., 1997; Kasschau *et al*., 1997). The discovery of RNA silencing prompted the idea that HCPro could suppress silencing, which was subsequently confirmed in tobacco for PVY HCPro (Brigneti *et al*., 1998) and for *Tobacco etch Potyvirus* (TEV) HCPro (Anandalakshmi *et al*., 1998; Kasschau and Carrington, 1998). Indeed, HCPro was the first viral protein identified as a suppressor of RNA silencing that blocks both virus-induced and transgene-induced RNA silencing. Expression of HCPro in tobacco (*N. tabacum*) and Arabidopsis increases miRNA and miRNA* accumulation, prevents the cleavage of miRNA targets, and induces developmental defects that partly resemble those of *dcl1* mutants (Mallory *et al.*, 2002; Kasschau *et al*., 2003; Dunoyer *et al*., 2004; Chapman *et al*., 2004).

Considerable study has been carried out aimed at understanding the mechanism of HCPro in suppression silencing but up to now, a clear relation between different results could not be established and a precise mechanism has not been reported. In particular somewhat contradictory results have been reported concerning HCPro's miRNA and siRNA binding activity, its ability to reduce accumulation of some classes of siRNAs and its interference with dsRNA processing, RISC activity and siRNA methylation.

A yeast two hybrid screen of tobacco proteins using TEV-HCPro as bait identified the interacting protein, rgsCaM (regulator of gene silencing calmodulin-like protein) (Anandalakshmi *et al*., 2000). Expression of rgs-CaM is induced in leaves of *N. tabacum* when HCPro is expressed, either from a transgene or by infection with a virus that encodes HCPro. Surprisingly, transient or transgenic over-expression of rgsCaM was shown to suppress silencing, indicating that rgsCaM may act as an endogenous suppressor of silencing presumably via a calcium-dependent regulatory pathway (rgsCaM has a C-terminal domain containing a calcium-binding motif). Whether an *in vivo* interaction occurs between rgsCaM and HCPro remain to be tested.

Interestingly, HCPro of *Lettuce mosaic Potyvirus* (LMV) has been reported to interact with the 20S proteasome *in vitro* and viral infection causes aggregation of the 20S proteasome to high molecular mass structures *in vivo*. HCPro inhibits also the putative 20S protein endonuclease activity *in vitro* but does not change its proteolytic activity. This finding may represent the existence of a novel mechanism of action for HCPro (Ballut *et al*., 2005). For the present, a model for the mechanism of HCPro as a silencing suppressor which encompasses all of the aforesaid findings has not been devised.

Table 4: Plant viral suppressors of silencing (reviewed by Voinnet, 2005a; Soosaar *et al*., 2005; Li and Ding, 2006)

Virus	Suppressor	characteristics and mechanism of suppression	Ref
ssDNA viruse (Geminiviruses)			
Beet curly top Curtovirus	L2	Interaction with and inactivation of Adenosine kinase (ADK)	(Wang et al., 2003, 2005)
Tomato yellow leaf curl Begomovirus	AC2	Interaction with and inactivation of Adenosine kinase (ADK)	(Wang et al., 2003, 2005)
Tomato yellow leaf curl Begomovirus	AC2	NLS	(Dong et al., 2003) (van Wezel et al., 2002)
Mungbean yellow mosaic Begomovirus	AC2	NLS, Zinc finger, DNA binding activity Transactivator of viral and cellular genes, miRNA binding	(van Wezel et al., 2002, 2003) (Trinks et al., 2005)
African cassava mosaic Begomovirus	AC2	Transactivator of viral and cellular genes miRNA binding	(Voinnet et al., 1999) (Trinks et al., 2005)
African cassava mosaic Begomovirus(CM) Srilankan cassava mosaic Begomovirus East African cassava mosaic Begomovirus (CM) Indian cassava mosaic Begomovirus	AC4 AC4 AC2 AC2		(Vanitharani et al., 2004)
Tomato Yellow Leaf Curl Begomovirus (Is)	V2	Target downstream of Dicer	(Zrachya et al., 2007)
satellite associated with Tomato Yellow Leaf Curl Begomovirus Y10 china isolate or with Tobacco Curly Shoot Virus Y35 isolate	βC1	DNA binding activity and NLS	(Cui et al., 2005)
satellite DNAB02 of Tomato leaf curl Java Begomovirus (ToLCJAV)	βC1		(Kon et al., 2007)
dsRNA viruses			
Rice dwarf Phytoreovirus	Pns10	suppresses local and systemic S-PTGS but not IR-PTGS	(Cao et al., 2005)
Negative strand RNA viruses			
Tomato spotted wilt Tospovirus	NSs	suppresses S-PTGS but not IR-PTGS	(Bucher et al., 2003) (Takeda et al., 2002)
Rice hoja blanca Tenuivirus	NS3	Binding to siRNA and miRNA	(Bucher et al., 2003) (Hemmes et al., 2007)
Positive ssRNA viruses			
Tobacco etch Potyvirus Potato Y Potyvirus Turnip yellow mosaic Potyvirus	HcPro	dsRNA binding, reduction of 21nt siRNA, interferes with siRNA and miRNA methylation	(Brigneti et al., 1998) (Kasschau et al., 1998) (Anadalakshmi et al., 1998)
Cucumber mosaic Cucumovirus Tomato aspermy Cucumovirus	2b	Blocks silencing signal, Interacts with and inhibits AGO1	(Brigneti et al., 1998) (Zhang et al., 2006)
Tomato bushy stunt Tombusvirus Cymbidium ringspot Tombusvirus Carnation italian ringspot Tombusvirus	P19	Binds to and sequesters 21nt siRNA and miRNA duplex and blocks spread of silencing signal, enhances RNA accumulation	(Voinnet et al., 1999)
Rice yellow mottle Sobemovirus	P1	Required for viral long-distance movement	(Voinnet et al., 1999)
Cocksfoot mottle Sobemovirus	P1		(Sarmiento et al., 2007)
Potato virus X (Potexvirus)	P25	Blocks silencing signal from spreading	(Voinnet et al., 2000)
Beet western yellows Polerovirus Cucurbit aphid-borne yellows Polerovirus	P0	???	(Pfeffer et al., 2002)
Barley yellow mosaic Hordeivirus (Barley stripe mosaic Virus)	γb	Size specific RNA binding (Cysteine-rich protein)	(Yelina et al., 2002) (Donald et al., 1996) (Merai et al., 2006)
Beet necrotic yellow vein Benyvirus	P14	Weak suppressor of silencing	(Dunoyer et al., 2002)
Beet yellows Closterovirus	P21	Binds to and sequestrates of 21nt siRNA and miRNA duplex, enhances RNA accumulation	(Reed et al., 2003) (Lu et al., 2004)
Citrus tristeza Closterovirus Beet yellow stunt Closterovirus (P22)	CP P20 P23	P20 and P23 suppress intracellular silencing (but CP does not) P20 and CP suppress intercellular silencing (export of the silencing signal), P23: RNA binding protein	(Lu et al ., 2004)
Grapevine leafroll-associated Closterovirus -2	P24		(Chiba et al., 2006)
Peanut clump Pecluvirus	P15	Binds ds- 21nt siRNA, upregulates some cellular mRNA, Dimerization (Cysteine-rich protein)	(Dunoyer et al., 2002, 2004) (Merai et al., 2006)
Tobacco rattle Tobravirus	16K	Cysteine-rich protein	(Liu et al., 2002)
Tobacco mosaic Tobamovirus Tomato mosaic Tobamovirus	P126 P130		(Kubota et al., 2003) (Ding et al., 2004) (Liu et al., 2005).
Turnip crinkle Carmovirus	P38	Coat protein, target DCL4 and DCL2	(Thomas et al., 2003) (Qu et al., 2003)
Cowpea mosaic Comovirus	S protein	Small coat protein	(Liu et al., 2004)
Turnip yellow mosaic Tymovirus	P69	Increases accumulation of miRNAs and DCL1 mRNA and increases miRNA-guided cleavage, prevents host RDR-dependent secondary dsRNA synthesis	(Chen et al., 2004)
Pothos latent Aureusvirus	P14	Suppresses S- and IR-PTGS has dsRNA binding activity (sequesters both long dsRNA and siRNA without size specificity)	(Merai et al., 2005, 2006)
Red clover necrotic mosaic Dianthovirus	RNA1+P27+P88+ 3'UTR of RNA2	Combination of RNAs and proteins acts as suppressor	(Takeda et al., 2005)
Sweet potato chlorotic stunt Crinivirus	P22 and RNaseIII	RNaseIII encoded by virus enhances suppressor activity of P22. Accumulation of siRNA is reduced.	(Kreuze et al., 2005)
Wheat soil-borne mosaic Furovirus	19K	Cysteine-rich protein	(Te et al., 2005)
Grapevine virus A (Vitiviruses)	P10	Suppression of local and systemic silencing, Binds to ss- and ds- siRNA and miRNA	(Zhou et al., 2006) (Chiba et al., 2006)
Apple chlorotic leaf spot Trichovirus	P50	suppressor of systemic silencing	(Yaegashi et al., 2007)

3-4. Pecluvirus encoded P15

Peanut clump Pecluvirus (PCV) encodes P15, a small cysteine-rich protein. Its deletion results in a significant decrease in accumulation of progeny viral RNA. Because the protein does not co-localize with the sites of viral replication, it was postulated that its indirect effect on PCV accumulation could result from suppression of a host defense system. Further experiments revealed that P15 is indeed a suppressor of RNA silencing (Dunoyer *et al*., 2002). P15 is targeted to peroxisomes in infected cells, owing to a C-terminal consensus SKL motif. Deletion of this motif did not alter the suppression of silencing mediated by P15 but decreased intercellular virus movement. The C-terminal region of P15 also contains four successive heptad sequences that are typical of coiled-coil-forming proteins. It was confirmed that P15 can self-associate *in vitro.* Point mutations affecting this putative coiled-coil domain abolished suppression of silencing by P15 (Dunoyer *et al*., 2002), suggesting that dimerization of P15 is necessary for silencing suppression. Recently it has been reported that P15 binds 21nt siRNA and miRNA duplexes in a size-specific manner (Merai *et al*., 2006).

4. Suppression by inhibiting DCL function: TCV encoded P38

Turnip crinkle Carmovirus (TCV) **coat protein** (**P38**) (Figure 4d) has several important roles in virus-host interactions. TCV P38 is needed for systemic movement in most hosts, and cell-to-cell movement in *N. benthamiana* (Hacker *et al*., 1992; Li *et al*., 1998). It has also been shown that P38 is the elicitor of gene-for-gene resistance in Arabidopsis line Di-17 (Kachroo *et al*., 2000) and that it specifically interacts with a transcription factor (NAC family in Arabidopsis) that seems associated with this resistance response (Ren *et al*., 2000). Finally, P38 is a strong silencing suppressor (Qu *et al*., 2003; Thomas *et al*., 2003) preventing the accumulation of detectable levels of siRNA in infiltrated leaves. The evidence indicates that P38 blocks generation of siRNAs derived from dsRNA processing by Dicer at an early initiation step of silencing but cannot revert established silencing. P38 is able to suppress PTGS induced by sense, antisense, and dsRNAs, and it prevents both local and systemic silencing (Qu *et al*., 2003). Similar suppressor activity has been observed with the CP of several other carmoviruses, including Carnation mottle virus and Cardamine chlorotic fleck virus (Qu and Morris, 2005).

The transgenic P38 Arabidopsis plants do not show the strong developmental abnormalities provoked by a number of other silencing suppressors (Dunoyer *et al*., 2004). *In vitro* assays showed that P38 binds long and small dsRNA in a size-independent manner and that the protein suppresses IR-PTGS (Merai *et al*., 2006). In Arabidopsis, TCV-derived siRNAs accumulated as a single, 22nt species, unlike other viral siRNAs that are mainly 21nt in length. The 22nt siRNA levels were strongly reduced upon infection of *dcl2* mutants and *dcl2dcl3* and *dcl2dcl4* double mutants but were not changed in *dcl3*, *dcl4*, or *dcl3dcl4* mutants, suggesting that DCL2 produces these viral 22nt siRNAs (Deleris, *et al*., 2006).

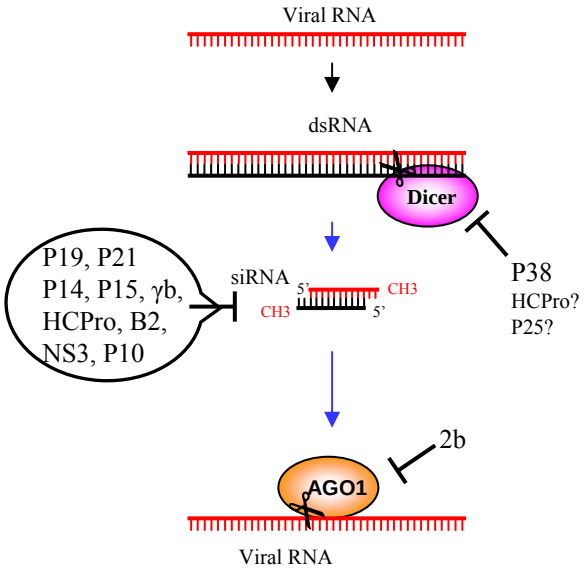


Figure 5: different suppressors act at different stages of silencing pathway.

Transgenic Arabidopsis plants expressing an inverted-repeat to silence chalcone synthase (CHS) mRNA have been transformed with P38 mRNA. In the resulting plant, expression of P38 restored CHS accumulation, significantly reduced 21nt CHS siRNA levels, and triggered accumulation of the normally less abundant 22nt siRNAs, suggesting that P38 suppresses DCL4. In the absence of DCL4, DCL2 produces 22nt siRNAs. In accordance with this hypothesis, endogenous DCL4-dependent tasiRNAs were specifically lost in P38 plants. Infection of Arabidopsis plants with a recombinant TCV virus in which the GFP reporter gene replaced the P38 sequence, resulted in accumulation of 21nt siRNAs. When *dcl4* mutant plants were infected with this recombinant virus, 21nt siRNAs were absent but 22nt siRNAs appeared. In transgenic P38-expressing plants and also in plant infected with TCV, DCL4-dependent silencing signals were inhibited, allowing virus entering and exiting through vascular bundles to establish systemic infection. Experiments with transgenic P38 plants revealed that P38 besides primary DCL4-antagonizing activities also suppresses the action of DCL2-dependent siRNAs. It is consistent with major antiviral roles for both enzymes (Deleris, *et al*., 2006) but the mechanism by which P38 suppresses DCL4 remains to be solved.

5. Suppression by inhibiting AGO1 activity: CMV encoded 2b

The ~15 KDa 2b protein of *Cucumber mosaic Cucumovirus* (CMV) was only recognized as a virus encoded protein when site-directed mutagenesis of the reading frame (Figure 4c) revealed that it was required for efficient movement of CMV in a host-dependent manner. CMV 2b mutants were incapable of systemically invading cucumber plants (Ding *et al*., 1994, 1995). 2b and HCPro were the first suppressors shown to abolish S-PTGS of GFP transgene expression in *N. benthamiana* (Anandalakshmi *et al*., 1998; Beclin *et al.,* 1998; Brigneti *et al.,* 1998). Stable expression assays demonstrated that the 2b protein produced cell non-autonomous suppression of silencing accompanied by reduction of siRNA accumulation, and inhibition of methylation of siRNA targeted transgenes (Guo *et al*., 2002). Grafting experiments and transient expression assays revealed that 2b suppresses the physical movement of the RNA silencing signals (Brigneti *et al.,* 1998; Lucy *et al*., 2000; Guo *et al*., 2002). A 2b-expressing interstock was sufficient to suppress intercellular signaling, which suggested that 2b either binds and sequesters the RNA silencing signal or inactivates the signal in some other stage (Guo *et al*., 2002). Several other experiments also indicated that 2b is implicated in cell-to-cell and in long distance movement of CMV (Shi *et al*., 2003; Wang *et al*., 2004). 2b is localized in the nucleus. Point mutations in its NLS (nuclear localization sequence) abolished the silencing suppression activity of the protein, suggesting that nuclear localization is necessary for 2b to interfere with RNA silencing (Lucy *et al*., 2000), but further experiments showed that nuclear localization was not sufficient. 2b from a mild strain of CMV (Q) was reported to have very little effect on miRNA-guided functions (Chapman *et al*., 2004). Recently, it was reported that 2b from a severe strain (FNY) interferes strongly with miRNA pathway in Arabidopsis (Zhang *et al*, 2006). 2b causes a significant increase in accumulation of miRNA, miRNA*

and tasiRNA passenger strands. Moreover, 2b inhibits miRNA guided cleavage of endogenous mRNAs but by a mechanism different from P19, because 2b binds neither ss-siRNA nor siRNA duplexes *in vitro*.

It was found that both perturbation of miRNA-guided cleavage of target RNAs and intensity of the phenotype in transgenic 2b plants were correlated with the severity of the viral strain from which the 2b protein sequence originated (Lewsey *et al*., 2007). Plants expressing 2b (of FNY) displayed developmental abnormalities with severity partially phenocopying *ago1* mutants. Severe 35S-2b lines died soon after emergence of true leaves (Zhang *et al*, 2006). 2b and AGO1 colocalized in *N. benthamiana* cells and co-immunoprecipitated in transient expression assays, in transgenic *Arabidopsis* plants and in CMV-infected plants. Pull-down experiments showed that the 2b-AGO1 interaction is specific and direct. A mutagenic study of AGO1 demonstrated that 2b interacts with AGO1 on the surface of the PAZ-containing module harboring the RNA binding groove and part of the PIWI domain (Zhang *et al*, 2006).

In vitro reconstitution of RISC using immunoprecipitated Flag-AGO1 (prepared from Flag-AGO1/ago1-36 transgenic plants) which was loaded with siRNA targeting PDS was able to cleave PDS mRNA at the expected position but pre-incubation of 2b with Flag-AGO1 before loading of the siRNA, inhibited PDS mRNA cleavage, suggesting that 2b indeed blocks RISC activity. Zhang *et al*. (2006) further showed that miRNAs and tasiRNAs co-immunoprecipitated with AGO1 in presence of 2b, which argues against the possibility that 2b prevents siRNA from being loaded into RISC. Therefore, the evidence indicates that 2b acts in suppression of silencing by blocking the AGO1 cleavage activity that is implicated in the antiviral pathway (Zhang *et al*, 2006).

6. Suppression by transactivation of host genes: Begomovirus encoded AC2

Geminiviridae are a family of unique small circular ssDNA plant viruses (Figure 4e) that replicate via dsDNA intermediates by a rolling circle mechanism in the nucleus. This dsDNA also serves as template for bidirectional transcription (Hanley-Bowdoin *et al*., 1999; Gutierrez, 2000). Geminiviruses of the genus *Begomovirus* express the small protein **AC2** (also called **C2**, **L2, AL2** or **TrAP**, Transcriptional Activator Protein), which activates transcription of late viral genes (Sunter *et al*., 1992; Haley *et al*., 1992). Consistent with its function as a transcriptional activator, three conserved domains have been recognized in this protein: a basic domain with a nuclear localization signal (NLS) at the N terminus, a central DNA binding domain with a non-classical zinc finger motif, and an acidic activator domain at the C terminus (Hartitz *et al*., 1999; Trinks *et al*., 2005). Studies on AC2 of *African cassava mosaic Begomovirus* (ACMV) and the homologous **C2** of *Tomato yellow leaf curl Begomovirus* (TYLCV) showed that these proteins are suppressors of RNA silencing (Voinnet *et al*., 1999; van Wezel *et al*., 2002; Dong *et al*., 2003). It has been shown that TYLCV C2 requires a functional NLS and the zinc finger domain to suppress silencing (van Wezel *et al*., 2002, 2003; Dong *et al*., 2003). Interestingly, attempts to produce transgenic plants constitutively expressing full length AC2/AL2 proteins failed, while

plants expressing a truncated form of *Tomato golden mosaic Begomovirus* (TGMV) AC2 lacking the transactivation activity were recovered (Sunter *et al*., 2001). Studies on *Mungbean yellow mosaic Begomovirus* (MYMV) have revealed that AC2 functions as a transactivator of the viral promoter and as a suppressor of RNA silencing. Analyses of mutants in the three above mentioned conserved domains revealed that silencing suppression and transactivation are functionally connected. Suppression of silencing by AC2 involves transactivation of host genes (Trinks *et al*., 2005). Transcription of ~30 host genes were strongly induced in response to AC2 from **MYMV** and its **ACMV** homologue in Arabidopsis protoplasts. Thus, it appears that the nuclear localized AC2 acts via a novel mechanism of silencing suppression which involves activating transcription of host genes. Among the AC2 induced genes is WEL1 (Werner Exonuclease-Like1) gene. Interestingly, the related protein WEX (Werner Exonuclease) (Glazov *et al*., 2003) is a positive effector of RNA silencing in Arabidopsis. There are indications that transient expression of WEL1 gene is sufficient to suppress RNA silencing in the *N. benthamiana* line 16c (Trinks *et al*., 2005).

On the other hand, AC2 of **TGMV** and L2 of *Beet curly top Curtovirus* (**BCTV**) have been shown to interact with and inactivate Adenosine Kinase (ADK), a cellular enzyme that phosphorylates Adenosine to produce 5'-AMP and is important for methyl cycle maintenance (Wang *et al*., 2003). It has been suggested that ADK activity is important in maintenance of RNA silencing (Moffatt *et al*., 2002) because loss of function of this protein produced a silencing suppression phenotype (Wang *et al*., 2005). ADK activity is reduced in infiltrated leaves expressing AC2 or L2 exhibiting silencing suppression. This indicates that AC2 of TGMV and L2 of BCTV might suppress silencing by a different mechanism than those AC2 that involve ADK inhibition because a mutation in the transactivation domain of TGMV AC2 did not affect suppressor activity of the protein and, moreover, AC2 does not bind to siRNA (Wang *et al*., 2005).

7. Other examples of silencing suppression of plant viral proteins

7-1. Potexvirus encoded P25

P25 is the largest of three 'triple gene block' proteins of potexviruses (Figure 4b) that are required for cell-to-cell movement of the virus (Beck *et al*., 1991; Angell *et al*., 1996). P25 is an RNA helicase (Kalinina *et al*., 2002) that can move from cell to cell and can modify plasmodesmata (Angell *et al*., 1996). It was initially thought that PVX did not encode a silencing suppressor. However, subsequent experiments demonstrated that P25 is indeed a silencing suppressor that prevents the movement of systemic silencing signals out of the primary infected cells (Voinnet *et al*., 2000). P25 of PVX is a relatively weak suppressor of RNA silencing compared to the P25 of three other potexviruses, Narcissus mosaic virus, Nandina virus X, and Viola mosaic virus, all of which were able to effectively reactivate a previously silenced GFP transgene (Voinnet *et al*., 1999). Transgenic P25 plants of *White clover mosaic*

Potexvirus (WClMV) were reported to phenocopy some silencing mutants (Foster *et al*., 2002). Random mutagenesis of the P25 gene revealed that suppression of silencing is necessary, but not sufficient, for cell-to-cell movement of PVX. Therefore, P25 carries a second function independent of silencing suppression but specifically required for movement (Bayne *et al*., 2005). P25 suppresses S-PTGS and IR-PTGS but siRNAs reduction is only observed in S-PTGS. This has led to the suggestion that the presence of siRNA in tissue undergoing suppression of IR-PTGS may be due to interference of P25 with the assembly or activity of the silencing effector complex (Bayne *et al*., 2005).

7-2. Combinations of viral proteins and RNAs as a suppressor of silencing

Red clover necrotic mosaic Dianthovirus (RCNMV) from Tombusviridae has a positive sense ssRNA genome which is divided into two RNA; RNA1, which encodes three proteins (P27, P88 both involved in replication and CP), and RNA2, which encodes the movement protein. RCNMV suppresses RNA silencing (S- and IR-PTGS and miRNA biogenesis) by using multiple viral components required for viral RNA replication (Takeda *et al*., 2005). RNA1 is essential for suppression activity but the proteins encoded by RNA1 are not sufficient. Silencing suppression requires the combination of P27, P88 and the 3' UTR of RNA2. *dcl1* mutants showed reduced susceptibility to the virus suggesting that DCL1 is a host factor involved in efficient infection of RCNMV (Takeda *et al*., 2005).

7-3. Suppressor protein encoded by negative strand RNA Tenuivirus (NS3)

In contrast to positive strand RNA plant viruses, negative strand RNA plant viruses replicate in both insect vector and plant host (Wijkamp *et al*., 1993; Falk and Tsai 1998) and may be a target for antiviral silencing in the insect vector as well as in plant host. *Rice hoja blanca Tenuivirus* (RHBV), a negative strand RNA plant virus infects rice and is transmitted by plant hoppers in which it also replicates (Falk and Tsai, 1998). The NS3 protein of RHBV was shown to be a suppressor of RNA silencing in plants that can suppress S-PTGS but not IR-PTGS (Takeda *et al*., 2002; Bucher *et al*., 2003). Recently, it has been reported that NS3 is also capable of suppressing RNA silencing in insect cells. Biochemical analyses showed that NS3 efficiently binds siRNA as well as miRNA molecules. Binding to NS3 is greatly influenced by the size of small RNA molecules, 21nt siRNA molecules being bound 100 times more efficiently than 26nt species. It was suggested that NS3 acts like P19 and P21 by sequestering siRNA and miRNA and inhibiting their assembly into RISC (Hemmes *et al*., 2007).

Aim of the research

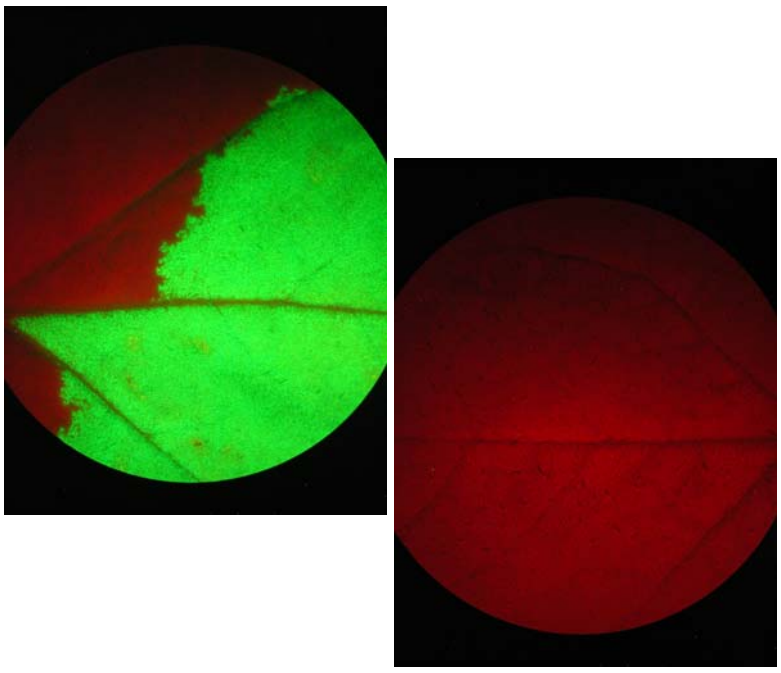
Fundamental research in virology such as study of the function of the viral genes is the basis for applied research such as the establishment of resistance against a virus. Poleroviruses are among the economically important viruses and amply merit the attention of phytopathologists. Research leading to a better understanding of these viruses and their interactions with host plant and vector will not only clarify fundamental questions but will also help to better defend economically important plants against such viruses. In addition, the findings drawn from virus biology can help to better our understanding of the molecular biology of more complex organisms such as plants and animals.

Upon my arrival in the laboratory, the function of polerovirus P0 had been elucidated but the mechanism by which it acts as a suppressor of silencing in plants remained to be discovered. During my thesis I have tried to understand the mechanism of action of P0 in silencing suppression. As mentioned in the introduction, one of the methods for studying the mechanism of action of a silencing suppressor is looking for its cellular partners in host plant. To this end, we carried out a screen of cDNA library of Arabidopsis by the yeast two-hybrid system and found a partner for P0. Then we validated this interaction and confirmed its requirement for viral infection. This interaction led us to discover a conserved motif in P0 of all poleroviruses indicating that P0 functions as an F-box protein (see Chapter one and Publication N.1).

The identity of P0's cellular partner suggested that P0 uses the plant protein degradation system that is ubiquitin-dependant to degrade a protein implicated in silencing pathway. Therefore, in the second stage, we looked for a target for P0 among the known protein components of the silencing pathway. Using P0 transgenic plants and transient expression assays, we identified AGO1 as a target for P0. Finally, we validated the interaction between P0 and AGO1 by biochemical methods (see Chapter two and publication N.2). The results have led us to propose a mechanism by which P0 acts as a suppressor of RNA silencing.

Results: Chapter One

The Silencing suppressor activity of P0 requires the F-box motif



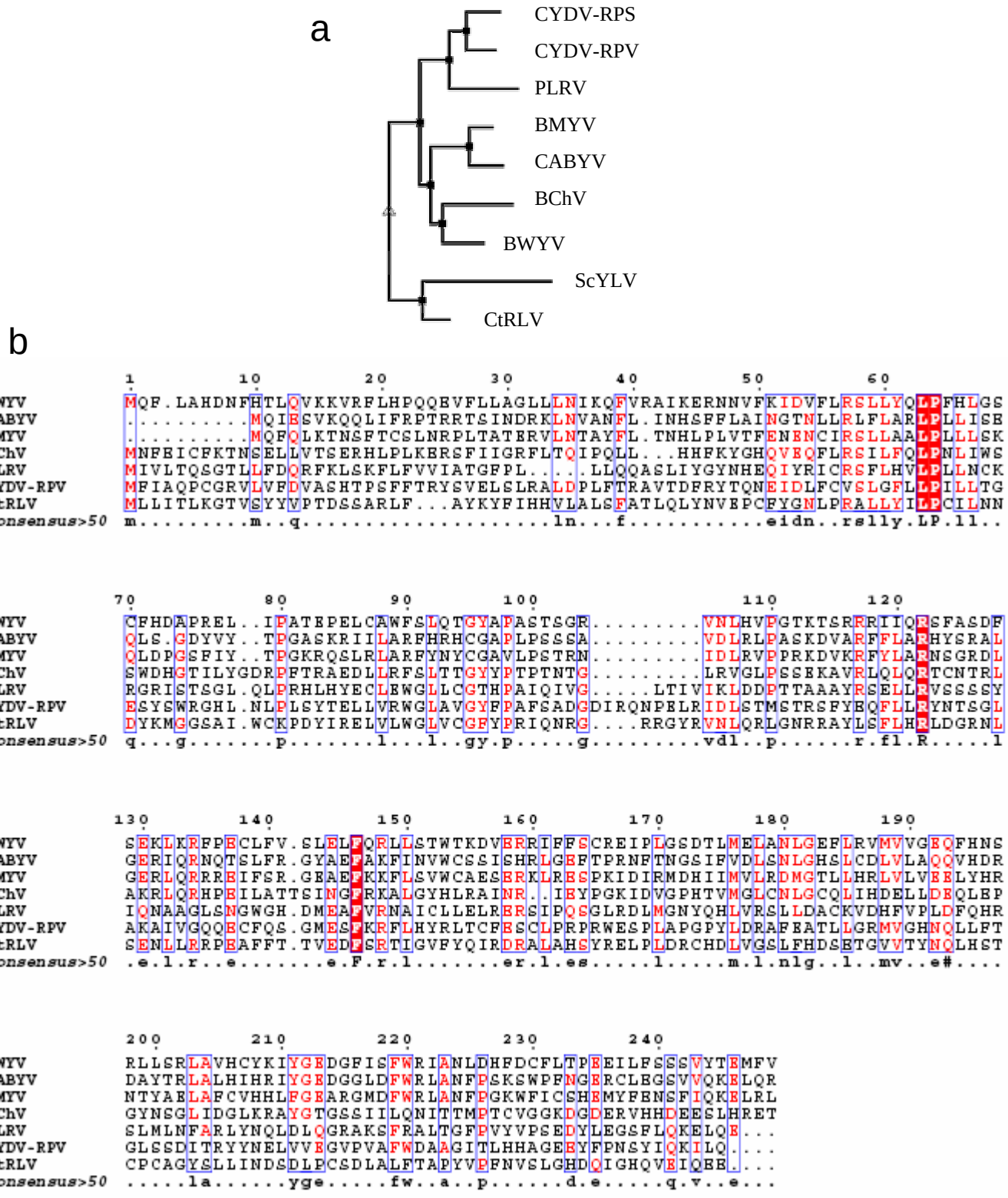


Figure 1-1: (a) The phylogenetical tree and distance between P0 of Polerovirus based on amino acid sequence. (b) Amino acid sequence alignment of Polerovirus P0s (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html).

Chapter one

The silencing suppressor activity of P0 requires the F-box motif

Introduction to P0

As the genome sequences of BWYV and then other poleroviruses became available, it became clear that P0 was by a wide margin the least conserved of the virus-coded proteins (Figure 1-1). Because all members of the polerovirus genus have basically similar biological properties except for host range, the sequence divergence among P0s led to the early idea that P0 might have a role in determining this property (Mayo *et al*., 1989; Veidt *et al*., 1992). Indeed, the studies of a group of 15 poleroviruses isolated from beet or rape have shown that the phylogenetic clustering based on the P0 sequence is predictive of many features of the host range (Hauser *et al*, 2000, 2002). Thus, the idea became rooted that P0 was implicated in virus-host interactions and that the ability of P0 to interact with a putative host partner protein or proteins would determine whether or not a particular plant could be infected by the virus.

Since it is encoded by the 5'-proximal gene on the viral RNA, P0 might be expected to accumulate to high levels in virus-infected plants. This is not the case, however, as P0 has never been detected by western blot in total proteins isolated from protoplasts or plants infected with any polerovirus (van der Wilk *et al*., 1997a; Smith and Barker, 1999; Pfeffer *et al*., 2002). The low accumulation of P0 *in planta* is probably due to the suboptimal translation initiation context of the P0 start codon in viral RNA (TTGATGC for P0 of BWYV; the ORF0 initiation codons of other poleroviruses are also in poor context). BWYV P0 could not be detected by western blot in total proteins from plants agroinfiltrated with a construction expressing ORF0 downstream of the viral non-coding sequence under control of the 35S promoter, but could be readily detected when the sequence around the translation initiation codon was altered to an optimal 'Kozak' context (A/GCCATGG; Kozak, 1999) (Pfeffer, *et al,* 2002). Furthermore, agroinfection of plants with constructs expressing full-length BWYV transcript altered so that the ORF0 initiation codon was in the Kozak context led to detectable levels of P0 in the inoculated leaves at early times post-agroinfection but infected upper leaves contained no detectable P0. Interestingly, analysis of the viral RNA in the upper leaves revealed that the region containing the ORF0 initiation codon had undergone second-site mutations that were predicted to dramatically lower P0 translation initiation rates (Pfeffer, *et al,* 2002). The evidence thus suggests that low level expression of P0 is important for optimal virus infectivity. Possible reasons why this may be so, will be discussed later.

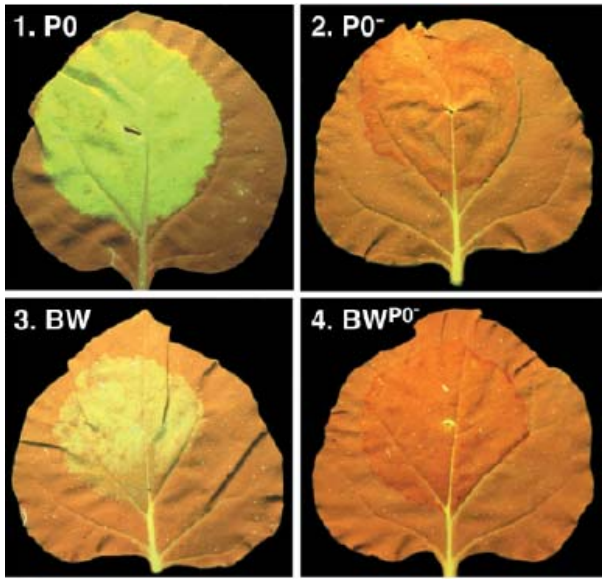


Figure 1-2: Silencing suppression activity of BWYV P0 studied by co-expression with GFP in *N. benthamiana* 16c. Patch agroinfiltrated with Agrobacterium harboring pBin-GFP plus pBin-P0 (panel 1), pBin-P0 frameshift mutant (panel 2), pBin-BWYV (panel 3) or Pbin-BWYV with mutant P0 (panel 4). Photographs were taken with long-wavelength UV light 5 days post infiltration (adapted from Pfeffer *et al.*, 2002).

The availability of infectious cloned polerovirus cDNA (Veidt *et al*, 1992; Leiser *et al*, 1992) provided a direct means of investigating the role of P0 in the context of a virus infection. Protoplast infection experiments with viral RNA transcripts carrying frameshift and deletion mutations in the 5' terminal part of ORF0, where there is no overlap with ORF1, revealed that the mutant viral RNA could be replicated in protoplasts but that the progeny RNA accumulated somewhat less efficiently than wild-type virus. Accumulation of these mutant viruses was also significantly (five to sevenfold) diminished in agroinoculated *N. clevelandii* (Ziegler-Graff *et al*., 1996). Sadowy *et al*. (2001b) also reported that PLRV mutants in P0 failed to detectably accumulate in agroinoculated potato leaf discs. Taken together, these results suggested that P0 is not strictly required for virus replication but that it might serve as a replication 'enhancer', be involved in virus movement, and/or exert a negative effect on a host virus defence system.

An important advance in our understanding of the function of P0 came from previous studies in our group when Pfeffer *et al*. (2002) discovered that P0 is a strong suppressor of post-transcriptional gene silencing (PTGS). Evidently, the discovery of the silencing suppressor activity of P0 provides an explanation for the effect of P0 knock-out mutations on virus pathogenicity. The transient co-expression of P0 with GFP by agroinfiltration on leaves of *N. benthamiana* 16c line containing a GFP transgene showed appearance of green fluorescence (Figure 1-2) and stabilization of GFP mRNAs, suggesting that P0 suppresses S-PTGS. The use of a non-translatable version of the P0 gene for co-expression with GFP did not provoke suppression of GFP silencing, indicating that P0 protein but not its RNA is responsible for suppression activity (Pfeffer *et al*., 2002). Co-agroinfiltration of GFP with BWYV full-length cDNA under control of the 35S promoter produced suppression of GFP silencing, but with lower efficiency than when P0 is expressed alone (Figure 1-2, Panel 3) because of a poor initiation codon context in the viral genome. Comparative experiments indicate that P0 of PLRV was a much less efficient suppressor than the P0s of BWYV and CABYV in this assay.

As mentioned earlier, members of the *Luteovirus* genus do not contain ORF0 but instead posses a short 3' proximal ORF (ORF6) which is absent in poleroviruses. In order to investigate the silencing suppression activity of luteoviruses, GFP was co-expressed with either P4 or P6 of BYDV by agroinfiltration in transgenic *N. benthamiana* 16c line. Only P4 showed a week suppressor activity. This could be explained by a silencing activity that became adapted to monocotyledon plants (BYDV infects monocots) and therefore, works less efficiently in dicotyledons such as *N. benthamiana.* Interestingly, P4 of BWYV does not display detectable silencing suppressor activity, making P0 the only polerovirus-encoded protein with such activity (Pfeffer, 2002). Therefore, if the week silencing suppressor activity putatively displayed by BYDV P4 is confirmed, this would indicate that P4 of the two genera do not play the same role, although 31% identity exists between P4 of BYDV and BWYV.

This earlier work also revealed that expression of P0 can enhance the pathogenicity of an unrelated virus and this activity is independent of other BWYV genes. For this purpose, *N. benthamiana* plants were mechanically inoculated with transcripts of a PVX-based vector in which the P0 coding region has been

inserted. Necrosis in the petioles and veins and then in mesophyll tissue was observed, followed by death of the upper leaves and eventually of the entire plant. In contrast, plants inoculated with transcripts of the PVX vector containing a frameshift-mutated version of P0 developed mosaic and chlorotic lesions similar to those observed following infection with the empty PVX vector. Therefore, P0 like many other suppressors of silencing can increase pathogenicity and virus accumulation of an unrelated virus (Pfeffer *et al*., 2002).

Other studies have shown that potato plants transformed with cDNA encoding P0 of PLRV display an altered phenotype resembling virus-infected plants (van der Wilk *et al*., 1997a; Franco-Lara *et al*., 1999; Prufer *et al*., 2006). A positive correlation was observed between levels of accumulation of the P0 transcripts and severity of the abnormal phenotype. In contrast, potato plants transformed with a modified, untranslatable ORF0 sequence were phenotypically identical to wild-type plants. These results suggest that the P0 protein but not P0 mRNA is involved in viral symptom expression.

To investigate its subcellular localization, BWYV P0 fusion proteins with GFP placed at either the N- or C-terminus were tested first for suppressor activity in transient expression assays. The results indicate that only P0GFP retained silencing suppressor activity. Preliminary confocal laser scanning microscopy showed cytoplasmic localization for the P0GFP particularly near the plasma membrane and nuclear envelope in tobacco BY2 cells (Pfeffer, 2002).

The mechanism by which P0 acts as a suppressor of silencing in plants remained to be discovered. Experiments with the first reported suppressor of PTGS, HCPro, used a yeast two-hybrid screen of cDNA from tobacco to identify a cellular partner, rgs-CaM (regulator of gene silencing-calmodulin-like), for HCPro (Anandalakshmi *et al*., 2000). We decided to employ a similar strategy to investigate the mode of action of P0 in suppression of silencing.

I. Yeast Two Hybrid Screen in Arabidopsis cDNA library for P0-cellular partner

CABYV and BWYV both infect Arabidopsis. Therefore, we cloned P0 of BWYV (**$P0^{BW}$**) into pGBKT7 (CLONTECH) carrying the GAL4 binding domain (BD) and used a cDNA library of Arabidopsis cloned in pGADT10 (CLONTECH) carrying the GAL4 activation domain (AD). The cells of *Saccharomyces cerevisiae* strain AH109 (CLONTECH) carrying the Histidine (H), Adenine (A) and *lacZ* reporter genes were double transformed with pGBKT7- $P0^{BW}$ and pGADT10-cDNA but none of 10^7 double transformants grew on selective medium. When P0 of CABYV (**$P0^{CA}$**) was used for screening, however, six of the 5×10^6 double transformants grew under strong selective condition (-HA). These plasmids were extracted, sequenced and analyzed by BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) against the complete Arabidopsis genome sequence. All six sequences were the same cDNA, corresponding to **ASK2** (At5g42190), Arabidopsis S-phase Kinase-related protein 2 (**SKP2**), which is an ortholog of the *S. cerevisiae SKP1* (suppressor of kinetochore protein) gene and a homolog of human SKP1 (S-phase

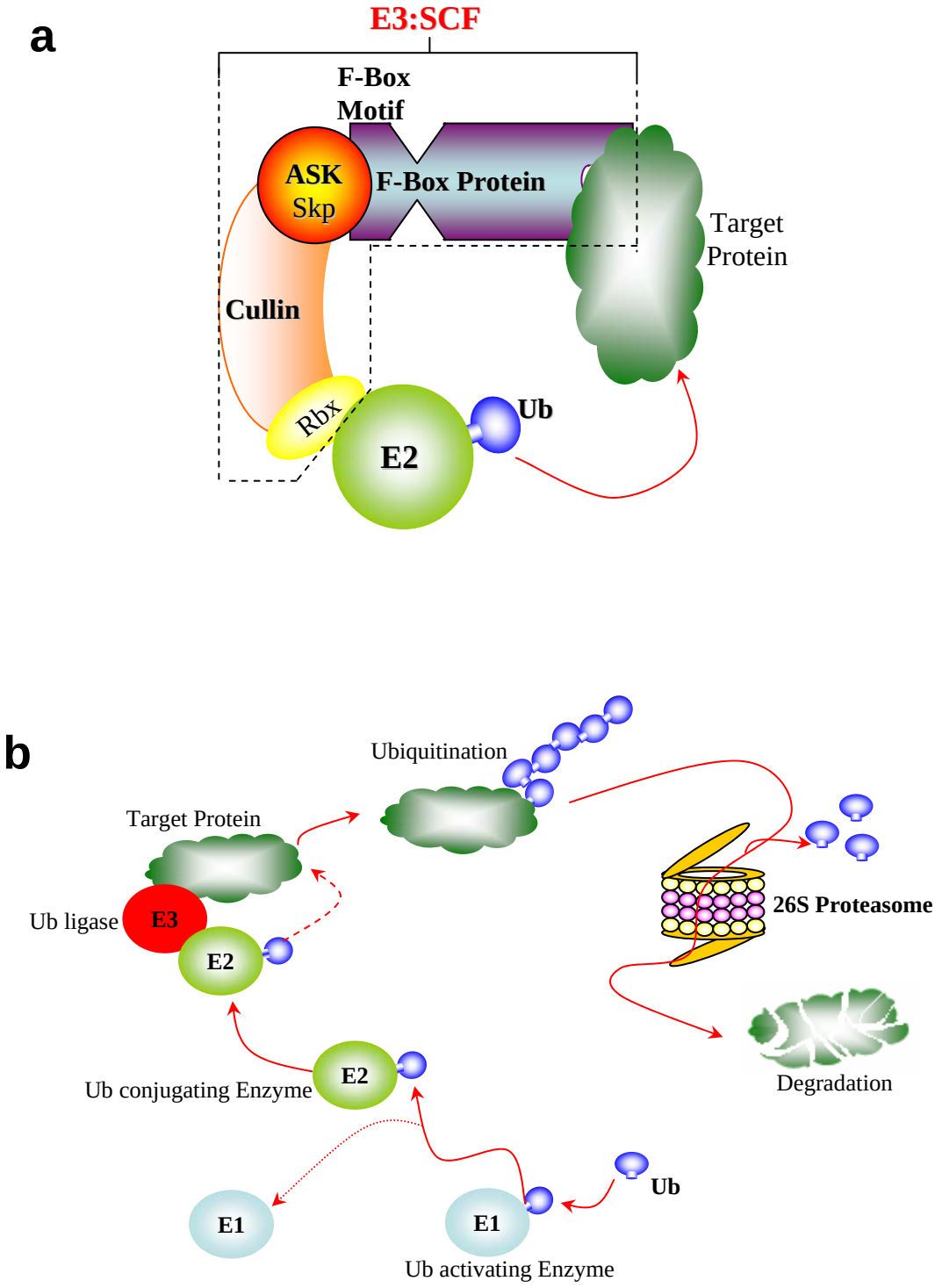


Figure 1-3: (a) The components of SCF E3 ligase. (b) Ubiquitin-Proteasome Pathway.

Kinase-associated protein). There are 21 ASKs in Arabidopsis but ASK1 and ASK2 are the most abundant and well studied members of this gene family (Farras *et al*., 2001; Risseeuw *et al*., 2003; Liu *et al*., 2004).

Introduction to ubiquitin-proteasome pathway

1. What is SKP?

SKP is a subunit of the **SCF** (SKP-Cullin-F box) complex (Figure 1-3a) which is a type of E3 (ubiquitin ligase) enzyme. The E3 enzymes are implicated in ubiquitination of cellular proteins and subsequent degradation by the 26S proteasome. Cellular proteins are in a dynamic state of synthesis and degradation. Plant growth, development and physiology are controlled by the selective removal of short-lived proteins. One important proteolytic pathway involves the small protein ubiquitin (**Ub**) and the 26S proteasome. In this pathway, Ub is attached to proteins destined for degradation in a three-step process via E1, E2 and E3 enzymes. The resulting ubiquitinated protein is then recognized and catabolized by the 26S proteasome (Figure 1-3b). Via this pathway up to 50% of the total protein is replaced by plants every week. This pathway is implicated in virtually all aspects of plant biology, including the cell cycle, embryogenesis, photomorphogenesis, circadian rhythms, hormone signaling, disease resistance and senescence (reviewed by Vierstra, 2003; Cardozo and Pagano, 2004; Smalle and Vierstra, 2004). To better understand the importance of P0's partner, ASK, I will describe the Ub-dependant proteasome pathway in plants (Arabidopsis) and the characteristics of its components.

2. Ubiquitin

Ub is a nearly ubiquitous, highly conserved 76-amino acid (8 KDa) eukaryotic protein which was discovered in the mid-1970s. Prokaryotes have no molecule that is functionally analogous to Ub (Pickart and Eddins *et al*., 2004). All Ub genes from higher plants encode almost identical proteins; these differ by only one amino acid from Chlamydomonas Ub, by two amino acids from *S. cerevisiae* Ub and by three amino acids from animal Ub (Callis *et al*., 1994). The Arabidopsis Ub gene family consists of 14 members (***UBQ***s) that can be divided into three types of Ub genes (Figure 1-4a): five **polyubiquitin genes** (*UBQ3, UBQ4, UBQ10, UBQ11* and *UBQ14*) that encode varying numbers of repeats of the 228 bp Ub coding region (Burke *et al.* 1988; Callis *et al*., 1994). There are also five **ubiquitin-like genes** (*UBQ7, UBQ8, UBQ9, UBQ12* and *UBQ13*), which are similar in structure to polyubiquitin genes, but that encode different numbers of tandem Ub repeats with additional non-ubiquitin amino acids at the C-termini (Callis *et al*., 1994). Finally, there are four **ubiquitin extension genes** (*UBQ1, UBQ2, UBQ5* and *UBQ6*) that encode an Ub monomer fused to one of two ribosomal subunit proteins (Callis *et al.,* 1990).

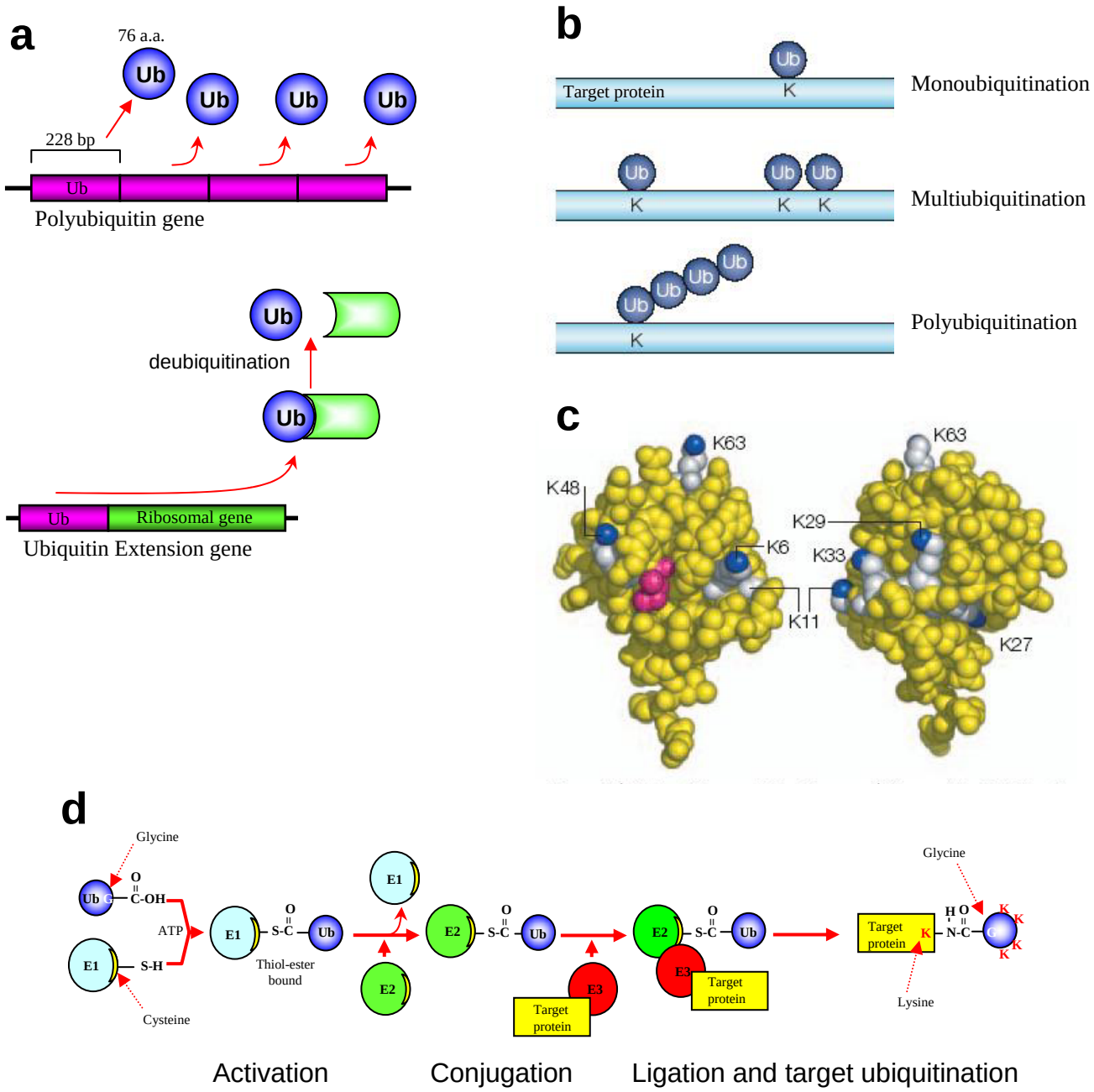


Figure 1-4: (a) different ubiquitin genes. (c) Diverse forms of Ub modifications on target protein. (K: Lysine residue) (c) Schematic structure of Ub showing position of seven Lys residues (Hicke *et al.*, 2005). The ubiquitin-binding domains contact ubiquitin by Ile44 (red). (d) Schematic three-step Ub conjugation to target protein (activation, conjugation and ligation).

The Ub moieties are then released from the initial translation products by deubiquitinating enzymes (DUBs), which are the proteases that cleave precisely after the Ub C-terminal Glycine (Wing, 2003).

Ub covalently attaches to Lysine (**K**) residues of target proteins (Figure 1-4d). Protein-attached Ub is a substrate for the attachment of further Ub residues, which leads to the formation of a polyubiquitin chain. Classically, polyubiquitination is a signal that directs proteins to the proteasome, where the Ub is recycled and the protein is degraded (Hershko *et* and Ciechanover, 1998). Ubiquitination can also remodel the surface of substrate proteins, thereby changing their stability, localization, or activity (Pickart and Eddins *et al*., 2004).

Diverse forms of Ub modifications exist (Figure 1-4b): **Monoubiquitination** is the attachment of a single Ub to a protein; **Multiubiquitination** occurs when several Lys residues of the target protein are tagged with single Ub molecules; and **Polyubiquitination** refers the addition of a Ub chain made of several Ubs that are linked through the C-terminal Glycine residue of each Ub unit and a specific internal Lys of the previously attached Ub. Monoubiquitination or multiubiquitination has been shown to be required for the entry of certain cargo proteins into vesicles at different stages of the secretory/endocytic pathway (Hicke, 2001) whereas polyubiquitination has been mainly associated with proteasomal degradation (Pickart and Fushman, 2004). In the case of polyubiquitination, there can be at least seven different linkages between Ubs, because there are seven internal Lysines in Ub (Figure 1-4c). Depending on the type of linkages, polyubiquitination can play different roles. Ub can also be removed from proteins, and different Ub hydrolases that regenerate free Ub have been identified and implicated in regulation of various cellular events (reviewed by Mukhopadhyay and Riezman, 2007).

A number of **Ub-related proteins** also exist in plants, such as RUB1 (or NEDD8), SUMO (Small Ubiquitin-related Modifier), APG8 (Autophagy-defective8), APG12 (Autophagy defective12), URM (Ubiquitin-related Modifier), and HUB (Homologous to Ubiquitin). Although most bear little sequence identity to Ub, they all contain the Ub fold with a similar flexible C-terminal extension. These tags attach to various targets (reviewed by Smalle and Vierstra, 2004).

3. Ubiquitination pathway

Free Ubs are attached to appropriate intracellular targets via an ATP-dependent three-stage E1→E2→E3 conjugation cascade (Figure 1-3b and 4d). The cascade begins with an **E1** (or Ub-activating enzyme) catalyzing the activation of the Ub molecule in its C-terminal **Glycine** (Gly) to a high-energy thiol ester intermediate and then binding the Ub directly via a thiol-ester linkage between the Ub Glycine and a **Cysteine** in the E1. This activation of Ub is ATP-dependant. The activated Ub is then transferred to a Cysteine in an **E2** (or Ub-conjugating enzyme) by transesterification. Finally, the Ub-E2 intermediate delivers the Ub to the ε-amino group of a **Lysine** residue in a substrate using an **E3**

(or Ub-protein ligase) as the recognition element. The end product is an Ub-protein conjugate (Smalle and Vierstra, 2004).

3-1. E1: E1s initiate the conjugation cascade and have no impact on target specificity. They are single ubiquitously expressed polypeptides that contain a positionally conserved **Cysteine** that binds Ub and a nucleotide-binding motif that interacts with either ATP or the AMP-Ub intermediate. Arabidopsis expresses only two E1 isoforms, one of which may be nuclear localized (Hatfield *et al*., 1997).

3-2. E2: Plants express a large family of E2 isoforms. For example, at least 42 E2 (or ***UBC***: Ub-carrier protein) genes exist in the Arabidopsis genome (Zeng *et al*., 2006). E2s are identified by a conserved 150 amino acid catalytic core that surrounds the active-site **Cysteine** buried within a shallow groove (Hamilton *et al*., 2001).

3-3. E3: As the last components in the Ub-conjugation cascade, E3s are responsible for identifying the many proteins that should be ubiquitinated. Consequently, they are the most numerous and diverse factors of the ubiquitination cascade. The Arabidopsis genome contains more than 1300 genes that encode putative E3 subunits (Smalle and Vierstra, 2004). Based on subunit composition and mechanism of action, four E3 types have been described in plants (Figure 1-5): (although some authors have classified them into two groups, HECT and RING-box containing APC and SCF.)

1. HECT (Homology to E6AP C Terminus)
2. RING (Real Interesting New Gene) and U-Box (UFD2 homology protein)
3. APC (Anaphase-Promoting Complex)
4. SCF (SKP1, Cullin/CDC53, and F-box protein complex)

3-3-1. HECT

HECT E3s are typically large proteins (>100 kDa) that can be recognized by the presence of a conserved 350-amino acid C-terminal region called the HECT domain. Human Papillomavirus E6-associated protein (E6AP) is the founding member of this E3 family. The HECT domain contains both an Ub binding site (Cysteine residue) and an E2 binding site (Pickart, 2001; Downes *et al*., 2003) (Figure 1-5a). In addition to the HECT domain, this E3 has an N-terminal protein-protein interaction domain and RING-finger, or coiled-coil domain. This part of the protein is involved in target recognition. Exceptional among Ub ligases, HECT E3s form a covalent bond with Ub before transferring it to the substrate protein and serve as the proximal Ub donor during the ligation reaction (Pickart, 2001). Seven HECT E3s (**UPL**1 to 7: Ub Protein Ligase) are encoded by the Arabidopsis genome (Schwarz *et al*., 1998; Downes *et al*., 2003).

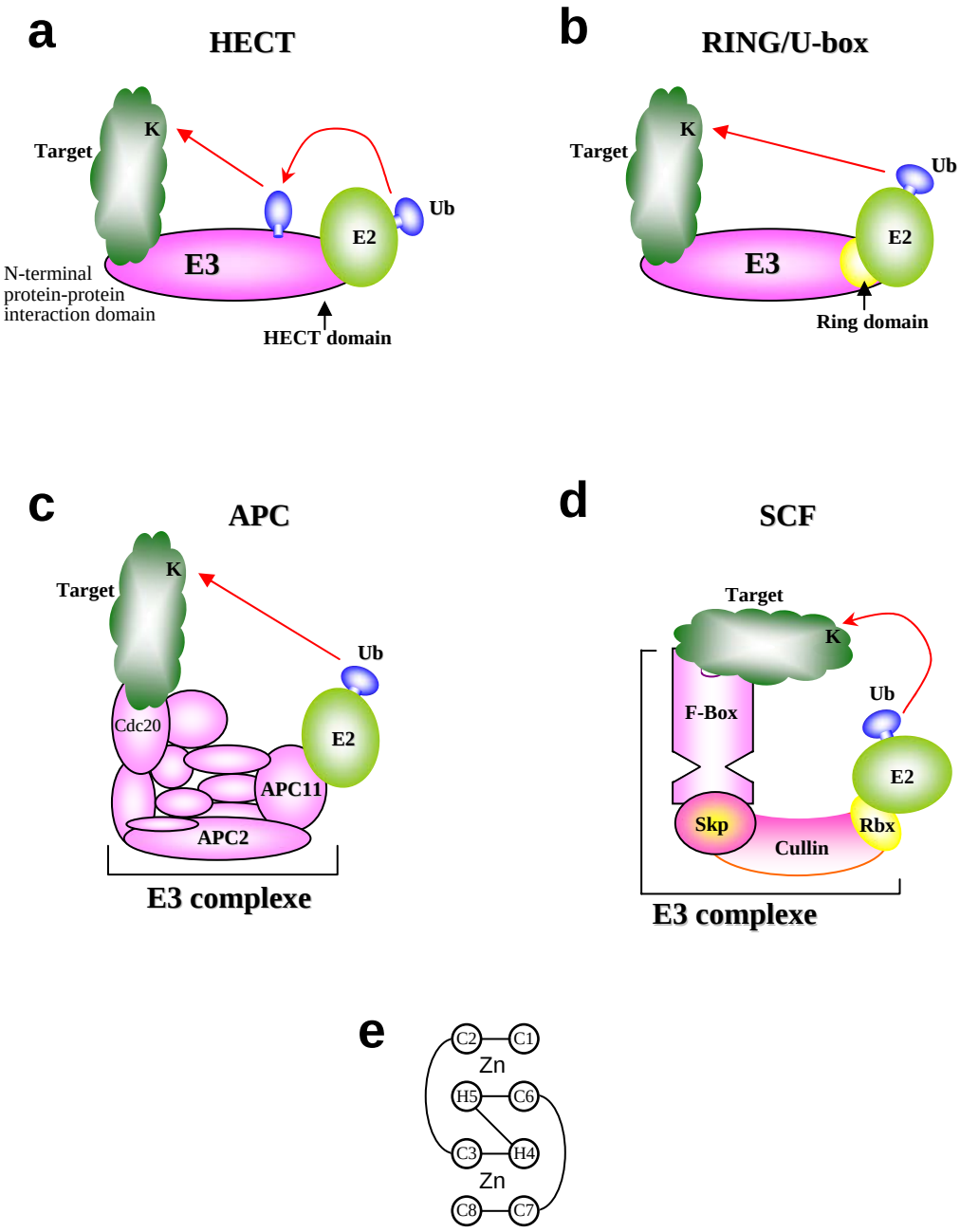


Figure 1-5: Schematic structure of four E3 ligases in Arabidopsis. (a) HECT. (b) RING/U-box. (c) APC. (d) SCF. (e) Schematic structure of RING finger domain. Eight residues forms a cross-brace. Residue 5 can be either Cysteine in RING-HC domains or Histidine in RING-H2 domains. (C: Cysteine and H: Histidine) (Vierstra, 2004).

Little is known about the functions of plant HECT E3s although there is evidence that one Arabidopsis isoform is necessary for trichome development (Downes *et al*., 2003).

3-3-2. RING/U-Box

The RING/U-Box E3s are a collection of polypeptides bearing either a RING-finger motif or a U-Box. The RING domain consists of a short motif rich in **Cysteine** (C) and Histidine (H) residues (C3HC4 or C3H2C3) that is a chelator of Zn^{2+} (Figure 1-5e) and the U-box domain is similar in structure to the RING domain, but does not use zinc ions to stabilize the motif. The Arabidopsis genome encodes approximately 480 RING finger-containing proteins and 64 proteins with a U-Box motif (Smalle and Vierstra, 2004). It has been demonstrated that the 70-amino acid RING finger binds directly to E2 (Seol *et al*., 1999) or it increases the probability of an interaction by bringing together the substrate Lysine and the E2-Ub intermediate (van Demark and Hill, 2002) (Figure 1-5b).

3-3-3. APC

The APC was the first multicomponent Ub ligase described and is required for the degradation of substrates controlling the metaphase-to-anaphase transition and the destruction of Cyclin B to allow the exit from mitosis (Peters, 1998). APC is a highly conserved complex consisting of eleven subunits (Gieffers *et al*., 2001; Capron *et al*., 2003) (Figure 1-5c). Two of the 11 components have homology to subunits in the SCF: APC2 (related to Cullin) and APC11 (an RING protein) (Tang *et al*., 2001). Presumably, they act similarly as scaffold for the other subunits (APC2) and by binding the Ub-E2 intermediate (APC11). In this case, the Arabidopsis E2 is the UBC19-20 subfamily (Criqui *et al*., 2002). Consistent with the crucial role of the APC in the cell cycle, mutations affecting several Arabidopsis *APC* genes block cell division (Blilou *et al*., 2002; Capron *et al*., 2003). APC action is controlled by phosphorylation/dephosphorylation of both the complex and targets (Harper *et al*., 2002). Most of the known targets of the APC are Cyclins (Fang *et al*., 1998; Hames *et al*., 2001). It has been reported that the APC recognizes two motifs in its substrates: the **D-box** (Destruction box: **R**KF**L**SLASN where the letters in bold are consensus) and the **KEN-box** (**KEN**IMRSENS) (Glotzer *et al*., 1991; Pfleger and Kirschner, 2000; Hames *et al*., 2001).

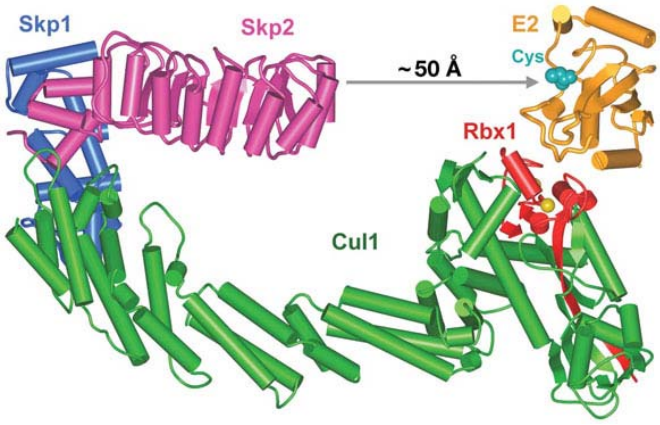


Figure 1-6: Model of a complete SCF ligase complex with its target protein (human SKP2) (Zheng *et al*., 2002).

3-3-4. SCF

SCF is the largest and best characterized E3. Its participation in plant development is extensive, affecting processes such as hormone response, photomorphogenesis, circadian rhythms, floral development, and senescence (reviewed by Moon *et al*., 2004; Smalle and Vierstra, 2004). Based on three of its core subunits, **SKP1**, **Cullin** (CUL/CDC53), and an **F-box** protein, this complex was named SCF. The fourth subunit is called **RBX** (Ring-Box protein or ROC1/HRT1) and contains a Ring-Finger motif with a particular folded protein domain that binds Zn^{2+} through a four-point arrangement of Cysteine and Histidine amino acids. In this E3 Ub ligase, this domain seems to be responsible for binding the E2 Ub conjugating enzymes. Cullin functions as a scaffold that simultaneously interacts at the N-terminus with SKP and at the C-terminus with RBX (Figure 1-5d). SKP acts as a connector and binds to one of many F-box proteins. Each F-box protein acts as an adaptor subunit of the complex and appears to be matched with a discrete number of specific substrates through a protein-protein interaction domain (reviewed by Smalle and Vierstra, 2004). The SCF complex is structurally conserved in human, mouse, Drosophila, *C. elegans*, plants, and yeast. The crystal structure of the SCF complex, which consists of CUL1, RBX1, SKP1, and human F-box protein SKP2, has been defined (Jackson and Eldridge, 2002; Zheng *et al*., 2002) (Figure 1-6).

Like RING/U-Box E3s, SCF E3s function as scaffolds that bring together the activated Ub-E2 complex and the target to promote conjugation without forming an E3-Ub intermediate. The Cullin-RBX-SKP1 subcomplex provides the Ub-transferase activity and the numerous F-box proteins confers target specificity (Smalle and Vierstra, 2004)

4. Subunits of SCF E3 ligase

4-1. CUL

In Arabidopsis, the five canonical Cullins are *CUL1, CUL2, CUL3A, CUL3B*, and *CUL4*. *CUL1* and *CUL2*, like animal *CUL1*, have been reported to be subunits of SCF complexes (Gray *et al*., 1999; Risseeuw *et al*., 2003; Moon *et al*., 2004). Gray *et al*. (1999) demonstrated that **CUL1** interacts with ASK1. Then Farras *et al*. (2001) co-immunoprecipitated ASK1 with CUL1 from Arabidopsis cell extracts and it is believed that CUL1 plays a very important role in general Arabidopsis development because null *cul1* mutants are embryonic lethal (Shen *et al*., 2002). A hypomorphic *cul1* mutant has recently been characterized and the evidence suggests that the morphological defects observed in this plant are caused by defective SCF complex formation (Moon *et al*., 2007).

However, the other four Cullins are also believed to form protein complexes with Ub-ligase activity. Animal studies indicate that CUL3 interacts with members of the BTB (Broad-complex, Tramtrack, Bric-a-Brac) family of proteins containing a conserved 100-residue protein motif known as a BTB domain

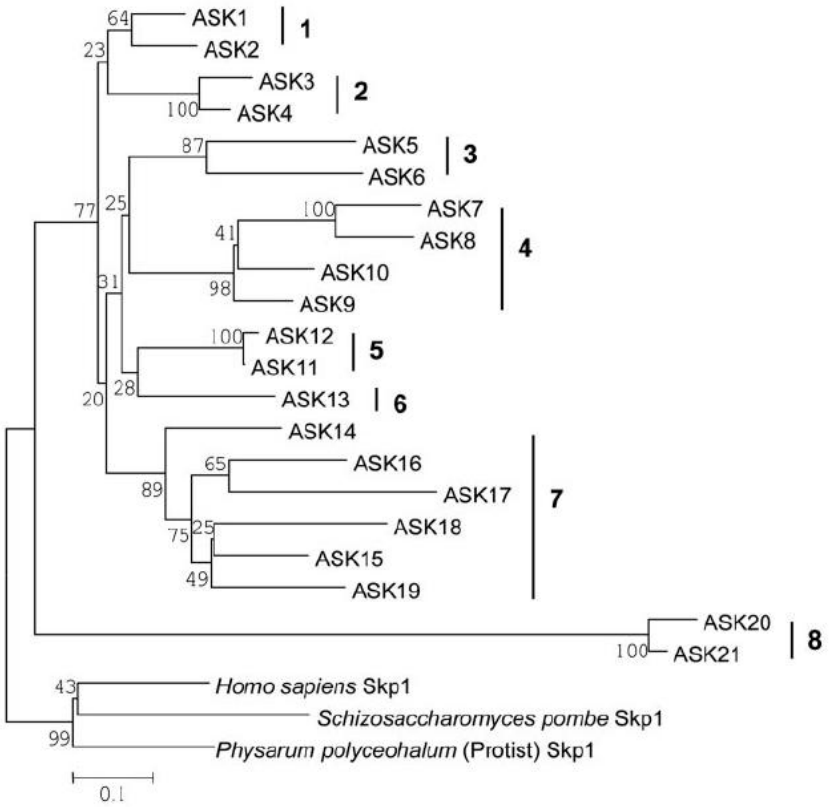


Figure 1-7: Phylogenetic relationships of the ASK genes based on amino acid sequence analysis. ASK genes have been classified into eight groups based on this tree (adapted from Zhao *et al*., 2003).

(Furukawa *et al*., 2003). The **CUL3/BTB** complex is similar to the SCF in that CUL3 serves as a scaffold. However, the SKP and F-box protein subunits have been replaced by a single BTB protein (Pintard *et al*., 2004). In plants, putative BTB proteins have also been characterized, including ETO1, which plays a role in the control of ethylene production (Wang *et al*., 2004). Both Arabidopsis **CUL3A** and **CUL3B** proteins are able to interact with RBX1 and with several plant BTB proteins (Dieterle *et al*., 2005; Weber *et al*., 2005), suggesting that they form similar CUL3-based E3 complexes. However, CUL3A loss-of-function mutants are viable and fertile and, exhibit only a mild phenotype (Dieterle *et al*., 2005). It has been demonstrated that the expression patterns of the *CUL3A* and *CUL3B* genes in Arabidopsis are largely overlapping and *cul3acul3b* loss-of-function double mutants are embryo lethal, suggesting functional redundancy between CUL3A and CUL3B (Thomann *et al*., 2005). Recently, the participation of Arabidopsis **CUL4** in a functional E3 ligase has been shown. CUL4 assembles with DDB1 (Damaged DNA-Binding1), DDB2, RBX1 and DET1 (De-Etiolated 1) *in vitro* and *in planta*. In addition, *cul4* mutants are severely affected in different aspects of development (Bernhardt *et al*., 2006). Thus apparently all Cullins participate in E3 ligase complexes.

The Cullin subunit is activated by the reversible attachment of **RUB1** (Related to Ubiquitin1), a protein with 75% sequence identity to Ub. Although the precise function of RUB1 modification is not clear, the cycling of RUB1 attachment and cleavage from CUL1 is necessary for SCF activity (Liu *et al*., 2002a). Arabidopsis encodes three RUB proteins, RUB1 and RUB2 are essential and regulate diverse processes throughout plant development (Bostick *et al*., 2004; Parry *et al*., 2004) by the function of ECR1 (E1-Conjugating enzyme-Related1) (Woodward *et al*., 2007).

4-2. RBX

The fourth essential subunit of SCF complexes (named in yeast and animal cells as *HRT1/ROC1/RBX1*) belongs to the ring finger domain RING-H2 protein family (Figure 1-5e) and interacts with both Cullin and E2. *In planta* association of RBX1 with CUL1 and ASK1 in Arabidopsis indicated that it is also part of plant SCF complexes. Mutation of the *RBX1* gene in budding yeast results in cell cycle arrest and the plant RBX1 is able to functionally complement it. There are two *RBX* genes in Arabidopsis (At5g20570 and At3g42830). *RBX1* is expressed in all plant organs while *RBX2* may only be expressed in particular cell types or at a very low level. Interestingly, higher *RBX1* mRNA accumulation was found in tissues containing actively dividing cells. Altered expression of *RBX1* causes severe defects in plant growth and development (Lechner *et al*., 2002).

4-3. SKP

In the Arabidopsis genome, there are 21 predicted *SKP1*-like genes (***ASK****s*) (see Figure 1-7 for a phylogenetic tree) (Farras *et al*., 2001; Gagne *et al*., 2002; Risseeuw *et al*., 2003). ASK1 is the first

Table 1-1: The Expression pattern of Arabidopsis ASKs tested by RT-PCR (Zhao *et al*., 2003; Marrocco *et al*., 2003). More + symbols for a higher expression level and − symbols for not-detected expression.

	locus	Seedling	Root	Stem	Leaf	Inflorescence	Silique
ASK1	At1g75950	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
ASK2	At5g42190	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +
ASK3	At2g25700	-	-	-	-	+	+ +
ASK4	At1g20140	+ +	+ +	+	+/-	+ + +	+ + +
ASK5	At3g60020	-	-	-	-	+	-
ASK6	At3g53060	-	-	-	-	-	-
ASK7	At3g21840	-	-	-	-	-	+
ASK8	At3g21830	-	-	-	-	-	+
ASK9	At3g21850	-	-	-	-	-	-
ASK10	At3g21860	+	+	+	+	+	+
ASK11	At4g34210	+	+	-	-	+	+
ASK12	At4g34470	+	+	+	+	+	+
ASK13	At3g60010	+	-	-	-	+ +	+
ASK14	At2g03170	-	-	-	-	+	-
ASK15	At3g25650	+	-	-	+	-	-
ASK16	At2g03190	-	-	-	-	-	+ +
ASK17	At2g20160	-	-	-	-	+	+ +
ASK18	At1g10230	+	+	+	+	+ +	+
ASK19	At2g03160	-	-	-	-	-	-
ASK20	At2g45950	+	+	+	+	+	+
ASK21	At3g61410	+	+	+	+	+	+

Table 1-2: Known F-box proteins and their function (reviewed by Lechner *et al*., 2006)

F-Box protein	Role in	Ref
TIR1 AFB1-3	Auxin signaling (targets Aux/IAA)	Ruegger et al., 1998; Gray et al., 1999; Dharmasiri et al., 2005a,b; Kepinski and Leyser, 2005
COI1	Jasmonate signaling, control of root growth, pollen fertility and defense response	Xie et al., 1998; Xu et al., 2002; Devoto et al., 2002, 2005
SLY1 SNE GID2	Gibberellin signaling (targets DELLA)	McGinnis et al., 2003; Dill et al., 2004; Strader et al., 2004; Fu et al., 2004; Ueguchi-Tanaka et al., 2005
EBF1 and 2	Ethylene signaling (targets EIN3)	Guo and Ecker, 2003; Potuschak et al., 2003; Gagne et al., 2004
TLP9	ABA signaling	Lai et al., 2004
UFO	control of flower development	Ingram et al., 1997; Samach et al., 1999; Zhao et al., 2001; Ni et al., 2004
ZTL FKF LKP2	control of flowering time and the circadian clock (targets TOC1)	Somers et al., 2000, 2004; Mas et al., 2003; Imaizumi et al., 2003, 2005; Schultz et al., 2001
EID1	photomorphogenesis	Dieterle et al., 2001; Marrocco et al., 2006
ORE9/MAX2	control of leaf senescence and lateral shoot branching	Woo et al., 2001; Stirnberg et al., 2002
ARABIDILLO1 and 2	control of latheral root development	Coates et al., 2006
SFB/SLF	control of self-incompatibility	Takayama and Isogai, 2005; Sijacic et al., 2004; Qiao et al., 2004a,b
SKP2A	control of cell cycle	del Pozo et al., 2002
SON1	defense response	Kim and Delaney, 2002

SKP1-like protein identified in Arabidopsis (Porat *et al*. 1998). As was said above, ASK1 has been shown to interact with CUL1 and to constitute a component of the SCF complex by interacting with diverse F-box proteins (Gray *et al*., 1999; Gagne *et al*., 2002). The phylogenetic analysis indicates that ***ASK1*** and ***ASK2*** are the most conserved among all *ASK* genes with respect to the yeast and human *SKP1* genes. In addition, among the *ASK* genes, *ASK2* is the most similar to *ASK1* in sequence (75.4% amino acid identity and 84.8% similarity) (Gagne *et al*., 2002). Moreover, ASK1 and ASK2 exhibit very similar expression patterns during vegetative and reproductive development (Table 1-1). Their mRNAs were detected in all major organs, although ASK2 expression was at lower levels than those of ASK1 (Zhao *et al*., 2003; Marrocco *et al*., 2003).

A null *ask1* mutant shows male sterility (Yang *et al*., 1999), reduction of leaf and petal sizes and alteration of floral organ identity (Zhao *et al*., 2001). There were no detectable defects in embryogenesis and early seedling development in the *ask1* mutant although some of the *ask1* phenotypes are consistent with a reduction in cell division (Zhao *et al*., 2001). Another study showed that the null *ask2* mutant has normal development throughout its life cycle. But the double *ask1ask2* mutations led to strong developmental defects at embryogenesis, resulted in seedling lethality and exhibited severe alterations in cell division, expansion and differentiation, whereas these defects were not detected in either *ask1* or *ask2* simple mutants (Liu *et al*., 2004). The expression patterns of ASK1 and ASK2 are consistent with their essential roles in embryogenesis and seedling development. These observations indicate that *ASK1* and *ASK2* may play redundant roles in controlling cell division and early development. In addition, the fact that the defects in the *ask1ask2* double mutant are much less severe than those in the *cul1* null mutant suggests that other *ASK* genes also may be important for early embryo development (Liu *et al*., 2004). So far, little is known about the role of the other ASK genes. Homozygous knockout mutants of ASK11, 12, 14 and 18 did not show differences in phenotype in comparison with wild-type plant (Takahashi *et al*., 2004). ASK1, 2, 11, and 19 interact with most of the F-box proteins investigated, whereas other ASKs interact with only a few F-box proteins (Gagne *et al*., 2002; Risseeuw *et al*., 2003).

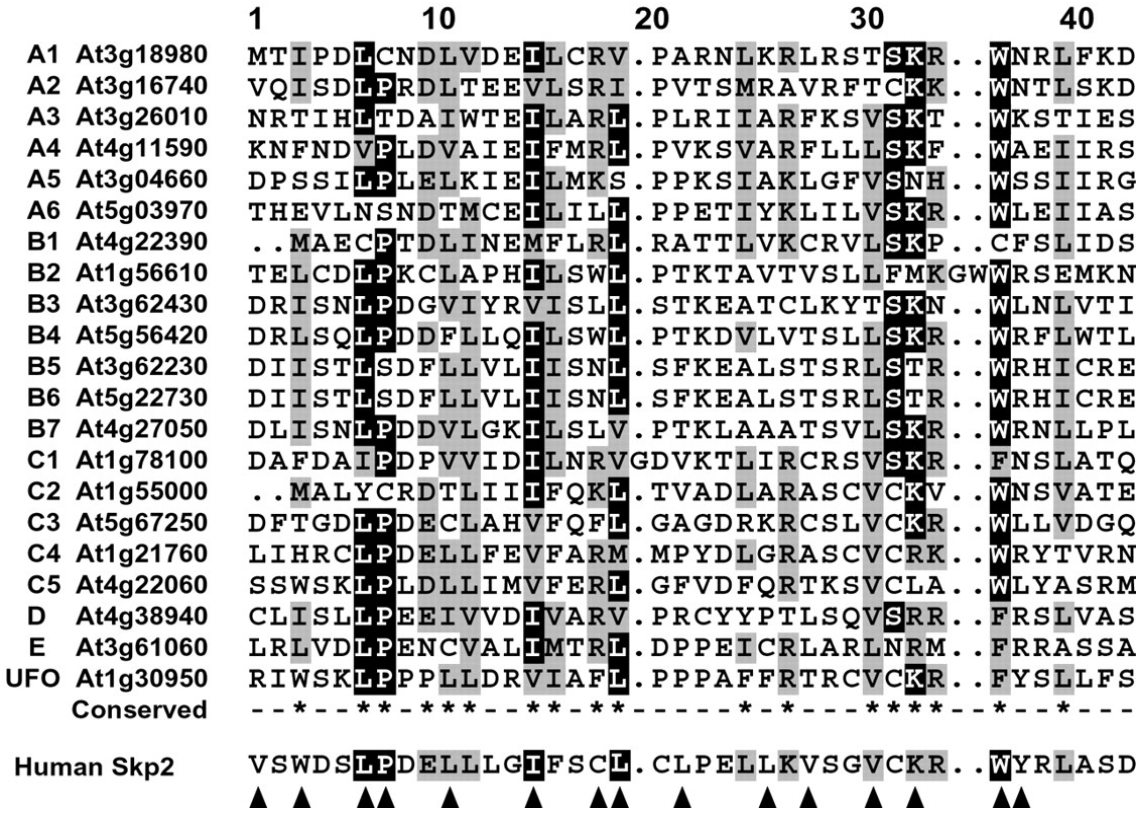


Figure 1-8: Sequence alignment of representative *Arabidopsis* F-box motifs. The 42-aa core F-box sequences from UFO, the human F-box protein Skp2, and from representatives of each of the 20 F-box protein groups (A1-E) from Arabidopsis were aligned. Dots denote gaps. Arrowheads mark the amino acids positions important for the Skp-F-box interactions between human Skp1 and Skp2 which is a F-box protein (adapted from Gagne *et al*., 2002).

4-4. F-box protein

SCF E3 provides an effective mechanism for recognizing many substrates simply by exchanging F-box subunits. The specificity of the SCF complex is conferred by the F-box subunit that recognizes the substrate. The name F-box was first given by Bai *et al.* (1996) on the basis of the presence of the protein-protein binding motif in Cyclin F. The Arabidopsis F-box gene UFO (Unusual Floral Organs) was the first F-box gene to be identified in plants (Samach *et al*., 1999). The Arabidopsis genome contains almost 700 F-box proteins encoding genes (Kipreos and Pagano, 2000) whereas only about 50 such genes in human and 14 in *S. cerevisiae* have been identified. The well characterized F-box proteins and their role in plant biology have been reviewed by Lechner *et al*. (2006) and Zeng *et al*. (2006) and they are recapitulated in Table 1-2. F-box proteins contain a loosely conserved F-box motif (~60 amino acids) near their N-terminus that anchors the subunit to the rest of the SCF complex by interacting with SKP. The first ~40 residues represent the core of the SKP-binding site and are followed by a ~20 residues variable domain with additional contacts that may help conferring a SKP binding preference (Figure 1-8) (Gagne *et al*., 2002). One of a number of possible protein-protein interaction motifs near the C-terminus of the F-box protein is implicated in substrate recognition.

In Arabidopsis, F-box proteins are classified into five major families based on amino acid sequence of F-box domain. Three of these families are further divided into 18 subfamilies, giving 20 distinct groups of proteins (Figure 1-8). Regarding the presence of 21 ASKs in Arabidopsis, it is demonstrated that there is an ASK specificity among the F-box proteins (Gagne *et al*., 2002).

On the other hand, a diverse array of C-terminal domains within the Arabidopsis F-box protein family have been identified including the well known LRRs (Leucine-rich repeat) and WD (Tryptophan-Aspartate) and various other domains such as Zinc fingers, Cyclin domains, Leucine Zippers, Ring fingers, TPRs (Tetratricopeptide repeats), Proline-rich domains, Kelch, Lectin binding, Armadillo, Jumonji-C and Tub, Actin and DEAD-like helicase (Figure 1-9). This diversity suggests that F-box proteins are capable of recognizing a wide variety of targets (Kipreos and Pagano, 2000; Gagne *et al*., 2002).

Most known targets of SCF complexes in animals and yeast need to be phosphorylated before F-box recognition (Deshaies *et al*., 1999; Hershko and Ciechanover, 1998; Jackson *et al*., 2000). In addition to phosphorylation, the binding of F-box proteins to substrates can also be promoted by other post-translational modifications such as glycosylation, methylation, ribosylation and acetylation (Cardozo and Pagano, 2004). Some F-box proteins can also direct auto-ubiquitination, possibly as a way to negatively regulate SCF E3 levels in the absence of substrate (Pickart, 2001; Kipreos and Pagano, 2000). In addition to targeting substrates for degradation, several mammalian and yeast F-box proteins have been shown to bind to and to modulate the activity of another protein independently of SCF (reviewed by Hermand, 2006). It has also been reported that the majority of yeast (*S. pombe*) F-box proteins are strongly induced

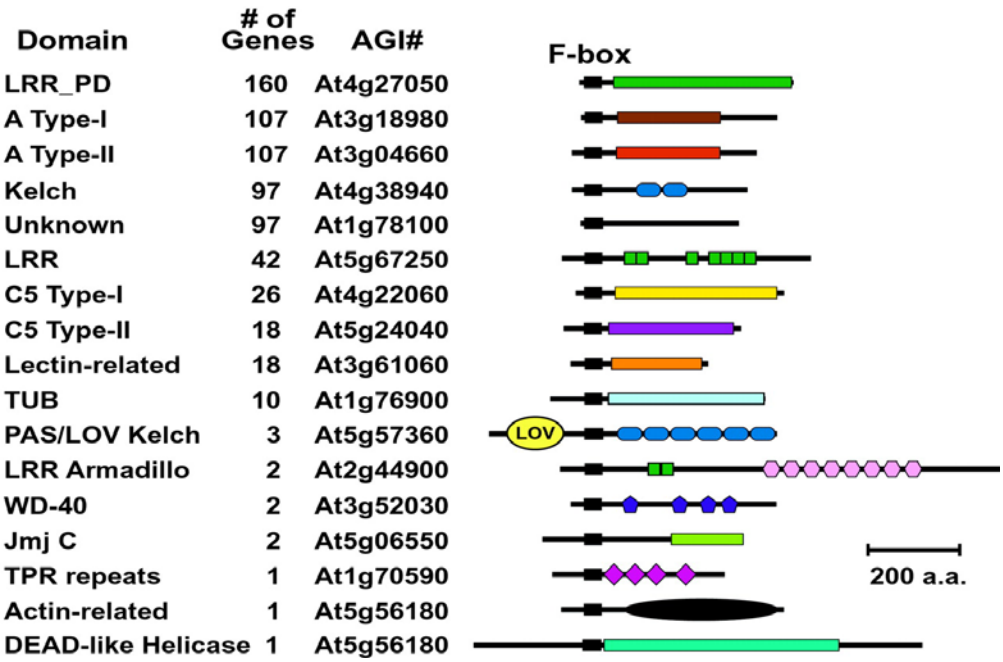


Figure 1-9: Diagrams of representative Arabidopsis F-box proteins with information on the structure and position of the C-terminal interaction domains. Shown on the left are the types of C-terminal domains, the number of F-box proteins predicted to have those domains, and the AGI number of the representative F-box protein on the right. (Adapted from Gagne *et al*., 2002).

by specific stresses such as oxidative stress (H_2O_2), osmotic stress (sorbitol), heat shock and response to Cadmium and Methyl Methane Sulfonate (Chen *et al*., 2003; Hermand, 2006).

Unlike other eukaryotes, plants can synthesize a remarkable number of SCF complexes. The Arabidopsis genome, encoding almost 700 F-box proteins, two RBX1 subunits, at least five Cullins and 21 ASKs could evidently generate an almost infinite array of distinct SCF ligases. It is also possible that members of the Cullin and SKP families interact with entirely new sets of substrate recognition factors (like that in the CUL3/BTB complex) to further expand specificity (Smalle and Vierstra, 2004).

Several other factors, such as SGT1 (Suppressor of G2 allele of SKP1) and CAND1 (Cullin-Associated and Neddylation-Dissociated1) have a regulatory effects on SCF activity. SGT1 which was identified in yeast as interacting with SKP1, possibly acts by promoting complex assembly with appropriate targets (Kitagawa *et al*., 1999). Conversely, CAND1, an inhibitor of the SCF, interacts with unmodified CUL1 and it has been proposed that dissociation of CAND1 from CUL1 by RUB1 promotes the binding of SKP1 and an F-box protein to CUL1 (Liu *et al*., 2002; Parry *et al*., 2004)

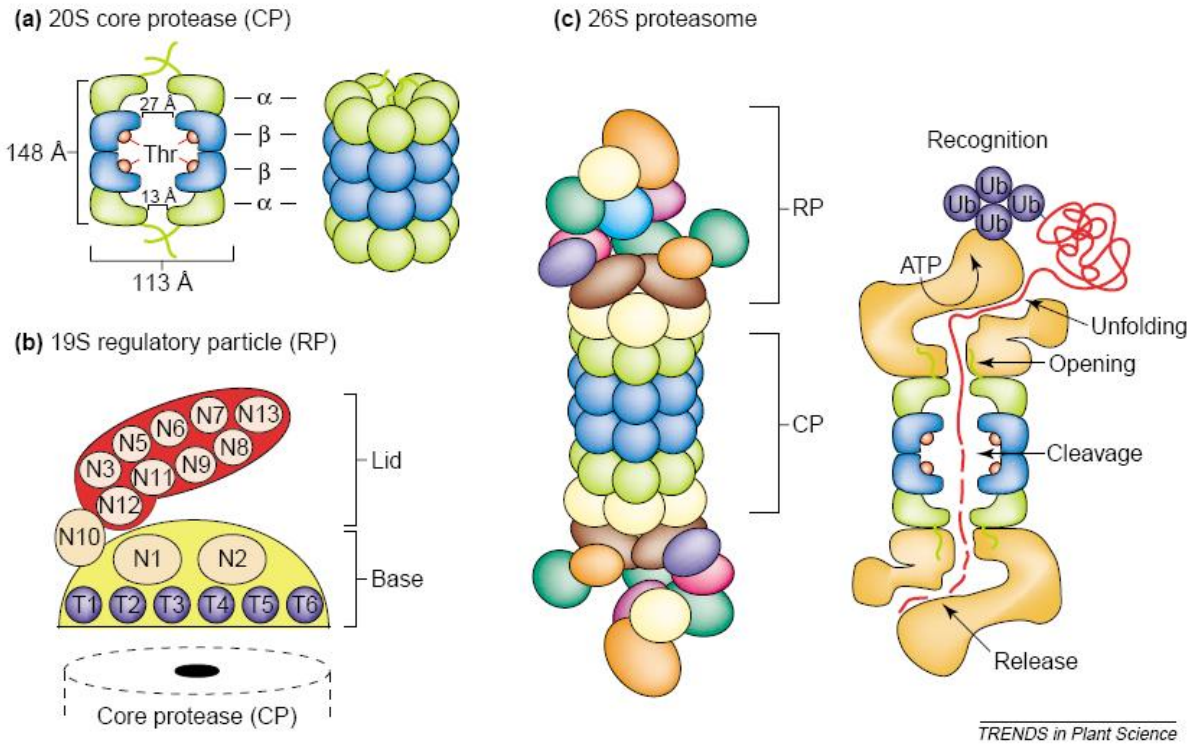


Figure 1-10: Structure of the 26S proteasome. (a) Organization of the core protease (CP) and (b) the regulatory particle (RP) with its Lid and Base subparticles. The N-terminal threonine residues that form the protease active sites in the b1, b2, and b5 subunits are indicated. Abbreviations: N, RP non-ATPase subunits; T, RP AAA-ATPase subunits. (c) Proposed structure that leads to the degradation of a ubiquitinated protein by the 26S proteasome (adapted from Vierstra, 2003).

5. The 26S Proteasome

The 26S proteasome is a 2-MDa ATP-dependent proteolytic complex that degrades ubiquitinated proteins (Voges *et al*., 1999; Hartmann-Petersen *et al*., 2003). Although most work on this complex is derived from animals and yeast, evidence is accumulating that the plant version is similarly organized (Fu *et al*., 1999; reviewed by Vierstra, 2003; Smalle and Vierstra, 2004).

The 26S proteasome contains 31 principal subunits arranged into two subcomplexes, the 20S cylindrical core protease (**CP**) and the 19S regulatory particle (**RP**) (Figure 1-10) (Groll and Huber, 2003). The CP is a broad spectrum ATP- and Ub-independent protease. It is created by the assembly of four heptameric rings of related α and β subunits. X-ray crystallographic analyses of the yeast and mammalian CPs revealed a large central chamber containing the protease active site (Groll *et al*., 1997; Unno *et al*., 2002) that has the capacity to cleave most peptide bonds. This active site of CP is very sensitive to the proteasome inhibitors, MG115, MG132, Lactacystin, and Epoxomycin (Yang *et al*., 2004). A small gate α subunit at entry and exit points of the channel restricts access to this chamber so that only unfolded proteins may enter and, in this way, the CP spatially separates proteolysis from the cellular milieu and restricts degradation to only those polypeptides that are unfolded and imported (Groll *et al*., 2000; Hartmann-Petersen *et al*., 2003).

The RP can be further divided into **lid** and **base** components (Figure 1-10); it associates with one or both ends of the CP and confers both ATP dependence and specificity for Lys48-linked polyUb chains to the particle. The Base contains a ring of six AAA-ATPase subunits (RPT1-6) along with three non-ATPase subunits (RPN1, 2 and 10). The lid contains the remaining eight non-ATPase subunits (RPN3, 5, 6, 7, 8, 9, 11 and 12) (Glickman *et al*., 1998). Collectively, the RP assists the CP in recognizing and unfolding appropriate substrates, removing the covalently bound Ubs and then directing the unfolded polypeptides into the lumen of the CP for breakdown (Hartmann-Petersen *et al*., 2003; Smalle and Vierstra, 2004).

The 26S proteasome is present in both the cytoplasm and nucleus of plant cells, with the highest amounts found in rapidly dividing tissues (Lee *et al*., 2003). During stress, the level of the complex also increases. In yeast, this upregulation is directed by a transcription factor, RPN4, that activates the expression of most 26S proteasome subunit genes (Xie and Varshavsky, 2001). Under normal conditions, RPN4 is rapidly degraded by the 26S proteasome, thus maintaining a low rate of 26S proteasome synthesis. But in situations with impaired 26S proteasome activity, RPN4 is stabilized, thus allowing subunit synthesis to rise. A similar, coordinated transcriptional upregulation of 26S proteasome genes is evident in Arabidopsis when 26S proteasome activity is diminished by mutation, suggesting that a similar negative feedback regulatory system exists (Yang *et al*., 2004).

6. Deubiquitinating enzymes (DUBs)

DUBs generate free Ub moieties from their initial translation products, recycle Ubs during breakdown of the polyUb-protein conjugates, and/or reverse the effects of ubiquitination. In Arabidopsis, at least 32 genes were identified that encode potential DUBs (Vierstra, 2003). RPN11 is a DUB in the 26S proteasome lid. All DUBs tested have remarkable specificity for Ub. They recognize the proximal Ub moiety and remove almost any amino acid or peptide attached to the C-terminal Glycine. Some DUBs can regulate a protein half-life by reversing the ubiquitination reaction, thus preventing their turnover by the 26S proteasome (Yan *et al*., 2000; Doelling *et al*., 2001; Smalle and Vierstra, 2004)

Ub/proteasome-dependent protein processing: A few cases have been discovered in which proteasomal degradation results in protein processing, thereby yielding proteins of different biological activity. This process is termed regulated Ub/proteasome-dependent processing (**RUP**) and is essential for the function of certain transcription factors. Examples are proteins of the mammalian NF-κB family and the yeast SPT23 and MGA2 proteins (reviewed by Rape and Jentsch, 2004). In the case of NF-κB (Nuclear factor kappa enhancer binding protein), p100 is the precursor of the p50, subunit of the NF-κB transcription factor. Phosphorylation of p100 in the C-terminal domain leads to selective degradation of this domain by an Ub/proteasome dependent reaction, while the N-terminal part, p50, is left intact and can be translocated into the nucleus and inhibit specific transcription. (Palombella *et al*., 1994; Chen, 2005).

Ub-independent protein degradation by the proteasome: There are a number of proteins that are degraded by the 26S proteasome in an Ub-independent manner. These includes ODC (Ornithine Decarboxylase), the first enzyme in the polyamine biosynthesis pathway, $p21^{Cip1}$, a Cyclin dependent kinase inhibitor, the α subunit of the T cell antigen receptor, c-Jun, IκBα, and Troponin (reviewed by Kahana, 2007).

7. Role of Ub-mediated protein degradation in resistance to pathogens

Several studies have demonstrated that Ub-mediated protein degradation plays a role in defense. For example, in plants, a homolog of the Ub ligase-associated protein, SGT1, is required for disease resistance (Austin *et al*., 2002; Azevedo *et al*., 2002; Liu *et al*., 2002b; Peart *et al*., 2002). In higher plants, some viral proteins are ubiquitinated (Dunigan *et al*., 1988). For example, Tobamovirus (TMV) movement protein is known to be polyubiquitinated and degraded by the 26S proteasome, a possible way to regulate viral spread and to reduce the damage caused by the protein (Reichel and Beachy, 2000). Mis-folded coat protein of TMV induces massive polyubiquitination in tobacco cells (Jockusch and Weigand, 2003). The movement protein of a Tymovirus (TYMV) is recognized as a substrate for polyubiquitination and subsequent rapid degradation by the proteasome (Drugeon and Jupin, 2002). In addition, perturbation of the Ub systems in plants can help viruses to multiply to elevated levels (Becker *et al*., 1993). Finally it

has been shown that functional NbSGT1 and NbSKP1 are required for resistance to TMV (Liu *et al*., 2002).

Several ubiquitination-related components have been reported to be induced after pathogen infection or by elicitors, for example, by the Avr proteins. The RING-finger-type E3 ligases ATL2 and ATL6 were rapidly induced in Arabidopsis plants treated with chitin elicitor (Salinas-Mondragon *et al*., 1999; Serrano and Guzman, 2004). Similarly, NbE1 was upregulated by infection with TMV and ToMV (Takizawa *et al*., 2005). Among F-box proteins that have been implicated in plant defense, Coronatine Insensitive1 (COI1) is required for plant response to jasmonic acid as well as defense against insects and pathogens (Devoto *et al*., 2005).

Inversely, examples are also known in which ubiquitination has been implicated in establishment of disease. Mutation in RING-finger-type E3 ligase in Arabidopsis resulted in enhanced disease resistance to *Erysiphe cichoracearum* with low-density inoculum (Ramonell *et al*., 2005). SON1 (Suppressor of Nim1) is another F-box protein that is implicated in plant-pathogen compatibility (Kim and Delaney, 2002). The *son1* mutant displays constitutive resistance against both the virulent Oomycete *Peronospora parasitica* and the bacterium *Pseudomonas syringae* pv. *tomato* strain DC3000, indicating that SON1-mediated ubiquitination negatively regulates plant defense (reviewed by Zeng *et al*., 2006). Thus, taken together, all components of Ub-mediated protein degradation pathways may have positive or negative effects in plant defense against pathogens.

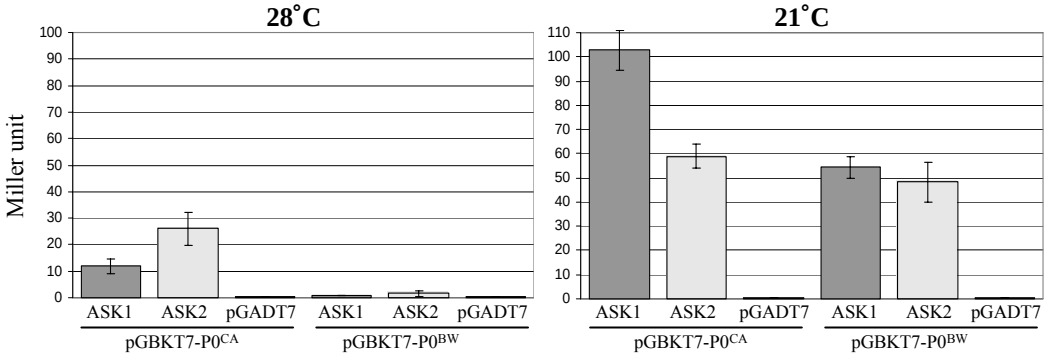


Figure 1-11: The interaction of $P0^{CA}$ and $P0^{BW}$ with ASKs by yeast two-hybrid quantitative test at two temperatures. Yeast strain Y187 doubly transformed with the indicated P0-ASK combinations was grown in –WL medium at the indicated temperature and β-galactosidase activity (Miller units) in the yeast extracts was measured using ONPG as substrate.

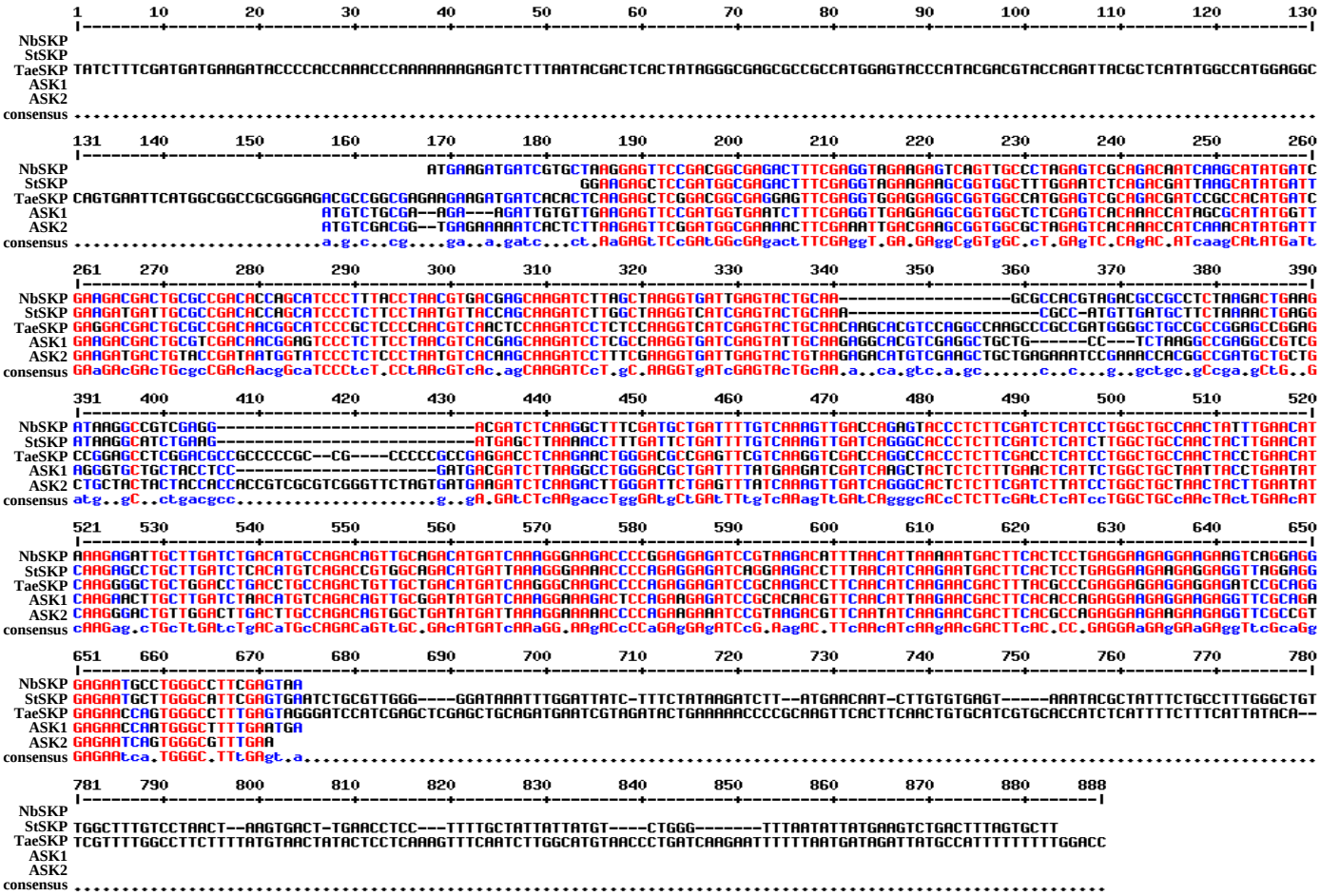


Figure 1-12: Sequence alignment of SKPs in different plants (http://bioinfo.genopole-toulouse.prd.fr/multalin/multalin.html). Nb: *N. benthamiana*, St: *Solanum tuberosum*, Tae: *Triticum aestivum*, ASKs from *A. thaliana*

A yeast two-hybrid screen in Arabidopsis cDNA library led us to identify ASK2 as a cellular partner for $P0^{CA}$**.** As mentioned above, phylogenetic analysis, expression pattern, analysis of Arabidopsis mutants and interaction with F-box proteins indicate that ASK1 and the very similar ASK2 are the most important ASKs among the 21 ASKs in Arabidopsis. Indeed, they are often referred to as 'master components' of the Arabidopsis SCF complex.

II. P0 interacts with both ASK1 and ASK2 in Yeast two-hybrid system

The first result of our screen was the strong interaction between $P0^{CA}$ and ASK2. Yeast colonies grew on medium without Adenine (A) and Histidine (H), two amino acids which are produced by reporter genes when two-hybrid interaction occurs. Such medium is referred to as –AHWL medium, where W (Tryptophan) is supplied by action of the *TRP1* gene carried by pGBKT7 and L (Leucine) by the *LEU2* gene carried by pGADT10. In this screen, four colonies containing the full-length ASK1 cDNA sequence grew in less stringent selection condition (–HWL) in the absence of only H as reporter gene but did not grow on –AHWL medium. When ASK1 was expressed from pGADT7 (see Figure 2-1) which contains a stronger promoter (full-length ADH1) with high relative protein expression level than pGADT10 a strong interaction between BD-$P0^{CA}$ and AD-ASK1 was observed (Figure 1B in publication N.1)

III. Interaction between $P0^{BW}$ and ASKs is temperature sensitive

Interaction of $P0^{BW}$ with ASK1 or ASK2 was observed at 21°C rather than 28°C. For details see publication N.1 (Figure 5 in Supporting Information). $P0^{CA}$ was able to interact with ASKs at both 21 and 28°C. The effect of temperature on the interaction between $P0^{CA}$ and $P0^{BW}$ and the ASKs was further investigated using yeast strain Y187, which carries only *LacZ* as a sensitive two-hybrid reporter gene. Y187 double transformants were grown at either 21°C or 28°C in –WL liquid culture and β-galactosidase activity in yeast extracts was assayed. The Y187 yeast doubly transformed with pGBKT7-$P0^{CA}$/pGADT7-ASK1 or pGBKT7-$P0^{CA}$/pGADT7-ASK2 displayed β-galactosidase activity at 28°C while the pGBKT7-$P0^{BW}$/pGADT7-ASK1 and pGBKT7-$P0^{BW}$/pGADT7-ASK2 double transformants did not. At 21°C, however, both $P0^{CA}$ and $P0^{BW}$ interacted with the ASKs and activated transcription of the reporter gene (Figure 1-11). The interaction of $P0^{CA}$ with ASK1 and ASK2 was stronger at 21°C than 28°C and the relative affinity of $P0^{CA}$ for the two ASKs was reversed. Whether the effect of temperature on the affinity between the P0s and the ASKs in yeast also applies to the P0-ASK interactions *in planta* is not known.

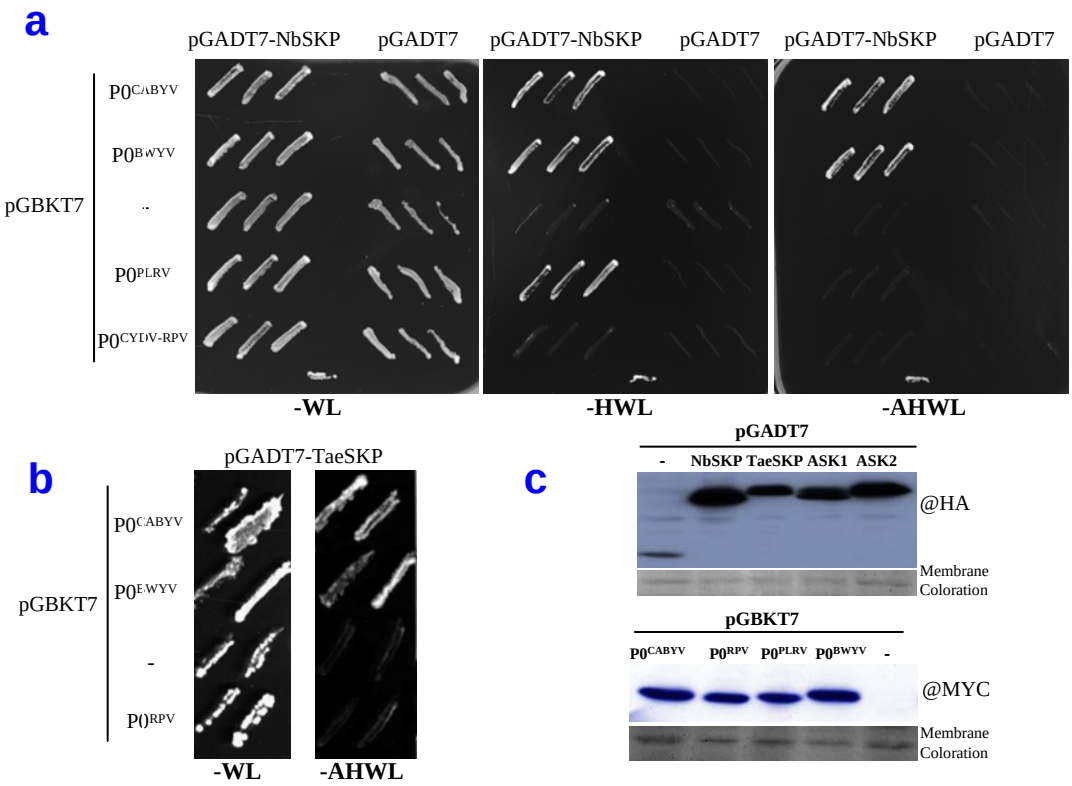


Figure 1-13: (a) Interaction of P0^{CABYV}, P0^{BWYV}, P0^{PLRV} and P0$^{CYDV-RPV}$ with NbSKP was investigated by yeast two-hybrid test using AH109 strain. Triple replicate double transformant clones were subcultured. (b) Similar yeast two-hybrid tests showed an interaction of P0^{CABYV}, P0^{BWYV}, but not P0$^{CYDV-RPV}$ with TaeSKP. Double replicate clones were subcultured. (c) western blot analyses on protein extracts of double transformed yeast to confirm the expression of fusion proteins in yeast. Antibody HA was used to detect the fusion proteins with AD in pGADT7 containing HA tag and antibody MYC was applied for detection of fusion proteins with BD in pGBKT7 containing MYC tag. Note that *N. benthamiana* is a host for BWYV and PLRV but not for CABYV and CYDV-RPV and the later virus is a virus infecting monocotyledons (such as wheat) wheras CABYV, BWYV and PLRV are viruses infecting dicotyledons.

Table 1-3: Interactions between different P0s and different SKP in Yeast two hybrid test

	CABYV P0	BWYV P0	PLRV P0	CYDV P0	pGBKT7
ASK1	●	●	○	○	○
ASK2	●	●	○	○	○
NbSKP	●	●	●	○	○
TaeSKP	●	●	ND	○	○
pGADT7	○	○	○	○	○

● Positive Interaction
● Weak Interaction
○ Without Interaction

Host Plant for corresponding virus

ND: not determinated

IV. P0 also interacts with both NbSKP and TaeSKP in yeast

In order to investigate the interaction between P0 and SKP protein from other host plants, we first conducted the amino acid sequence alignment of ASK1, ASK2, SKP of *N. benthamiana* (NbSKP) and SKP of *Triticum aestivum* (bread wheat, TaeSKP) (Figure 1-12). Strong homology of sequence was observed, in particular, NbSKP shares 74% similarity and 83% identity with ASK1 and 79% similarity and 67% identity with ASK2.

Using the primers designed from the published sequence we cloned *NbSKP* and *TaeSKP* from total RNA extracted from *N. benthamiana* and *T. aestivum* leaves, respectively, in vector pGADT7. Sequenced clones had the same sequence as was published (Ciaffi *et al*., 2005: *TaeSKP* accession N. AJ577364 and Liu *et al*., 2002: *NbSKP* accession N. AF494084). Yeast two-hybrid tests using the P0 of CABYV, BWYV, PLRV and CYDV-RPV (in pGBKT7) showed a strong interaction between $P0^{CA}$ and $P0^{BW}$ with NbSKP (Figure 1-13a) and TaeSKP (Figure 1-13b) although wheat is not a host for BWYV and CABYV. P0 of PLRV also interacted with NbSKP but with less efficiency (grew on –HWL medium only). However, P0 of CYDV-RPV did not interact with any tested SKP (ASK1, ASK2, NbSKP and TaeSKP) even on –HWL medium although wheat is a host for CYDV-RPV (Figure 1-13 a and b). The expression of the P0 and SKP fusion proteins was confirmed by Western blot analysis (Figure 1-13c). Table 1-3 shows the recapitulated results of yeast two-hybrid tests for P0s and SKPs. We conclude that interaction between ASK and P0 does not strictly correlate with host specificity. In particular, the absence of interaction between $P0^{CYDV}$ and TaeSKP raises the question of the function and/or mode of action of $P0^{CYDV}$. Up to now, nothing is known about this protein encoded by a cereal infecting polerovirus.

V. *In vitro* and *in planta* interactions of P0 with both ASK1 and ASK2

Using *in vitro* pull-down and *in planta* BiFC assays we showed that the interaction between $P0^{CA}$ and ASKs is direct. (For details see publication N.1, Figures 1C and 2)

```
     BWYV-P0: FKIDVFLRSLLYQLPF.HLGSCFHDAPREL..IPATEPEL.CAWFSLQ  VNLHVPGTKTSRRRIIQR
    CABYV-P0: INGTNLLRLFLARLPL.LISEQLS.GDYVY..TPGASKRIILARFHRH  VDLRLPASKDVARFFLAR
     BMYV-P0: FENENCIRSLLAALPL.LLSKQLDPGSFIY..TPGKRQSLRLARFYNY  IDLRVPPRKDVKRFYLAR
     BChV-P0: HQVEQFLRSILFQLPN.LIWSSWDHGTILYGDRPFTRAEDLLRFSLTT  LRVGLPSSEKAVRLQLQR
     PLRV-P0: EQIYRICRSFLHVLPL.LNCKRGRISTSGL.QLPRHLHYECLEWGLLC  IKLDDPTTAAAYRSELLR
CYDV-RPV-P0: NEIDLFCVSLGFLLPI.LLTGESYSWRGHL.NLPLSYTELLVRWGLAV  IDLSTMSTRSFYEQFLLR
     CtRLV-P0: CFYGNLPRALLYILPC.ILNNDYKMGGSAIWCKPDYIREL.VLWGLVC  VNLQRLGNRRAYLSFLHR

     F-box:      KPFPLL.RLPIELLRKILSRLD      PIDLLRLRLVSKKWRSLVDSLNIWFK     FIL
                 SS SIS DM LKIIKEVFKHMP      FKERFSFSLLCRRTKRIIKK KFKIR     KLE
                    RF  N  EDV  N IRK S      L  IIK  FT  NL Q LRD  LFKD
                           A     EY E       I   VN       F          R
                                 Q
```

Figure 1-14: The F-box motif sequence in P0 of Poleroviruses aligned with the consensus F-box motif sequence of 234 F-box proteins. A minority of F-boxes contain small insertions at various locations. The four lines below the first line of the F-box consensus sequence show observed substitutions of residues in F-box proteins. Frequency of underlined residues is over 40% and that of non-underlined is 10-40% in F-box proteins (Kipreos and Pagano, 2000).

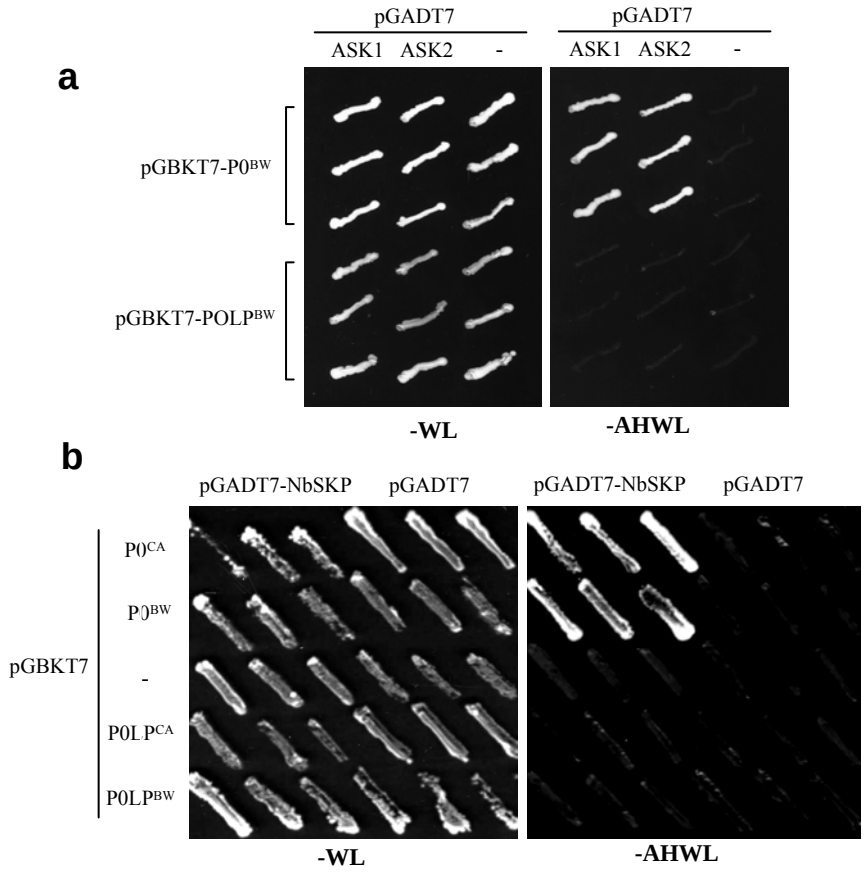


Figure 1-15: The P0 F-box motif mutant (LP→AA) has lost its ability to interact with SKP. (a) Mutant $P0^{BW}$ in which LP was substituted with AA did not interact with ASK1 and ASK2 in yeast two-hybrid system. Triple replicate colonies were subcultured at 21ºC. (b) Similar loss of interaction of P0LP mutants with NbSKP. SD/-WL is non-selective and SD/-AHWL is selective medium for interaction. (-) represent empty vector.

VI. P0s of poleroviruses contain an F-box motif essential for interaction with ASKs

The direct interaction of P0 with SKP led us to the hypothesis that P0 is an F-box protein because as was mentioned above, in an SCF complex, SKP interacts with Cullin on one hand and diverse F-box proteins on the other hand. Therefore, we sought to discover a conserved F-box motif in P0. The amino acid sequence alignment of P0 from different poleroviruses showed a conserved F-box like motif in their N-termini (Figure 1-14). In particular, a short LPxxI/L sequence is conserved in virtually all P0s. Since the F-box motif is generally responsible for interaction with SKP we next determined whether P0 mutated in the F-box motif can interact with ASKs and if this sequence has a biological activity when P0 is expressed from virus.

Deletions of the F-box motif or substitution of the LP residues by AA (Alanine) in P0 of CABYV resulted in loss of the interaction with ASK1 and ASK2 (see publication N1 for details) and with NbSKP (Figure 1-15) in the yeast two-hybrid system and also in a BiFC *in planta* assay (see publication N.1 and Figure 2). Similar results were obtained with comparable mutants of $P0^{BW}$ (Figure 1-15).

VII. Incorporation of P0 into the SCF complex

The presence of a functional F-box sequence in P0 suggested that P0 is incorporated into an SCF complex. To investigate this hypothesis, we used a yeast triple-hybrid system (bridging assay) to show that interaction between CUL1 and P0 is possible only in presence of ASK1 or ASK2 (For details see publication N.1). Therefore, P0's interaction with ASK does not block the ability of the P0-ASK complex to interact with another component of the SCF complex. This result is similar to the findings of Risseeuw *et al*. (2003) who have demonstrated that cellular F-box proteins can interact with CUL1 in presence of ASK1 or ASK2 in bridging assay.

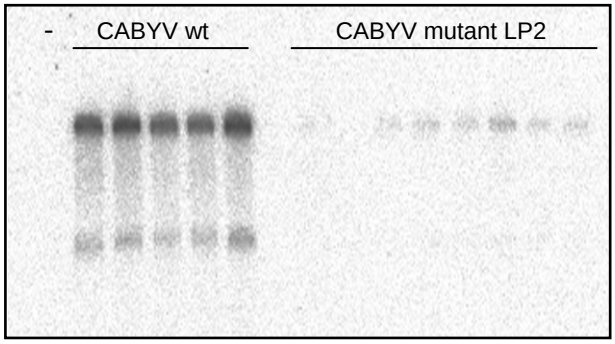


Figure 1-16: CABYV containing mutant P0 (mutated in the F-box motif) accumulated ~10 fold less efficiently than wild type. Northern blot analyses of total RNA extracted 14 dpi from plants agroinfiltrated with wild-type or mutated CABYV is shown. Each lane presents an independent Arabidopsis plant. (-) is a non-inoculated plant. Probe was designed for detection of the 3' of CABYV RNA.

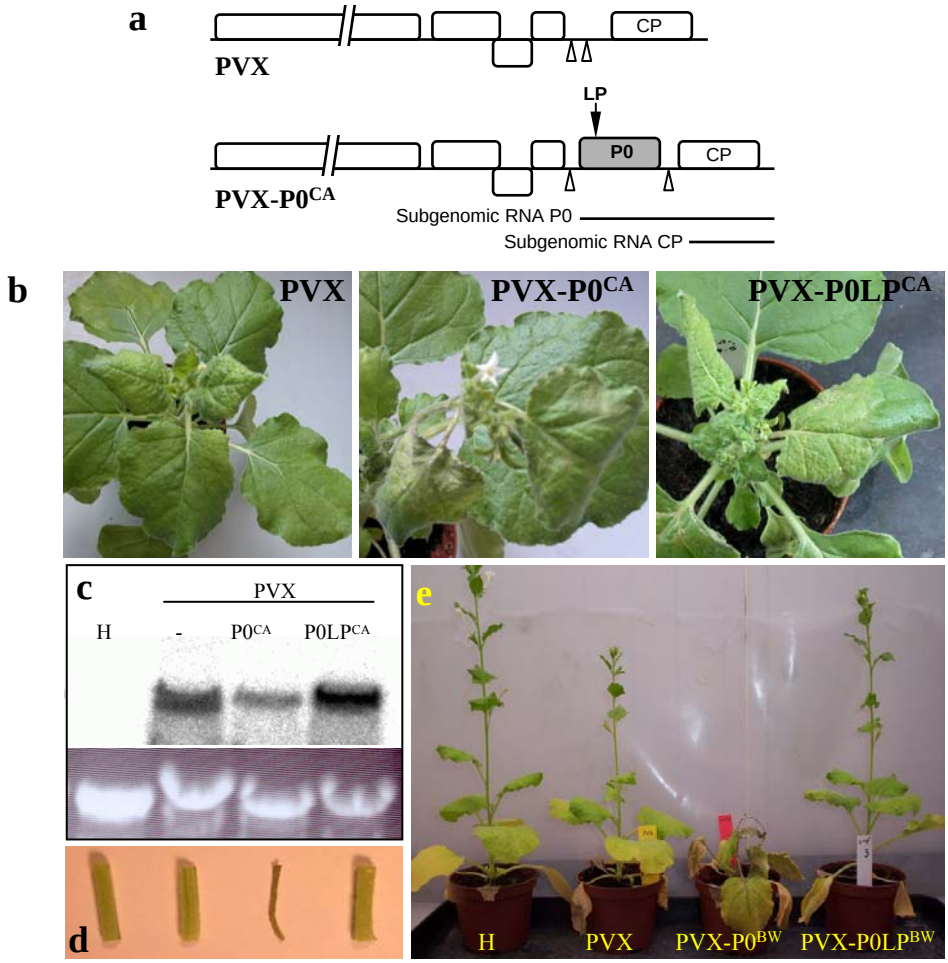


Figure 1-17: The LP F-box mutation diminishes the necrotic response provoked by infection of *N. benthamiana* with PVX chimera expressing $P0^{CA}$. (a) Structure of PVX and PVX-$P0^{CA}$. The position of the LP mutation (LP→AA) in PVX-$P0LP^{CA}$ is indicated by an arrow. The duplicated promoters (Δ) drive synthesis of the subgenomic RNA. (b) Symptoms provoked on systemically infected leaves (12 dpi) following inoculation with the indicated transcript. (c) Northern blot detection of the progeny of viral genomic RNA in systemically infected leaves following inoculation with the indicated transcript. Total leaf RNA was extracted 12 days post-inoculation. H: RNA from a leaf of a non-inoculated plant. A loading control (rRNA) is shown below.(d). Effect of infection on petiole of plants infected with the transcripts as indicated in (c). (e) Overview of plants 18 days post infection by indicated transcripts.

VIII. The F-box motif is required for P0-mediated viral pathogenicity

To test the importance of the F-box motif in P0 for viral function we introduced a mutation in this motif in the viral genome. A viral construct (LP2) was produced containing a mutation in $P0^{CA}$ in which LPLLI was substituted with MFMQF, so as to keep unchanged the amino acid sequence of P1, which is encoded by an overlapping open reading frame. Arabidopsis plants were agroinfiltrated with agrobacterium harboring wild-type pBIN-CABYV or pBIN-CABYV LP2 mutant. Total RNA extraction was carried out on leaf tissues five weeks after inoculation and northern blot analysis was performed using a complementary RNA complementary probe to detect the 3' terminal part of CABYV RNA (Figure 1-16). Results showed that the mutant virus accumulated ~10 fold less efficiently than the wild-type virus. This result was consistent with previous results obtained in our laboratory using BWYV with knockout mutations in the 5' terminal part of ORF0. Accumulation of these mutant viruses was also significantly (five to sevenfold) diminished in agroinoculated *N. clevelandii* (Ziegler-Graff *et al*., 1996). Therefore, the F-box motif in P0 has an important biological effect for viral RNA accumulation.

Other earlier work in our laboratory revealed that $P0^{BW}$ is a pathogenicity factor capable of enhancing the pathogenicity of an unrelated virus and this activity is independent of other BWYV genes. It has been demonstrated that *N. benthamiana* plants mechanically inoculated with transcripts of a PVX-based vector (pP2C2S) in which the $P0^{BW}$ coding region has been inserted, showed necrosis in the petioles and veins and then in mesophyll tissue followed by death of the upper leaves and eventually of the entire plant (Pfeffer *et al*., 2002). We repeated this experiment with a CABYV P0LP mutant and compared the resulting symptoms with those provoked by wild-type P0 and empty PVX vector. We observed strong necrotic symptoms in infection with PVX-$P0^{CA}$ but plants infected with PVX-$P0LP^{CA}$ mutant showed symptoms similar to plant infected with PVX transcripts, including mild mosaic without any necrotic lesions (Figure 1-17). We conclude that the pathogenicity enhancement provoked by P0 is strongly related to its F-box motif. We repeated this experiment for $P0^{BW}$ and $P0LP^{BW}$ and obtained similar results as for $P0^{CA}$ and $P0LP^{CA}$ (For details see Publication N.1 and Figure 6 in supporting information and Figure 1-17e here).

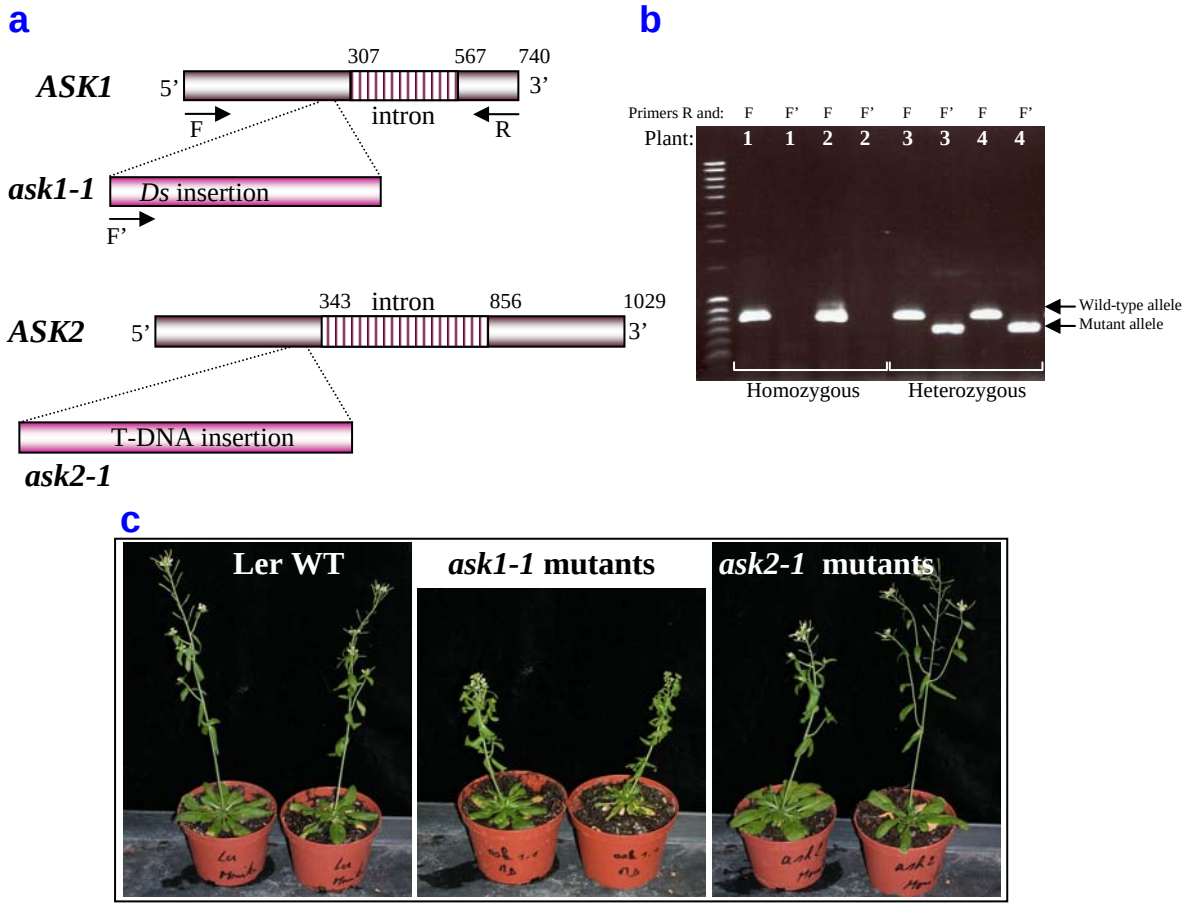


Figure 1-18: (a) Schematic structure of ASK1 and ASK2 genes in Arabidopsis and their insertion position in the corresponding mutants (Liu *et al*., 2004). For genotyping of homozygous *ask1-1* mutant among mixed homozygous and heterozygous plants we carried out two PCR reactions on DNA extracted from one leaf of each plant using R primer (reverse for both PCR) and F primer (forward to detect wild-type allele) or F' primer (forward to detect insertion mutant allele). (b). An example of gel analysis shows products of PCRs for four plants, two homozygous and two heterozygous *ask1* mutants. (c) Phenotype of ask mutants and wild-type Arabidopsis L*er* plants

IX. ASK1 and ASK2 are redundant for action of P0 in viral infection

If the interaction between P0 and ASK1 and ASK2 plays a role in the mechanism by which the virus evades the host defense response, we would predict that plants carrying null mutations in ASK1 and ASK2 should display reduced susceptibility to polerovirus infection. As mentioned above, Arabidopsis double mutants in both ASK1 and ASK2 die at the early seedling stage but *ask1* and *ask2* plants are viable (Gray *et al*., 1999; Yang *et al*., 1999; Zhao *et al*., 1999; Liu *et al*., 2004). The homozygous *ask1* mutant is male sterile and the *ask1* mutant must be maintained by a heterozygous plant (Yang *et al*., 1999). Two week-old Arabidopsis *ask1* homozygous mutants were selected by genotyping (Figure 1-18a,b). These plants and *ask2* homozygous mutants (Liu *et al*., 2004) (Figure 1-18c) were inoculated with BWYV and CABYV by aphid transmission (Veronique Brault, INRA Colmar, France). We could not use the agroinfiltration method because ASK1 and ASK2 are required for delivery of the T-DNA by agrobacterium for function of its VirF F-box protein (Tzfira *et al*., 2004). The susceptibility of the mutants to infection was measured by ELISA four weeks post-inoculation and the virus titer was compared to that of a wild-type Arabidopsis ecotype Landsberg *erecta* (L*er*) inoculated in parallel.

Several independent experiments were carried out but no significant differences in viral infection levels between the *ask1* and *ask2* simple mutants and wild-type plants were observed. As ASK1 and ASK2 both interact with P0 in yeast, it is likely that there will be functional redundancy between ASK1 and ASK2 as well as with other ASKs in Arabidopsis.

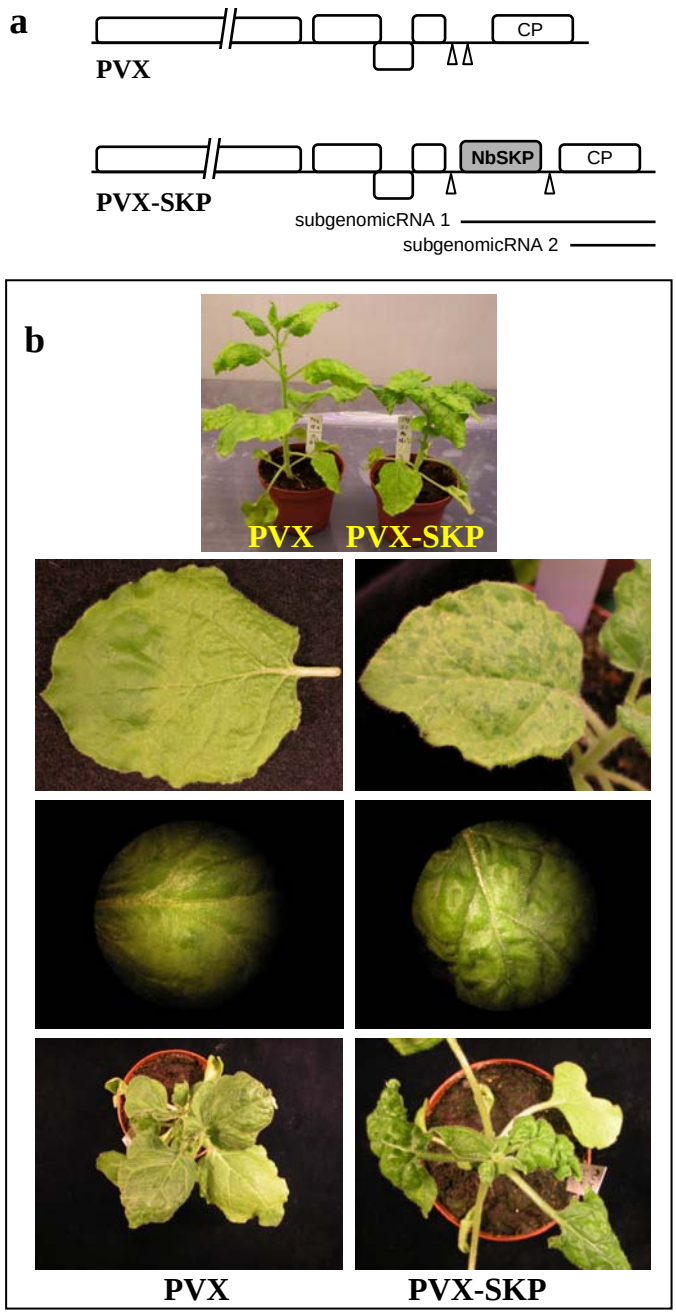


Figure 1-19: (a) genomic organization of PVX and PVX-SKP (four 5' terminal nucleotides of NbSKP deleted) (b) The symptoms on plants infected with PVX or PVX-SKP (see text). A close up of the symptoms is shown in the middle panel. The roughened leaf surface is visible on leaf of plant infected with PVX-SKP.

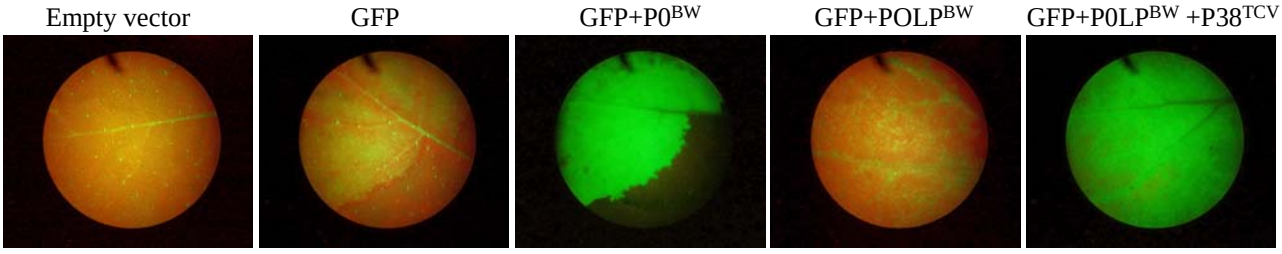


Figure 1-20: Florescence levels of GFP on leaves of *N. benthamiana* 16c (containing GFP transgene) 5 days after agroinfiltration with agrobacteria harboring indicated genes.

X. NbSKP is essential for polerovirus infection in *N. benthamiana*

The non-conclusive results of the viral infection experiments with the Arabidopsis *ask1* and *ask2* mutants led us to design a similar experiment in *N. benthamiana*. As noted above, $P0^{CA}$ and $P0^{BW}$ interact with NbSKP in yeast two-hybrid system. Therefore we decided to deplete the mRNAs encoding *N. benthamiana* SKP or SKPs (if there are SKP family genes in this plant) using virus-induced gene silencing (VIGS) and then test the efficiency of poleroviral infection. To this end, we cloned the NbSKP entire cDNA sequence except for the four 5'-terminal nucleotides into PVX in order to avoid NbSKP translation (Figure 1-19a) and then inoculated the PVX-SKP transcripts to *N. benthamiana* plants. As a control, plants were inoculated in parallel with transcript of the empty PVX vector. Symptoms of PVX infection appeared on upper leaves by 8 dpi. The mosaic symptoms on upper leaves of plants infected with PVX-SKP were more severe than the mild mosaic on leaves of plants infected with PVX (Figure 1-19b). The plants infected with PVX-SKP had a roughened leaf surface with long petioles, epinasty in upper leaves and stunted plant size. SKP silencing was recorded in a time course experiment in which disappearance of SKP transcripts and proteins were analyzed in parallel by RT-PCR and Western blot, respectively. SKP mRNA was found to be reduced in PVX-SKP plants to about 5% of that of PVX control plants from 16 to 30 days post-infection (dpi). Diminution of SKP protein levels was somewhat delayed; it started at 16 dpi and continued to almost complete disappearance at 30 dpi. Inoculation of BWYV was carried out by aphid transmission at 16 dpi by PVX or PVX-SKP before the plants became too big for viral inoculation, and BWYV titer on upper leaves was measured by ELISA two weeks after. Results (see publication N.1 and Figure 3) showed that plants silenced for the SKP gene(s) were resistant to BWYV. Therefore, SKP, the cellular partner of P0, is required for virus infection by polerovirus. Similar results were obtained with CABYV infection.

XI. Mutation in the F-box motif inhibits P0's suppression of gene silencing

We have shown that P0 is an F-box protein that interacts with SKP in plant in an SCF complex and this incorporation is important for virus pathogenicity. On the other hand, P0 is a suppressor of silencing. We next asked whether the F-box activity of P0 is necessary to its silencing suppressor function. To answer this question we used a Patch Test assay in which pBIN-GFP was co-expressed with pBIN-$P0^{BW}$ or pBIN-$P0LP^{BW}$ by agroinfiltration in leaves of *N. benthamiana* 16c containing GFP transgene (Ruiz *et al*., 1998). We observed that mutation in the F-box motif resulted in loss of the silencing suppression activity. By co-expression with another suppressor of silencing, P38 of TCV, we confirmed that the P0 mutant protein is stable. Absence of inhibition of silencing was therefore not due to instability of the protein but to a real loss of activity. Figure 1-20 shows the fluorescence levels of GFP co-expressed with different proteins in the Patch Test. For details and molecular analysis see publication N.1 and Figure 4).

Publication N.1:

F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function. Proc. Natl. Acad. Sci. USA, February 7, 2006, 103(6):1994-1999.

F-box-like domain in the polerovirus protein P0 is required for silencing suppressor function

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Plants employ small RNA-mediated posttranscriptional gene silencing as a virus defense mechanism. In response, plant viruses encode proteins that can suppress RNA silencing, but the mode of action of most such proteins is poorly understood. Here, we show that the silencing suppressor protein P0 of two *Arabidopsis*-infecting poleroviruses interacts by means of a conserved minimal F-box motif with *Arabidopsis thaliana* orthologs of S-phase kinase-related protein 1 (SKP1), a component of the SCF family of ubiquitin E3 ligases. Point mutations in the F-box-like motif abolished the P0–SKP1 ortholog interaction, diminished virus pathogenicity, and inhibited the silencing suppressor activity of P0. Knockdown of expression of a SKP1 ortholog in *Nicotiana benthamiana* rendered the plants resistant to polerovirus infection. Together, the results support a model in which P0 acts as an F-box protein that targets an essential component of the host posttranscriptional gene silencing machinery.

E3 ubiquitin ligase | RNA silencing | viral pathogenicity | viral suppressor

Posttranscriptional gene silencing (PTGS) in plants is an example of a widespread phenomenon in metazoa in which RNA transcripts are degraded in a sequence-specific manner through the intervention of homologous short (21–24 nt) RNAs known as siRNAs (1, 2). The presence of double-stranded RNA (dsRNA) in the cytoplasm triggers PTGS. siRNAs are generated from the dsRNA by members of the Dicer family of dsRNA-specific endonucleases and are loaded onto an Argonaut-containing multicomponent complex known as RISC (RNA-induced silencing complex), where they act as guide RNAs to mediate degradation of RNA sequences complementary to the siRNAs.

PTGS is important in host defense against viruses, and it is now recognized that many plant viruses encode silencing suppressor proteins, which can counter this defense response (3–5). There is no sequence homology between known silencing suppressor proteins of different virus genera, suggesting that they intervene at different steps in the PTGS pathway. However, with the exception of a group of silencing suppressors exemplified by P19, which binds siRNAs or related molecules and is thought to disrupt PTGS by sequestering these species (6–8), relatively little is known about the mode of action of silencing suppressor proteins.

The Poleroviruses (family *Luteoviridae*) are a group of plant viruses with a small (≈5.6 kb) plus sense-RNA genome and icosahedral virions that are phloem-limited in their hosts (9). P0, the ≈29-kDa protein encoded by the 5′-proximal gene on polerovirus genomic RNA (Fig. 1*A*), is a potent silencing suppressor (10). In this work, we show that P0 interacts by means of an F-box-like domain with *Arabidopsis thaliana* S-phase kinase-related protein 1 (SKP1) orthologs. SKP1 is a core component of the SCF family of E3 ubiquitin ligases and serves to tether the rest of the complex to an F-box protein, which provides specificity in binding to ubiquitin ligase substrate proteins (11, 12). Point mutations in the F-box-like motif of P0 that abolished the P0–SKP1 interaction also abolished the silencing suppressor activity of P0 and diminished viral pathogenicity. Finally, knockdown of SKP1 ortholog levels in *Nicotiana benthamiana* rendered the plants resistant to Polerovirus infection. Together, these observations support a model in which P0 functions as a virus-coded F-box protein to direct an essential component of the host's PTGS-based virus defense system to the E3 ubiquitination ligase machinery.

Results

P0 interacts with *Arabidopsis* SKP1-Related (ASK1) and ASK2 in Yeast. The Poleroviruses Beet western yellows virus (BWYV) and Cucurbit aphid-borne yellows virus (CABYV) infect *Arabidopsis*. Yeast were transformed with constructs expressing P0 from either BWYV ($P0^{BW}$) or CABYV ($P0^{CA}$) fused to the GAL4 DNA binding domain (BD), and the resulting fusion proteins were used as bait in two-hybrid screens of a cDNA library expressing *A. thaliana* proteins fused to the GAL4 activation domain (AD). With $P0^{BW}$ as bait, none of the 10^7 double transformants tested grew on selective medium. When $P0^{CA}$ was the bait, six of the 5×10^6 double transformants grew under strong selective conditions. All contained the same prey sequence, corresponding to *ASK2* (At5g42190), which is an ortholog of the *Saccharomyces cerevisiae Skp1* gene.

SKP1 is a core subunit of the multicomponent SCF (SKP1/Cullin1/F-box/RBX1) E3 ubiquitin ligase. The E3 ubiquitin ligases are a large and diverse group of proteins and protein complexes that can direct ubiquitination of specific target proteins as a signal for their degradation by the 26S proteasome (11, 12). SKP1 orthologs are found in many organisms, including higher plants, where SCF-mediated ubiquitination/proteolysis of target proteins regulates numerous pathways (13). The SKP1 subunit in the SCF complex acts as a specific adapter linking the Cullin1 (CUL1) scaffold protein to one of a large family of F-box proteins, which in turn selectively binds to a target protein. The target is then ubiquitinated by an E2 ubiquitin-conjugating enzyme, which docks at the RBX1 subunit of the SCF complex. Genes for 21 SKP1 orthologs have been identified in *Arabidopsis*, but ASK2 and the closely similar ASK1 are the most abundant and interact with many F-box proteins (14–16). Although in our original screen no double transformants containing *ASK1* were

Conflict of interest statement: No conflicts declared.

Abbreviations: PTGS, posttranscriptional gene silencing; SKP1, S-phase kinase-related protein 1; ASK, *Arabidopsis* SKP1-like; YFP, yellow fluorescent protein; CFP, cyan fluorescent protein; BD, binding domain; CABYV, Cucurbit aphid-borne yellows virus; BWYV, Beet western yellows virus; PVX, Potato virus X; AD, activation domain; CUL1, Cullin1; pi, postinoculation; YN, N-terminal YFP; YC, C-terminal YFP; VIGS, virus-induced gene silencing.

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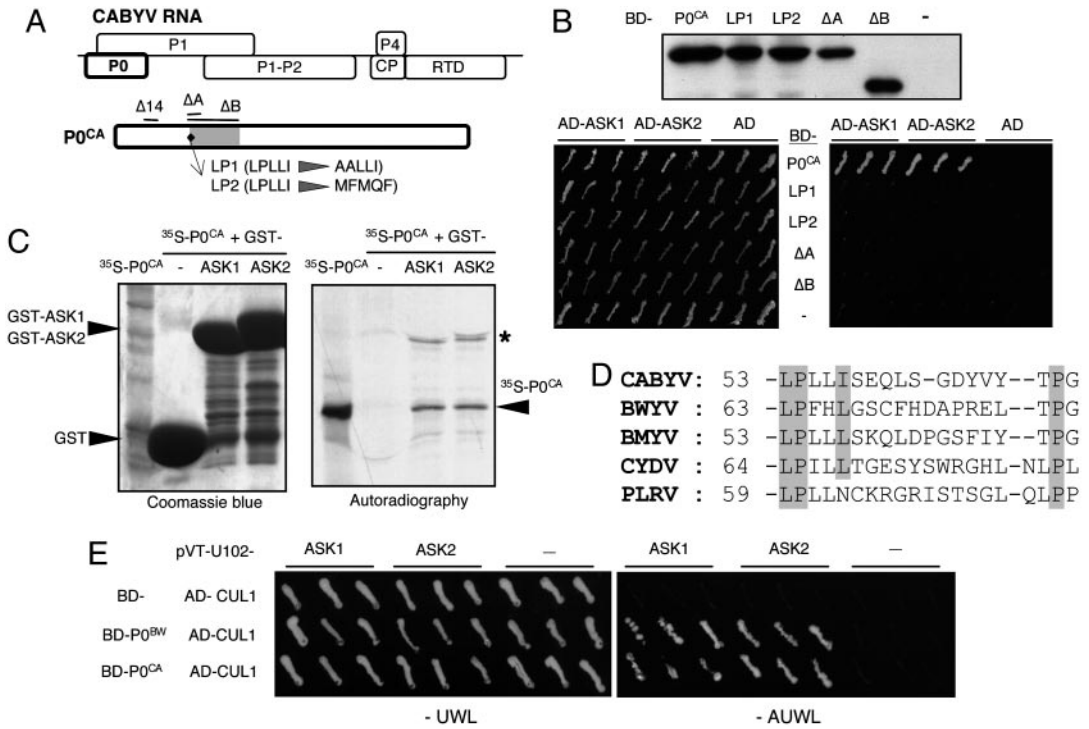

Fig. 1. P0^{CA} interacts with ASK1 and ASK2 via an F-box domain. (*A*) Genetic organization of CABYV RNA. Labeled rectangles symbolize important genes. The genetic organization of BWYV RNA is identical. The blow-up image shows the positions of deletions (above) and point mutations (below) in P0. Shading indicates the notional position of the ≈60-residue F-box domain. (*B*) Three independent colonies of yeast transformed with bait and prey plasmids expressing the indicated fusion proteins were replicate-streaked on nonselective medium (*Left*) and on medium selective for a strong two-hybrid interaction (*Right*). *Upper* is a Western blot showing accumulation of the WT and mutant P0^{CA} fusion proteins in total yeast protein extracts. (*C*) Pull-down of [^{35}S]methionine-labeled P0^{CA} by glutathione *S*-transferase (GST) and GST–ASK1 and GST–ASK2 fusion proteins immobilized on glutathione-Sepharose beads. Proteins immobilized on the beads were visualized by Coomassie blue staining (*Left*) and ^{35}S-P0^{CA} by autoradiography (*Right*). An aliquot of the input ^{35}S–P0^{CA} was loaded in the leftmost lane of the gel. The minor bands marked by an asterisk in the two right-hand lanes of the autoradiogram comigrate with the GST–ASK1 and GST–ASK2 bands (*Left*) and may be ^{35}S–P0^{CA}, which has remained associated with the fusion proteins under denaturing conditions. (*D*) Sequences of P0 of CABYV, BWYV, Beet mild yellowing virus (RefSeq accession no. NC 003491), Cereal yellow dwarf virus-RPV (NC 004751), and Potato leafroll virus (NC 001747) near the LPxxI/L motif and a downstream proline conserved in many plant F-box domains. (*E*) A yeast-bridging assay shows that interaction between P0^{BW} and P0^{CA} and AtCUL1 requires ASK1 or ASK2 as a bridging component. Three independent yeast AH109 colonies triply transformed with plasmids expressing the indicated BD-P0 and AD-CUL1 fusion proteins plus either empty pVT-U102 or pVT–U102 expressing ASK1 or ASK2 were replicate-streaked on a dropout plate under nonstringent (−UWL) and stringent (−AUWL) conditions selecting for interaction between BD–P0 and AD–CUL1. The plates were incubated at 21°C for 7 days.

obtained, an interaction with P0^{CA} was observed when AD-ASK1 was expressed in yeast from a vector (pGADT7) with a stronger promoter (Fig. 1*B*).

The high degree of conservation of SCF components between yeast and plants (15) raised the possibility that a yeast protein could participate in the observed two-hybrid interactions. To determine whether P0^{CA} can associate with ASK1 and ASK2 in the absence of other proteins, glutathione-Sepharose beads loaded with GST–ASK1, GST–ASK2, and GST (expressed in *Escherichia coli*) were incubated with [^{35}S]methionine-labeled P0^{CA} translation product. After washing, bound proteins were eluted from the beads and analyzed by SDS/PAGE. Coomassie blue staining of the gel revealed the GST, GST–ASK1, and GST–ASK2 in the eluates (Fig. 1*C Left*), whereas autoradiography detected ^{35}S-labeled P0^{CA} only in the eluates from the beads loaded with GST–ASK1 and GST–ASK2 (Fig. 1*C Right*). We conclude that association between P0^{CA} and the ASKs is direct.

An F-box-like motif in P0 is necessary for interaction with ASK1 and ASK2. Its interaction with ASK1 and ASK2 suggested that P0^{CA} might contain an F-box, the ≈60-residue domain that is generally situated near the N terminus of an F-box protein (11). The F-box consensus has no strictly invariant residues and contains gaps so that it is often difficult to reliably identify an F-box from the sequence alone. However, inspection of the P0^{CA} sequence detected the short motif LPLLI (residues 53–57; Fig. 1*D*), which matches the start of the F-box consensus sequence (LPxxI/L), the most highly conserved part of the domain in plant F-box proteins (15). A similarly positioned motif is present in the P0s of other poleroviruses (Fig. 1*D*), even though overall sequence identity among different P0s is low (9).

To investigate the significance of the LPLLI sequence, mutant forms of P0^{CA} were tested for interaction with ASK1 and ASK2 in the two-hybrid system. In mutants ΔA and ΔB, 10 or 62 residues were deleted starting with the LPLLI sequence. In mutants LP1 and LP2, the wild-type (WT) sequence was replaced by AALLI and MFMQF, respectively (Fig. 1*A*). The LP2 mutation does not change the amino acid sequence of P1, which is encoded by an overlapping ORF in viral RNA (Fig. 1*A*). All of the mutant proteins were stable in yeast but none reacted with ASK1 or ASK2 in the two-hybrid assay (Fig. 1*B*).

P0^{BW} Interacts with ASK1 and ASK2. P0^{BW} also contains an F-box-like motif (LPFHL; Fig. 1*D*), but no yeast double transformants containing ASK1 or ASK2 were obtained during the two-hybrid screen with P0^{BW} (see above) or when double-transformant yeast expressing BD-P0^{BW} and either AD-ASK1 or AD-ASK2 were incubated at 28°C (see Fig. 5, which is published as supporting information on the PNAS web site). Incubation of the plates at 21°C, however, permitted growth of the double transformants on

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selective media (Fig. 5). The BD-P0^{CA}/AD-ASK1 and BD-P0^{CA}/AD-ASK2 double transformants also grew well at the lower temperature (Fig. 5). A P0^{BW}LP1 mutant (LPFHL replaced by AAFHL) did not interact with ASK1 or ASK2 in yeast at either 21°C or 28°C (data not shown).

ASK1 and ASK2 Can Incorporate P0 into a Trimeric Complex with CUL1. To determine whether P0^{BW} and P0^{CA} can form trimeric complexes with *A. thaliana* CUL1 and an ASK, bridge assays were carried out with BD-P0 and AD-AtCUL1 fusion proteins expressed together in yeast along with ASK1 or ASK2. Growth under selective conditions occurred when either ASK1 or ASK2 was provided as a bridging component but not when the ASKs were omitted (Fig. 1*E*). Thus, the association between ASK and P0 does not interfere with docking of the complex with CUL1.

P0^{CA} and ASK1 Interact *in Planta*. The interaction between P0^{CA} and ASK1 *in planta* was investigated by using bimolecular fluorescence complementation (17, 18). In this assay, the yellow fluorescence protein (YFP) is expressed as N-terminal (YN) and C-terminal (YC) nonfluorescent fragments. Restoration of YFP fluorescence occurs when the two fragments are brought into proximity by an interaction between two proteins that have been fused to the YN and YC fragments, respectively. Plasmids expressing P0^{CA}–YN and YC–ASK1 were cobombarded into epidermal cells of etiolated mustard seedlings. To identify transformed cells, the bombardment mix also contained a plasmid expressing the cyan fluorescent protein (CFP) fused to the parsley common plant regulatory protein 2 (CPRF2), which localizes to the nucleus (17). A YFP signal was observed in 26 of 41 transformed cells examined. The fluorescence was most intense in the nucleus (Fig. 2*A*), as observed for a control interaction between YN–ASK1 and YC–EID1 (empfindlicher im dunkelroten Licht), a pair of proteins known to interact in the nucleus (17) (Fig. 2*D*). In the case of the P0^{CA}–YN/YC–ASK1 interaction, a faint YFP signal was also present in the cytoplasm (Fig. 2*A*). A similar result was obtained when the fusion partners were reversed: cobombardment with P0^{CA}–YC and YN–ASK1 gave rise to YFP fluorescent nuclei in 34 of 56 transformed cells examined (data not shown).

To confirm the role of the F-box motif in the interaction, we examined cells cobombarded with P0^{CA}LP1–YN and YC–ASK1. Although 33 of the 41 transformed cells examined did not exhibit detectable YFP fluorescence, a faint YFP signal could be discerned in the nuclei of eight transformed cells (Fig. 2*B*). This result resembles the situation for cells bombarded with P0^{CA}–YN and YC, where weak nuclear fluorescence was observed in only 9 of the 50 transformed cells examined (Fig. 2*C*). We conclude that the weak YFP signal sometimes obtained with P0^{CA}LP1 is nonspecific and that P0 interacts via its F-box motif with ASK1 in plant cells.

F-Box Motif Is Required for P0-Mediated Viral Pathogenicity. We next asked whether the P0–SKP1 interaction is important for virus pathogenicity. As previously observed for BWYV (19), a CABYV mutant carrying a 14-nt deletion in the P0 ORF at a position upstream of the F-box domain (Δ14; Fig. 1*A*) was hypovirulent, accumulating ≈10 times less progeny viral RNA than plants infected with WT CABYV (data not shown). Similar low levels of progeny viral RNA accumulation were observed for a CABYV mutant carrying the LP2 mutation (data not shown).

The effect of the F-box mutation also was studied when P0 was expressed from an heterologous virus. In *N. benthamiana* infected with a Potato virus X (PVX)–P0^{BW} chimera (see Fig. 6*A*, which is published as supporting information on the PNAS web site), accumulation of progeny viral RNA (Fig. 6*B*) in upper leaves was accompanied by severe necrosis of vascular tissue and death of the upper part of the plant (Fig. 6*C*). This result is in contrast to the mild leaf mosaic symptoms produced by PVX infection (Fig. 6*C*) but resembles the symptoms induced by other silencing suppressor proteins when expressed in the PVX background (20–22). When P0^{BW}LP1 was substituted for P0^{BW} in the chimera, the systemically infected leaves displayed only a few necrotic flecks (in addition to the mosaic symptoms typical of a PVX infection), and the plants survived (Fig. 6*C*). Collectively, these experiments implicate the association between P0 and plant SKP1 orthologs in the mechanism by which P0 enhances virus pathogenicity.

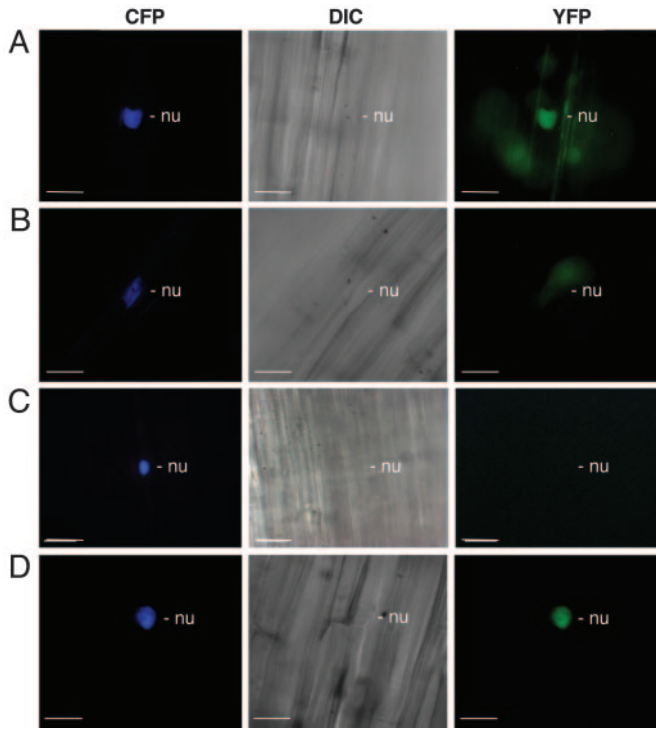

Fig. 2. Visualization of the interaction between P0 and ASK1 *in planta* using bimolecular fluorescence complementation. Three-day-old dark-grown mustard seedlings were transformed by particle bombardment with combinations of plasmids expressing different YN- and YC-fusion proteins. To identify transformed cells, a plasmid expressing the CFP fused to the parsley common plant regulatory protein 2 (CPRF2), which localizes to the nucleus (nu), was included during bombardment. Images were recorded 5 h after bombardment by using CFP-specific and YFP-specific filters. Shown are typical cells bombarded with plasmids expressing P0^{CA}–YN and YC–ASK1 (*A*), P0^{CA}LP1–YN and YC–ASK1 (*B*), P0^{CA}–YN and YC (*C*), and YN–ASK1 and YC–EID1 (*D*), a pair of proteins known to interact (17). A differential interference contrast (DIC) image is shown between each pair of fluorescent images. Regions of diffuse YFP fluorescence in *A* and *B* that are not confined to a single cell are background. (All images are at the same magnification; scale bar: 40 μm.)

Depletion of SKP1 Ortholog(s) in *N. benthamiana* by Virus-Induced Gene Silencing (VIGS) Induces Resistance to BWYV. If the P0–SKP1 interaction is important for virus pathogenicity, we reasoned that plants which produce less SKP1 should display heightened resistance to polerovirus infection. *Arabidopsis* lines carrying null mutations in both *ASK1* and *ASK2* are nonviable (23). Therefore, VIGS (24, 25) was used to lower SKP1 accumulation levels in the BWYV host *N. benthamiana*. The cDNA of an *N. benthamiana* SKP1 ortholog was cloned, and the encoded protein (NbSKP1) was shown to interact with P0^{BW} and P0^{CA} in the yeast two-hybrid system (data not shown). For the VIGS experiment, the sequence encoding all but the four N-terminal amino acids of NbSKP1 was inserted into the PVX genome. A long rather than a short NbSKP1 cDNA fragment was used to trigger

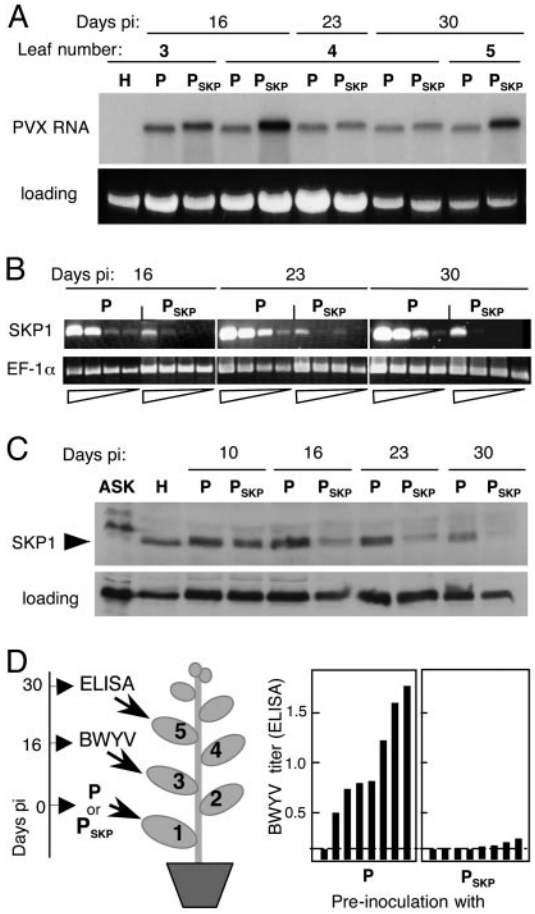

Fig. 3. Depletion of SKP1 in *N. benthamiana* by VIGS provokes resistance to BWYV. (*A*) Northern blot analysis of PVX (P) and PVX-NbSKP1 (P_{SKP}) progeny RNA in upper leaves of *N. benthamiana* at different times pi using a ^{32}P-labeled RNA probe complementary to the 3′-terminal PVX RNA sequence. Leaf positions are indicated in *D*. Lane H is RNA from a healthy plant. (*B*) Inhibition of NbSKP1 transcript accumulation by VIGS. Reverse transcription followed by PCR with primers specific for the 3′ noncoding region of NbSKP1 mRNA was carried out on total RNA from leaf 4 (16 and 23 days pi) or 5 (30 days pi) of P- or P_{SKP}-infected plants. In the dilution series, the amounts of cDNA template used for PCR were 1/10, 1/50, and 1/250 of that used in each left-hand lane. Products of RT-PCR amplification of a portion of the Elongation Factor 1α (EF-1α) sequence from the same RNA samples are shown as a control. (*C*) Inhibition of SKP1 accumulation by VIGS. NbSKP1 levels in leaf 3 were monitored at different times after inoculation with P or P_{SKP} by Western blot using an SKP1-specific antiserum. The left-hand lane (ASK) was loaded with a protein extract from *A. thaliana* and lane H with protein from healthy *N. benthamiana*. (*D*) BWYV titer in leaf 5 of plants that had been preinoculated with PVX or PVX–NbSKP1. The plants were aphid-inoculated with BWYV 16 days after infection with PVX or PVX–NbSKP1 and tested by ELISA for BWYV at 30 days. (*Right*) Bars represent the ELISA A_{405} value for each plant. The ELISA background is indicated by the horizontal dashed line. The cartoon in *Left* shows the relative positions of the leaves subjected to the different treatments.

VIGS in these experiments because we regard it as probable that, as in *Arabidopsis*, the SKP1-like molecules of *N. benthamiana* are encoded by a multigene family, and expression of a long cDNA fragment should increase the probability that transcripts of other members of the family that could otherwise complement NbSKP1 function will be targeted for silencing as well.

The PVX–NbSKP1 chimera transcript (P_{SKP}) was rub-inoculated to a lower leaf (leaf 1, Fig. 3*D*) of young plants. As a control, plants also were inoculated with transcript of empty PVX vector (P). Symptoms of virus infection appeared on upper leaves of the inoculated plants by 8 days postinoculation (pi), and similar amounts of progeny viral RNA could be detected by Northern blot of total RNA extracted from the symptomatic leaves (Fig. 3*A*). Semiquantitative RT-PCR revealed that NbSKP1 mRNA accumulation in the P_{SKP}-infected plants was reduced at 16–30 days pi to ≈5% of the levels observed in the P-infected plants (Fig. 3*B*). Western blot analysis using a SKP1-specific antiserum confirmed that NbSKP1 protein levels in the P_{SKP}-infected plant were diminished (Fig. 3*C*). Starting at ≈15 days pi, the P_{SKP}-inoculated plants developed a phenotype that is presumably a consequence of SKP1 depletion, including crinkling and epinasty of upper leaves and slight corkscrewing of the stem.

To test the effect of the lower NbSKP1 levels on BWYV infection, the leaves in position 3 of P- and P_{SKP}-infected plants were inoculated at 16 days pi with aphids viruliferous for BWYV, and the leaves at position 5 were tested for virus infection by ELISA 2 weeks later (30 days pi). Viruliferous aphids rather than agro-infection with an infectious cDNA clone were used to deliver the BWYV inoculum because an F-box protein–SKP1 interaction has been implicated in uncoating of T-DNA in the plant cell nucleus before its integration into the host genome (26). High levels of BWYV were present in seven of the eight plants preinoculated with PVX (P), but virus levels were near background in the plants preinoculated with P_{SKP} (Fig. 3*D*). We conclude that not only P0, but also its interaction partner SKP1, is required for efficient BWYV infection.

Mutation of the P0 F-Box Motif Inhibits Suppression of Gene Silencing. The above observations support a model in which interaction between P0 and one or more plant SKP1 orthologs is required for efficient polerovirus infection, but they fall short of establishing a direct link between the P0–SKP1 interaction and the silencing suppressor activity of P0. To address this question, we compared the ability of $P0^{BW}$ and $P0^{BW}$LP1 to suppress RNA silencing induced by ectopic expression of a foreign transcript in an agro-infiltration assay (27). The assay employs *N. benthamiana* line 16c, which expresses GFP from a transgenic locus. Infiltration of a leaf with Agrobacteria harboring a pBin–GFP binary construct results in initial high levels of expression of GFP transcript in the infiltrated zone, which subsequently triggers PTGS-mediated degradation of the transcript. By 5 days postinfiltration, most of the GFP transcript expressed from the transgene and the agro-infiltrated pBin-GFP in the patch was degraded (Fig. 4*A*, lane 7), and GFP transcript-specific siRNAs appeared (Fig. 4*B*, lane 7). When Agrobacteria harboring a plasmid-expressing $P0^{BW}$ were coinfiltrated into a leaf along with the pBin–GFP-containing Agrobacteria, high levels of GFP transcript (Fig. 4*A*, lane 1) and low levels of GFP-specific siRNA were observed in the patches (Fig. 4*B*, lane 1). Note that the $P0^{BW}$ transcript is also abundant (Fig. 4*C*, lane 1) even though, like the GFP transcript, it is ectopically expressed and is expected to be a trigger for and a target of PTGS. The silencing suppressor protein encoded by the transcript, however, would "protect" it from degradation.

When $P0^{BW}$LP1 was substituted for WT $P0^{BW}$ in the agro-infiltration assay, GFP transcript levels (Fig. 4*A*, lane 4) were almost as low as in patches infiltrated with Agrobacteria containing the empty vector pBin61 (Fig. 4*A*, lane 7). Accumulation of $P0^{BW}$LP1 transcript was also low (Fig. 4*C*, lane 4), as expected if the LP1 mutation abolishes P0s silencing suppressor activity. Similar results were obtained when the silencing suppressor activities of $P0^{CA}$ and $P0^{CA}$LP1 were compared (data not shown).

The foregoing observations are consistent with the hypothesis that a functional F-box motif is required for P0 silencing suppressor activity. The possibility remains open, however, that the LP1 mutant is for some reason less stable than the WT

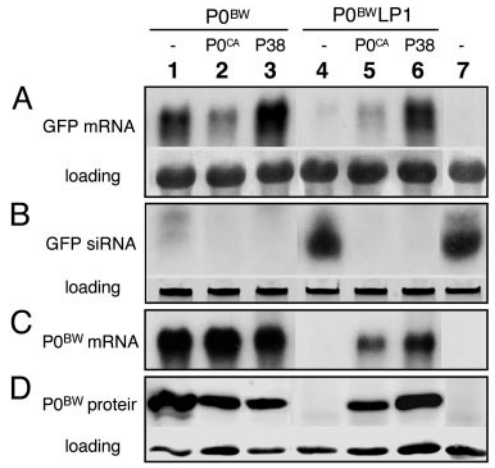

Fig. 4. The LP1 mutation inhibits the silencing suppressor activity of $P0^{BW}$ but does not destabilize the protein. Leaves of *N. benthamiana* line 16c were infiltrated with a mixture of Agrobacteria strains containing pBin–GFP plus either empty vector (pBin61, lane 7) or pBin61 expressing the silencing suppressor protein(s) indicated at the top (lanes 1–6). Total RNA was extracted 5 days later from the agro-infiltrated patches and analyzed for the presence of GFP transcript (*A*), GFP transcript-derived siRNAs (*B*), and $P0^{BW}$ transcript (*C*) by Northern blot using specific ^{32}P-labeled probes. Total protein was extracted from the same patches and analyzed for $P0^{BW}$ protein by Western blot using a $P0^{BW}$-specific polyclonal antiserum (*D*). Loading controls are shown below each blot.

protein *in planta*, even though a functional F-box is usually a destabilizing protein motif (11). Thus, an unlikely but possible alternative scenario would be that failure of $P0^{BW}$LP1 to suppress silencing is because of low accumulation levels rather than loss of silencing suppressor activity. To test this hypothesis, we added to the agro-infiltration assay mix a binary vector expressing a second RNA silencing suppressor, P38 of Turnip crinkle virus (TCV) (28). Addition of pBin–P38 stabilized the GFP and the $P0^{BW}$LP1 transcripts (Fig. 4 *A* and *C*, lane 6) so that they accumulated to ≈65% of the level observed in patches agro-infected with WT pBin–$P0^{BW}$ plus pBin–P38 (Fig. 4 *A* and *C*, lane 3). Importantly, Western blot analysis of protein in the patches revealed that $P0^{BW}$LP1 accumulated in amounts comparable with those observed for $P0^{BW}$ (Fig. 4*D*, compare lanes 3 and 6). Similar results were obtained when $P0^{CA}$ was used as the secondary silencing suppressor. Although the degree of protection afforded by $P0^{CA}$ was lower than with P38, the $P0^{BW}$ and $P0^{BW}$LP1 proteins accumulated to similar levels (compare Fig. 4*D*, lanes 2 and 5). Together, these experiments establish that the inability of $P0^{BW}$LP1 to suppress RNA silencing is a consequence of loss of silencing suppressor activity rather than instability of the mutant protein, and we can therefore conclude that the F-box motif is required for the silencing suppressor activity of P0.

Discussion

Many animal viruses exploit the cell's ubiquitination/proteolysis machinery to inhibit host responses or more generally alter the cellular environment to favor infection (29). These viruses typically act at the ubiquitination step, either by expressing a novel E3 ligase with appropriate properties from the virus genome or by altering the specificity of a host E3 ligase. Examples of the latter strategy are the Vif protein of *HIV-1* (HIV-1) and the Adenovirus proteins E4orf6 and E1B55K, which direct their target proteins (APOBEC3G and p53, respectively) to a Cul5-containing SCF-like complex (30, 31). None of these viral proteins, however, is a conventional F-box protein, and, indeed, only one viral protein other than P0 has been demonstrated to interact with a SKP1 ortholog. This protein is the Faba bean necrotic yellows virus protein CLINK, which may deregulate the host cell cycle in favor of viral DNA replication by targeting a pRB-like protein (32).

The most straightforward interpretation of our findings is that polerovirus silencing protein P0 acts as an F-box protein that targets an essential component of the host small RNA-dependent RNA degradation virus defense pathway. We cannot rule out the possibility that the P0–SKP1-ortholog complex recognizes and inactivates its target protein by simple sequestration, but, given the ability of the P0–ASK complex to assemble with AtCUL1, we regard it as more likely that P0 incorporates its substrate protein into an SCF complex for ubiquitination. Addition of ubiquitin chains to a target protein by the SCF generally destines it for degradation by the proteasome, although other scenarios that do not involve target degradation cannot be eliminated (33). It is furthermore possible that the P0–SKP1 complex could act indirectly, perhaps as an antagonist of a cellular F-box protein, which normally degrades a negative regulator of the silencing pathway. Discrimination among these various possibilities should be facilitated once the ultimate target or targets of P0 in the silencing pathway is identified. It also will be interesting to determine whether other viral silencing suppressor proteins employ an E3 ubiquitin ligase (although not necessarily an SCF E3 ligase) and ubiquitin-mediated processes to target proteins of the PTGS pathway in the cytosol.

F-box proteins generally interact with their targets via a C-terminal domain that is often, but not always, a known protein-interaction motif such as a leucine-rich repeat (LRR) or a kelch domain (11). No such motif is present in P0, but $P0^{CA}$ and $P0^{BW}$ both contain a C-terminal-proximal sequence (K/R)IYGEDGX_3FWR (related sequences are present in other polerovirus P0s), which could represent a previously undescribed type of substrate interaction domain. Our future studies will address the problem of determining which component of the host silencing machinery is the target of the putative SCF^{P0} E3 ubiquitin ligase.

Materials and Methods

Gene Constructs, Virus Infection, and Agro-Infection. Plasmid constructions are described in detail in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site. Production and inoculation of infectious chimera transcripts of PVX were as described in ref. 10. *A. thaliana* were infected with CABYV by agro-inoculation (34). *N. benthamiana* were infected with BWYV by using 30 viruliferous *Myzus persicae* per leaf and a 4-day inoculation access period (35). The aphids were confined to a single leaf by using a clip-on cage. BWYV levels were assayed by double antibody-sandwich ELISA on leaf tissue extracts with a BWYV-specific antiserum (Loewe Biochemica, Sauerlach, Germany). Western blot analysis of NbSKP1 levels used an antiserum raised against a peptide corresponding to the N-terminal sequence of ASK1 (36). The loading control was obtained with an anti-Cdc2 (PSTAIRE) polyclonal antibody (Santa Cruz Biotechnology). Detection of NbSKP1 transcript by semiquantitative PCR is described in *Supporting Materials and Methods*.

Agro-infiltration of leaves of *N. benthamiana* line 16c (34) and Northern and Western blot analysis were performed as described in refs. 10 and 37 with TRIzol (Invitrogen) used for siRNA extraction. Radioactivity in bands was quantified with a Phosphorimager Bas1000 (Fujix, Kyoto, Japan).

Two-Hybrid Assay. Two-hybrid screening of an *A. thaliana* cDNA library (Clontech) was carried out in AH109 as described by the Clontech Matchmaker Protocol with a first round of selection on dropout plates lacking histidine, tryptophan, and leucine (−HWL) followed by more stringent selection on plates lacking

adenine as well as the three above-mentioned amino acids (−AHWL). BD–P0 fusion proteins were detected by Western blot using a monoclonal antibody (Roche) directed against a myc epitope encoded by the spacer sequence between the BD and P0 coding regions. In bridging assays, ASK1 and ASK2 were expressed from pVT–U102 (38).

Pull-Down Experiments. GST–ASK1, GST–ASK2, and GST in *E. coli* extracts were immobilized on glutathione-Sepharose beads, which then were incubated with [^{35}S]methionine-labeled P0^{CA}. A detailed description is provided in *Supporting Materials and Methods*.

Bimolecular Fluorescence Complementation. The P0^{CA} and P0^{CA}LP1 coding sequences were isolated as BamHI–EcoRI restriction fragments and cloned into pENTR3C (Invitrogen). The resulting entry vectors were used to introduce the P0 sequences into the split YFP destination vectors by Gateway Scientific (St. Louis) technology to obtain P0–YN, P0–YC, and P0LP1–YN constructs. ^{35}S–CPRF2–CFP, YN–ASK1, YC–ASK1, and YC–EID1 are described elsewhere (17). The different constructs were transformed into 3-day-old, dark-grown mustard seedlings by particle bombardment (17). Images were recorded 5 h after bombardment with an Axioskop II fluorescence microscope (Zeiss) by using CFP- and YFP-specific filters and 9-ms and 2-s exposure times, respectively.

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Supporting Information

Files in this Data Supplement:

Supporting Figure 5
Supporting Figure 6
Supporting Materials and Methods

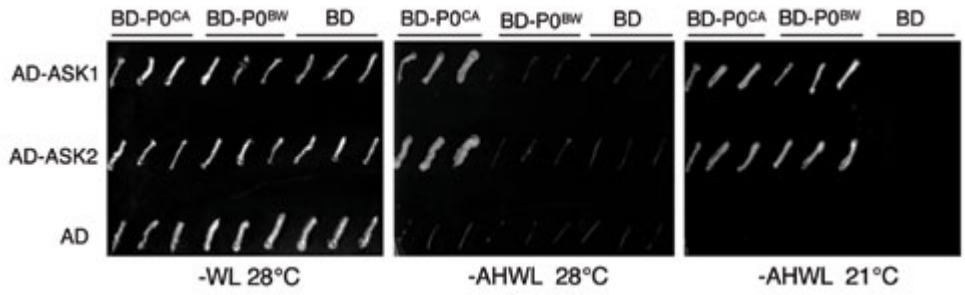

Fig. 5. The interaction between $P0^{BW}$ and ASK1 and ASK2 is temperature sensitive. Yeast AH109 double transformed with bait and prey plasmids expressing the indicated fusion proteins was grown under nonstringent conditions (–WL plate) or on –AHWL plates to select for a strong two-hybrid interaction. Three independent colonies were replicate-streaked onto the plates, which then were incubated at the indicated temperature.

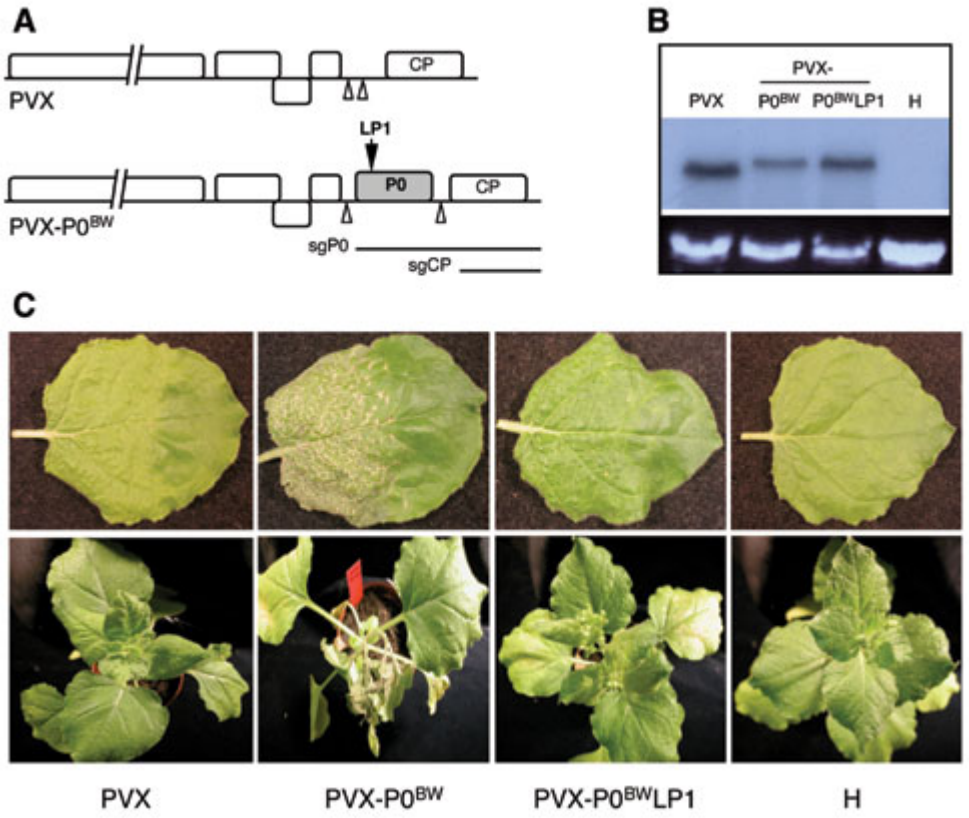

Fig. 6. The LP1 F-box mutation diminishes the necrotic response provoked by infection of *N. benthamiana* with a Potato virus X (PVX) chimera expressing $P0^{BW}$. (*A*) Structure of PVX and PVX_P0^{BW}. The position of the LP1 mutation in PVX_P0^{BW} LP1 is indicated by an arrow. The duplicated promoters driving synthesis of the subgenomic RNAs expressing the coat protein (sgCP) and P0 (sgP0) are indicated by triangles. (*B*) Northern blot detection of progeny viral genomic RNA in systemically infected leaves after inoculation with the indicated transcript. Total leaf RNA was extracted 12 days postinoculation (pi). The bands have mobility corresponding to PVX and PVX-$P0^{BW}$ genomic RNA. H, RNA from a leaf of a noninoculated plant. A loading control is shown below. The low levels of PVX_P0^{BW} RNA relative to PVX_P0^{BW} LP1 RNA in the infected tissue may be due to the onset of necrosis in the PVX_P0^{BW}-infected tissue when the samples were taken. (*C*) Symptoms provoked on systemically infected leaves (12 days pi) and whole plants (18 days pi) after inoculation with the indicated transcript. H, a noninoculated plant.

Supporting Materials and Methods

Gene Constructs. Plasmids containing infectious full-length cDNA of Beet western yellows virus (BWYV)–FL1 (1) [recently renamed Turnip yellows virus (TuYV)] (2) and Cucurbit aphid-borne yellows virus (CABYV) (3) were used as PCR templates to generate DNA fragments containing the

P0 coding regions flanked with appropriate restriction sites for insertion into pGBKT7 (Clontech), pBin61 (4), and pP2C2S (5). The *Nicotiana benthamiana* S-phase kinase-related protein 1 (SKP1) ortholog NbSKP1 (AF494084) was amplified from total RNA extracted from leaves by reverse transcription followed by PCR using primers based on the published sequence (6). PCR fragments containing the *Arabidopsis* SKP1-like (ASK1), ASK2, and NbSKP1 coding sequences were inserted into pGADT7 (Clontech), pGEX-2TK (Pharmacia), or pP2C2S using restriction sites built into the primers used for PCR. Site-directed mutagenesis was carried out by overlap-extension PCR. pBin–P38 was provided by O. Voinnet, and pBin61 and pP2C2S were gifts from D. Baulcombe.

Semiquantitative PCR. For detection of NbSKP1 transcript by semiquantitative PCR, 6 μg of total RNA was used as a template for reverse transcription (Invitrogen) with primer SK100, complementary to nucleotides 906–927 in the NbSKP1 3' noncoding region or EF100 complementary to nucleotides 867–888 of the *Nicotiana tabacum* Elongation Factor-1α (EF-1α) coding sequence (AF120093). PCR was carried out on different dilutions of the resulting cDNA using the primers SK100 and SK101 (corresponding to nucleotides 513–538 of NbSKP1) and EF100 plus EF101, corresponding to nucleotides 254–275 of the EF-1α coding sequence. The PCR products of 150 and 674 nucleotides, respectively, were resolved by agarose gel electrophoresis.

Pull-Down Experiments. *Escherichia coli* (Rosetta; Novagen) was transformed with pGEX2TK-ASK1, pGEX2TK_ASK2, or empty vector and grown at 37°C until A_{600} = 0.5. Synthesis of glutathione *S*-transferase (GST) fusion proteins was induced by addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the bacteria were incubated for an additional 4 h at 28°C. Bacteria were harvested, resuspended in NET buffer (100 mM NaCl/1 mM EDTA/20 mM Tris, pH 8) plus 1 mM DTT, 0.5% Nonidet P-40, and a protease inhibitor mixture (Roche), and sonicated. GST-tagged proteins were isolated by affinity chromatography on glutathione-Sepharose beads (Pharmacia). [^{35}S]methionine-labeled P0^{CA} was produced by coupled *in vitro* transcription and translation of pGBKT7_P0^{CA} using a TNT_Quik kit (Promega). The translation product was diluted into PBS and incubated for 2 h at 4°C with the beads. After washing with NET buffer modified to contain 350 mM NaCl, immobilized proteins were eluted from the beads with 10 mM glutathione in 50 mM Tris (pH 8), precipitated with acetone, taken up in gel-loading buffer, and subjected to SDS/PAGE. Total proteins in the gel were visualized by Coomassie blue staining, and ^{35}S_P0^{CA} was identified by autoradiography.

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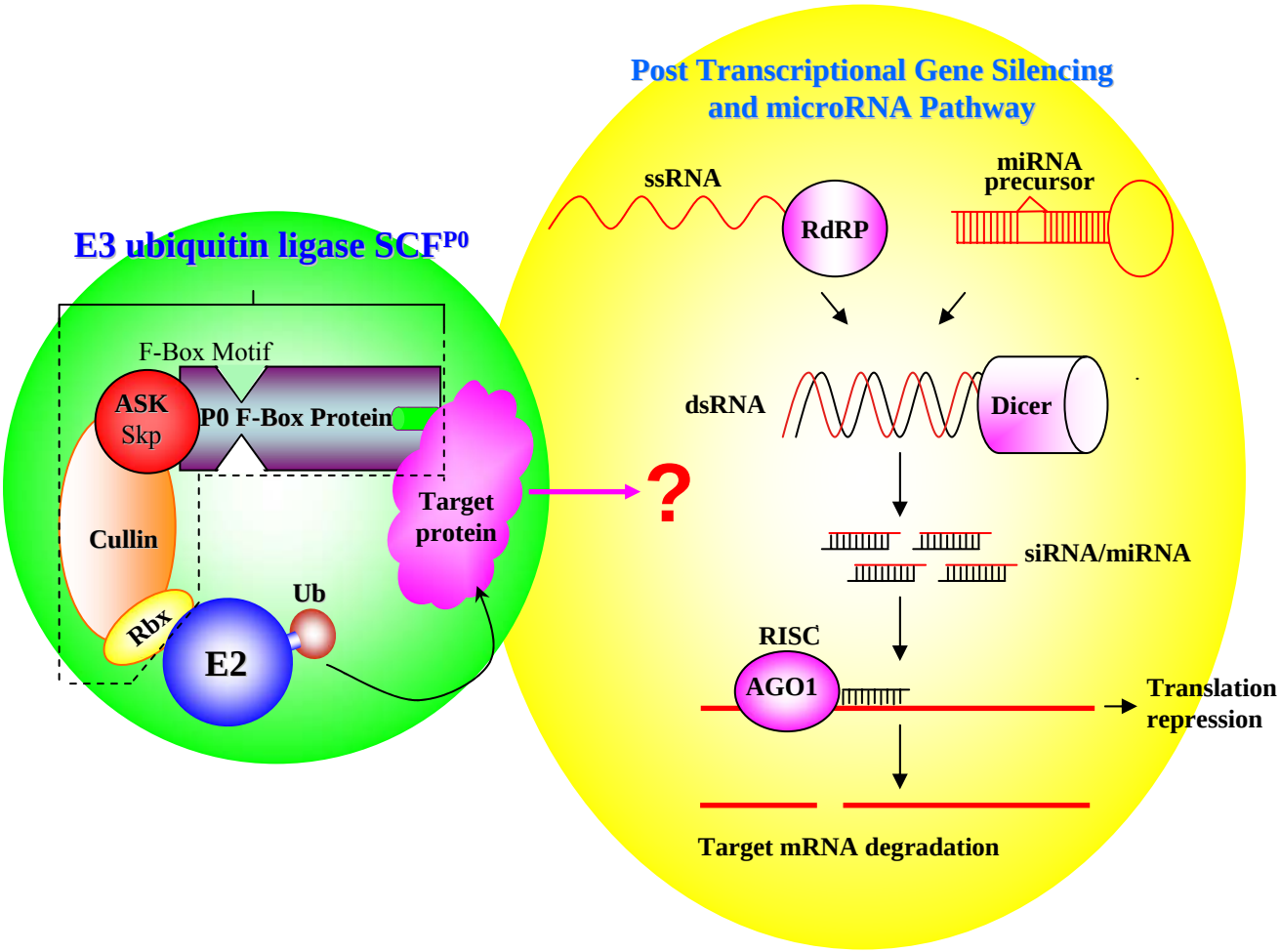


Figure 1-21: A model in which P0 acts as an F-box protein, recruiting the plant post-translational modification system to overcome plant post-transcriptional gene silencing system. In this model P0 interacts with SKP and composes a SCF^{P0} complex to address an essential component of the silencing pathway for ubiquitination and eventually degradation by 26S proteasome.

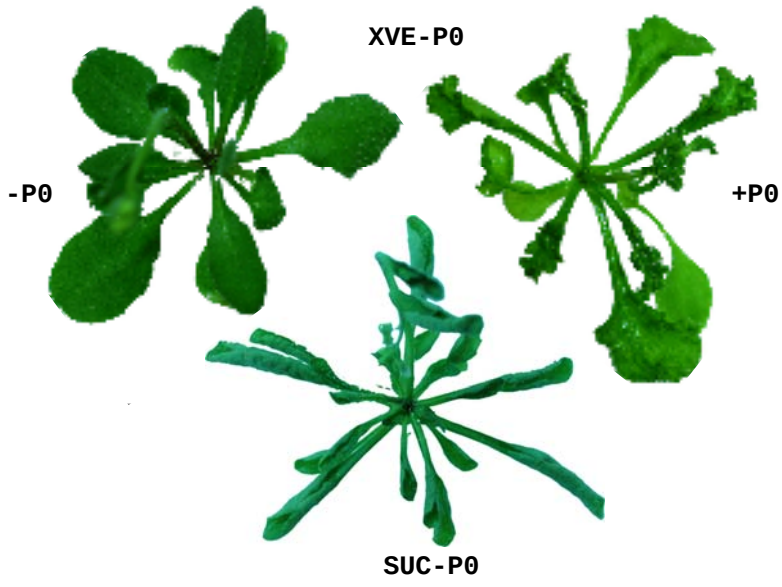
Conclusion

We have shown that the polerovirus silencing suppressor protein P0 interacts directly with ASK1 and ASK2 of Arabidopsis and SKP of *N. benthamiana*. P0 has an F-box-like sequence and a point mutation in its F-box motif which inhibits the P0-SKP interaction also abolishes P0`s silencing suppressor activity. Interaction with SKP in plants is necessary for establishment of infection and viral pathogenicity.

Our data support a model in which P0 acts as an F-box protein, recruiting the plant post-translational modification system to overcome the plant post-transcriptional gene silencing system. In this model P0 interacts with SKP to constitute an SCF^{P0} complex which could address an essential component of the silencing pathway for ubiquitination and, presumably, degradation by 26S proteasome (Figure 1-21). However, we can not role out the possibility that the ubiquitination could result in modification of the target protein's activity rather than leading to its degradation.

Chapter Two

P0 Targets ARGONAUTE1 to Suppress RNA Silencing



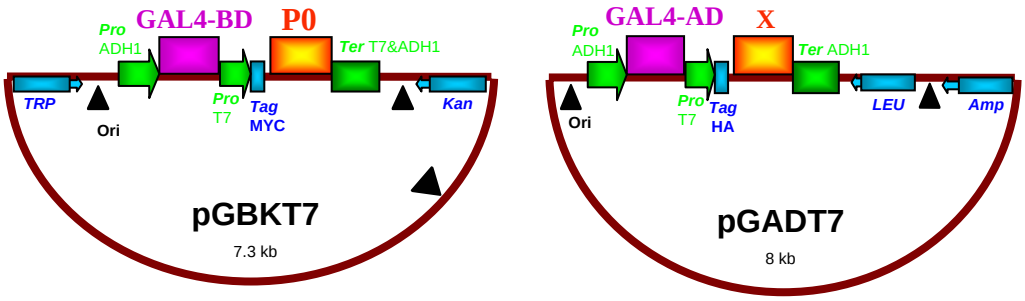


Figure 2-1: bait and prey plasmids in yeast two hybrid system

Table 2-1: The genes from PTGS pathway whose interactions with P0 were tested in the yeast two-hybrid system. (-): double transformed yeast AH109 strain with indicated genes did not grow on –AHWL or –HWL. ND: interaction was not determined.

Interaction tests have been performed by Talyne ELMAYAN from H. Vaucheret laboratory, INRA Versailles, FRANCE.

Gene	$P0^{CA}$	$P0^{BW}$	$P0LP^{CA}$	$P0LP^{BW}$
HYL1	-	-	ND	ND
HEN1	-	-	ND	ND
RDR6	-	-	ND	ND
SGS3	-	-	ND	ND
SDE3	-	-	-	-
DCL2	-	-	-	-
AGO1	-	-	-	-
PAZ domain of AGO1	-	-	-	-
PIWI domain of AGO1	-	-	-	-

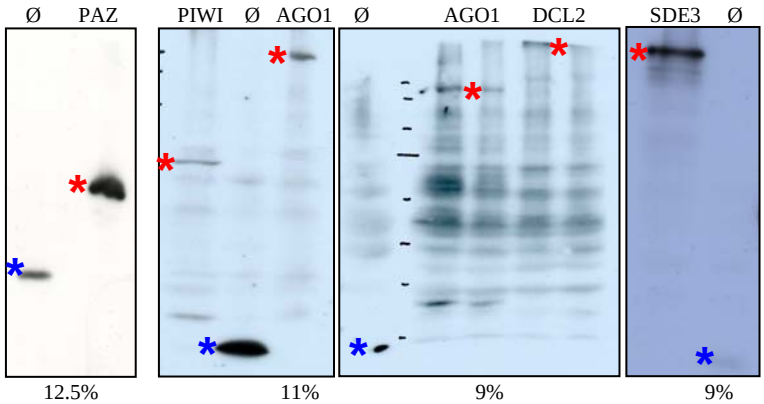


Figure 2-2: Expression of different proteins in double transformed yeast clones was confirmed by detection in Western blot using anti-HA antibody. Red stars show the corresponding bands and blue stars indicate the AD fused to the HA tag. Concentration of acrylamide in each gel is noted below.

Chapter Two

P0 targets ARGONAUTE1 to suppress RNA silencing

We have shown that P0 interacts with SKP, a component of the SCF ubiquitin E3 ligase, and acts as an F-box protein. Furthermore, its interaction with SKP and/or its F-box activity is indispensable for silencing suppression and viral pathogenicity. We proposed a model in which P0, as an F-box protein, targets an essential component of RNA silencing to be ubiquitinated and degraded by 26S proteasome. As a next step, we asked which protein in the RNA silencing pathway is the target of P0.

I. Attempts to find the target of P0 using the yeast two-hybrid system

To find a target protein that interacts with P0 we performed another screen of the *A. thaliana* cDNA library with the yeast two-hybrid system. In view of the similarity between ASK1 and yeast SKP1, we hypothesized that P0 could interact also with yeast SKP1 and that this could lead to ubiquitination of the target protein and its subsequent degradation in yeast cell. If this were to occur we would not be able to detect an interaction with the target protein in this system. Therefore, as a bait (in pGBKT7 carrying BD; see Figure 2-1) we used the P0LP mutant of CABYV, which is not capable of interacting with SKP. We did not find any plausible candidates among the obtained prey (At5g57800, At1g08830, At5g46110, At2g16540, At2g16590, At2g23980, At1g08110 and At5g03760) that have been implicated in the RNA silencing pathway.

The Arabidopsis cDNA library used in these experiments is not 100% representative of all expressed mRNAs in all tissues and in all stages of plant development. Secondly, long transcripts are likely to be under-represented. Therefore, we cloned some available candidate genes known to be important in silencing pathway into pGADT7 to test their interaction with P0 (Table 2-1). Some of these tests were carried out in collaboration with Talyne ELMAYAN from H. Vaucheret's laboratory (INRA Versailles, France). None of the tested proteins (HYL1, HEN1, RDR6, SGS3, SDE3, DCL2, AGO1, and the PAZ and PIWI domains of AGO1) interacted with $P0^{CA}$ or $P0^{BW}$ nor with P0LP mutants of both viruses, although the expression of the proteins in yeast was confirmed by detection in Western blot (Figure 2-2). It should be emphasized, however, that the failure to detect an interaction in this assay does not rigorously rule out the possibility that it occurs in plants. Several reasons may account for the absence of an interaction in yeast such as, for instance, the absence of the proper post-translational modification that is often required for recognition of its target by an F-box protein.

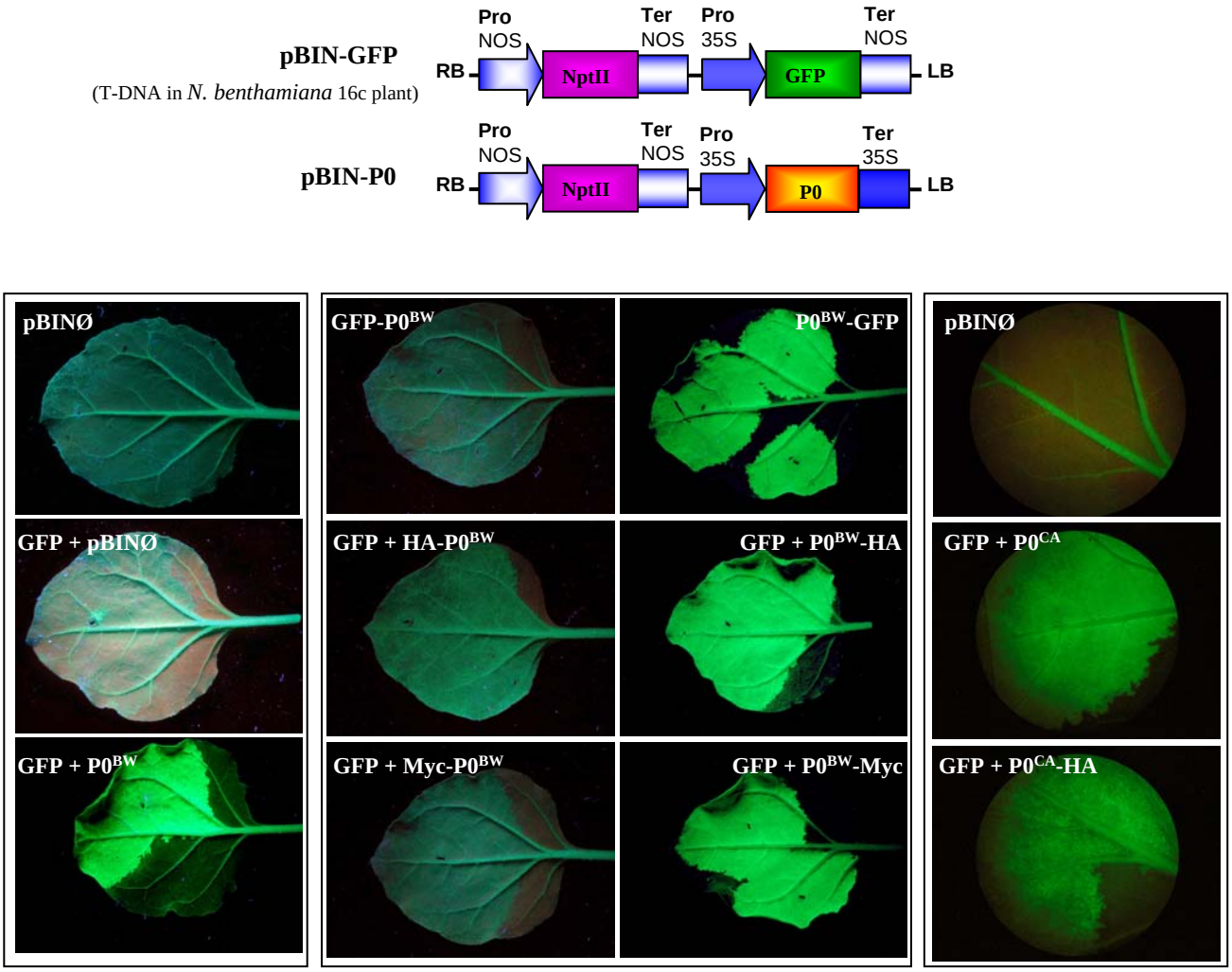


Figure 2-3: N-terminal fusion to P0 abolishes its suppressor activity. Patch Test with co-expression of pBIN-GFP with either wild-type pBIN-P0 or its N- or C-terminal tagged fusion protein on leaves of *N. benthamiana* 16c plants (constructions are shown top of the photos). Photos were taken under UV lamp at five days post Agroinfiltration.

II. N-terminal fusion to P0 abolishes its suppressor activity

In a series of experiments, using the Patch Test and co-expression of GFP with either wild-type P0 or N- or C-terminal tagged versions of P0 on leaves of *N. benthamiana* 16c, we discovered that an N-terminal fusion to P0 (but not a C-terminal fusion) abolished its silencing suppressor activity (Figure 2-3). Western blot analyses of the suppressor proteins showed instability of the N-terminal fusions of P0, indicating a loss of activity and therefore, degradation of its own mRNA.

In view of the fact that in the yeast two-hybrid system the binding domain of GAL4 is a N-terminal fusion to P0 (Figure 2-1), we hypothesized that a similar problem could be encountered in our previous yeast two-hybrid tests, i.e. the N-terminal fusion to P0 could inhibit its interaction with a target protein although it does not affect its interaction with SKP. Therefore, we altered the pGBKT7 vector so as to be able to produce a C-terminal fusion of the BD to $P0^{CA}$ (with the help of Herfried Eisler). We repeated the yeast two-hybrid tests for DCL2, AGO1, and the PAZ and PIWI domains of AGO1 with the new vector but still did not observe any interaction, although both P0s interacted with ASK1 and ASK2 as before.

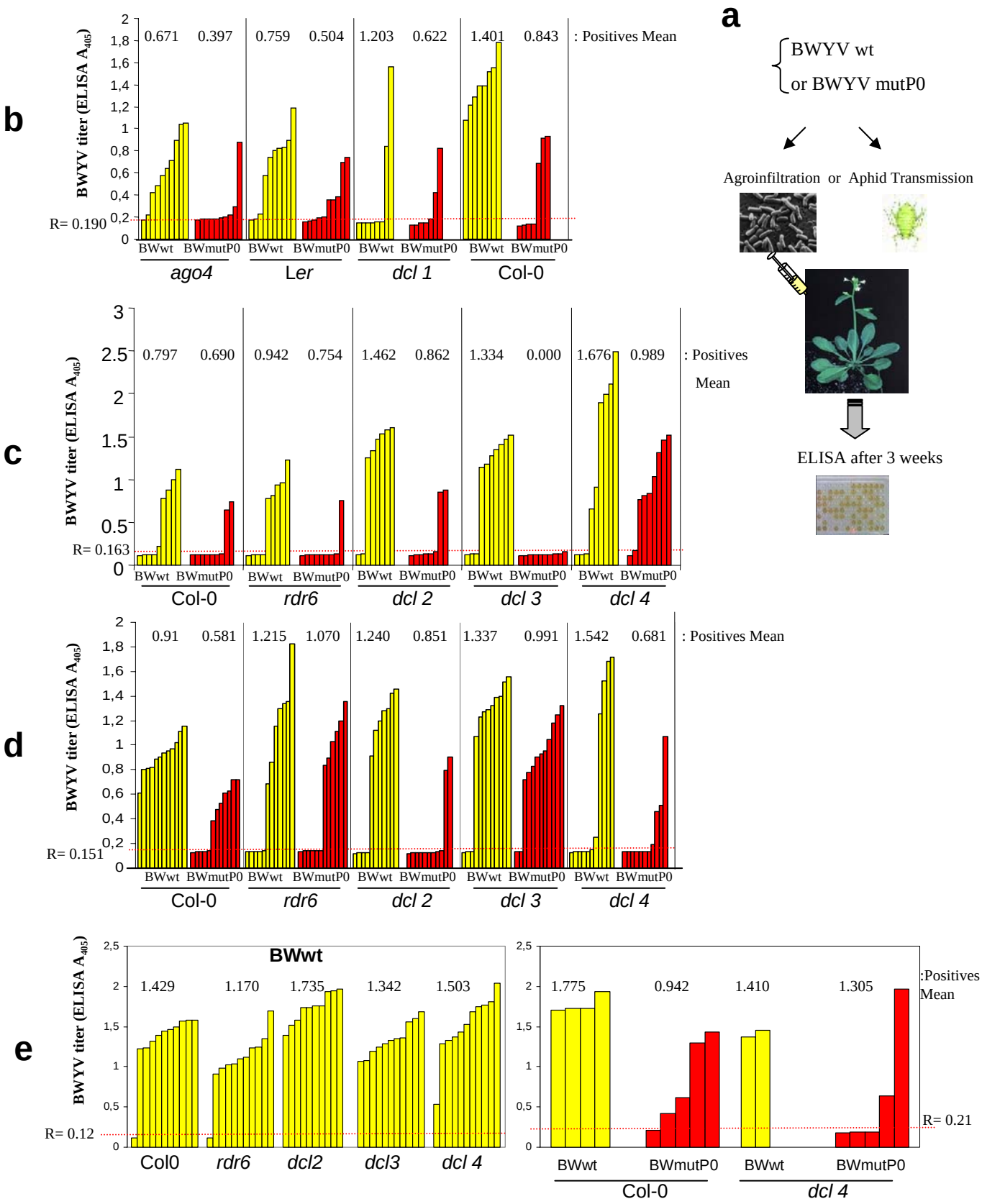


Figure 2-4: virus infection assay on Arabidopsis mutated in genes implicated in RNA silencing. (a) Protocol of experiment. (b, c, d and e) Infection rate measured by ELISA (A_{405}) on leaves of independent infected plants. Inoculation was performed by agroinfiltration (b, c and d) or by aphid transmission (e). Each bars in the graph corresponds to individual plants. R is the infection threshold calculated independently in each experiment (see text).

III. BWYV infection assay on Arabidopsis plants mutated in genes implicated in silencing

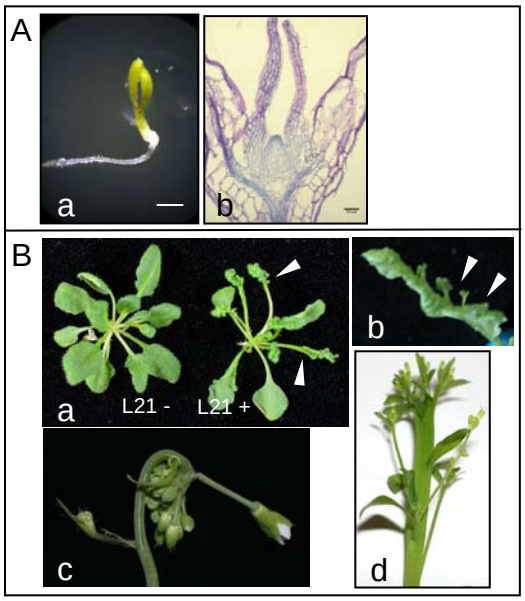
In another approach to determine which protein in the RNA silencing pathway is a target of P0, we investigated the efficiency of BWYV infection on various available Arabidopsis mutants: *rdr6, dcl1, dcl2, dcl3, dcl4* (Deleris *et al*., 2006) and *ago4* (Zilberman *et al*., 2003). In addition to wild-type BWYV, we also used a BWYV mutant which does not express P0 (mut P0: without a start codon: TGATG→GGATC, plus a deletion of 26 nts from nucleotide 123 in ORF0) and accumulates much less efficiently than wild-type virus (Ziegler-Graff *et al*., 1996). As a control, we infected both wild-type and mutated virus on wild-type Arabidopsis (Col-0 or L*er*). Infections were carried out on three week-old plants by agroinfiltration or by aphid transmission (Veronique Brault Laboratory, INRA Colmar, France). Virus titer was measured by ELISA after three weeks in duplicate replication. The experiments were repeated three times independently. The results of different experiments are recapitulated in Figure 2-4. Although usually the infection levels in the mutant plants were higher than in wild-type plants (except in the first experiment), we could not draw any strong conclusions from these results because (1) infection rates on different plants in independent experiments were rather variable, and (2) in some mutant plants such as *dcl1*, the abnormal phenotype of the mutant inhibited the efficacy of agroinfiltration. (3) Finally, as mentioned in the introduction, *DCLs, AGOs* and *RDRs* all belong to multigene families and functional redundancy could explain the absence of any strong effect on viral infection. Therefore it would be preferable to do such experiments on multiple mutant plants. Unfortunately, however, such mutant plants are often nonviable or were not available (case of *dcl2-3-4*).

IV. P0 suppresses IR-PTGS and interferes with a step downstream of Dicer

We have also used different assays for suppressor activity to gain insight into which component of the silencing pathway is targeted. Previous work using the Patch Test and co-expression of sense GFP with P0 revealed that P0 suppresses S-PTGS (Pfeffer *et al*., 2002). We designed an experiment to determine if P0 also suppresses IR-PTGS. In the assay pBIN-P0^{BW} is co-expressed in leaves of *N. benthamiana* (WT) with a pBIN construct containing an inverted repeat of 400 nucleotides from the 5' of GFP gene (GFFG) plus pBIN-GFP. The fluorescence levels of GFP and molecular analyses showed that P0 also suppresses IR-PTGS (see Publication N.2 and Figure 1). Furthermore, accumulation of primary siRNAs was not affected by P0. We therefore can conclude that P0 suppresses PTGS by interfering with a step downstream of DCL (for detail see publication N.2).

Publication N. 2:

The Polerovirus F-box protein P0 targets ARGONAUTE1 to suppress RNA silencing. Current Biology, Accepted 20 July 2007.

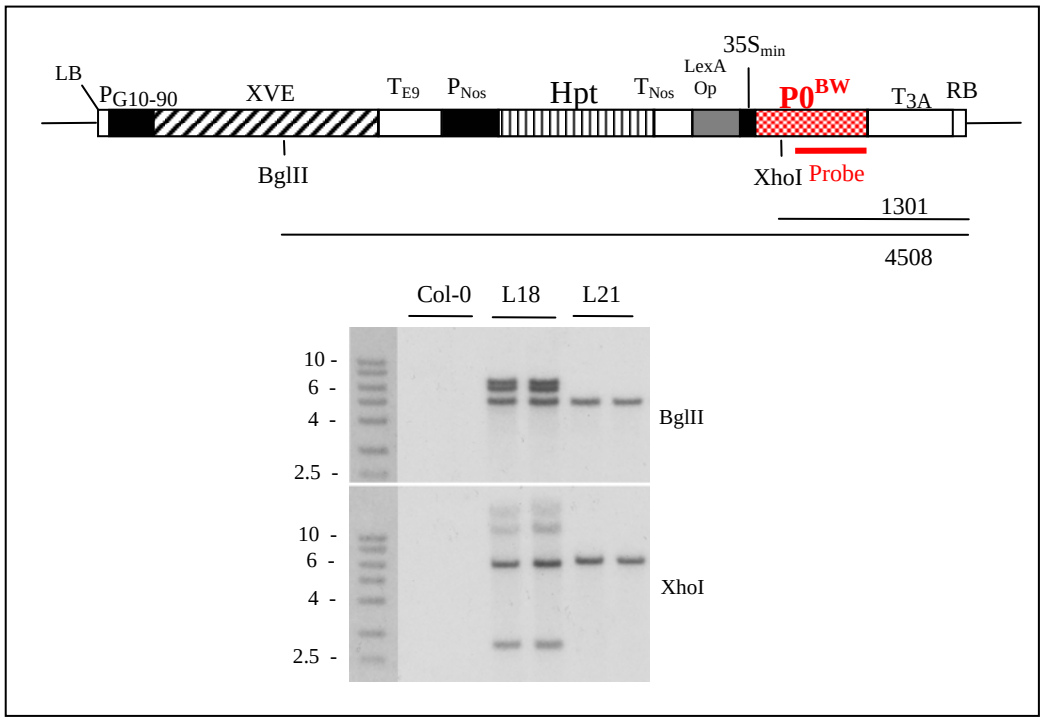


S1:

BWYV-encoded P0 protein induces developmental defects in Arabidopsis.

(A) Growth of 35S-P0^{BW} seedlings stops early in development. Seedlings start to appear two weeks after germination and remain green for about two more weeks before degenerating (panel a). Scale bar: 500μm. Longitudinal section showing the presence of an apical meristem and the first pair of leaves of a 4 week old seedling (panel b). Scale bar: 50 μm.

(B) The observed abnormalities on the leaves of induced XVE-P0^{BW} L21 plant affected both leaf margin and lamina with adaxial (upward) curling and severe crumpling of newly developing leaves (panel a). A pronounced serration 'tooth' was generally formed at each margin of the source-sink boundary (see arrows). Occasionally, a few leaf enations were observed (panel b, arrows) and trichomes appeared on the abaxial side of young leaves (not shown). Some flowering stems from estradiol-induced XVE-P0^{BW} plant also showded stem curling (panel c) and fasciated stem with fused flowers (panel d).



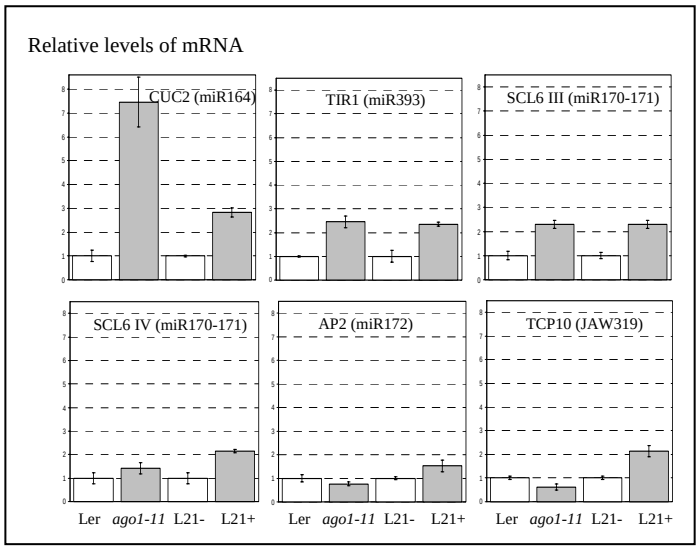
S2:

Southern blot of DNA extracted from XVE-P0^{BW} plants (L18 and L21) and from control Col-0 plants.

A schematic diagram of the T-DNA construct of XVE vector [1] containing the P0^{BW} gene (in red) is provided. The probe is given as a red line below the construct and the black lines represent the predicted minimum length of the fragments after *Bgl*II and *Xho*I digestion. Genomic DNA was extracted using DNeasy® plant mini kit (Qiagen) and digested with *Bgl*II or *Xho*I. Hybridisation was performed using the indicated randomly primed probe complementary to the 3' terminal 500 nucleotides of the P0^{BW} gene.

S3. Analysis of the expression of some miRNA-target genes in induced versus non-induced XVE-P0^{BW} L21 plants and in ago1-11 versus L*er* accession by real-time quantitative PCR.

RNA were extracted 5 days after chemical treatment from the younger developing leaves showing aberrant phenotype or the corresponding leaves from non-treated plants. Quantifications were first normalized by comparison to *ACTIN2* then to the values of *Ler* control plants (for *ago1-11*) or to those of non-treated plants (L21-). Among the twelve known miRNA-target genes analysed, six genes showed a strong up-regulation of their transcript accumulation following P0 induction (see Figure 2) whereas six other genes exhibited either little accumulation (*CUC2, TIR1, SCL6 III*) or no deregulation (*AP2, SCL6IV* and *TCP10*) of their transcript levels following P0 induction (this figure). With the exception of *CUC2*, these genes were also only weakly or not at all up-regulated in the *ago1-11* mutant background. Error bars represent the standard deviation from three replicates.

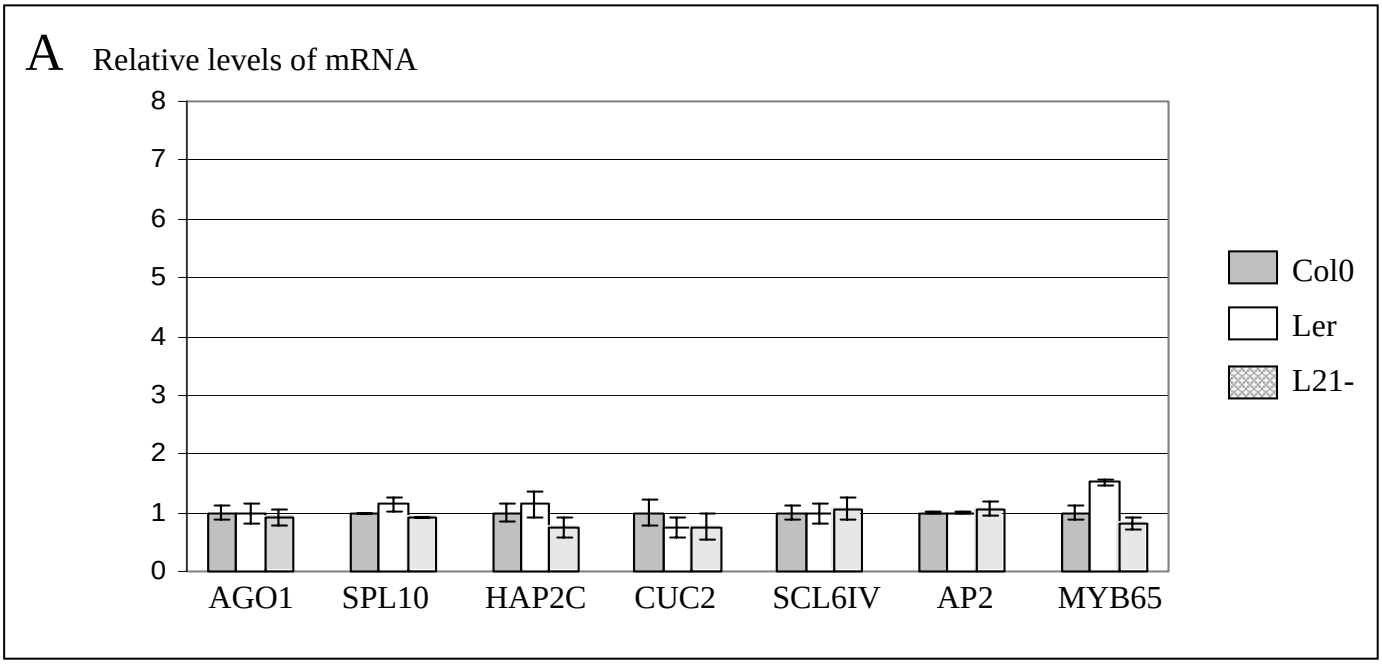


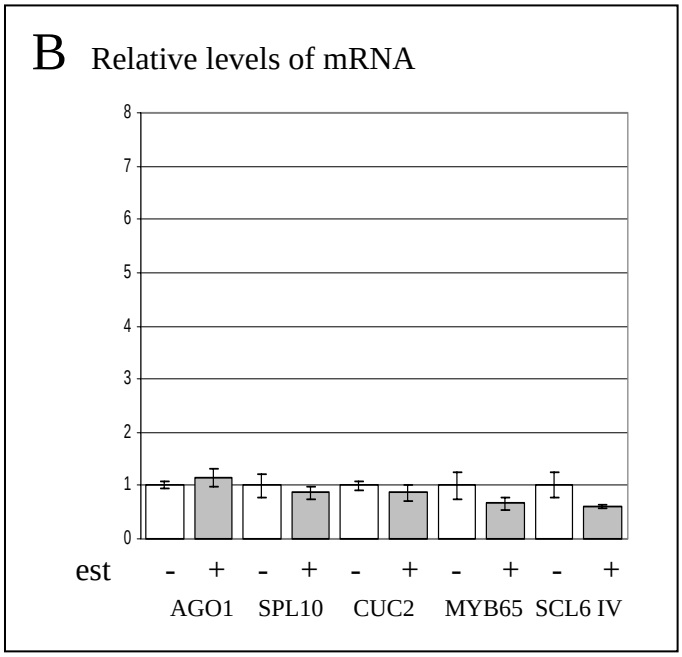
S4: Analysis of the transcription levels of several mi-RNA target genes by real-time quantitative PCR:

A. Comparison of expression in three lines: Col-0, L*er* and XVE-P0^{BW}

B. Effect of the estradiol treatment on Col-0 seedlings. -: no treatment, +: estradiol

Quantifications were first normalized by comparison to *ACTIN2* then to the values of Col-0 control plants. Error bars represent the standard deviation from three replicates.





S5:

Experimental procedures

Quantitative PCR

Total RNA was extracted using Tri-Reagent (Sigma) from pooled developing leaves of *in vitro* plantlets and treated with an RNase-free DNase (Qiagen). cDNA was synthesized using the Superscript III reverse transcriptase (Invitrogen) with oligo dT primer. Control reactions were performed without reverse transcriptase. Real-time quantitative PCR reactions were performed in 96-well optical reaction plates on a Bio-Rad i-cycler apparatus using PCR master mix (Eurogentec) containing SYBER® Green I fluorescein reporter with gene specific primers. Samples were pre-heated at 95°C for 10 min to activate Hot Star Taq DNA polymerase, and PCR was then performed by 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 15s. A melting curve was performed at the end of the amplification by steps of 1°C from 95°C to 50°C to control for the absence of primer-dimers. For each cDNA synthesis, quantification was performed in triplicate.

ACTIN2 (At3g18780) was used as an internal standard for equalization of RNA levels using the primers described by [2]. *GADPH (At1g42970)* was tested as a second standard gene to validate the *ACTIN2* gene using the primers given by Czechowski et al.[3]. As neither of these mRNAs displayed significant changes in accumulation levels upon P0 induction (data not shown), we have chosen to use *ACTIN2* for normalization of the data. Quantifications were then normalized to that of non-induced plantlets for P0 plants or of Ler plants for *ago1-11* allele. Error bars represent the standard deviation from three replicates. To assess P0 transgene induction, RT-qPCR was performed using the following primers: 5'-tgagcaatttcacaactcccgtct-3' (forward) and 5'-tcatacaaacatttcggtgtagacc-3' (reverse). To analyse the effect of P0 induction on cellular mRNA in transgenic lines, twelve miRNA target genes were investigated. For *AGO1, SPL10, CUC2, MYB65, ARF17* and *SCL6III*, the primers were those described by Vazquez et al. [2]. *DCL1 and TIR1* primers are those given by Vaucheret et al. [4]. and Navarro et al. [5], respectively. For *HAP2C, AP2, TPC10* and *SCL6IV*, the primers were designed to produce a PCR fragment covering the miRNA cleavage site. The primer sequences are as follows: *HAP2C (At1g72830)*: 5'-aagtcatccttggctactactagttct -3' and 5'-actagtttagacaagagatccatggta-3'; *AP2 (At4g36920)*: 5'-attctcactgtttccggcggctga-3' and 5'-tctcatgagaggaggttggaagccat-3'; *TCP10 (At2g31070):* 5'-attgcttaatagtcagcaacaacaagtgt-3' and 5'-tgatgatgatgatgatccgacgtcgtt-3'*SCL6-IV (At4g00150)*: 5'-ataacagagcagctggttaaggcagca-3' and 5'-atcttgaagatgagggaataagggttta-3'.

In vitro pull-down assays and co-immunoprecipitation experiments

Recombinant P0 clones were obtained by inserting an *Eco*RI-*Bam*HI PCR fragment containing $P0^{BW}$ or $P0^{CA}$ into pGEX2TK. *Escherichia coli* (Rosetta; Novagen) were transformed with these vectors and grown at 37°C until A_{600}=0.5. Synthesis of glutathione S-transferase (GST) fusion proteins was induced by addition of 1 mM isopropyl- -D-thiogalactopyranoside (IPTG), and the bacteria were incubated for an additional 3h at 37°C. Bacteria were harvested, resuspended in extraction/binding buffer (100 mM NaCl, 10 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 0.2% Triton X100, 5mM DTT) containing an EDTA-free protease inhibitor mixture (Roche) and 100 mg/L fresh lysozyme solution, incubated 10 min on ice and sonicated. GST-tagged proteins were isolated by affinity on glutathione-Sepharose 4 beads (Pharmacia) and eluted from the beads with 10 mM glutathione in 50 mM Tris pH 8 for 3h at room temperature.

Three grams of Flag-AGO1 fresh leaf tissue were ground in liquid N_2 and resuspended in 6 mL extraction/binding buffer plus an EDTA-free protease inhibitor cocktail. After 10 min centrifugation at 15000 rpm the supernatant was filtered through a 0.2 µm syringe filter and the Flag-AGO1 proteins were immobilised on anti-Flag M_2 affinity beads (Sigma). The eluted GST-tagged proteins were added to the beads along with the Flag-AGO1 proteins. Incubation was carried out using extraction/binding buffer containing the EDTA-free protease inhibitor cocktail at 4°C overnight. All washing steps were repeated 3 times in a PBS buffer containing 0.25M NaCl. Finally the beads were taken up in PAGE loading buffer, heated at 100°C and the proteins were analysed by western blot.

References :

1. Zuo, J., Niu, Q.W., and Chua, N.H. (2000). Technical advance: An estrogen receptor-based transactivator XVE mediates highly inducible gene expression in transgenic plants. Plant J *24*, 265-273.
2. Vazquez, F., Gasciolli, V., Crete, P., and Vaucheret, H. (2004). The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing. Curr Biol *14*, 346-351.
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4. Vaucheret, H., Vazquez, F., Crete, P., and Bartel, D.P. (2004). The action of ARGONAUTE1 in the miRNA pathway and its regulation by the miRNA pathway are crucial for plant development. Genes Dev *18*, 1187-1197.
5. Navarro, L., Dunoyer, P., Jay, F., Arnold, B., Dharmasiri, N., Estelle, M., Voinnet, O., and Jones, J.D. (2006). A plant miRNA contributes to antibacterial resistance by repressing auxin signaling. Science *312*, 436-439.

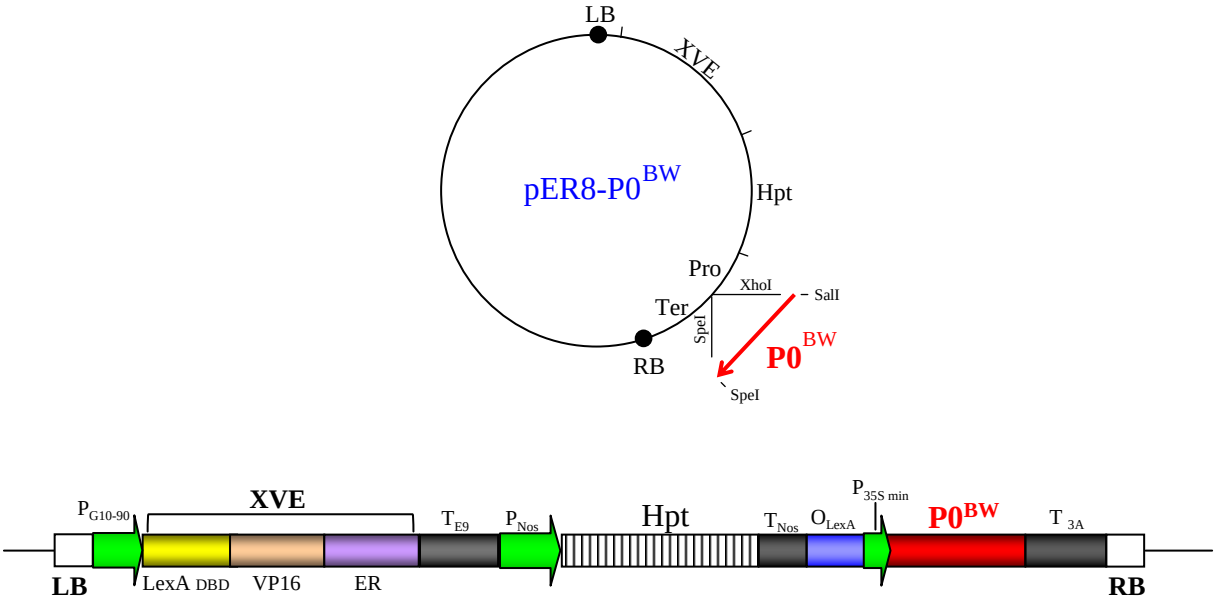


Figure 2-5: Structure of the plasmid pER8-P0^{BW} and its T-DNA. Promoter P_{G10-90} controls XVE. XVE: DNA sequence encoding a chimeric transcription factor which is assembled by fusion of the DNA-binding domain of the bacterial repressor LexA (X), the acidic transactivating domain of VP16 (Herpes simplex virus protein 16; V) and the regulatory region of the human estrogen receptor (ER). T_{E9}: rbcS E9 Terminator sequence. P_{Nos}: Nopaline synthase promoter sequence. Hpt: Hygromycin phosphotransferase II. T_{Nos}: Nopaline Synthase terminator. O_{LexA}: eight copies of the LexA operator sequence. P^{35S} minimal promoter. T_{3a}: rbcS 3A Terminator. Properties of this vector allow transgene to be expressed at the desired level and period by adding and adjusting estradiol concentration and incubation time (Zuo et al., 2000).

As mentioned in **Publication N.2**, numerous attempts to obtain transgenic lines expressing P0 of BWYV under the control of the CaMV 35S promoter failed. Although transformed seedlings were obtained, they were unable to develop much beyond embryonic stage. To overcome this difficulty, we used an inducible estrogen receptor-based transactivator XVE (pER8) system (Zuo *et al*., 2000) (Figure 2-5) to express $P0^{BW}$ in plants after the embryonic stage by treatment with estradiol. Among eighteen analyzed T2 lines of Arabidopsis transformed plants we concentrated on two lines; L21 (containing one copy of P0 and showing abnormal phenotype in ~90% of plants) and L18 (containing at least three copies of P0 and showing abnormal phenotype in ~75% of plants) (Figure S2 in supporting data of publication N.2)

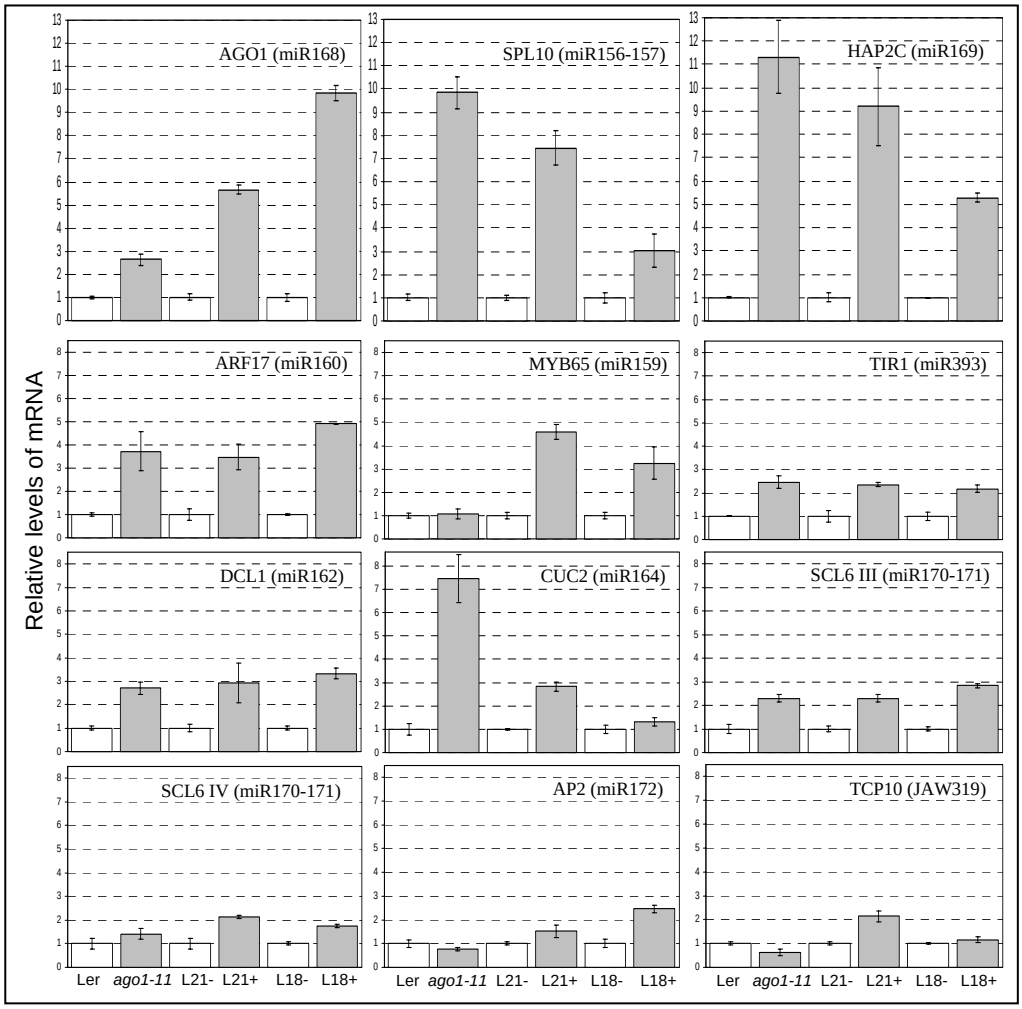


Figure 2-6: Upregulation of several miRNA-targeted endogenous mRNAs in L21 and L18 induced plants in comparison with *ago1-11* mutant. The values of *ago1-11* and induced L21 and L18 plants (+) were calculated in relation to the values of Ler and non-induced L21 and L18 (-), respectively, that were arbitrarily fixed to 1.

Table 2-2: Available results on upregulation of tested mRNA by micro-array analyses on an *ago1-11* hypomorphic mutant (Ronemus *et al*., 2006) or by qPCR on *ago1* knockout (*ago1-3*) and hypomorphic (*ago1-26* and *ago1-27*) lines (Vaucheret *et al*., 2004). First number in the column of *ago1-11* corresponds to the ratio for 9 day and the second one to 21 day old mutant plants ND: not determined. The results of two tests cannot be directly compared because of using different approaches.

Genes ↓ plants→	***ago1-11***	***ago1-26***	***ago1-27***	***ago1-3***
AGO1	1.9/1.8	4.2	4.8	29
SPL10	8.6/4.8	7.5	3.9	36.5
HAP2C	14.3/5.9	ND	ND	ND
ARF17	ND	2.1	3.1	32.6
MYB65	-2.1/-1.5	1.7	1.3	31.7
TIR1	2.1/-1.1	ND	ND	ND
DCL1	1.7/-1.3	2.3	2	27
CUC2	3.9/1	1.2	0.7	35.5
SCL6III	ND	1.3	1.8	34.7
SCL6IV	-1.4/-1.3	ND	ND	ND
AP2	-1.5/1.2	ND	ND	ND
TCP10	-1.2/1.4	ND	ND	ND

V. Another inducible P0 line (L18) has the same pattern of upregulation of miRNA-targeted transcripts

In parallel with the XVE-P0^{BW} L21 plants, we investigated the upregulation of miRNA-targeted endogenous mRNA in L18 plants to rule out the possibility that an effect of transgene locus is responsible for these upregulations. Based on the results obtained from the time scale analyses of the P0 transcripts in L21 plants, total RNA was extracted from newly grown leaves of L18 plants five days after transferring on inducible medium (+Est). RT-qPCR using primers surrounding the miRNA cleavage site was performed in parallel with that of L21. As explained in publication N.2, the results of qPCR for different mRNAs were normalized by mRNA of the housekeeping *Actin2* gene (Vaucheret *et al*., 2004) and then the relative level of each mRNA was calculated in induced versus non-induced XVE-P0^{BW} L18 plants (for which the values were arbitrarily fixed to 1). The experiment was repeated independently three times. The results showed that transcript upregulation in L18 follows the same pattern as in L21 (Figure 2-6) although the upregulation was somewhat less than that observed in L21 except for AGO1 and ARF17 mRNAs (which had higher relative levels in L18 than those in L21). These results confirmed the previous results with L21 and we suggested that upregulation of endogenous mRNAs (which are targets of miRNAs) is due to the effect of P0 on RISC. Available results on upregulation of tested mRNAs by qPCR (Vaucheret *et al*., 2004) or by micro-array analyses (Ronemus *et al*., 2006) on *ago1* knockout (*ago1-3*) and hypomorphic (*ago1-26*, *ago1-27* and *ago1-11*) mutants are recapitulated in Table 2-2. The results globally confirm that P0 plants, in addition to phenotypically resembling hypomorphic *ago1* mutants (Figure 2-7c), have miRNA-target RNA upregulation rates similar to these mutants.

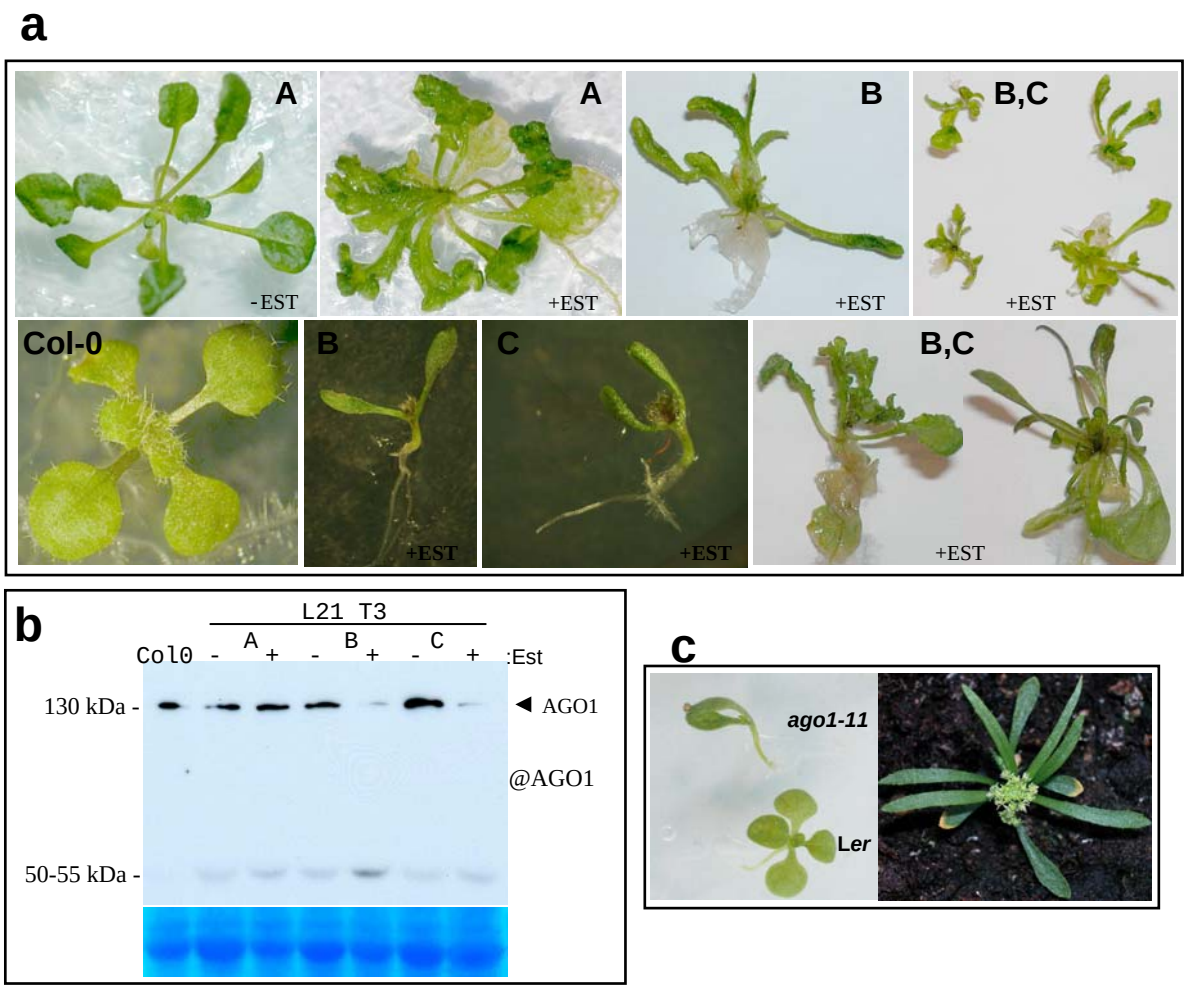


Figure 2-7: Stronger abnormalities in phenotype of T3 generation of L21 plants after induction of P0 expression. The seeds of three T2 plants (A, B and C) were cultured in the absence (-Est) or presence (+Est) of estradiol. Stronger abnormalities (particularly small plant size, delay in growth and development, curled leaves and increase in number of lateral roots with reduced length of the main root) were observed, especially in B and C plants (a). Western blot analysis (b) showing the correlation between the stronger phenotype and AGO1 stability. AGO1 was detected using a polyclonal anti-AGO1 antibody raised against a N-terminal peptide (D. Baulcombe Laboratory). (c) phenotype of an *in vitro ago1-11* plantlet compared to L*er* plant and a mutant *ago1-11* grown on soil for 8 weeks.

VI. Stronger abnormalities of induced P0 plants in the next generations

In additional experiments with the P0 transgenic plants, seeds of three T2 plants of XVE-$P0^{BW}$ L21 (plants A, B and C) were cultured in the absence or presence of estradiol. Stronger abnormalities in phenotype (particularly smaller plant size with a delay in development, more strongly curled leaves and the more lateral roots) were observed, particularly in the B and C plants (Figure 2-7a). In order to see if there is a link between the intensity of the phenotype and AGO1 accumulation, we analyzed by Western blot the presence of AGO1 in these plants (Figure 2-7b). Interestingly, when P0 expression was induced in line B and C, which exhibited the stronger phenotype, the accumulation of AGO1 was strongly reduced. Conversely, in line A which showed milder leaf deformations, AGO1 was more stable. Although P0 protein expression was not monitored, these results suggest a correlation between the severe phenotype and degradation of AGO1 protein in the induced plants. The phenotype of T3 plants resembled homozygous *ago1-11* plants (Figure 2-7c).

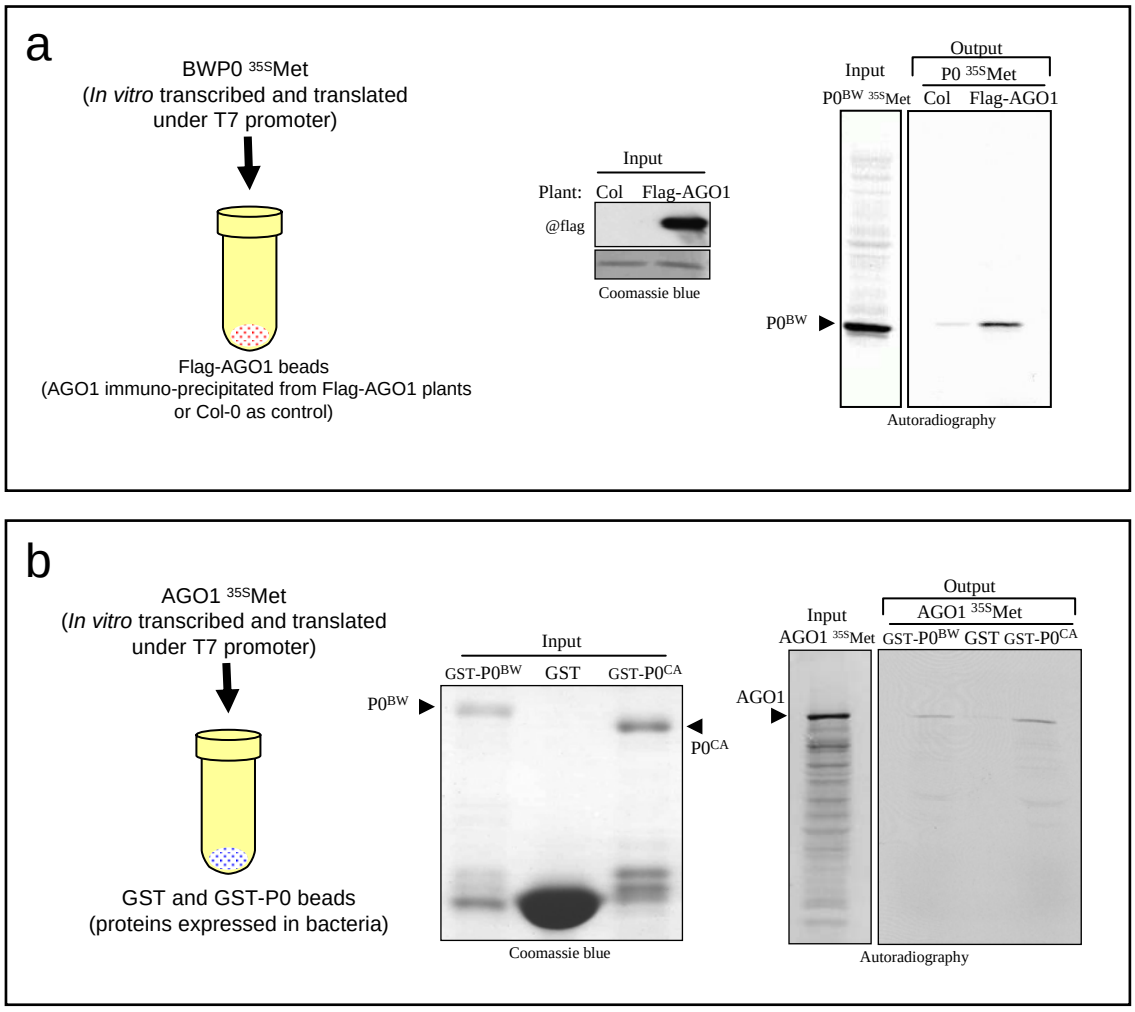


Figure 2-8: Direct interaction of P0 with AGO1 detected *in vitro* by pull-down assay. In the first experiment (a), Flag-AGO1 proteins extracted from Flag-AGO1/*ago1-36* plants were fixed on anti-Flag beads. Then *in vitro* translated $P0^{BW}$ labeled with ^{35S}Methionine was added to the reactions. After incubation, the beads were washed, SDS-PAGE was performed. Then AGO1 protein was detected by Western blot using anti-Flag antibody (SIGMA) and P0 was detected by autoradiography. In the second experiment (b), $P0^{CA}$ and $P0^{BW}$ were produced in *Escherichia coli* as fusion proteins with GST and fixed on Glutathione Sepharose 4B beads. As a control, GST protein was used in parallel. Then ^{35S}Methionine-labeled Flag-AGO1 was added. The beads were washed after incubation and SDS-PAGE was performed. The P0s and GST proteins were visualized by gel coloration and the AGO1 protein was detected by autoradiography.

VII. Direct interaction of P0 with AGO1 *in vitro*

Indirect findings that P0 suppresses silencing by interfering with RISC and then the discovery that AGO1 is degraded in the presence of P0 suggest that P0, as an F-box protein, targets AGO1 to be ubiquitinated and then degraded by proteasome. This fact that the F-box proteins interact directly with their targets prompted us to look again for an interaction between P0 and AGO1. In parallel with the pull-down assay which has been explained in publication N.2, two other independent pull-down experiments with different origin of proteins were carried out.

In the first experiment, Flag-AGO1 proteins extracted from Flag-AGO1/*ago1-36* plants (Baumberger and Baulcombe, 2005) were fixed on anti-Flag beads (SIGMA). As a control, protein extracts from Col-0 plants were treated with beads in parallel. Then *in vitro* translated $P0^{BW}$ labeled with ^{35S}Methionine was added. After incubation, the beads were washed and the co-sedimented proteins were analyzed by SDS-PAGE. AGO1 was detected by Western blot using anti-Flag antibody and P0 was detected by autoradiography (Figure 2-8a). The results showed a direct interaction of P0 with AGO1.

In the second experiment, $P0^{CA}$ and $P0^{BW}$ were produced in *Escherichia coli* as fusion proteins with GST (Glutathione S-Transferase) and fixed on Glutathione Sepharose 4B beads (GE Healthcare, Sweden). As a control, GST protein was used in parallel. Then ^{35S}Methionine-labeled Flag-AGO1 was added to the beads. After incubation, the beads were washed and SDS-PAGE was performed. P0s and GST proteins were visualized by gel coloration and AGO1 protein was detected by autoradiography (Figure 2-8b). These results also confirmed the result of the other pull-down experiments. We conclude that P0 interacts directly with AGO1.

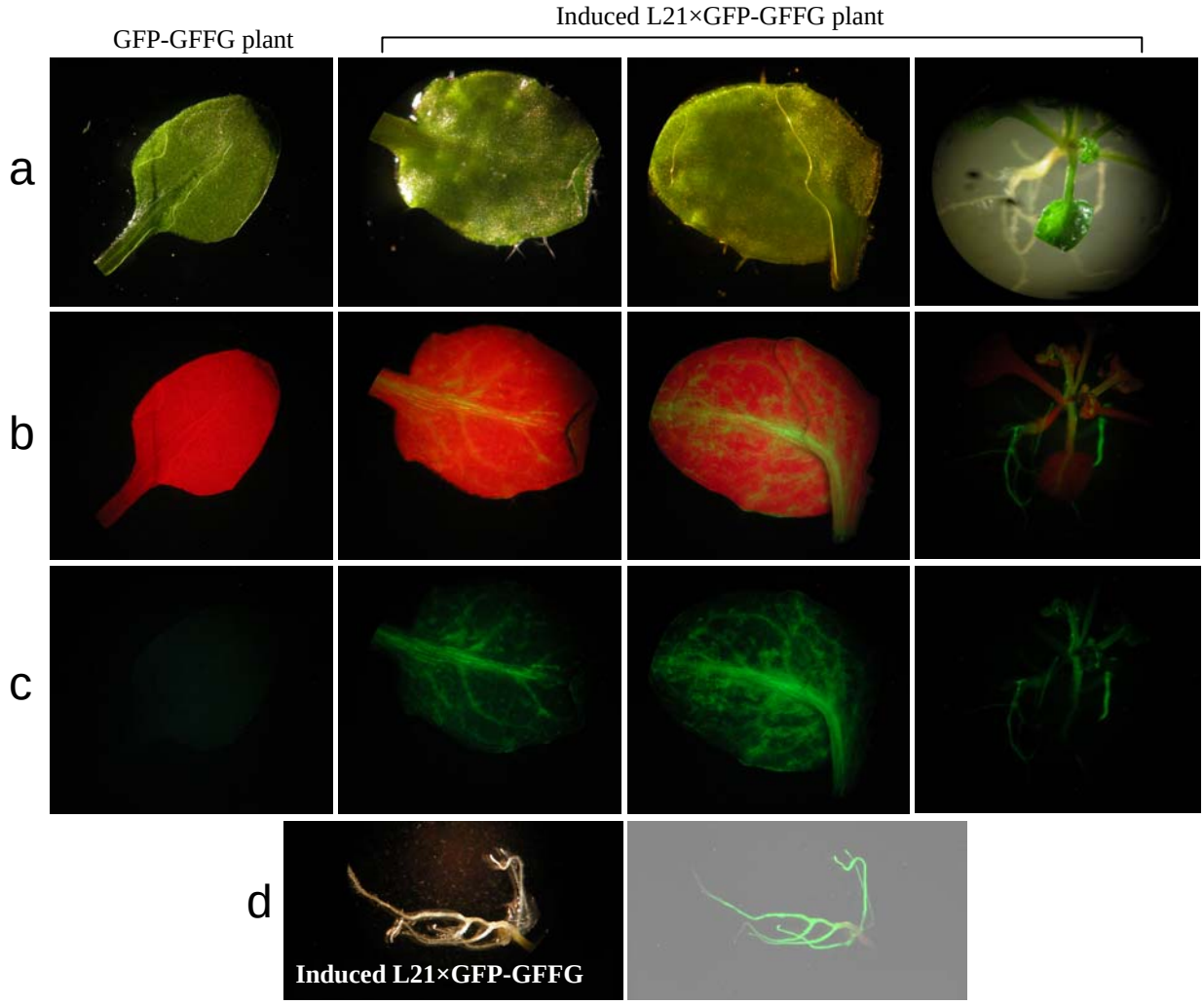


Figure 2-9: P0 suppresses IR-PTGS in P0 plants crossed with plants carrying GFP-GFFG transgenes. Photos are of plantlets 7 days after transfer on medium containing estradiol for induction of P0 expression under normal light (a) and under UV with two different filters (b and c). (d) the roots under normal light (left) or under UV light (right) (see text).

VIII. Suppression of constitutive IR-PTGS in P0 expressing plants

To further investigate *in planta* function of P0, we crossed L21 T2 plants with Arabidopsis plants carrying a constitutive GFP gene plus an inverted repeat construct (35S-GFFG) to silence GFP (Dunoyer *et al*., 2006). Selected crossed progeny plants were transferred to fresh medium containing estradiol. As a control the same treatment was done on GFP-GFFG parental plants. Green fluorescence appeared under UV light at 7 days post induction in the crossed plants, particularly in veins and neighboring tissues (Figure 2-9) with the strongest effect in newly grown leaves. We also noticed that roots of these plants exhibited a green fluorescence which was more pronounced than the roots of GFP-GFFG plants (Figure 2-9). We conclude that P0 expressed from the transgene suppresses established IR-PTGS. One possible explanation for this finding would be that P0 is functionally active only in phloem tissues which is in agreement with the phloem restriction of polerovirus although this hypothesis is difficult to reconcile with the finding that P0 suppresses S-PTGS and IR-PTGS in parenchyma cells of *N. benthamiana* in the Patch Test. Another possible explanation is that P0 is only expressed in phloem tissues in our transgenic plants, although expression from the XVE promoter is thought to be non-cell-specific. This second possibility needs to be tested by immuno-histological experiments.

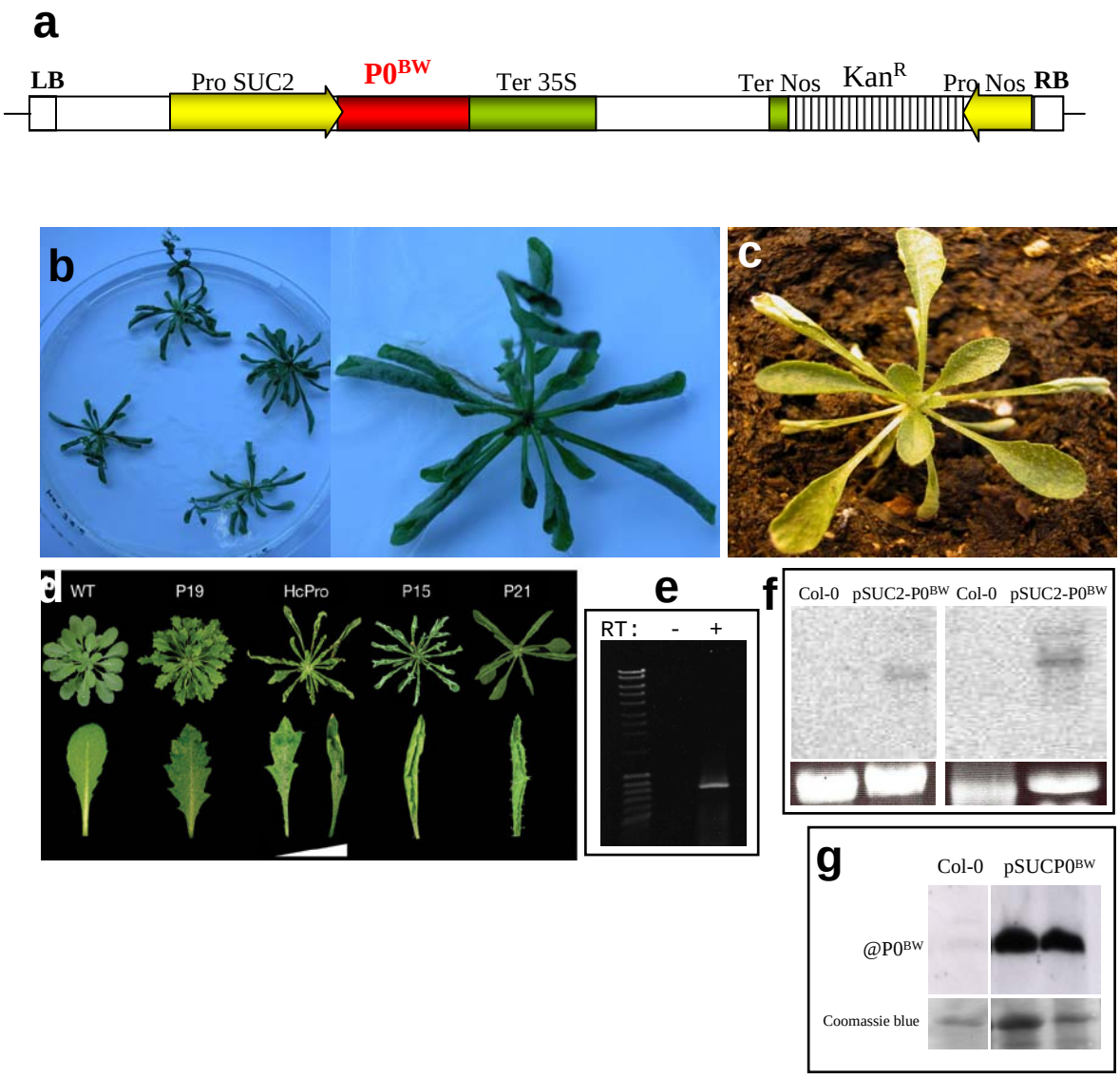


Figure 2-10: (a). T-DNA cassette for transforming Arabidopsis with P0^{BW} under control of SUC2 promoter. Abnormal phenotype of SUC2-P0^{BW} T2 plants with curled leaves on medium (b) and one week after transfer on soil (C). (d) similar phenotype in plants encoding different silencing suppressor proteins (adapted from Voinnet, 2005a). Detection of P0 mRNA by RT-PCR (e) and by Northern blot (f) in plant total RNA from SUC2-P0^{BW} plants and control Col-0 plants. Detection of P0 protein by Western blot using an anti-P0^{BW} antibody (g) in three week-old *in vitro* pSUC2-P0^{BW} plants.

IX. Arabidopsis plants expressing P0 under control of a phloem specific promoter (SUC)

The mortality of 35S-P0 plants impelled us to use other promoters to express P0 *in planta*. In parallel with the inducible XVE-$P0^{BW}$ plants, we transformed Arabidopsis Col-0 plants with $P0^{BW}$ under control of the Arabidopsis phloem specific SUC2 promoter (SUC- $P0^{BW}$) (Figure 2-10a). The Arabidopsis *SUC2* gene (At1g22710) encodes a plasma-membrane sucrose-H+ symporter. The DNA sequence of the SUC2 promoter has been determined (Sauer and Stolz, 1994). Using the reporter genes, GUS (beta-glucuronidase, Truernit and Sauer, 1995) and GFP (Imlau, 1999; and also using a fluorescent secondary antibody (Stadler and Sauer, 1996) the tissue specificity of the SUC2 promoter has been studied. In Arabidopsis, the SUC2 promoter directs gene expression with high specificity to the phloem of all green tissues such as rosette leaves, stems, and sepals. During leaf development, gene expression is first observed in the tips of young rosette leaves. In older leaves the expression proceeds from the tips to the base of the leaves following sink/source transition. Expression has been also observed in sink tissues such as roots.

As was mentioned in the introduction, poleroviruses are restricted to the phloem of their hosts. Specific expression of P0 only in phloem tissue would be similar to its natural expression pattern in the context of a viral infection. All of the transformed T1 plants showed normal phenotype. Only one line, among eight T2 lines tested, showed an abnormal phenotype (Figure 2-10b) with curled leaves ("cigarette shape") and the frequency of this phenotype was 100% in this line. Similar phenotypes have been reported in plants expressing various silencing suppressor proteins such as $HCPro^{TuMV}$, $P15^{PCV}$ and $P21^{BYV}$ (Figure 2-10d) (Dunoyer *et al*., 2004; Voinnet, 2005a). Two weeks after transferring the plants to soil, the phenotype was no longer visible. In such plants we detected P0 mRNA by RT-PCR (Figure 2-10e) and northern blot (Figure 2-10f) and P0 protein by Western blot with an anti-$P0^{BW}$ antibody (Figure 2-10g). The relative level of P0 mRNA using RT-qPCR compared to Col-0 or SUC-$P0^{BW}$ without addition of reverse transcriptase (RT) was ~4000 times. These plants, despite having a phenotype, did not show significant differences in relative levels of miRNA-targeted mRNAs compared to Col-0. This is presumably because we extracted total RNA from the whole leaf tissue and this would dilute the effect of P0 which is expressed only in phloem. Southern blot analyses (with the help of Diane Bortolamiol) showed the presence of at least three copies of P0 gene in the SUC-$P0^{BW}$ line.

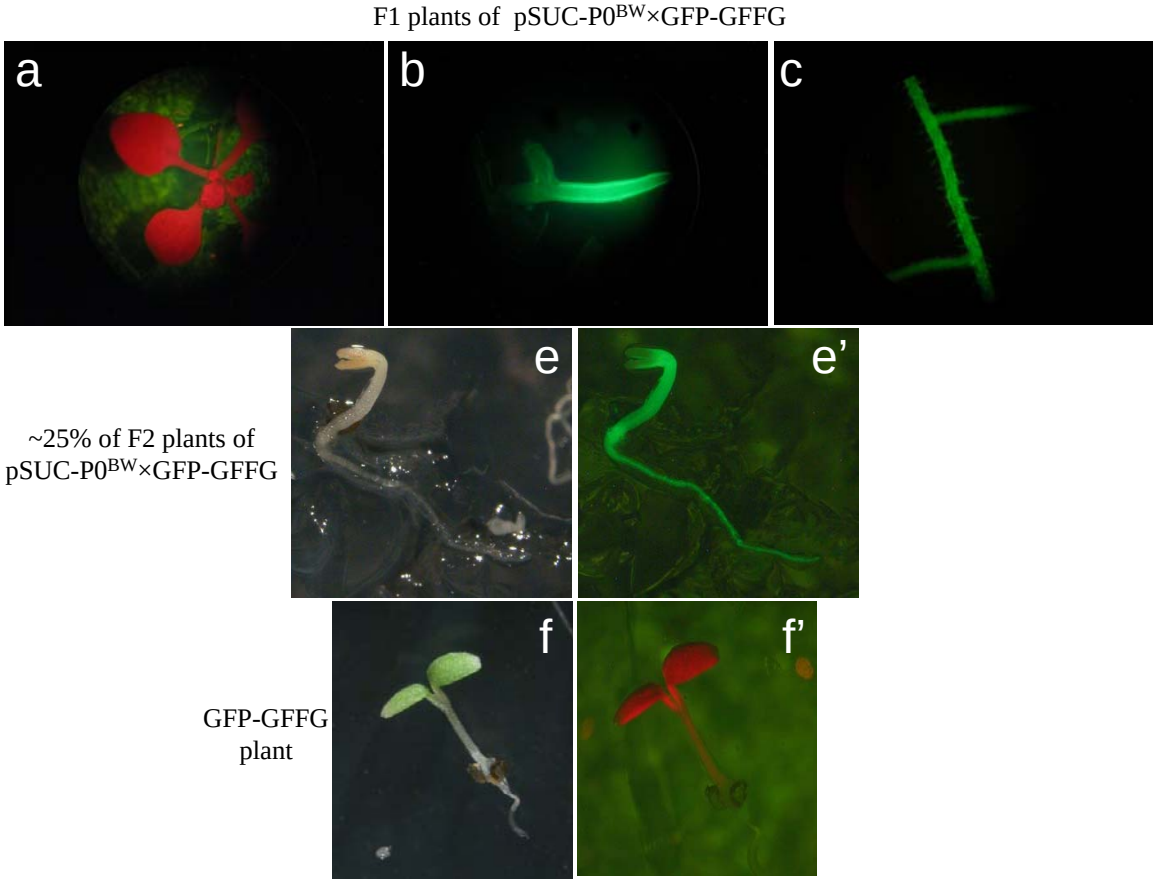


Figure 2-11: SUC-P0^{BW} Arabidopsis plants crossed with 35S-GFP-GFFG plants show suppression of IR-PTGS only in roots of 10% of F1 plants. The green fluorescence of GFP under UV light is visible in roots (b and c) but not in aerial part of crossed plant (a) and not in roots of 35S-GFP-GFFG plant (f'). In F2 generation ~25% of plants show green fluorescent roots and ~25% of the plants stopped growing after germination (e) showing green fluorescence in hypocotyl and hyporhize of a plant that stopped growing (e'). Control 35S-GFP-GFFG plant of the same age is shown in (f and f'). Photos in (e) and (f) were taken under normal light and the others under UV light. (e) and (e') were taken at a 4× magnification compared to (f) and (f').

We further investigated the SUC-P0^{BW} line by crossing it with an Arabidopsis plant carrying a silenced GFP reporter gene (35S-GFP and the 35S-GFFG inverted repeat, Dunoyer *et al*., 2006) to determine whether P0 in this background could suppress silencing induced by IR-PTGS. About 10% of the F1 plants showed strong green fluorescence only in roots, but this disappeared with time (no more fluorescence in roots after four weeks). 25% of the crossed F2 plants showed green fluorescence in roots (Figure 2-11b,c). Interestingly, ~25% of the rest of the plants stopped growing after germination. These plants typically had green fluorescent hypocotyls and hyporhizes (Figure 2-11e,e'). The existence of three copies of P0 in the parental SUC-P0^{BW} plants could result in auto-silencing and consequently low expression of P0. Presumably, during crossing and then segregation in the F2 generation some plantshave lost one or two copies of P0 and strong expression of the remained copy results in plant mortality similar to that observed in 35S-P0^{BW} plants.

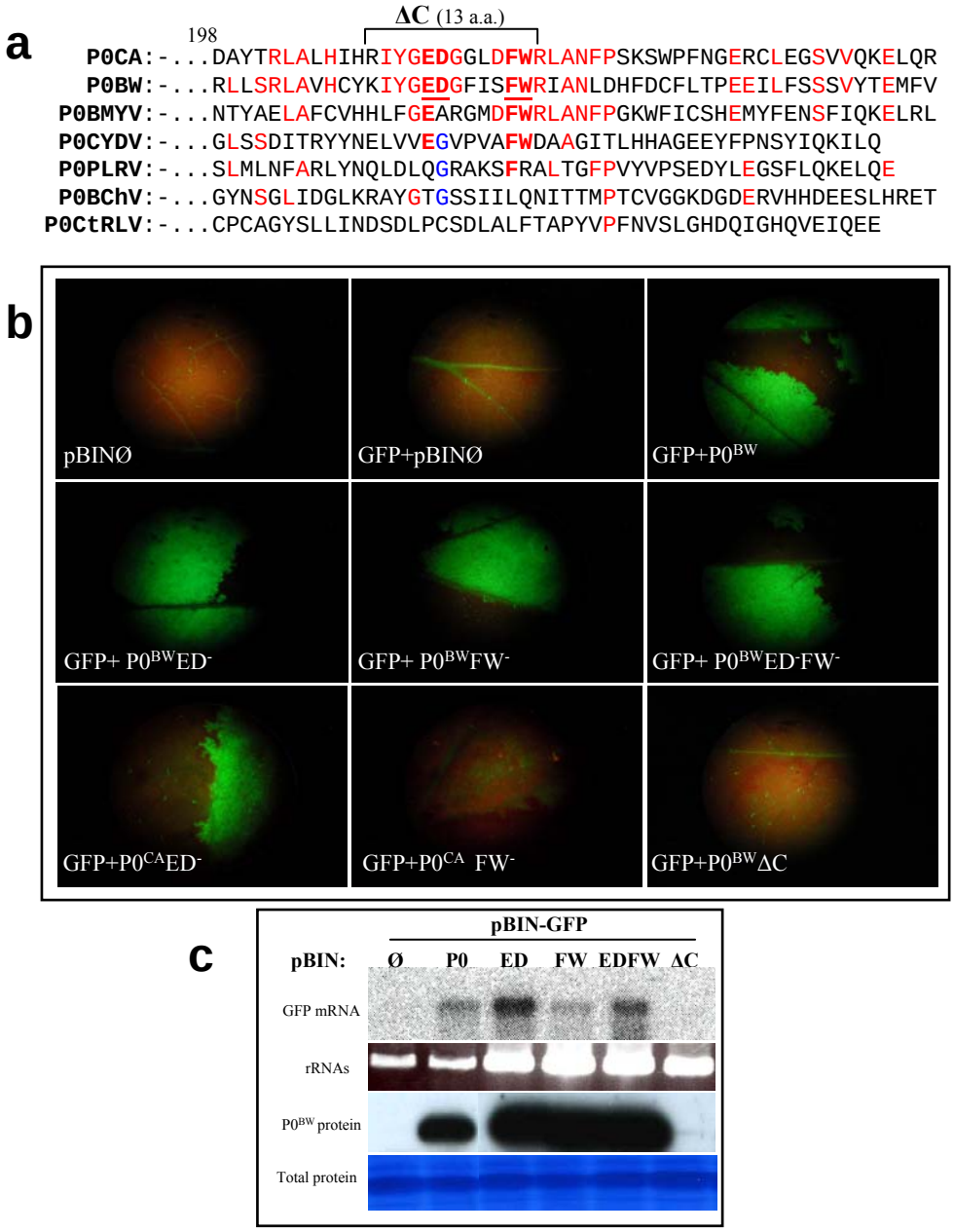


Figure 2-12: (a). Conserved domain near the C-terminus of P0s. (b and c) Patch Test for investigation of suppressor activities of the C-terminal mutants of $P0^{BW}$ and $P0^{CA}$. Agroinfiltration was carried out on leaves of *N. benthamiana* 16c with agrobacterium harboring a GFP plasmid and the indicated vectors. (b) shows green fluorescent levels of patches under UV light five days after agroinfiltration. The $P0^{BW}\Delta C$ and $P0^{CA}FW$ mutants lost their suppressor activities. (c) Molecular analyses of total RNA and total protein extracts of infiltrated patches for $P0^{BW}$ mutants. GFP mRNA were detected using a probe produced to detect 5' part of GFP mRNA. In the presence of silencing suppression activity, GFP mRNAs were protected. Wild-type P0 and mutant proteins were detected using an anti-$P0^{BW}$ antibody.

X. Looking for a C-terminal domain in P0 implicated in interaction with the target protein (AGO1)

As mentioned in Chapter 1, in addition to the F-box motif implicated in interaction with SKP, typical F-box proteins have a C-terminal region carrying one of a number of possible divergent protein-protein interaction domains (Figure 1-7) by which they interact with target proteins. The most frequent C-terminal domains are LRRs (Leucine-rich repeat) and WD (Tryptophan-Aspartate) (Kipreos and Pagano, 2000; Gagne *et al*., 2002). P0 does not contain similar motifs but the amino acid sequence alignment of $P0^{CA}$ and $P0^{BW}$ showed a short conserved domain near their C-termini (Figure 2-12a) that shares a few conserved amino acids with P0 of other poleroviruses. Therefore, we hypothesized that this domain may be implicated in interaction with AGO1. To test this hypothesis, we created a deletion of 13 amino acids (ΔC) and three Alanine substitution mutations (ED→AA, FW→AA and EDX_4FW→AAX_4AA) in $P0^{BW}$. Then we cloned these mutants in pBIN61 vector for agroinfiltration with pBIN-GFP in the Patch Test (on leaves of *N. benthamiana* 16c) to test their suppressor activities. All three construct containing point mutations maintained suppressor activity similar to that of wild-type P0 but the P0ΔC mutant lost its suppressor activity (Figure 2-12b). To verify that the sequence of the point mutants had not altered *in planta*, total RNAs were extracted from the infiltrated patches and RT-PCR was carried out. Sequencing of PCR products showed that the mutant sequences were conserved. Similar $P0^{CA}$ mutants were created (except for EDX_4FW) and assayed in the Patch Test. P0ΔC was inactive as suppressor (like $P0^{BW}$ΔC). $P0^{CA}ED^-$ mutant was similar to wild-type P0 in silencing suppression (like $P0^{BW}ED^-$) but the $P0^{CA}FW^-$ mutant had lost its suppressor activity (unlike $P0^{BW}FW^-$) (Figure 2-12b). Molecular analyses by northern blot showed that changes in GFP mRNA levels correlate with observed green fluorescence levels (Figure 2-12c). Therefore, both $P0^{CA}$ΔC and $P0^{BW}$ΔC and $P0^{CA}FW^-$ were candidates for further investigation.

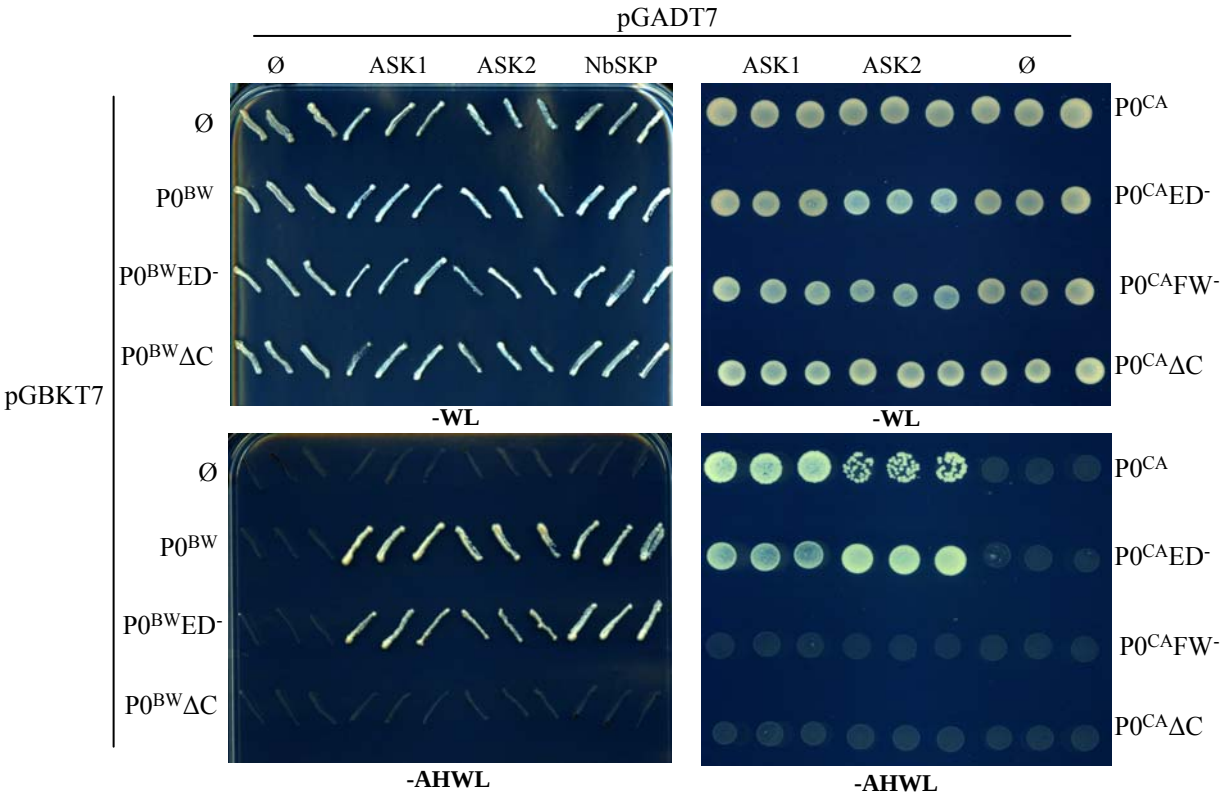


Figure 2-13: Interaction between ASKs and C-terminal mutants of $P0^{BW}$ (left panels) and $P0^{CA}$ (right panels) in a yeast two hybrid system. The $P0^{BW}ED^-$ and $P0^{CA}ED^-$ mutants behave like wild-type P0s and the $P0^{BW}\Delta C$, $P0^{CA}\Delta C$ and $P0^{CA}FW$ mutants do not interact with ASKs. The mutants that showed no silencing suppression activity in the Patch Test, were unable to interact with ASK proteins. SD/-WL is non-selective medium and SD/-AHWL is selective medium for interaction. Ø represent empty vector.

In the next step, we wished to determine whether these mutants interact with ASKs. To answer this question we cloned them into the pGBKT7 vector and assayed their interaction with ASK1 and ASK2 in the yeast two-hybrid system. None of the three mutants interacted with ASKs. Presumably, deletion in ΔC mutant and the amino acid changes in the FW^- mutant have changed the protein conformation so that they were no longer capable of interacting with ASKs (Figure 2-13a).

Taken together, we cannot conclude that the sequence deleted in ΔC is implicated in interaction with target (AGO1) protein because the mutants that lost the suppressor activity also lost their ability to interact with ASKs. Possibly, P0 needs to interact with ASK to compose an SCF^{P0} ubiquitin ligase before being able to interact with its target. Therefore, more attempts will be required to find an eventual C-terminal P0 mutant that interacts with ASKs but is no longer capable of acting as a suppressor of silencing.

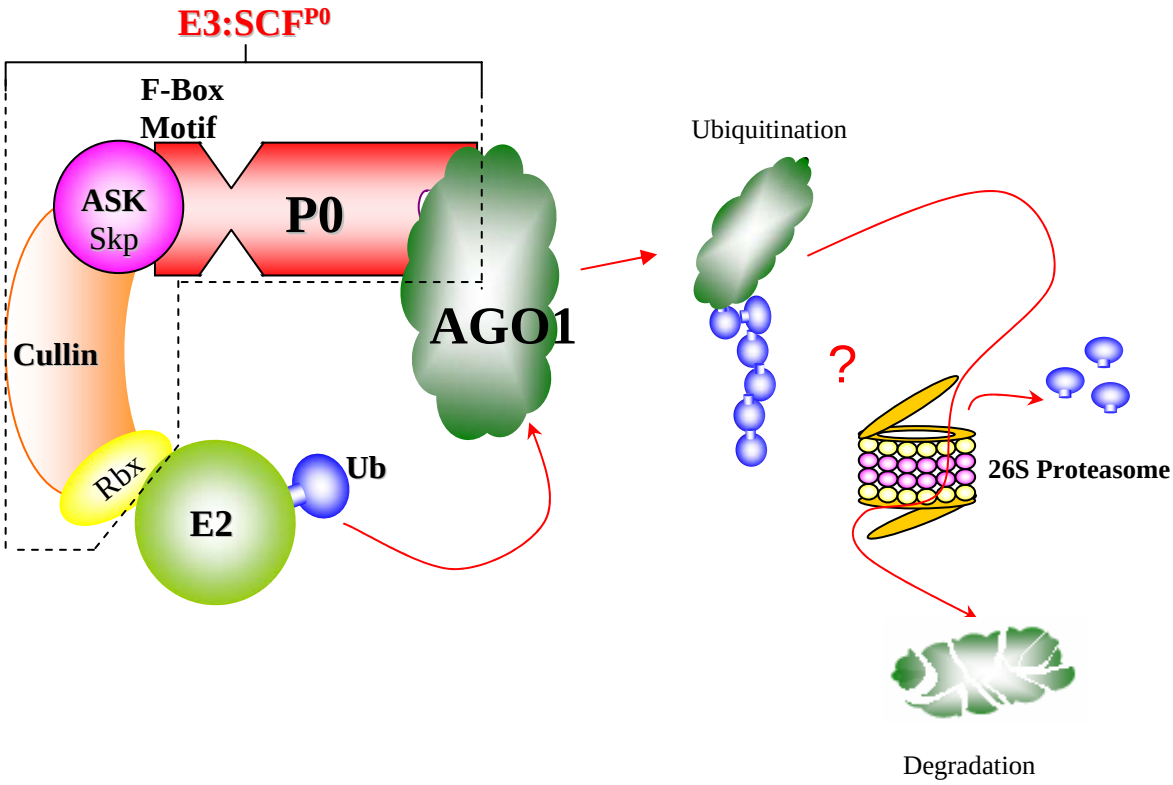
Conclusion

In this chapter, we have shown that the polerovirus P0 protein suppresses RNA silencing by interfering at a step downstream of DCL activity. Then we have shown that Arabidopsis plants expressing P0 under control of the 35S promoter are impeded in their development process and do not survive. Furthermore, P0 expression under control of an inducible promoter results in abnormal phenotypes. It was observed that several miRNAs in induced P0 plants accumulated less abundantly than in non-induced plants. miRNA-targeted endogenous transcripts were also upregulated in the presence of P0. Therefore, we conclude that P0 must be targeting an essential common component of the antiviral silencing and miRNA pathways such as RISC. Subsequently, we showed that AGO1 protein undergoes degradation in the presence of P0 in both transient expression (co-expresssion in *N. benthamiana* leaves) and in crossed P0-FlagAGO1 plant. After that, we confirmed a direct interaction between P0 and AGO1 proteins *in vitro* and *in planta*. All these data are in favor of AGO1 being a target of P0. This is the first example of a suppressor of silencing that acts in an SCF complex to promote ubiquitination of an essential component of the silencing pathway and its degradation, thereby, inhibiting the plant antiviral defense.

Progeny of plants expressing P0 under control of an inducible promoter showed more dramatic phenotypes, resembling strong *ago1* mutants. We also observed an abnormal phenotype in an Arabidopsis line expressing P0 under control of a phloem specific promoter, a phenotype similar to other silencing suppressor expressing plants. P0 expressing plants are capable of suppressing IR-PTGS.

Main Conclusion

Discussion and perspectives



Main Conclusion, Discussion and Perspectives

I. P0: a viral suppressor of silencing with a novel mode of action

Silencing suppression mechanisms

RNA silencing is an evolutionarily conserved system that functions as an antiviral mechanism in plants and animals. To counteract RNA silencing, viruses express silencing suppressor proteins. Viral suppressors of silencing are diverse in sequence and structure. They evolved independently over time in a bilateral interaction between virus and host. When I started my thesis there was relatively little information available about the suppression mechanism of viral suppressors but during the last three years the modes of action of several such proteins have been characterized. Among the best known suppressor proteins, it has been demonstrated that P19 and P21 are dsRNA-binding proteins that interact physically with siRNA duplexes *in vitro* as well as *in vivo*. They inhibit RISC assembly by siRNA sequestration (Vargason *et al*., 2003; Lakatos *et al*., 2006). Small dsRNA binding function has also been demonstrated for several other suppressor proteins. Other studies have shown that suppressor proteins can target and block an essential protein of silencing pathway. Thus, P38 blocks DCL4 and DCL2 activities in Arabidopsis (Deleris *et al*., 2006) and 2b inhibits cleavage function of AGO1 (Zhang *et al*., 2006).

The aim of this thesis was to elucidate the mechanism of action of P0 in RNA silencing suppression. Screens of Arabidopsis cDNA library using the yeast two-hybrid system had previously been used to find cellular partners for viral suppressor proteins. Using this method, it has been shown that (1) $HCPro^{TEV}$ interacts with rgsCaM (regulator of gene silencing calmodulin-like protein), which may act as an endogenous suppressor of silencing (Anandalakshmi *et al*., 2000), (2) $P19^{TBSV}$ interacts with members of the ALY gene family, proteins that are RNA processing factors involved in RNA transport and also act as transcription cofactors (Uhrig *et al*., 2004; Park *et al*., 2004), (3) $P38^{TCV}$ interacts with TIP (TCV-interacting protein), probably a transcription factor, to prevent nuclear localization of TIP (Ren *et al*., 2000, 2005), and (4) $AC2^{TGMV}$ and $L2^{BCTV}$ interact with and inactivate ADK (Adenosine Kinase), a cellular enzyme that phosphorylates Adenosine to produce 5'-AMP, which is important for methyl cycle maintenance (Wang *et al*., 2003). By applying this approach, we found that both $P0^{BW}$ and $P0^{CA}$ interact with SKP proteins, which are subunits of the SCF complex in the ubiquitin-dependent protein degradation pathway. This interaction led us to discover a conserved F-box like motif in P0 that is indispensable for its silencing suppression activity. In addition, the P0-SKP interaction, or the presence of the SKP

protein in plants, is necessary for viral pathogenicity and establishment of infection. In contrast to the other studies, our findings lead to an almost complete model for the action of P0 in silencing suppression. In this model P0 acts as an F-box protein in the plant cell, interacts with SKP and constitutes an SCF^{P0} complex to address an essential component of the RNA silencing pathway for ubiquitination and subsequent degradation by the 26S proteasome. Using the yeast bridging assay, we have shown that P0 interacts with CUL1 in the presence of ASK1 or ASK2 but the incorporation of P0 into a functional SCF complex in the presence of its target protein still remain to be demonstrated and the structural details are not yet known. Using transgenic plants expressing P0, we investigated the effect of P0 on the miRNA pathway. Some miRNA were down-regulated and some miRNA-targeted transcripts showed increased accumulation. This led to the suggestion that P0 acts at the level of RISC, an hypothesis that we confirmed by showing that AGO1 disappears in the presence of P0 *in planta*. Finally we have shown by *in vitro* pull-down assay and *in planta* BiFC tests that P0 interacts directly with AGO1 in Arabidopsis. These data allow us to conclude that P0 functions as a suppressor of silencing by targeting AGO1 and destabilizing it via the incorporation into an SCF complex. Only the last steps of this model remain to be confirmed: the P0-mediated ubiquitination of AGO1 and the implication of 26S proteasome in its degradation. These steps could be tested using chemicals that inhibit the function of 26S proteasome.

Silencing suppression of polerovirus P0 functions at the same level as CMV 2b protein but the mechanism is different. While both target AGO1, 2b does not provoke its degradation but inhibits its slicer activity (Zhang *et al*., 2006). P0, on the other hand, apparently does not inhibit AGO1's slicer activity but instead blocks the silencing process by marking the enzyme for degradation by the cellular proteolytic machinery. 2b interacts with a region upstream of the PAZ domain, the PAZ domain and part of the PIWI domain of AGO1 (Zhang *et al*., 2006). Molecular characterization of the interaction between P0 and AGO1 remains to be elucidated. Typical F-box proteins interact with their targets by means of their C-terminal region but we have not yet identified the domain of P0 implicated in the interaction with AGO1. It is noteworthy that many F-box proteins only interact with phosphorylated targets (Cardozo and Pagano, 2004). Whether AGO1 undergoes a posttranslational modification which is necessary for the interaction with P0 remains to be answered. In summary, P0 is the first protein among plant viral suppressors to be able to provoke the degradation of an essential component of the silencing pathway. The findings with P0 and 2b provide additional evidence that not all silencing suppressors target dsRNA. Finally, although we propose AGO1 degradation as the principal means by which P0 silences suppression, we cannot rule out the possibility that P0 has another target protein in addition to AGO1 or also acts by a different mechanism in parallel.

Exploitation of the host ubiquitin/proteasome machinery by pathogens

Just as plants encode F-box proteins implicated in defense and which can confer resistance to pathogens (Zeng *et al*., 2006), it is now becoming clear that pathogens can also encode their own F-box proteins or manipulate the host ubiquitin-proteasome system to favor their infection (Banks *et al*., 2003; Lechner *et al*., 2006). We have demonstrated that polerovirus P0 is an F-box protein that uses host ubiquitin-proteasome machinery to overcome the post-transcriptional silencing process. The first known viral F-box protein was CLINK (Cell cycle link) encoded by Faba been necrotic yellows Nanovirus (Aronson *et al*., 2000). CLINK interacts with MsSKP1, an alfalfa SKP1 homologue, and interferes with the plant cell cycle (Lageix *et al*., 2007) but its direct implication *in vivo* with the cellular RBR (Retinoblastoma Related) protein remains to be confirmed. Another example of a pathogen encoding F-box protein is the plant bacterium, *A. tumefaciens*, which encodes an F-box protein called VirF that interacts with ASK1 and ASK2 (Tzfira *et al*., 2004). VirF is specifically required during the infection process and is involved in turnover of both the host protein VIP1 and the bacterial protein VirE2. VirF may thus contribute to the uncoating of the T-DNA before its integration into the plant genome. Another recent finding showed that the phytopathogenic bacterium *Ralstonia solanacearum* encodes a family of seven type III secretion system (T3SS) effectors (GALA: because of their conserved GAxALA sequence) that are F-box proteins capable of interacting with different ASKs. An *R. solanacearum* strain in which all seven GALA effector genes have been deleted or mutated was no longer pathogenic on Arabidopsis and less virulent on tomato. The F-box domain is essential to the virulence function of GALA7. It is proposed that these effectors promote disease by degrading host defense proteins through the action of an SCF complex in plant and using ubiquitin-proteasome machinery (Angot *et al*., 2006). Animal viruses have also been reported to manipulate the host ubiquitin-proteasome machinery either by encoding a novel E3 ligase with appropriate properties or by altering the specificity of a host E3 ligase (Banks *et al*., 2003; Barry and Fruh, 2006). Up to now, plant viral proteins that could act as an E3 ligase have not been reported although several proteins secreted by phytopathogenic bacteria can mimic host E3 ligase in plant cells to inactivate plant defenses (Janjusevic *et al*., 2006). These examples indicate that many pathogens hijack the ubiquitin-proteasome machinery to establish infection in their hosts. Our results indicate that P0 represents a novel variation on the above themes by acting as an F-box protein in plants that, presumably, uses the ubiquitin-proteasome system to suppress the RNA silencing pathway.

II. Deleterious effects of expression of P0 in transgenic plants

Previous studies in the laboratory demonstrated that P0 protein is expressed below detection leves during a poleroviral infection and that this is a consequence of the suboptimal translation initiation context of the P0 start codon in viral RNA (Pfeffer *et al*., 2002). A mutation to optimize the P0 translation initiation efficiency in BWYV RNA was not stable during virus multiplication *in planta*. Instead, the P0 initiation context in the progeny was frequently replaced by a less efficient one, indicating that there is selection against over-expression of P0 from the viral genome. On the other hand, poleroviruses require P0 to suppress host RNA silencing since it has been demonstrated that null mutations in the P0 gene strongly diminish or abolish viral RNA accumulation during infection (Ziegler-Graff *et al*., 1996; Sadowy *et al*., 2001). We observed similar low accumulation of viral RNA with CABYV carrying a P0 gene mutated in its F-box motif. Our studies on transgenic Arabidopsis plants encoding P0 suggest that low accumulation of P0 during a poleroviral infection is a compromise strategy to counteract the host defence response without serious damaging the cellular environment required for viral replication.

Arabidopsis plants expressing P0 under the control of 35S promoter were not viable; transformed plants expressing $P0^{BW}$ germinated but did not develop further than the cotyledon stage and only stayed alive for 3-4 weeks. Quantitative RT-PCR showed that the maximum expression of $P0^{BW}$ in oestradiol-induced plant (3 dpi) was less than half of the expression level observed in 35S-$P0^{BW}$ seedling before they died. Therefore, although induced XVE-$P0^{BW}$ plants show a strong abnormal phenotype, the expression of P0 in these plants is lower than in 35S-$P0^{BW}$ plants and we believe that we were unable to obtain plants with higher expression levels of P0 because of toxic effects during development, presumably due to leaky expression of small amounts of P0 in such plants even in the absence of induction. This was confirmed at least in part by further experiments on XVE-$P0^{BW}$ plants.

Similar mortality after emergence of true leaves was also observed in 35S-2b plants with 2b from a severe strain of CMV (Zhang *et al*., 2006) and also in 35S-AC2 plants (Sunter *et al*., 2001). The lethality of P0 is evidently an indication that P0 targets an essential protein implicated in plant development. Interestingly, Arabidopsis plants transformed with the P0LP mutant under control of the 35S promoter are viable and do not show an abnormal phenotype, suggesting that the lethal effect of P0 is related to its F-box function and to the targeting for degradation of cellular protein(s) that are essential for development.

Does P0 target another AGO protein?

A. thaliana encodes nine other AGO proteins in addition to AGO1 and the targeting of at least some of these other AGO proteins by P0 is very likely, particularly as the PAZ and PIWI domains are highly conserved in all ten members. One piece of evidence for this hypothesis is that constitutive expression of P0 is lethal but Arabidopsis null *ago1* mutants are viable, although they have an abnormal phenotype. However, homozygous *ago1ago10* mutants are embryo-lethal (Lynn *et al*., 1999) and AGO10 has 75% similarity with AGO1. Evidently, if P0 targeted AGO10 as well as AGO1, this could explain why the constitutive expression of P0 is lethal. Some support of the idea that P0 can target other AGO proteins comes from the observation that P0 has silencing suppressor activity in *N. benthamiana* and interacts with NbSKP in the two hybrid assay, suggesting strongly that P0 can target one or more AGOs in this plant. It has been shown that NbAGO1 has approximately 80% similarity with AtAGO1 (Jones *et al*., 2006). Finally, Baulcombe *et al*. (2006) have reported recently that P0 is able to destabilize other *A. thaliana* AGO proteins in a Patch Test, confirming our assumption.

The phenotype and the miRNA pathway in plants expressing P0

Inducible XVE-P0^{BW} plants showed an abnormal phenotype comparable to that observed in Arabidopsis plants expressing other silencing suppressors such as HCPro, P19, P21, 2b and P15 expressed under control of the 35S promoter (Kasschau *et al*., 2003; Dunoyer *et al*., 2004; Chapman *et al*., 2004; Zhang *et al*., 2006; Voinnet, 2005a). There is a clear correlation between the occurrence of such morphological defects and alteration of the miRNA pathway in plants, and the mentioned suppressor proteins all affect this pathway by one or another mechanism. However, plants expressing P38, which inhibits DCL4 and DCL2 activities in Arabidopsis, do not display developmental abnormalities and it has been demonstrated that P38 has no impact on the miRNA pathway (Dunoyer *et al*., 2004). Analysis of miRNA and miRNA* levels and accumulation of miRNA-targeted transcripts in induced P0 plants showed that P0 affects the miRNA pathway. Reduced miRNA levels and upregulation of some endogenous mRNAs levels were similar to a hypomorphic *ago1* mutant. It has also been reported that potato plants transformed with P0^{PLRV} show an altered phenotype resembling virus-infected plants (van der Wilk *et al*., 1997a; Prufer *et al*., 2006). In these plants a positive correlation was observed between levels of accumulation of P0 transcripts and severity of the abnormal phenotype, although P0 protein could not be detected. The defects in XVE-P0^{BW} plants are probably more complex than those observed in Arabidopsis mutants in which miRNA-directed functions are compromised, suggesting that other silencing pathways could also be affected. This point merits to be investigated in more depth.

We found that the accumulation level of several miRNA in induced XVE-P0^{BW} plants paralleled the level observed in the *ago1-11* mutant. It has been reported that, in hypomorphic *ago1* mutants (like *ago1-11* in our experiments), the compromised AGO1 function does not provoke a substantial change in miRNA accumulation, whereas in null *ago1* mutants a dramatic drop in some of the miRNAs has been observed (Vaucheret *et al*., 2004). AGO1 protein is believed to stabilize miRNAs (Vaucheret *et al*., 2004) and incomplete degradation of AGO1 or partially resistant AGO1 in specific cell-types in induced XVE-P0^{BW} plants could be an explanation for the mild effect of P0 on the accumulation of some of the tested miRNAs. On the other hand, another consequence of the dysfunction of RISC is the up-regulation of DCL1 mRNA that is controlled by miR162 (Xie *et al*., 2003). This would result in more DCL1 protein and, presumably, more miRNA production, which could compensate for the primary destabilization of the miRNA. In particular, it is noteworthy that some miRNA (miR160, miR164 and miR167) remained stable in the presence of P0. Similarly, tasiRNA255 did not undergo any significant change in induced XVE-P0^{BW} plants whereas there were below detectable levels in the *ago1-11* mutant. A possible explanation may be that, since we always induced P0 in two-week old plantlets, substantial production of tasiRNAs may already have occurred before induction of P0 or that the primary cleavage product made by AGO1 before P0 induction is stable enough to produce tasiRNA by the action of RDR6 and DCL4. Finally, it should be noted that normal accumulation of tasiRNAs in some hypomorphic *ago1* mutants such as *ago1–27* has been reported (Vazquez *et al*., 2004).

We cultured the seeds of three T2 plants of XVE-P0^{BW} on inducible medium. Two of three lines produced plants with reduced size and a more abnormal phenotype that phenocopied homozygous *ago1-11* mutants. Interestingly, AGO1 protein was more extensively degraded in these plants and we conclude that the more severe phenotype is a consequence of increased degradation of AGO1 (which could be a consequence of a higher expression of P0 although this remains to be tested). The fact that the abnormalities observed in the progeny of the three T2 plants were not uniform may reflect different levels of expression of P0 in these lines and it is possible that different levels of transgene methylation may play a role. It has been reported in P0^{PLRV} transgenic plants that phenotypic variation were accompanied by the changes in the methylation of P0 DNA sequence (van der Wilk *et al*., 1997a). On the other hand, Vaucheret *et al*. (2004) have reported that perturbation in AGO1 function in Arabidopsis results in developmental defects that, as the plants grew, became more variable from plant to plant and from one leaf to another.

In addition, culture of the seeds directly on inducible medium results in P0 induction and AGO1 degradation during germination. Because of the importance of AGO1 in development, particularly in early stages of growth, its precocious degradation would presumably have dramatic consequences and a stronger phenotype even if the degradation of AGO1 was not complete. Normally, degradation of AGO1 protein would result in dysfunction of RISC so that in response, there would be upregulation of endogenous miRNA-targeted mRNAs in plant. Because the mRNA of AGO1 is

regulated by miR168 (Vaucheret *et al*., 2004, 2006), degradation of AGO1 provoked by P0 will be partially compensated by enhanced AGO1 mRNA accumulation so that it may well be impossible to completely eliminate AGO1 by expression of P0 *in planta*, in accordance with our observation. It is interesting to speculate, on the other hand, that if other AGO proteins are also targeted by P0, any of these AGOs which do not employ a miRNA-mediated negative feedback loop to regulate their accumulation would be expected to be highly susceptible to P0-mediated degradation.

III. P0 and host specificity

Analysis of available sequence data shows that the members of Polerovirus are display considerable sequence homology in their genomes except for ORF0. One of the functions orignally proposed for the ORF0 gene product P0 was a role in determining host specificity (van der Wilk *et al*., 1989; Sadowy *et al*., 2001b). For example, the host range of BMYV and TuYV is similar except that BMYV infects beet species whereas brassica species are non-hosts. The opposite applies for TuYV. Exchanging ORF0 sequences between the two poleroviruses in order to investigate potential host range determinants would be interesting although the presence of overlapping sequences between ORF0 and ORF1 represents a serious difficulty.

Using the Patch Test and co-infiltration on leaves of *N. benthamiana*, Pffefer *et al*. (2002) have reported that, although $P0^{PLRV}$ is a suppressor of silencing, it is much less efficient that $P0^{BW}$ or $P0^{CA}$. We have shown that $P0^{PLRV}$ interacts with NbSKP in the yeast two-hybrid system but not as strongly as do $P0^{BW}$ and $P0^{CA}$ (yeast colonies double-transformed with $P0^{PLRV}$ and NbSKP grew only on –HWL medium). In addition, $P0^{PLRV}$ did not interact with ASK1 and ASK2. *N. benthamiana* (but not *A. thaliana*) is a host for PLRV, suggesting that each P0 may have evolved to overcome RNA silencing in its host plant. It would be interesting to test whether $P0^{PLRV}$ interacts with SKP of potato and whether this P0 employs the same mechanism of silencing suppression as do $P0^{BW}$ and $P0^{CA}$. As mentioned above, potato plants transformed with $P0^{PLRV}$ displayed several abnormalities such as yellowing and rolling of the leaves, slow and severely stunted growth of the plant and inhibited root formation. Several of the obtained transgenic plants were not able to produce viable tubers (van der Wilk *et al*., 1997a). These observations suggest that $P0^{PLRV}$ interferes with the miRNA pathway and thus would probably target AGO homologs in potato but confirmation of this hypothesis will require more studies.

IV. Phloem Restriction of Polerovirus

The phloem limitation of Polerovirus is not due to their inability to replicate in other cells as it was shown for PLRV (Barker and Harrison, 1982) and BWYV (Veidt *et al*., 1992) that these viruses can replicate efficiently in mesophyll protoplasts. Replication of PLRV in phloem tissues as well as in mesophyll cells of transgenic plants constitutively expressing PLRV full-length cDNA (under control of 35S promoter) has also been demonstrated (Schmitz *et al*., 1997; Franco-Lara *et al*., 1999).

It has been reported that poleroviruses and enamovirus can spread more extensively into mesophyll cells when co-inoculated with certain other viruses such as PVX, PVY, TMV and PEMV-2 (an umbravirus) (Demler *et al*., 1996). Furthermore, mechanical transmission of BWYV and PLRV, which is normally impossible, has been achieved from doubly-infected plants or by co-inoculation with an umbravirus (Falk *et al*., 1979; Mayo *et al.,* 2000; Ryabov *et al*., 2001). When recombinant strains of CMV or PVX expressing umbravirus-encoded ORF4 (encoding MP) were tested, the CMV construct, but not the PVX construct, complemented PLRV mechanical transmission (Ryabov *et al*., 2001). Moreover, the CMV construct mutated in its 2b protein lost the ability to complement PLRV mechanical transmission, suggesting the need of the silencing suppressor 2b to fulfill this function. Thus expression of the umbravirus ORF4 protein alone is not sufficient. This effect is likely due to differences in the mechanism by which PVX and CMV suppress silencing. Indeed, we know that CMV 2b inhibits AGO1 activity (Zhang *et al*., 2006) whereas PVX P25 is likely to act differently.

Pfeffer *et al*. (2002) have reported that P0 does not suppress long-distance silencing. This means that P0 is cell-autonomous and must be expressed in the destination cell to suppress silencing there. Our proposed mechanism of silencing suppression is consistent with this fact. In the antiviral silencing pathway, the first step that restricts virus replication is dicing by Dicers. It has been proposed that PTGS is highly active in the cells of the vascular bundle sheath, which control access to the phloem tissues (Marathe *et al.,* 2000). Silencing suppressor proteins that target upstream steps in the silencing pathway (such as inhibition of DCL activity by P38 of TCV) can apparently overcome the putative strong Dicer activity in bundle sheath cells, allowing the virus to exit the phloem (Deleris *et al*., 2006). The fact that P0 targets a protein downstream of Dicer would have the consequence that the viral RNA would be susceptible to the aforesaid strong Dicer activity, which would produce viral siRNA. These siRNA could then enter the surrounding cells and establish a silenced state that would tend to confine the infection to the phloem compartment.

Finally, it should be noted that the present data do not rule out the possibility that Polerovirus may lack a movement protein which functions at the phloem-bundle sheath cell boundary. Indeed, work with the PEMV-2 encoded movement protein, which allows systemic infection with PEMV RNA-1,

suggests a requirement for a movement protein, perhaps in addition to a specialized silencing suppressor protein, in enabling exit from the phloem. It should be noted, however, that earlier attempts to demonstrate silencing suppression activity of Umbravirus P4 were unsuccessful (Ryabov *et al*., 2001). Inoculation of Polerovirus on plants expressing silencing suppressor proteins (such as P38 plants) that employ different mechanisms of suppression, or on plants mutated in genes implicated in RNA silencing (such as multiple mutants of Dicers), may help to determine the importance of silencing in restricting Poleroviruses to the phloem.

Materials and Methods

Materials and Methods

Biological Materials

I. Plant materials

1. *Arabidopsis thaliana* (L.) Heynh. (mouse ear cress) ecotype [Colombia (Col) and Landesberg *erecta* (L*er*)] is a small flowering plant that is widely used as a model organism in plant biology. Arabidopsis is a member of the Brassicaceae family, which includes cultivated species such as cabbage and radish. Arabidopsis is not of agronomic significance, but it offers important advantages for basic research in genetics and molecular biology. Its small genome (125 Mb) was sequenced in the year 2000. We used this model plant to study to mechanism of Polerovirus P0 in RNA silencing suppression.

2. Arabidopsis mutants:

Mutant	Locus	Ecotype	Mutagen	Position	References
ask1-1	At1g75950	Ler	Ds transposon insertion	end of first exon at nucleotide 237	Yang *et al*., 1999
ask2-1	At5g42190	Ler	T-DNA insertion	end of first exon at nucleotide 318	Liu *et al*., 2004
ago1-11	At1g48410	Ler	EMS	A to T conversion at the splice acceptor site of intron 14	Kidner and Martienssen, 2005
ago4-11	At2g27040	Ler	EMS	mutation destroyed a splice acceptor site, causing deletion of almost the entire PIWI domain	Zilberman *et al*., 2003
dcl1-9	At1g01040	Col-0	T-DNA insertion	exon 19, second DRB domain	Jacobsen *et al*., 1999
dcl2-9	At3g03300	Col-0	T-DNA insertion	exon 9, DEAD helicase-DUF	Xie *et al*., 2004
dcl3-9	At3g43920	Col-0	T-DNA insertion	exon 7, DEAD helicase-DUF	Xie *et al*., 2004
dcl4-9	At5g20320	Col-0	T-DNA insertion	exon 23, before second DRB domain	Xie *et al*., 2004
rdr6-15	At3g49500	Col-0	T-DNA insertion	exon 2	Xie *et al*., 2004

Because the homozygous *ask1-1* and *ago1-11* mutants are sterile, the *ask1-1* and *ago1-11* alleles must be maintained in heterozygous plants. The homozygous mutants were screened by genotyping using PCR for *ask1-1* with forward primer: 5'-ccggtatatcccgttttcg-3' and reverse primer 5'-aacataaggaagaagaaactgg-3' (Figure 1-18a, forward primer for wild-type allele of ASK1: 5'-atgtctgcgaagaagattgtg-3') and for *ago1-11* with forward primer: 5'-taggcaggagctcattcagg-3' and reverse primer 5'-caggatggcatcaagttcata-3'.

3. *A. thaliana* 35S-GFP×35S-GFFG transgenic line (Dunoyer *et al*., 2006) was used for crossing with P0 plants.

4. *Nicotiana benthamiana* (Solanaceae) wild-type and transgenic 16c line (containing 35S-GFP) (Ruiz *et al*., 1998) were used in the Patch Test and co-expression assay.

II. Bacteria

1. *Escherichia coli* XL1-blue strain (STRATAGENE)

This strain was used in transformation of different vectors for amplification during almost all cloning. This strain *recA*$^-$ limits recombination risks. It contains a mutation in the lactose operon and encodes β-galactosidase deleted at its N-terminus that can be complemented by α-peptide of β-galactosidase encoded by a pBluescript type cloning vector.

2. *E. coli* MC1022 strain

This strain was used in transformation of vectors with large inserts for amplification during cloning. It was derived from *E. coli* K12 and allows α-complementation with the N-terminal fragment of β-galactosidase encoded by pBluescript type vectors.

3. *E. coli* Rosetta strain (Novagen)

This strain was used for expression of Glutathione S-Transferase (GST) fusion proteins. Rosetta strain is a BL21 derivative and, contains DE3, a λ prophage carrying the T7 RNA polymerase gene and $lacI^q$. T7 RNA polymerase from a lac promoter is induced by IPTG (Isopropyl-β-D-thiogalactopyranoside). The strain is designed to enhance the expression of eukaryotic proteins that contain codons rarely used in *E. coli*. The strain also contains pLysS plasmid carrying chloramphenicol resistance and phage T7 lysozyme, effective at attenuating activity of T7 RNA polymerase, for better inhibition of expression under non-induced conditions. These strains are recommended for use with expression plasmids carrying the Ampicillin resistance marker bla.

4. *Agrobacterium tumefaciens* strain GV3101 (pMD90)

This strain was used for infiltration of leaves of *N. benthamiana* in the Patch Test and leaves of Arabidopsis for viral infection and also for transformation of Arabidopsis plants. It contains a Rifampicin resistance gene on its chromosome. It also contains a disarmed Ti-plasmid with *vir* genes which allow *in trans* integration to the plant genome of an T-DNA with right and left borders (RB and LB). The T-DNA is supplied by a binary pBIN vector. The bacterium strains are stocked at -80ºC in 15% glycerol.

III. Yeast Strains

Saccharomyces cerevisiae: (CLONTECH)

strain	Reporter gene	Origin of UAS	Origin of TATA	UAS regulated by	Expression level
AH109	*HIS3* *ADE2* *LacZ*	GAL1 GAL2 MEL1	GAL1 GAL2 MEL1	GAL4	High High Low
Y187	*LacZ*	GAL1	GAL1	GAL4	High

These yeast strains were used in the yeast two-hybrid system. The GAL4 transactivation domain (AD) and GAL4 DNA binding domain (BD) are supplied from two yeast vectors. The vectors are constructed to encode each candidate protein as a fusion to the GAL4 activation domain (AD) or binding domain (BD). Then they are double transformed in to the appropriate yeast strain. Positive interaction between two proteins leads to the transcriptional activation of a reporter gene containing a binding site for GAL4 and allowing the yeast colony to grow in the absence of corresponding amino acid. A *LacZ* reporter gene allows quantifying the interaction level (Keegan *et al*., 1986). The yeast strains are stocked at -80ºC in 25% glycerol. (UAS: upstream activating sequence)

IV. Vectors

1. pBluescribe (+/-)

This vector (3.2 Kb) was used for cloning of DNA fragments for sequencing and transferal into pBIN61 vector as well as for *in vitro* transcription and preparing RNA probes. This vector was derived from pUC19 and contains the Ampicillin resistance gene (β-lactamase), ColEI origin of replication, *LacZ* gene encoding α peptide of β-galactosidase and phage T3 and T7 promoter sequences that allow transcription of the coding sequence inserted in the cloning cassette in either direction depending on the promoter and RNA polymerase.

2. pGEX2TK

This vector was used to express GST fusion proteins. It contains *lac* I^q gene allowing its amplification in any *E. coli* host, a *tac* promoter for chemically inducible, high level expression by IPTG and the Thrombin protease recognition sites for cleaving the desired protein from the fusion product. It also has a recognition sequence for the catalytic subunit of cAMP-dependant protein Kinase obtained from heart muscle. The protein Kinase site is located between the GST domain and an expanded multiple cloning site. Expressed protein can be directly labeled using protein Kinase and ^{32}P γ-ATP and readily detected by autoradiography. pGEX2TK contains Ampicilin resistance gene.

3. pBIN61

This vector was used extensively for expression of different genes in *N. benthamiana* as well as for transformation of P0 in Arabidopsis. pBIN61 (Voinnet *et al*., 2000) is a 12.9 Kb binary vector derived from pBIN19 (Bevan, 1984). It carries within the T-DNA sequence an expression cassette under the control of a CaMV 35S promoter and a 35S terminator sequence. The vector carries also a Kanamycin resistance gene [under an eukaryotic promoter (NOS promoter)] between the border sequences for plant selection and a second Kanamycin resistance gene under a prokaryotic promoter for bacterial selection.

4. Yeast two-hybrid vectors: pGBKT7 and pGADT7 (CLONTECH)

vector	Domain/Tag	promoter	Protein expression	Detection in Western Blot	Resistance in bacterium	Selection gene in yeast
pGBKT7	GAL4 BD/MYC	ADH1 (700 bp)	High	+++	Kan	TRP1 (W)
pGADT7	GAL4 AD/HA	ADH1 (full-length)	High	+++	Amp	LEU2 (L)
pGADT10	GAL4 AD		very low	not detectable	Amp	LEU2 (L)

These vectors were used in yeast two hybrid system for testing protein-protein interaction or for screens of a cDNA library of Arabidopsis (For more details about vectors see Figure 1-1)

Methods

I. Cloning of DNA fragment in vectors

1. PCR

PCR was carried out by using 50 ng of plasmid or 100 ng of genomic DNA. The *Taq* DNA polymerase (Invitrogen) or, when amplification required high fidelity, the HiFi *Taq* DNA polymerase (Roche) was used as follows:

Normal PCR reaction:

DNA	50-100 ng
Primers	0.5 µM
PCR buffer	×1
$MgCl_2$	1.5 mM
dNTP	0.2 mM
Taq DNA Polymerase	0.5U
H_2O	Up to 20 µl

HiFi PCR reaction:

DNA	50-100 ng
Primers	0.5 µM
HiFiTaq DNA Polymerase	0.5U
H_2O	Up to 20 or 30 µl

A basic PCR was performed using the following cycles:

Pre-denaturation at 94˚C for 2 min

Denaturation at 94˚C for 20 sec Annealing at 50˚C for 30 sec Elongation at 72˚C for 45 sec	Repeated 30 cycles

Final elongation at 72˚C for 15 min

The conditions were optimized for each pair of primers and the length of the desired product. PCRs were performed in an Eppendorf Mastercycler apparatus.

2- Analysis of DNA fragments on Agarose gel

The DNA fragments were separated by electrophoresis in horizontal agarose gels. The concentration of agarose varied from 0.8 to 2.5% depending on the size of DNA fragments. The gels were prepared by boiling agarose in TBE (Tris-Borate-EDTA) buffer (0.5×). DNA samples were prepared by adding loading buffer (20% Glycerol, 1 mM Na_2EDTA, 50 mM Tris pH 7.5 and 0.4% Bromophenol blue) and loaded on gel. The gel was run in TBE buffer (0.5×) at 100V. Then they were stained by Ethidium Bromide (1 mg/l) for 15 min and, after rinsing with H_2O, were photographed on a short wavelength UV trans-illuminator (GelDoc 1000, BioRad).

For isolation of DNA fragment from gels, low melting point (LMP) agarose was used for preparation of gel. After electrophoresis, DNA was visualized on a long wavelength UV trans-illuminator (TM40, UVP) and the desired fragments were excised using a razor blade. They were then purified by heating to 65˚C for 5 min in LMP buffer (200 mM Tris-HCl pH 7.5 and 1 mM EDTA) followed by phenol and phenol/chloroform extraction and ethanol precipitation in the presence of 200 mM NaCl.

3. DNA digestion by restriction enzymes

One µg of DNA was generally digested in a 20 or 50 µl reaction containing 0.1 mg/l BSA, the appropriate enzyme buffer 1× and 5U restriction enzyme. Reactions were generally carried out at 37˚C for 1.5h. Then phenol/chloroform extraction followed by ethanol precipitation was performed.

To prevent re-circularization of plasmid during the ligation step, the linearized plasmids were dephosphorylated by Shrimp Alkaline phosphatase (CIP) so that the 5' terminal phosphate was removed. This reaction was performed by adding 0.5 µl of SAP (1U/µl) at the end of the restriction enzyme digestion followed by incubation at 55˚C for 30 min.

4. Ligation

T4 DNA ligase catalyses the formation of a phosphodiester bound between the 3' OH terminus and 5' phosphate terminus of two DNA molecules. Ligation of purified DNA insert fragment into linearized plasmid (100 ng) was performed in a 3:1 molecular ratio in 20 µl reaction containing 0.1 mg/l BSA, the T4 DNA ligase buffer (250 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$, 5 mM DTT, 5 mM ATP and 25% PEG 8000) and 5U T4 DNA ligase with incubation overnight at 16˚C. Ligation product was extracted by phenol/chloroform, precipitated with ethanol and resuspended in 15 µl H_2O. 1-2 µl ligation was used for transformation of bacteria.

5. Transformation of bacteria

Electroporation: In this method, 1-2 µl of plasmid was added to 40 µl of electro-competent bacteria cells. The mixture was transferred to the cuvette and loaded to in a cell-electroporator apparatus (BioRad) for electroporation with 125µF capacitance, 200Ω resistance and 2.5V voltage. After electroporation, cells were mixed with 1 ml of LB medium and incubated at 37˚C (*E. coli*) or at 28˚C (*A. thumefaciens*) for 30 min. The transformed cells were selected on solid LB medium containing appropriate antibiotics after incubating overnight at 37˚C (*E. coli*) or 48h at 28˚C (*A. tumefaciens*).

Heat-shock transformation: 5 µl of plasmid was mixed with 50 µl of competent cells and then incubated on ice for 20 min. The cells were heat-schocked at 42˚C for 45 sec and chilled on ice for 2 min. The cells were mixed with 1 ml of LB medium and incubated for 30 min at 37˚C. Then the cells were spread onto solid LB medium containing appropriate antibiotic for selection of transformants.

6. Culture of bacteria

The culture medium used for *E. coli* was LB and for *A. tumefaciens* was YEB. Agar (1.5%) was added for solid medium preparation.

YEB medium : pH 7.2

Beef extract	0.5 %
Yeast extract	0.1%
Bacto-Peptone	0.5%
Saccharose	0.5%
+$MgSo_4$ (after autoclaving)	2 mM

LB medium : (Luria-Bertoni) pH 7.4

Bacto-Tryptone	1%
Yeast extract	0.5%
NaCl	0.5%

One colony of transformed bacteria was cultured in 3 ml medium containing the appropriate antibiotic with incubation overnight at 37˚C (*E. coli*) or 48h at 28˚C (*A. tumefaciens*) with shaking.

7. Extraction of Plasmid

The amplified plasmid from 3 ml bacterium culture was extracted by an Alkaline Lysis method. Cells were sedimented and the medium was discarded. 100 µl of Solution I was added and the cells were resuspended by vortex. Then 200 µl of solution II was added and gently mixed by inverting the tube. After 5 min, 150 µl of solution III was added and again agitated by inverting. Centrifugation was performed at 15000 rpm for 5 min and the supernatant was transferred to a new tube. Then phenol/chloroform purification was carried out and plasmids were precipitated with ethanol and resuspended in 50 µl H_2O.

The characterization of positive recombinant colonies was performed by enzymatic restriction analysis or by PCR. The selected positive plasmids were treated by RNase (10 ng/µl) in TE buffer (10 mM Tris-HCl and 1 mM EDTA) in 50 µl reaction and with incubation at 37˚C for 30 min. DNA was then extracted by phenol/chloroform and precipitated in ethanol in the presence of 2M Ammonium acetate. Finally, the plasmids were taken in 50 µl H_2O from which 50 ng was used for sequencing.

Solution I	Solution II	Solution III
25 mM Tris-HCl pH 8	200 mM NaOH	58.8 g/200ml Potassium Acetate
10 mM EDTA	1% SDS	23 ml Acetic Acid
50 mM Glucose		

II. Working with Yeast

1. Yeast Transformation

There are several methods used to introduce DNA into yeast, including the electroporation and the Lithium Acetate (LiAc)-mediated method. We used the LiAc-mediated method as described in the CLONTECH manual. In this method, yeast (*S. cerevisiae* strain AH109) competent cells are prepared as a preculture of 20 ml YPD medium (CLONTECH, composed of 20 g/l Difco peptone, 10 g/l yeast extract, 2% glucose and 20 g/l agar for plates, pH 6.5) incubated at 28ºC overnight and then half of them are inoculated into 50 ml YPD and incubated for 4h at 28ºC with shaking until A_{600}= 0.6. The yeast cells were sedimented by centrifugation at 4000 rpm for 3 min and resuspended in 2 ml sterile H_2O. Centrifugation was performed at 6500 rpm for 1 min, and the cells were washed by gentle resuspension and centrifugation two times with H_2O and two times with LiAc/TE 1× (10×: 1M Litium Acetate, 100 mM Tris and 10 mM EDTA, pH 7.5). Finally, the cells were taken up in LiAC/TE 1× (300-500 µl depending on number of transformations). For transformation, 5 µl carrier DNA (Salmon sperm DNA, 10 mg/ml, after boiling 5 min and chilling 3 min on ice) was mixed with 1 µl of each plasmid (pGBKT7 and pGADT7 derivates) and then 50 µl yeast cells and 350 µl PEG/LiAc/TE (1.6 ml PEG 50% + 0.2 ml LiAc/TE 1× + 0.2 ml H_2O) were added. The mixture was mixed by slowly inverting the tube several times. The transformation was performed by incubation at 30 ºC for 30 min and then at 42 ºC for 20 min. Finally, 700 µl sterile H_2O was added. After centrifugation for 1 min at 6500 rpm and discarding the supernatant, the cells were taken up in 100 µl H_2O and spread on appropriate plates (containing SD medium supplemented with all amino acids except **L**: Leucine for selection of pGADT7 and **W**: Tryptophan for selection of pGBKT7). The plates were incubated three days at 28ºC. The interaction assay was performed by subculturing transformed colonies on medium SD-HWL or SD-AHWL. The growth of colonies in the absence of **H**istidine implies a weak interaction between candidate fusion proteins to AD and BD and the growth in the absence of **A**denine and **H**istidine shows a stronger interaction between them. SD is a standard medium for yeast without amino acids and composed of 6.7 g/l Yeast nitrogen base, 2% glucose and 20 g/l agar for plates, pH 5.8).

2. Yeast two-hybrid screen (CLONTECH Manual)

We used the yeast two-hybrid system to identify cellular partners of P0 in an Arabidopsis cDNA library. To this end, the pGBKT7-P0 vector (encoding BD-P0 fusion protein) was transformed into yeast strain AH109. The transformed yeast colonies were selected on SD-W medium. Then a 50 ml YPD culture started with one transformed colony was grown until A_{600}=1.5-2 by incubation at 28ºC. This culture was used as an inoculum for 500 ml YPD culture followed by incubation until A_{600}=0.3-0.4. For most yeast strains a suspension containing 1×10^6 cells/ml will give an A_{600} of 0.1. The yeast cells were sedimented by centrifugation at 4000 rpm for 10 min and taken up in 2×50 ml LiAc/TE 1×. The suspension was incubated 30 min at RT and then centrifuged at 4000 rpm for 10 min. The cells were then resuspended in 5 ml LiAc/TE ×1.

Transformation was carried out in 15 ml Falcon tubes. 100 µl carrier DNA (Salmon sperm DNA, 10 mg/ml), 1.5 µl bank DNA (cloned in pGADT10 containing GAL4 AD) and 1 ml of yeast cells were mixed in each tube then incubated 10 min at RT. 2.8 ml fresh LiAc/TE/PEG50% was added and incubated 60 min at 30ºC. Then 430 µl Dimethylsulfoxide (DMSO) was added and incubation was continued at 42ºC for 20 min. Then, the tubes were placed on ice for 3 min followed by centrifugation at 1500 rpm for 7 min at RT. The cells were taken up in 30 ml YPD and incubated 1

h at 30ºC and then centrifuged again. The cells were washed in 20 ml H_2O and taken in 20 ml SD selective medium (–AHWL or –HWL) containing 10 µg/ml Tetracyclin. Samples were taken for cell titration and the rest of the cells were kept at 4ºC overnight. The day after, cells were sedimented by centrifugation at 2000 rpm for 10 min and 10 ml of supernatant was removed. Then the cells were resuspended in the remaining liquid and spread homogenously on 25 plates (15 cm diameter plates) containing SD-HWL or SD-AHWL, depending on the experiment. The plates were incubated 3-4 days at 28ºC. The resulting colonies were subcultured in selective medium. Their DNA was extracted and transferred to *E. coli* and sequenced.

Cell titration was carried out with 20 µl of yeast cells (equivalent of 1/1000 of transformants) as follows:

20µl cells + 180µl H_2O = 200µl (equivalent of 10^{-3} of transformants).
↓
40µl + 160µl H_2O = 200µl →100µl was cultured on first plate (equivalent of 10^{-4})
↓
40µl +160µl H_2O = 200µl →100µl was cultured on second plate (10^{-5})

Numbers of colonies were counted on the first and second plates to estimate the number of transformed yeast cells.

3. DNA extraction from Yeast

5 ml of yeast culture was centrifuged at 4000 rpm for 5 min and the yeast cells were taken up in 250 µl DNA extraction buffer (10 mM Tris-HCl pH 8, 1 mM EDTA, 100 mM NaCl, 1% SDS and 2% Triton). About 250 µl of glass beads (0.5 mm diameter) and 250 µl of phenol/chloroform were added and then mixed by vortex for 3 min. After centrifugation at 12000 rpm for 5 min, 200 µl of supernatant was transferred to a new tube. DNA was precipitated with 3 volume ethanol in the presence of 1/10 volume 4M Ammonium acetate. Finally, after 15 min centrifugation at 14000 rpm and washing with 70% ethanol, DNA was taken up in 40 µl H_2O.

4. Protein Extraction from Yeast

5 ml of yeast culture was centrifuged at 13000 rpm for 5 min and the yeast cells were washed with H_2O and taken up in 150 µl of extraction buffer (1.85 M NaOH, 7.5% β-mercaptoethanol). 150 µl of 55% TCA was added followed by incubation for 10 min on ice. After centrifugation at 15000 rpm for 15 min at 4ºC, as much supernatant as possible was discarded and 25 µl PBS and 25 µl PAGE×2 were added. After denaturation at 65ºC for 10-15 min, several µl of 1N NaOH were added to restore the neutral pH of the loading solution (blue color).

5. β-galactosidase assay

2.5 ml of yeast cells (strain Y187) was cultured in selective medium until A_{600}=0.5 (incubation overnight). 1.5 ml of culture was centrifuged at 5000 rpm for 3 min. After discarding the supernatant, 0.5 ml Z buffer (100 mM Na_xPO_4 pH 7.2, 10 mM KCl, 1 mM $MgSO_4$ in H_2O plus β-Mercaptoethanol (360 µl/100ml just before use) and 200 µl/100ml ether saturated in H_2O was added then mixed by vortex for 1 min. The mixture was centrifuged 1 min at 13000 rpm and the tubes were left open in a fume hood to evaporate ether (20 min). Vortexing was carried out to put the cells in suspension and then the tubes were incubated 5 min at 30°C (tubes open).
For the reaction, 100 µl ONPG (4 mg/ml in Z buffer, fresh) was added and incubated at 30°C for 1h maximum in darkness. When the color turned to yellow the reaction was stopped by adding 250 µl 1M Na_2Co_3. Then it was centrifuged at 13000 rpm for 5 min.
The absorbance measurement was carried out on the supernatant at A_{420nm}. Z buffer was used as a standard (blank). The calculation of β-galactosidase activity (Miller units) was as follows:

$$\text{Activity} = 1000 \times A_{420nm} / (A_{600nm} \times \text{Volume} \times \text{Time})$$

A_{420nm} : Absorbance read after reaction
A_{600nm} : Absorbance of culture of initial cells
Volume: volume used for test (2ml)
Time: duration of reaction in minute

III. Protein Extraction from plant and Western blot analyses

1. Protein extraction from plant tissue

Fresh plant tissue was ground in protein denaturing buffer or Laemmli buffer (1W/1V) in a chemical hood and then denatured by heating at 95˚C for 5 min. After 5 min centrifugation at 5000 rpm, the supernatant was transferred to a new tube to run on a gel.

Protein denaturing and loading buffer (PAGE×2): (Laemmli)

Tris-HCl pH 6.8	125 mM
SDS	5%
β-mercaptoethanol	10%
Glycerol	20%
Bromophenol Blue	0.1%

2. Gel for protein Analyses

Stacking and resolution denaturing gels were prepared as follows:

	Resolution gel: 12.5%	**Stacking gel:**
40% Acryl-bisacryl (38.9:1.1)	3.16 ml	0.5 ml
Resolution buffer ×3	3.3 ml	-
Stacking buffer ×5	-	1 ml
H_2O	3.5 ml	3.5 ml
Ammonium Persulfate (APS) 25%	50 µl	40 µl
TEMED	10 µl	8 µl

	Resolution buf ×3 (pH 8.8)	**Stacking buf ×5** (pH 6.8)
Tris-HCl	6.8 g	18.5 g
SDS 20%	7.5 ml	6.25 ml
H_2O	to 500 ml	to 250 ml

The protein extracts, along with a protein ladder (Euromedex) were run on the gel. The electrophoresis migration buffer was composed of 25 mM Tris-HCl, 0.2 M Glycine and 0.1% SDS. Migrations were carried out at 80-120 V and 30 mA. For staining, the gel was incubated 20 min in solution composed of 10% acetic acid, 25% Ethanol and 0.5 g/l Coomassie blue. Distaining was carried out in 10% acetic acid. In some experiments the gel was dried during 2h at 80ºC in vacuum conditions.

3. Western blot

For Western blots, an electroblot apparatus (BioRad) was used to transfer the proteins from the gel on to a nitrocellulose membrane or a Immobilon™-P polyvinylidene fluoride (PVDF) microporous membrane (Millipore) which was prewet in 100% ethanol. The transfer was carried out in transfer buffer (25 mM Tris-HCl, 0.2 M Glycine) at 500 mA and 80 V for 2h at 4ºC. The membrane was blocked with 2.5% milk powder in Phosphate buffer saline (PBS) containing 0.5% Tween20® overnight at 4ºC. The membrane was then incubated with the primary antibody for 3h at RT and washed 3 times for 5 min in PBS-Tween. It was then incubated with the secondary antibody conjugated with horse radish peroxidase (HRP, Santa-Cruz) for 2h at RT and washed again as described. Proteins of interest were revealed by chemiluminecence with Lumi-Light^{PLUS} Western Blotting kit (Roche) by exposition on to an autoradiography film. To estimate the loading of total proteins the membrane was stained for 20 min in a Coomassie blue solution or with Red Ponceau (SIGMA) solution (also compatible with immuno-reactions) and then distained as described for gel or with PBS for Red Ponceau. The PBS buffer (×10) pH 7.4 is composed of KH_2Po_4 (2.04 g/l), Na_2HPo_4 (14.24 g/l), KCl (2.01 g/l) and NaCl (87.66 g/l).

IV. Nucleic acid analysis

1. DNA extraction from Arabidopsis

Genotyping of Arabidopsis plants required DNA extraction. To this end, fresh plant tissues (50-100 mg) were placed in 1.5 ml eppendorf tubes containing 10-20 glass beads (1 mm, Biospec) and frozen in liquid N_2. Using a "dentist" machine, the samples were ground (10 sec, 2 times), 300 µl of CTAB buffer (2% CTAB, 1% PVP, 100 mM Tris-HCl pH 8.0, 20 mM EDTA and 1.4 M NaCl) was added followed by agitation and incubation for 10 min at 65°C. An equal volume of chloroform was added and agitated by vortex. After centrifugation at 14000 rpm for 5 min at RT, the supernatants were transferred into new tubes and an equal volume of isopropanol was added. After 10 min incubation at RT, the DNA was precipitated by centrifugation for 15 min at 15000 rpm. After washing with 1ml 70% ethanol and drying, DNA was dissolved in 50 µl of 10 mM Tris pH 8.0. 1-2 µl was used for PCR.

2. RNA extraction

Fresh tissue from infiltrated *N. benthamiana* leaves was ground on ice in polysome extraction buffer (1W/3V) described by Jackson and Larkins (1976). This buffer is composed of 200 mM Tris, pH 9, 400 mM KCl, 35 mM $MgCl_2$, 25 mM EGTA and 200 mM sucrose). Then, phenol and phenol/chloroform extractions were carried out. After ethanol precipitation, high molecular weight RNAs were separated from low molecular weight RNA by precipitation with 5% PEG 8000 and 0.5 M NaCl. The small RNA-containing supernatant was then precipitated with 3 volumes of ethanol whereas high molecular weight RNA (pellet) was recovered by precipitation with 3M sodium acetate.

RNA from *in vitro A. thaliana* plantlets was extracted using Tri-Reagent (Sigma) according to the manufacturer's instructions. In this method, 200 mg of plant tissue was ground in liquid nitrogen. The resulting powder was homogenized in 1ml Trizol®, a mono-phasic solution of phenol and guanidine isothiocyanate, and then 200 µl chloroform was added. The mixture was agitated by vortex and incubated 10 min at RT. Centrifugation was performed at 4°C at 14000 rpm for 15 min, the upper phase was transferred to a new tube and 1 volume Isopropanol (0.6 ml) was added. After incubation for 10 min at RT, centrifugation at full speed precipitated the RNAs. After washing with 70% ethanol (1ml), RNAs were taken up in 50 µl RNAse-free H_2O. RNA samples were adjusted to the same concentration by spectrophotometry and their quality was assessed by loading about 0.5 µg on a 1% Agarose gel.

3. Blot for high molecular weight RNA

A denaturing agarose gel (1%) was prepared by melting 1.7g of agarose in 125 ml of deionized water. 17 ml of HEPES buffer 10× (200 mM HEPES, 10 mM EDTA and KOH 9.5 g/l, pH 7.8) was added and the solution was allowed to cool down. Then 28 ml of formaldehyde 37% was added and the solution mixed and poured to set the gel. Three volume of RNA loading buffer were mixed with one volume of RNA sample (2-5 µg of total RNA) and then the samples were denaturated at 65°C for 5 min and chilled on ice for 5 min.

RNA loading buffer:	
HEPES buffer 10×	500 µl
Formaldehyde	800 µl
Formamide deionized	2.5 ml
Glycerol 50%	250 µl
Ethidium Bromide	1µg/ml
Bromophenol blue	a little bit

After loading the samples, the gel was run at 80 V for 3-4 h in HEPES buffer 1× and photographed under UV light to confirm equal loading. RNAs were transferred on a neutral nylon membrane Hybond-NX (Amersham) by capillarity overnight or with a vacuum pump for 1h in 20× SSC (NaCl 175.3 g/l and Na_3Citrate 88.2 g/l). Then the membrane was rinsed with 4× SSC and RNA was UV cross-linked to the membrane twice in Stratalinker apparatus (1200 Joules X100), photographed under UV light to check for efficient RNA transfer.

4. Blot for small RNA

Denaturing 17.5% polyacrylamide gels were prepared by mixing 12.6 g urea, 13.1 ml of 40% acrylamide/bisacrylamide 19:1 and 1.5 ml of 10× TBE. After dissolving the urea at 37°C, H_2O was added up to 30 ml. Ammonium persulphate 25% (80 µl) and TEMED (20 µl) were added just before pouring the mixture into the gel frame. RNA samples (25 µg) were prepared in loading buffer and denatured as described for high molecular weight RNA. After a 30 min pre-run of the gel at 400 V in 0.5× TBE, the wells were washed by pipeting. RNA samples were loaded and the gel was run at 400 V for 2-3 h until the blue dye exited the gel. RNAs were transferred on a neutral Hybond-NX nylon membrane (Amersham) in 0.5× TBE using a BioRad electroblot apparatus for 1h at 300 mA (80 V) at 4°C. The membrane was treated as described for high molecular weight RNA.

5. Probe Preparation

RNA blots were hybridized with either RNA or DNA probes. The **RNA probes** were prepared by *in vitro* transcription of a sequence under control of T3 or T7 phage RNA polymerase promoters in linearized vector (like pBluescript) and in the presence of labeled UTP as follow:

H_2O	8.5 µl
Transcription buffer 10×	2 µl
Mix (rATP, rCTP, rGTP) 10mM	1 µl
DTT 0.1M	2.5 µl
BSA (10mg/ml)	0.5 µl
DNA (linearized) 1µg/µl	1µl
RNasine (Fermentas) 40U/µl	1µl
T3 or T7 RNA Polymerase (Promega)	1 µl
^{32}P-rUTP 40 µCi	2 µl
Afer 15 min add rUTP 0.5mM	0.5 µl

Incubation for 1.5h at 37ºC and then addition of:

H2O	70 µl
DNase buffer 10×	10 µl
DNase (RNase-free, Promega) 1U/µl	1 µl

The reaction was incubated at 37ºC for 30 min and then 100 µl phenol/chlorophorm was added and the solution was vortexed and centrifuged. The upper phase was precipitated with ethanol in the presence of 100 µl 4M Ammonium acetate to avoid precipitation of non-incorporated ^{32}P-UTP. The pellet was washed with 70% ethanol, dried and the labeled RNAs were taken up in 20 µl RNase-free H_2O. DNase buffer (10×) is composed of 400 mM Tris-HCl pH 7.5, 60 mM $MgCl_2$ and 100 mM NaCl.

DNA probes were prepared by end-labelling (New England Biolabs) using 3 µl of 10 µM antisense oligonucleotide that was mixed with 9 µl H_2O. Then, 2 µl of T4-Polynucleotide Kinase (PNK) buffer (10×), 5 µl of ^{32}P γ-ATP and 1 µl of T4-PNK enzyme (10 U/µl) were added and then final volume was adjusted to of 40 µl. The reaction was incubated at 37ºC for 1h. The reaction mix was

supplemented with 20 µl H_2O and non-incorporated nucleotides were removed by loading onto a G25 sephadex (Amersham) mini-column (prepared by centrifugation of 300 µl G25 sephadex at 2000 rpm for 2 min) followed by centrifugation at 2000 rpm for 2 min. The purified probe was denatured by heating 5 min at 95ºC and chilled on ice for 5 min before hybridization.

6. Hybridization of RNA probes

The membranes were prehybridized for at least 1h and, hybridized with the appropriate probe overnight and then washed. The method is different for the high molecular weight (HMV) RNA blots and the small RNAs blots:

	HMW RNA blots	**small RNAs blots**
Hybridization buffer	Formamide 50%, SSC (20×) 5%, SDS 0.1%, Na phosphate 50 mM pH 6.5, Denhardt's solution (100×) 8%, Salmon sperm DNA 250 µg/ml and yeast RNA 500 µg/ml	Perfect Hyb Plus buffer (SIGMA)
Pre-hybridization (1h)	60ºC	42ºC
Hybridization (overnight)	55ºC	42ºC
First washing step (15 min and two times)	65ºC with SSC 2× plus SDS 0.1%	55ºC with SSC 2× plus SDS 2%
Second washing step (15 min)	65ºC with SSC 0.2× plus SDS 0.1%	55ºC with SSC 1× plus SDS 1%

1M Na phosphate, pH 6.5, was prepared from 255 mM Na_2HPO_4 and 745 mM NaH_2PO_4. Small RNA blots were rehybridized with a probe complementary to U6 to provide loading controls. RNAs were detected either by autoradiography or by using phosphorimager plate and scanning.

V. *In vitro* Transcription and mechanical infection with virus

This method was used for mechanical inoculation of PVX and its recombinants. The pP2C2S vector allows the PVX genome to be transcribed *in vitro*. The linearized pP2C2S (using *SpeI*) (Pfeffer *et al*., 2002) was transcribed as follow:

Transcription buffer 5×	4 µl
rATP 100 mM (Promega)	1.5 µl
rCTP 100 mM	1.5 µl
rUTP 100 mM	1.5 µl
rGTP 100 mM	0.15 µl
CAP: ^{7m}G(5')ppp(5')G 15 mM (New England BioLabs)	4 µl
RNase inhibitor 40 U/µl	0.5 µl
DTT 0.1 M	1 µl
BSA 10 mg/ml	1 µl
DNA (linearized) 1-2 µg/µl	1 µl
T3/T7 RNA Polymerase mix (Promega)	1.5 µl
H_2O to	20µl

The reaction was incubated at 37ºC for 30 min and then 1.5 µl of rGTP 100 mM and 0.5 µl of T3/T7 RNA Polymerase mix (Promega) were added. The reaction was incubated again at 37ºC for 1.5h. RNA quality was assessed by visualization on 0.8% agarose gel. Then inoculum was prepared by mixing 10 µl 0.5M KH_2PO_4, pH 7.4, 8 µl of 0.5% Macaloid, 2 µl of yeast RNA, 1 µg/µl *in vitro* transcription product and H_2O to 100 µl. 50 µl of this inoculum was rubbed on two leaves of a *N. benthamiana* plant.

VI. *In vitro* Translation

In vitro transcription and translation of DNA was carried out for production of labeled proteins using a TNT Promega kit of rabit reticulocyte or wheat germ extract. The reaction mixture was prepared as follows, and then incubated 1.5h at 30ºC.

TNT mix	40 µl
^{35}S-Methionine	1.5 µl
DNA (pGBKT7)	1 µl
H_2O	7.5 µl
Total volume	50 µl

VII. Pull-down assay

This method was used to show direct interaction of P0 with ASK or AGO1. In two independent experiments P0s and ASKs were produced as a GST fusion protein. To this end, they were cloned into pGEX2TK vector and then electroporated in *E. coli* (Rosetta strain). Selection of transformed bacteria was performed using Ampicillin for (pGEX2TK) and Chloramphenicol whose resistance gene is situated on another plasmid in this strain.

Preparation of bacteria cells: 2 ml of an overnight preculture of one colony was inoculated into 50 ml LB containing antibiotics and then incubated at 37ºC with shaking. After 2-3 h incubation until A_{600}=0.5, synthesis of GST fusion proteins was induced by addition of 1mM IPTG (Isopropyl-β-D-thiogalactopyranoside). The bacteria were incubated for additional 3h at 37ºC (until A_{600}= 1-1.5) and the bacteria then sedimented by centrifugation at 5000 rpm for 10 min. The cells were resuspended in 3 ml NET buffer (100 mM NaCl, 1 mM EDTA and 20 mM Tris-HCl pH 8) and centrifuged again. After discarding the supernatant, the pellet of cells was conserved at -80 ºC.

Extraction and purification of protein: The bacteria cells (equivalent of 17-25 ml culture) were taken up in extraction buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 100 mM NaCl and 0.2% Triton ×100) and then 5 mM DDT, 3 µl of antiprotease EDTA-free (Roche, 1 tablet/ml) and 100 µg/ml Lysozyme (freshly prepared) were added and the cells incubated 10 min on ice. Sonication was performed at 40% Duty cycles with 30 pulses and then centrifugation was performed at 15000 rpm for 10 min at 4ºC. The supernatant was transfered into new tubes and incubated with Glutathione Sepharose 4B beads (GE Healthcare, Sweden) for 2h at 4ºC on a wheel. The glutathione sepharose was initially prepared by centrifugation of 0.5 ml of stock (containing 20% ethanol) at 500× g for 3 min. The beads were washed with 5 ml 1× PBS and taken up in 1 ml 1× PBS from which 100-200 µl was used for each bacterial extract. Non-fixed proteins were eliminated by washing three times with 100 mM PBS-NaCl.

For the P0-ASK pull-down assay, the GST-ASK proteins fixed on the beads were incubated with ^{35S}Met-labelled P0 protein in 1 ml PBS 1× with 0.5% Nonidet P-40 and protease inhibitor at 4ºC for 2h. After washing with NET buffer containing 350 mM NaCl, immobilized proteins were eluted from beads with 300 µl elution buffer (10 mM Glutathione in 50 mM Tris pH 8) for 2-3 h at RT. Proteins were then precipitated with 100% acetone (10 volume), washed with 80% acetone, taken up in PAGE loading buffer and subjected to SDS-PAGE. The GST-ASK proteins in the gel were visualized by Coomassie blue staining, and labeled-P0 proteins were identified by autoradiography on the dried gel.

In the basis P0-AGO1 pull-down assay, the GST-P0 proteins after purification on Glutathion Sepharose 4B beads were eluted from the beads as described above. The GST-P0 proteins in the elution buffer were then allowed to interact with Flag-AGO1 proteins fixed on anti-Flag M_2 affinity beads (SIGMA). This interaction was performed in extraction buffer (described above) complemented with DDT and protease inhibitor at 4ºC overnight. Three washing steps were performed using PBS buffer complemented with 300 mM NaCl. Finally, the beads were taken up in PAGE loading buffer, heated at 100ºC and the proteins were analyzed by Western blot.

Immunoprecipitation of Flag-AGO1 protein from Flag-AGO1/*ago1-36* Arabidopsis plants (Baumberger and Baulcombe, 2005) was performed by grinding three grams of fresh leaf tissue in liquid nitrogen and taking it up in 6 ml extraction buffer (described above) complemented with DDT and protease inhibitor. After 10 min centrifugation at 15000 rpm, the supernatant was filtered through a 0.2 µm filter and the Flag-AGO1 proteins were immobilized on anti-Flag beads by incubation at 4ºC for 2-3 h. The washing steps were performed as previously indicated using PBS buffer complemented with 100 mM NaCl.

VIII. RT-quantitative PCR

This method was used to quantify several endogenous mRNA in P0-expressing transgenic Arabidopsis plants. Total RNA was extracted using Tri-Reagent (Sigma) from pooled developing leaves of *in vitro* Arabidopsis plantlets and treated with RNase-free DNase (Qiagen kit). cDNA was synthesized by mixing 1 µg total RNA, 1 µl of 50 µM oligo dT primer, 1 µl of 10 mM dNTP mixture and H_20 to 13 µl. Then the mixture was heated at 65ºC for 5 min and chilled on ice for 5 min for RNA denaturing. 4 µl of Superscript III reverse transcriptase 5× buffer (Invitrogen), 1µl of 0.1M DDT, 1 µl of RNase 40 U/µl inhibitor and 1 µl of 200 U/µl Superscript III reverse transcriptase were added and the reaction was incubated at 50ºC for 1h. Control reactions were performed without reverse transcriptase. Real-time quantitative PCR reactions (20 µl) were performed in 96-well optical plates on a BioRad i-cycler apparatus using PCR master 2× mix (Eurogentec) containing SYBER® Green I fluorescein reporter with gene specific primers. Samples were pre-heated at 95°C for 10 min to activate Hot Star *Taq* DNA polymerase, and PCR was then performed by 40 cycles of denaturation at 95°C for 15s, annealing at 60°C for 30s and extension at 72°C for 15s. A melting curve was performed at the end of the amplification by steps of 1°C from 95°C to 50°C to control for the absence of primer-dimers. For each cDNA synthesis, quantification was performed in triplicate. ACTIN2 was used as an internal standard for equalization of RNA levels. GAPDH was tested as a second standard gene to validate the ACTIN2 gene. As neither of these mRNAs displayed significant changes in accumulation levels upon P0 induction, we chose to use ACTIN2 for normalization of the data. Quantifications were then normalized to that of non-induced plantlets for P0 plants or of L*er* plants for *ago1-11* mutant. Results were analyzed using Relative Expression Software Tool-Multiple Condition Solver (REST-MCS-version 2) (http://www.gene-quantification.de/download.html). Error bars represent the standard deviation from three replicates.

qPCR was performed using the following primers:

Actin2 (At3g18780)	Forward	5'-gcaccctgttcttcttaccg-3'	Vazquez *et al*., 2004
Actin2	Reverse	5'-aaccctcgtagattggcaca-3'	
AGO1 (At1g48410)	F	420: 5'-aaggaggtcgaggagggtatgg-3'	Vazquez *et al*., 2004
AGO1	R	585: 5'-aaattgctgagccagaacagtagg-3'	
AP2 (At4g36920)	F	1255: 5'-attctcactgtttccggcggctga-3'	
AP2	R	1441: 5'-tctcatgagaggaggttggaagccat-3'	
ARF17(At1g77850)	F	5'-agcacctgatccaagtccttctatg-3'	Vazquez *et al*., 2004
ARF17	R	5'-tggtgaatagctggggaggatttc-3'	
CUC2 (At5g53950)	F	5'-gcaccaacacaaccgtcacag-3'	Vazquez *et al*., 2004
CUC2	R	5'-gaatgagttaacgtctaagcccaagg-3'	
DCL1 (At1g01040)	F	5'-gatccattcctaagcgaagtttcagag-3'	Vaucheret *et al*., 2004
DCL1	R	5'-gcccgagcaacataaagatccatag-3'	
GAPDH (At1g13440)	F	5'-ttggtgacaacaggtcaagca-3'	Czechowski *et al*., 2005
GAPDH	R	5'-aaacttgtcgctcaatgcaatc-3'	
HAP2C (At1g72830)	F	1417: 5'-aagtcatccttggctactactagttct -3'	
HAP2C	R	1581: 5'-actagtttagacaagagatccatggta-3'	
MYB65 (At3g11440)	F	5'-gatggttcctgatagccatacagttac-3'	Vazquez *et al*., 2004
MYB65	R	5'-taggcatcaacagagtcaaggagatc-3'	
$P0^{BW}$	F	5'-tgagcaatttcacaactcccgtct-3'	
$P0^{BW}$	R	5'-tcatacaaacatttcggtgtagacc-3'	
SCL6 III (At3g60630)	F	5'-accaagaccagtcagcggtaatc-3'	Vazquez *et al*., 2004

SCL6 III	R	5'-agtgtcgtcgttgttgttgttaagg-3'	
SCL6-IV (At4g00150)	F	794: 5'-ataacagagcagctggttaaggcagca-3'	
SCL6-IV	R	999:5'-atcttgaagatgagggaataagggttta-3'	
SPL10 (AT1g27370)	F	2431: 5'-gtgggagaatgctcaggaggc-3'	Vazquez *et al*., 2004
SPL10	R	2530: 5'-gagtgtgtttgatcccttgtgaatcc-3'	
TCP10 (At2g31070)	F	1323:5'-attgcttaatagtcagcaacaacaagtgt-3'	
TCP10	R	1466: 5'-tgatgatgatgatgatccgacgtcgtt-3'	
TIR1 (AT3g62980)	F	5'-gcctctctctatctggcctcttgac-3'	Navarro *et al*., 2006
TIR	R	5'-agggcagctctctggtctcgagtcc-3'	

IX. Agroinfiltration

1. Culture: *A. tumefaciens* strain GV3101 was transformed with pBIN61 recombinant plasmid by electroporation and then cultured on solid YEB medium with antibiotics at 28ºC for 48h. One colony of Agrobacterium was cultured in 3 ml liquid YEB medium containing appropriate antibiotics (preculture) at 28ºC for 48h with shaking. For agroinfiltration the main culture (5-50 ml) contained YEB medium with antibiotics and enriched with 10 mM MES, pH 5.7, and 20 µM Acetosyringone (solublized in DMSO) for induction of Agrobacterium *vir* genes and was inoculated with 0.2 ml of preculture and incubated at 28ºC with shaking overnight.

2. Preparation of the infiltration medium: Agrobacterium culture was centrifuged 15 min at 5000 rpm at RT. Then liquid medium was discarded and infiltration medium (10 mM MES, 10 mM $MgCl_2$ and 150 µM Acetosyringone) was added. The Agrobacterium cells were resuspended by vortex and incubated 3-4 h at RT. A_{600} was adjusted to 0.5 by dilution in infiltration medium and infiltration on leaves of plant was done using a 2ml syringe without needle.

X. DAS-ELISA

Double antibody sandiwich-ELISA was performed for evaluation of viral infection in plants. First, a 96-well ELISA plate (Nunc) was coated with a specific antiviral antibody at appropriate dilutions [1/400 for both CABYV (BioRad) and BWYV (Loewe Biochemica GmbH)] in coating buffer (1.6 g/l Na_2Co_3 and 2.92 g/l $NaHCo_3$, pH 9.6). The plates were incubated 3-4 h at 37ºC, and washed 3 times with PBS-Tween20 (0.05%) (washing buffer, pH 7.4). The samples were prepared by grinding fresh plant tissue in Extraction buffer (2% PVP360-500G in PBS-Tween) (1W/2.5V). The debris were eliminated by centrifugation 3 min at 3000 rpm and supernatants were added to ELISA plates and incubated at 4ºC overnight. After washing 3 times, the specific antiviral antibody conjugated to Alkaline phosphatase prepared in conjugating buffer (2% PVP and 0.2% BSA in PBS-Tween) at appropriate concentration (CABYV: 1/800 and BWYV: 1/400) was added to the ELISA plates and incubated 3-4 h at 37ºC. A final 3 times washing step was performed and phosphate tablets (SIGMA) were solubilized in substrate buffer (Diethanolamine 97 ml/l in H_2O, pH 9.8) and then added to the ELISA plates. The plates were incubated in the dark at 37ºC and ELISA values were read at A_{405}. The infection threshold (R) was calculated by R=X+3SD (Hill and Jackson, 1984) where X is the mean of ELISA values in the corresponding non-infected plants and SD is the standard deviation of its replicates.

XI. Transformation of Arabidopsis by Floral dip

Arabidopsis thaliana (ecotype Col-0) plants were transformed as described by Bechtold and Pelletier (1993). In this method, Arabidopsis plants were grown under 16h photoperiod conditions and the first flowering stem was cut to allow development of several flowering stems. Agrobacterium (strain GV3101) harboring a binary vector with the gene of interest was cultivated 24h in YEB (250 ml) containing appropriate antibiotics at 28ºC. Then bacteria cells were sedimented by centrifugation at 5000 rpm for 15 min at RT and resuspended in 250 ml transformation medium (0.5× MS medium,

5% sucrose, 0.45 ml/l Silvet L-77 and 200 µM Acetosyringone). The flowers of the Arabidopsis plants were dipped into the mixture for 90 sec and then the plants were maintained in a small growth chamber to maintain humidity during 48h in the dark.

XII. Arabidopsis genetic crosses

Arabidopsis flower buds were emasculated to avoid self-fertilization before crosses. The day after, these female flowers were fertilized by providing the pollen of another genotype onto the carpels.

XIII. *In vitro* culture of Arabidopsis plants

For *in vitro* culture, Arabidopsis seeds were sterilized with 75% ethanol for 2 min then with 10% bleach +Tween® (one drop/tube) for 15 min under agitation. The seeds were rinsed with sterile H_2O three times and spread on culture medium. They were then vernalized for 48h at 4ºC before placing in a growth chamber (16h photoperiod and 20-22ºC).

Medium was prepared by mixing Murashige and Skoog (MS) medium (MO255: with vitamins and MES; Duchefa) with 3% sucrose and 7 g/l agar. pH was adjusted to 5.7 by KOH and the medium was autoclaved at 120°C for 20 min. Antibiotics were added after cooling.

Carbenicillin was used to inhibit Agrobacterium growth. Selection of transgenic plants was carried out on medium containing appropriate antibiotics and induction of XVE-P0^{BW} plants was performed by transferring two-week-old plantlets to fresh medium supplemented with 5 µM estradiol (prepared in DMSO) (induction medium) or DMSO alone (control medium). Final concentration of antibiotics and chemicals are recapitulated in the following Table.

	Final concent. for plant	**Final concent. for *E. coli***	**Final concent. for *Agrobacterium***
Kanamycin	50µg/ml	35µg/ml	100µg/ml
Hygromycin	15µg/ml		
Carbenicillin	0.5µg/ml		
Tetracycline in ethanol		10µg/ml	
Rifampicilin in DMSO			100µg/ml
Spectinomycin			100µg/ml
Chloramphenicol in ethanol		30µg/ml	
Estradiol in DMSO	5µM		
Basta® (Phosphinothricin)	10mg/l		

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