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Contribution to the study of the *Agrobacterium rhizogenes plast* genes, *rolB* and *rolC*, and their homologs in *Nicotiana tabacum*

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Contribution to the study of the *Agrobacterium rhizogenes plast* genes, *rolB* and *rolC*, and their homologs in *Nicotiana tabacum*

Agrobacterium rhizogenes induces hairy roots on plants by transfer of a DNA fragment (the T-DNA) to its host genome. All *A. rhizogenes* T-DNAs have a conserved fragment that carries the root locus (*rol*) genes *rolA, rolB* and *rolC*; these are necessary and sufficient for the hairy root phenotype. *rolB* and *rolC* belong to the *plast* T-DNA gene family with strong effects on plant growth, the basic mechanism of these genes is still unknown. Interestingly, several *Nicotiana* species, including *N. tabacum*, contain T-DNA sequences (cT-DNAs) that result from ancient natural transformation events. We have shown that *N. tabacum* contains two cT-DNA fragments: one carries genes *torf8, trolA, trolB, trolC* and in some cultivars *torf13*, the other carries *torf14* and *tmis*. Of the *trol* genes, only *trolC* is intact. Controlled expression of *trolC* and its bacterial homolog A4-*rolC* in tobacco and Arabidopsis caused very similar growth modifications, showing conservation of the *trolC* function in tobacco. Some modifications, in particular increased uptake of sucrose, resemble those induced by the distantly related *plast* gene *6b*, suggesting that they are correspond to the basic *plast* gene functions. We have extended our *plast* studies to the *rolB* gene which induces leaf necrosis. We could show that *rolB*-induced necrosis is not a hypersensitive response (HR) and rather resembles a senescense phenomenon, as it can be blocked by cytokinins. An earlier reported RolB tyrosine phosphatase activity could not be confirmed under carefully controlled conditions. Finally, we have discovered the first *plast*-like genes outside *Agrobacterium* (and *Nicotiana*) in an ectomycorrhizal basidiomycete, *Laccaria bicolor*.

Etude de deux gènes *plast* d'*Agrobacterium rhizogenes, rolB* et *rolC* et leurs homologues chez *Nicotiana tabacum*

Agrobacterium rhizogenes induit des chevelus racinaires sur un grand nombre d'espèces végétales par transfert d'un fragment d'ADN (ADN-T) vers le génome de son hôte. Tous les ADN-T d'*A. rhizogenes* possèdent un fragment conservé portant des gènes d'induction de racines (gènes *rol*) *rolA, rolB* et *rolC*. *rolB* et *rolC* appartiennent à la famille des gènes *plast* des ADN-T, qui provoquent des effets de croissance importants, leur mécanisme de base reste à ce jour inconnu. Il a été démontré que plusieurs espèces de *Nicotiana*, dont *N. tabacum*, contiennent des séquences d'ADN-T (cT-DNAs) résultant d'évènements de transformation anciens naturels. Nous avons montré que *N. tabacum* contient deux fragment d'ADN-T, l'un porte les gènes *torf8, trolA, trolB, trolC* et dans certains cultivars *torf13*, l'autre porte *torf14* et *tmis*. Des gènes *trol*, seul *trolC* est intact. L'expression contrôlée de *trolC* et du gène bactérien A4-*rolC* dans le tabac et Arabidopsis produisent des modifications de croissance très similaires, montrant par cela une grande conservation de la fonction de *trolC* dans le tabac. Certaines modifications, en particulier une accumulation accrue de saccharose, ressemble à celles induites par le gène *plast 6b*, dont la séquence est éloignée de celles des gènes *rolC*, suggérant qu'elles font partie des fonctions originales des gènes *plast*. Nous avons élargi nos études des gènes *plast* au gène *rolB* qui induit des nécroses foliaires. Nous avons montré que cet effet n'est pas une réponse d'hypersensibilité (HR), il pourrait s'agir d'un phénomène de sénescence, puisque la nécrose peut être bloquée par la cytokinine. L'activité tyrosine phosphatase rapportée dans la litérature n'a pas pu être confirmée dans des conditions expérimentales rigoureuses. Finalement, nous avons découvert les premiers gènes de type *plast* en dehors des Agrobactéries (et Nicotianae) dans un basiodiomycète formant des ectomycorrhizes, *Laccaria bicolor*.

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DISCUSSION AND PERSPECTIVES

MATERIALS AND METHODS

Abbreviations

3-AT	3-aminotriazole
ABP1	auxin binding protein
AD	activation domain
AMP	adenosine monophosphate
APS	ammonium persulfate
ASLA	*Allium sativum* leaf agglutinin
AtKAPα	*Arabidopsis thaliana* karyopherin α
ATP	adenosine triphosphate
BA	6-benzyl adenine
BD	binding domain
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
CDPKs	calcium-dependent protein kinases
CGA	chlorogenic acid
CHSA	chalcone synthase A
chv	chromosomal virulence genes
cT-DNA	cellular T-DNA
cv.	cultivar
CYC	Cycloidea
dex	dexamethasone
dip	day(s) post infection/ infiltration/induction
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
dNTP	desoxyribonucleic triphosphate
Dof	DNA binding with one finger
DTT	dithiothreitol
EDTA	ethylene diamine tetra-acetate
EST	expressed sequence tags
GA	gibberellic acid
GFP	green fluorescent protein
GSS	genomic survey sequences
GST	glutathione S-transferase
GUS	β-glucuronidase
h	hour(s)
HEPES	4-(2-hydroxyethyl piperazine)-1-ethane sulfonic acid
HR	hypersensitive response
IAA	indole-3-acetic acid
IAM	indole-3-acetamide
ICS	isochorismate synthase
ILA	indole-3-lactate
iPePP	isopentenyl pyrophosphate
iPMP	isopentenyl adenosine monophosphate
ipt	isopentenyl transferase
IPTG	Iisopropyl-β-D-thiogalactopyranoside
Kb	kilo base
KDa	kilo dalton
L/W/H/A	Leucine/ Tryptophan / Histidine/ Adenine
LB	left border
LB	Luria-Bertoni
MCS	multiple cloning site
MES	2-(N-morpholino) ethanesulfonic acid
min	minute
mis	mikimopine synthase
MS	Murashige and Skoog
NAA	naphthaleneacetic acid

Ng	*Nicotiana glauca*
NLS	nuclear localisation signal
Nt	*Nicotiana tabacum*
nt	nucleotide
NtBBF1	*N. tabacum rolB* domain B factor 1
NtSIP1	*Nicotiana tabacum* 6B–interacting protein 1
OD	optical density
OG	oligogalacturonides
ORF	Open Reading Frame
PAL	pPhenylalanine ammonia-lyase
pBin	binary plasmid
PCF	Proliferating Cell Factor
PCNA	Proliferating Cell Nuclear Antigen gene
PCR	polymerase chain reaction
PEG	poly ethylene glycol
pNPP	p-nitrophenylphosphate
PR	Pathogenesis-Related
pRi	Root-inducing plasmid
PS3	sulfated laminarin
PTGS	post-transcriptional gene silencing
pTi	Tumor-inducing plasmid
PTPases	protein tyrosine phosphatases
PVDF	polyvinylidene fluoride
qRT-PCR	quantitative reverse transcription PCR
Raf	Rat fibrosarcoma
RB	Right border
RNA	Ribonucleic acid
RNAi	RNA interference
rol	root locus
ROS	reactive oxygen species
ROX1	*rolB*-overexpressed1
RT	Room temperature
RT-PCR	reverse transcription PCR
SD	synthetic defined
SDS	sodium dodecyl sulfate
ss	single-stranded
SSC	salt sodium citrate
ß-meg	ß-megaspermin
STS	stilbene synthase
Taq	*Thermus aquaticus*
TB1	teosinte branched1
TBS	Tris buffered saline
TCL	thin cell layer
TCP	Teosinte branched1, Cycloidea, PCF
T-DNA	transferred-DNA
TE	Tris-EDTA
TEMED	NNN'N'-tetramethylethylenediamine
tet	tetracycline
T_L-DNA	left T-DNA
tmr	tumor morphology rooty
tms	tumor morphology shooty
T_R-DNA	right T-DNA
Tris	Tris(hydroxymethyl)aminomethane
UTR	untranslated regions
UV	ultraviolet
VIP1	virE2-interacting protein 1
vir	virulence
YEB	yeast extract buffer

INTRODUCTION

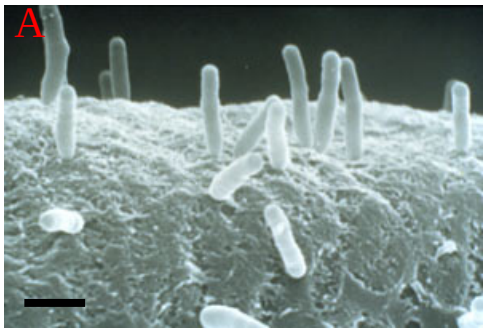

Figure 1. Agrobacterium and growth abnormalities.
A) Electron microscopy image of *Agrobacterium tumefaciens* (Bar: 1µm) B) Crown gall disease caused by *A. tumefaciens*, C) Hairy root disease induced by *A. rhizogenes.*

I. Agrobacterium

The genus Agrobacterium is a gram-negative soil bacterium and belongs to the family Rhizobiaceae. Some species in this genus are plant pathogens such as *A. tumefaciens*, *A. vitis*, *A. rubi* and *A. rhizogenes*, others are non-pathogenic such as *A. radiobacter* (Figure 1A). *A. tumefaciens* and *A. rhizogenes* are the best known species, they can infect several dicotyledonous and some monocotyledonous plants (De Cleene and De Ley, 1976, 1981; Porter, 1991) and cause abnormal proliferation of plant cells at the site of infection which is manifested by the formation of tumors called "crown galls" in the case of *A. tumefaciens* or by the appearance of adventitious roots called "hairy roots" in the case of *A. rhizogenes* (Binns and Costantino, 1998) (Figure 1B and C). *A. rubi* causes cane gall disease and *A. vitis*, which is restricted to grape and a few other plant species (Gelvin, 2003), causes tumours on the crown of grape vines as well as necrotic lesions on grape roots (Burr and Otten, 1999). These pathogenic bacteria present a major agricultural problem because they infect wounded plants including grafted plants, such as fruit trees or vines.

1. General concepts

The mechanism of infection by Agrobacterium is very special. Indeed, it is a natural phenomenon in which bacteria genetically transform infected plant cells. The transformation process depends on the presence of a large size plasmid, 200-800 kilo base (kb) (Zaenen *et al.,* 1974, Costantino *et al.,* 1994; Broothaerts *et al.,* 2005) in the bacterium called pTi (Tumor-inducing plasmid) in the case of *A. tumefaciens* and *A. vitis*, or pRi (Root-inducing plasmid) in the case of *A. rhizogenes*. These plasmids carry one or more DNA fragments called T-DNA (Transferred-DNA), which are transferred into the plant cell, and the virulence (*vir*) genes that encode the trans-acting factors necessary for T-DNA processing (Sheng and Citovsky, 1996; Zhou *et al.,* 1999).

In plant biotechnology the characteristics of pTi have been widely exploited in order to transform plants by disarmed Agrobacterium strains in which the native T-DNA region has been removed from the Ti plasmid and a recombinant T-DNA region (including the DNA segment of interest) usually resides on a small, autonomous binary plasmid that functions in *trans* with respect to the *vir* genes.

It has been also demonstrated that Agrobcaterium is capable of transforming other eukaryotic species such as fungi (Piers *et al.,* 1996; De Groot *et al.,* 1998) and even human cells (Kunik

et al., 2001) and this application of the bacterium has begun to be widely used.

1.1. Crown gall

Crown galls are tumors, which often arise on the lower stem part in contact with the soil (crown), but they also develop on roots and branches. In woody plant species that are propagated by grafting scions onto rootstocks, the tumors are formed at the graft junction. The grafting causes wounds that are usually covered by soil and thus provides an excellent entry point for the Agrobacterium cells. The crown galls represent a serious problem in vineyards, as well as in almond, plum, apple and peach nurseries. The mechanism of induction of tumor formation is not yet well known, but it seems to be mainly related to the production of plant hormones, auxin and cytokinin, encoded by the T-DNA genes in infected plant cells (Zhu *et al.,* 2000a; Terakura *et al.,* 2007).

1.2. Hairy roots

The hairy root disease is manifested by the production of highly branched ageotropic adventitious roots from the site of *A. rhizogenes* infection. Transformed roots can be regenerated into fertile plants which, in many species, have a characteristic morphology, called the "hairy root" phenotype that includes stunted growth, shortened internodes, wrinkled leaves, reduced apical dominance and very abundant and only partially geotropic roots (Tepfer 1984; Spano *et al.,* 1987). These plants contain Ri T-DNA sequences which are transmitted to their offspring in a Mendelian fashion (Costantino *et al.,* 1984; Tepfer, 1984). Leaf explants from hairy root plants rapidly and characteristically differentiate roots on a hormone-free culture medium (Benvenuto *et al.,* 1983). The hairy roots are composed only of transformed cells whereas the tumors induced by *A. tumefaciens* contain a mixture of transformed and untransformed cells (Chilton *et al.,* 1982; Bercetche *et al.,* 1987).

1.3. T-DNA

In both Ri and Ti plasmids, the T-DNA is flanked by two 25 base pair (bp) long imperfect direct repeats, termed border sequences (Yadav *et al.,* 1982). The right border (RB) is essential for efficient tumorigenesis/rhizogenesis and acts in a polar fashion, directing the transfer of sequences to its left (Shaw *et al.,* 1984, Wang *et al.,* 1984). In contrast to the right

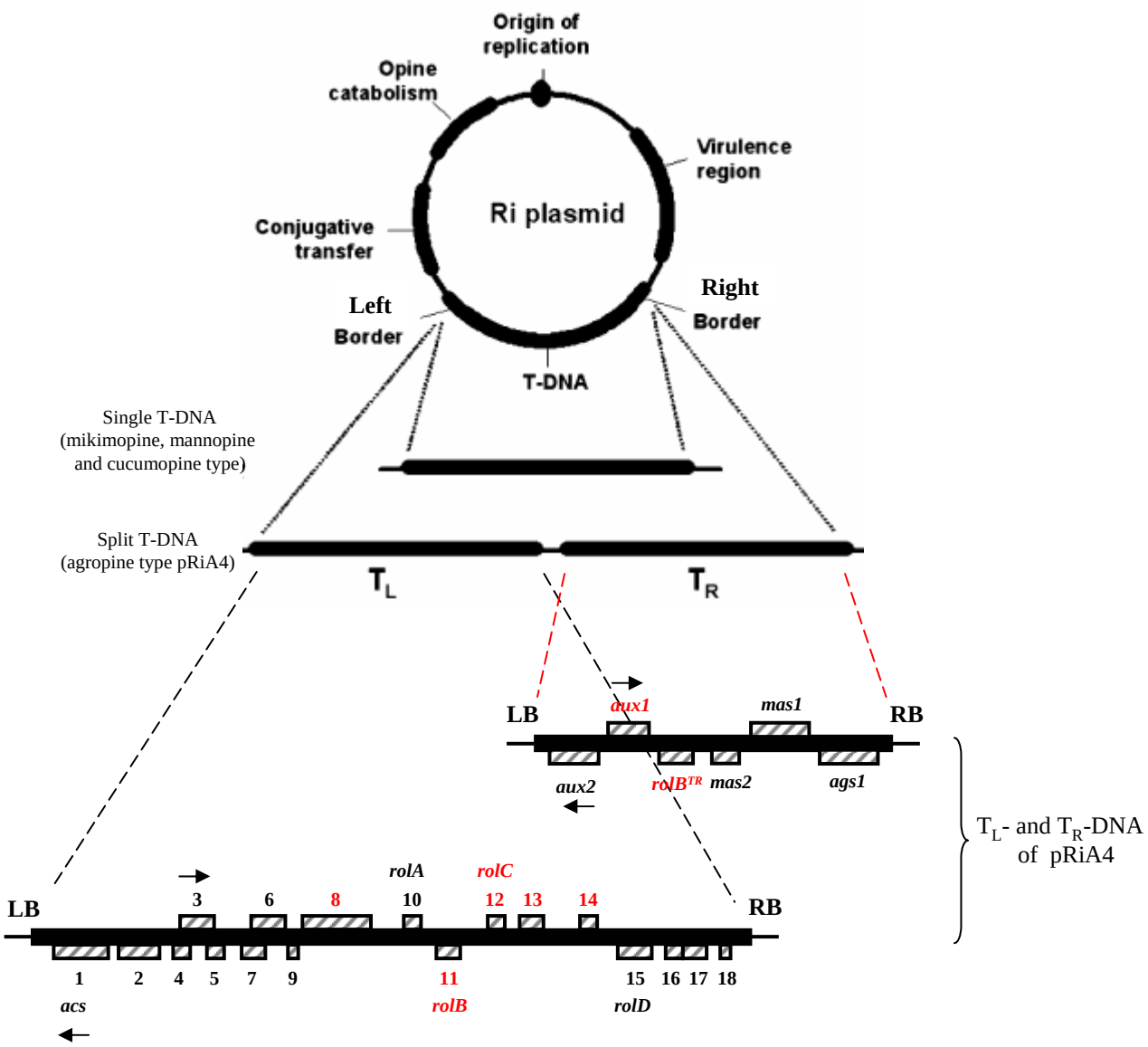

Figure 2. Structure of the Ri-plasmids of *A. rhizogenes*.
T_L- and T_R-DNA shown here are from strain A4. The ORFs (1-18) are shown as open boxes with oblique lines above or below the lines according to their orientation. The arrows indicate the orientation (5' to 3') of ORFs. The genes presented in red belong to the *plast* gene family. *acs*: agrocinopine synthase gene, *mas*: mannopine synthase gene, *ags*: agropine synthase gene, LB: Left Border, RB: Right Border.

border, the left border (LB) is dispensable for tumorigenesis/rhizogenesis (Joos *et al.,* 1983). In some Agrobacterium strains a second T-DNA may be present (White *et al.,* 1985) (Figure 2). In this case the two T-DNAs (left T-DNA or T_L-DNA and right T-DNA or T_R-DNA) are called "split" T-DNA (Veena and Taylor 2007). T_L-DNA and T_R-DNA, ranging in size from ~15–20 kb, are transferred and integrated independently into the host plant genome (White *et al.,* 1985; Slightom *et al.,* 1986).

2. Infection process

The description of the infection process by Agrobacterium, described below, is inspired from several reviews (Winans, 1992; Costantino et al., 1994; Ziemienowicz, 2002; Tzfira and Citovsky, 2002; 2006; McCullen and Binns, 2006; Lacroix et al., 2006; Veena and Taylor, 2007; White and Winans, 2007; Pitzschke and Hirt, 2010). Due to the lack of space, we will not cite all the documents that have led to the current knowledge of this process.

The transformation process involves several groups of genes belonging to both bacterial and plant genomes. They include a) the chromosomal virulence genes (*chv*) on the bacterial chromosome, which are necessary for the recognition and the attachment of the bacterium to the plant cell, b) the virulence (*vir*) genes, localized in a region of about 30-40 kb on pRi and pTi and responsible for the T-DNA processing within the bacterium and its transport and integration into the plant genome and c) some plant genes important for T-DNA transport in the cytoplasm of the plant cell and its integration into the genome.

The *vir* genes are organized on 6 mono- or poly-cistronic loci (*virA*: 1 ORF (Open Reading Frame), *virB*: 11 ORFs, *virR*: 2 ORFs, *virD*: 5 ORFs, *virE*: 2 ORFs, *virG*: 1 ORF). It has been demonstrated that some strains of Agrobacterium contain additional *vir* genes such as *virF*, *virH* and *tzs*. These genes are not essential for the T-DNA processing but they increase the virulence and the host range of the bacterium (Winans, 1992).

The transformation process begins with the attachment of the bacterium to plant cells and is followed by the induction of *vir* genes, the production of T-DNA and its integration into the host genome (see below). A model of these steps is shown in Figure 3.

2.1. Attachment of the Agrobacterium to the host cell

Recognition of plant cells by Agrobacterium and the establishment of a physical contact

between them is a very important step in the infection process since the abolition of the interaction results in avirulence of the bacterium (Figure 3, step 1).

The molecules of the plant cell that are recognized by the bacterium are localized on the cell wall and are related to vitronectin, a protein found in the extracellular matrix of animal cells and known to serve as a receptor for several bacteria. Several chromosomal loci (*attR, chvA, chvB, pscA* and *cel*), that are expressed constitutively in the bacterium, have been found to be necessary for the attachment of the bacterium to the plant cell. The product of *attR* is implicated in the biosynthesis of acetylated polysaccharides capable of the recognition of plant cell wall proteins and interaction with them (Matthysse *et al.,* 2000). The interaction leads to the synthesis of cellulose filaments that reinforce the contact. The stabilization of the attachment is ensured with other genes including *pscA* (or *exoC*), *chvA* and *chvB*, which are involved in the synthesis of periplasmic β-1,2 glucan, as well as the *cel* gene that produces the cellulose fibrils (Cangelosi *et al.,* 1989; Weising and Kahl, 1996).

2.2. Virulence (*vir*) gene expression

In nature, Agrobacterium attacks mainly wounded tissue. A wound site secretes compounds such as phenols and sugars and creates a low pH condition. These conditions provide a favourable environment for the bacterium and induce *vir* gene expression (Figure 3, steps 2 and 3).

The *vir* gene activation by plant factors requires two genes, *virA* and *virG* which are constitutively expressed at a basal level, but can become highly induced in a feed-forward manner (Winans *et al.,* 1988). The VirA protein is a membrane-bound receptor and VirG is the intracellular response regulator (Wolanin *et al.,* 2002). On signal sensing, the histidine kinase VirA activates VirG through transferring its phosphate to VirG, thereby activating VirG to function as a transcription factor. Phosphorylated VirG then binds at specific 12 bp DNA sequences of the *vir* gene promoters and activates their transcription (Brencic and Winans, 2005). Consequently the T-DNA processing is initiated.

2.3. T-DNA processing

2.3.1. Single-stranded (ss) T-DNA production

After induction of the *vir* genes, Agrobacterium generates a single-stranded (ss) T-DNA

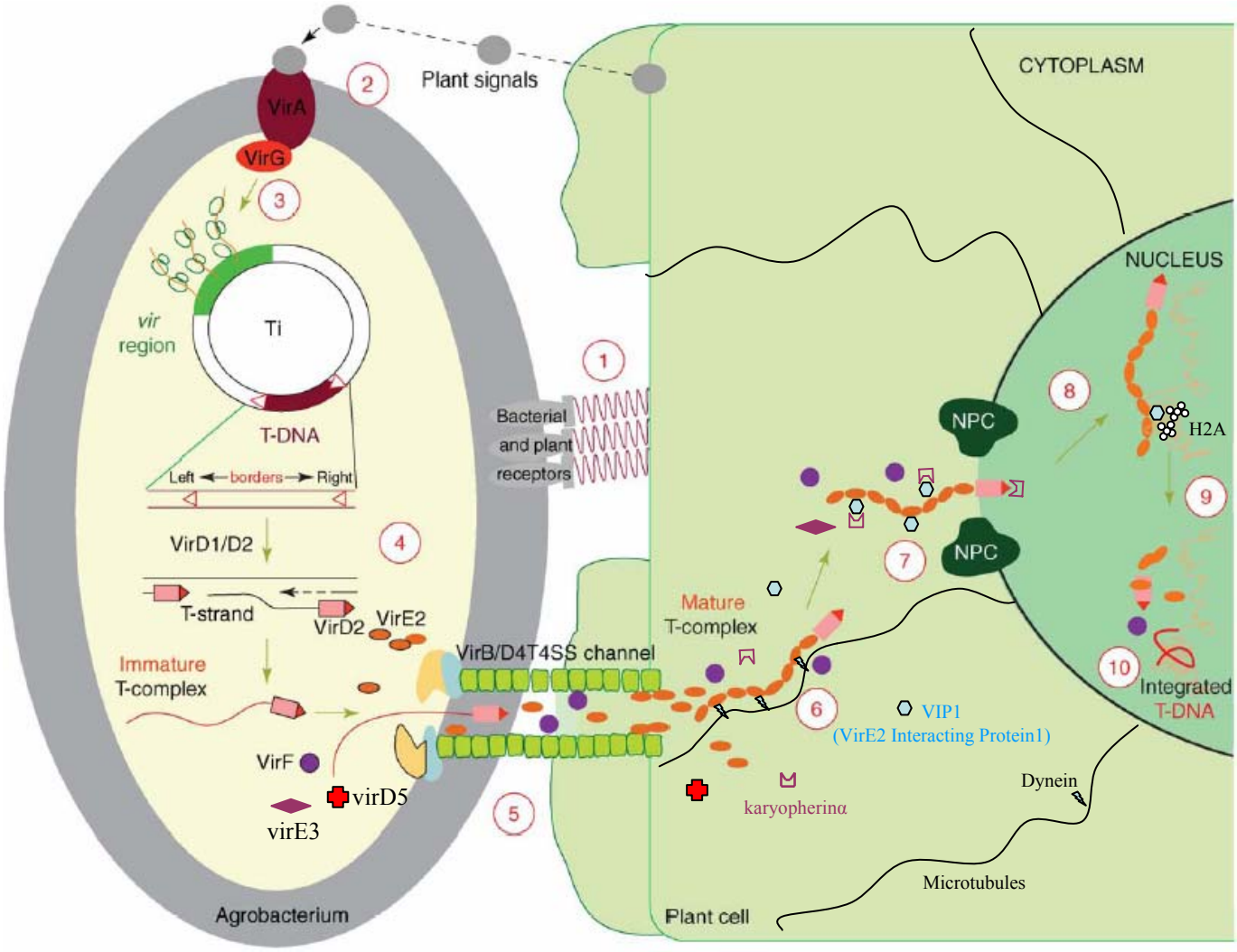

Figure 3. A model for Agrobacterium-mediated genetic transformation. The transformation process is summarized by 10 major steps: **1)** Recognition and attachment of the Agrobacterium to the plant cell **2)** Perception of specific plant signals by the Agrobacterium VirA/VirG complex **3)** Activation of the *vir* genes **4)** Formation of a T-DNA/VirD1,D2 protein complex (immature T-complex) and **5)** Its transport together with several other Vir proteins into the plant cytoplasm **6)** Association of VirE2 to the T-strand in the plant cytoplasm, formation of the mature T-complex and its movement through the cytoplasm via microtubule and dynein molecules **7)** Translocation of mature T-complex through Nuclear Pore Complex (NPC) via host (karyopherin α and VIP1) and bacterial factors (VirD2 and VirE2) **8)** Inside the nucleus, T-DNA is recruited to the point of integration, most likely via the interaction of VIP1 with the histone H2A, and **9)** stripped of its escorting proteins via VirF and host proteasomal degradation machinery **10)** and integrated into the host genome (adapted from Tzfira and Citovsky 2010, with some modifications).

molecule (T-strand) that is the lower strand of the T-DNA region of the pTi/ pRi (Stachel *et al.,* 1986). In the presence of VirD1 protein, VirD2 cleaves the lower strand at the right and left sequences and binds covalently to the 5' end of the nicked DNA. The nicked DNA is then displaced from the plasmid, producing the VirD2-ssT-DNA complex (immature T-complex) (Figure 3, step 4).

2.3.2. Transporter complex

The VirD2-ssT-DNA complex and the other Vir proteins (VirE2, VirE3, VirF, VirD5) are then exported to the plant cell through a transporter complex formed by VirBs (11protein) and VirD4 (Vergunst *et al.,* 2005) (Figure 3, step 5). The VirB/D4 complex closely resembles the bacterial type IV secretion system and contains a "core" part anchored in the bacterial cell wall and the external pilus, called T-pilus (Christie *et al.,* 2005) (Figure 4). The core part of the complex is composed of VirD4 and all of the VirB proteins whereas the T-pilus is formed primarily of VirB2. VirB2, 4, 11 and VirD4 are the ATPases that provide the energy for T-complex assembly and its translocation in the bacterial membrane.

2.3.3. T-DNA intracellular transport and nuclear targeting

Inside the plant cell, the T-DNA is found as a mature T-complex, in which the 5' end is associated with VirD2 and the entire length is coated with VirE2 proteins (Figure 3, step 6). These proteins inhibit degradation of the T-DNA by the plant cell nucleases. In the cytoplasm this complex must find its way toward the nucleus. Some bacterial and plant proteins have been found to be involved in this movement. VirD2 and VirE2 contain nuclear localisation signal (NLS) sequences that allow the T-complex to be directed to the nucleus (Tinland *et al.,* 1995). VirE3 is suggested to aid nuclear localisation of VirE2 (Lacroix *et al.,* 2005). Using biophysical particle tracking methods and fluorescently labelled VirE2–ssDNA complexes, it has been demonstrated that the T-complex is delivered to the cell nucleus with the assistance of the host intracellular transport machinery (microtubules and dynein molecules).

The large size of the mature T-complex (~15.7 nm outer diameter, Abu-Arish *et al.,* 2004) suggests an active mechanism for its nuclear import, which is thought to be mediated by the nuclear-import machinery of the host cell. Indeed, VirD2 and VirE2 have been shown to interact with host proteins for their nuclear import. VirD2 interacts with AtKAPα, a member of the Arabidopsis karyopherin α family (also termed importin α) and VirE2 interacts with the

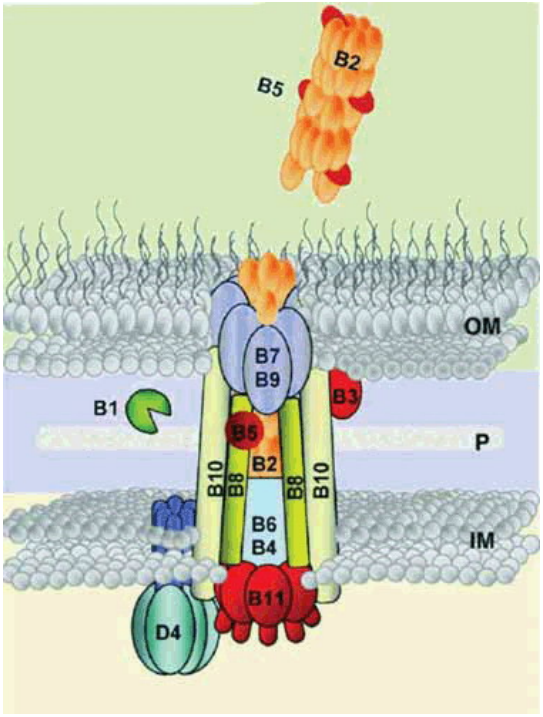

Figure 4. A model showing the Agrobacterium VirB/D4 complex. The core part of the complex is composed of VirD4 and all of the VirB proteins whereas the T-pilus is formed primarily of VirB2. IM, inner membrane; P, periplasm; OM, outer membrane (Christie, 2004).

plant VirE2-interacting protein 1 (VIP1) and the bacterial VirE3 protein. VIP1 and VirE3 act as molecular adaptors between VirE2 and importin α, enabling VirE2 to be translocated to the nucleus (Figure 3, step 7). Therefore the combined action of the bacterial and host proteins is required for shuttling of the mature T-complex into the plant cell nucleus.

2.3.4. T-DNA integration in the plant genome

After import of the T-complex into the nucleus, several steps are necessary before the T-DNA integrates into the host genome. These steps also require a collaboration between the bacterial and plants proteins. The coating proteins of the T-complex have to be at least partially removed and the T-complex should be targeted to its site of future integration (Figure 3, steps 8, 9 and 10). The VirF protein of Agrobacterium is exported to the plant cell and plays a key role in T-DNA uncoating (Tzfira *et al.,* 2004b). In the nucleus, VirF, in concert with the host proteasome machinery is involved in degradation of the T-complex proteins and therefore facilitates the release of the T-DNA and its subsequent chromosomal integration (Schrammeijer *et al.,* 2001; Tzfira *et al.,* 2004 a, b). The integration of T-DNA into the plant genome occurs by illegitimate recombination (Gheysen *et al.,* 1991), but very little is known about the precise mechanism of integration and the proteins involved in this process. T-DNA enters the nucleus as a single-stranded molecule and then most likely becomes double stranded because the conversion to a transcriptionally competent form requires the synthesis of a complementary DNA on the T-strand (Narasimhulu *et al.,* 1996). However it is not clear whether the T-DNA integrates through the single-stranded or double-stranded form. As VirD2 is covalently linked to the T-DNA strand and probably stays attached to the T-DNA up to the integration step, it has been suggested that it has some function in the integration process. The integration of the 5' end of the T-strand is generally precise and only a few 5' nucleotides are usually deleted on T-DNA integration into the plant genome (Tinland *et al.,* 1995). This may be the result of the protection from exonucleases that VirD2 offers to the 5' end of the T-strand.

Among the host cell proteins the importance of some histone proteins as well as histone-modifying enzymes in T-DNA integration has been well documented in recent years. It has been demonstrated that the H2A protein is capable to interact with VIP1 and therefore it may help the T-complex to target the integration site (Zu *et al.,* 2003; Li *et al.,* 2005; Tenea *et al.,* 2009). Since none of the T-complex bacterial proteins possess the DNA repair functions needed for T-DNA integration this seems also to be heavily dependent on host plant proteins.

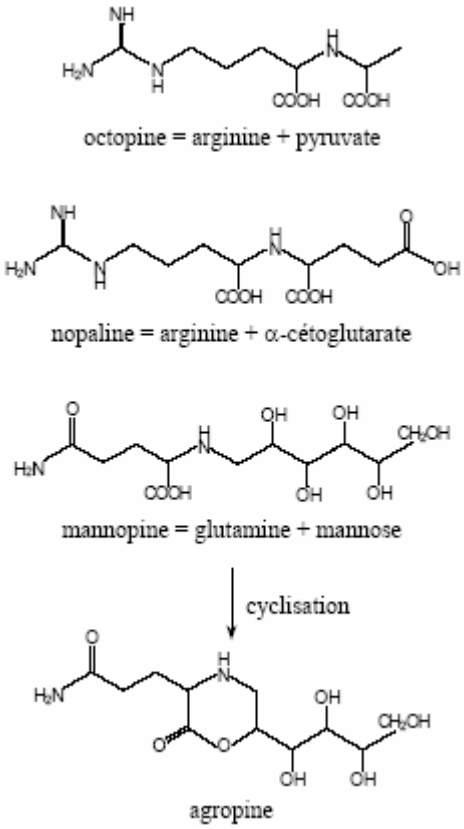

Figure 5. The structure of four opines. These metabolites, found in crown gall and hairy roots, result from the condensation of an amino acid and a sugar or an α-keto acid.

In this regard, several DNA repair proteins (e.g. DNA ligase IV) have been found essential for T-DNA integration in plant cells (Mysore *et al.,* 2000; Friesner *et al.,* 2003).

II. The Agrobacterium T-DNA genes

After having discussed the infection process, I will present the most important T-DNA genes on Ti and Ri plasmids. It should be noted that among these genes, the genes whose expression leads to the neoplastic growth of the plant tissues resulting in tumors or hairy roots are called oncogenes (Binns and Costantino, 1998).

1. Opine synthesis genes

Opines are low-molecular-weight compounds whose synthesis is directed by genes present on the T-DNA of pRi or pTi. They result from the condensation of an amino acid and a sugar or an α-keto acid (Figure 5). They serve as a source of nitrogen and carbon for the bacterium (Hong *et al.,* 1997). The genes for the catabolism of these compounds are also encoded by Ti or Ri plasmids but are not located on the T-DNA (Costantino *et al.,* 1984; Tepfer 1989). Each strain of Agrobacterium produces a specific opine molecule and this characteristic had been used for classification of different strains (Petit *et al.,* 1983). However, at present the classification of strains is based on ribosomal DNA sequences. In *A. tumefaciens,* strains C58, T37 and AKE10 contain a single T-DNA in their pTi and carry an agrocinopine synthase and a nopaline synthase gene. Strains Ach5 and A6, with a split T-DNA, contain an octopine synthesis gene on the T_L-DNA. Both carry, furthermore, three other opine genes, the mannopine and agropine synthesis genes on the T_R-DNA. In *A. rhizogenes,* strains 1724, 8196 and 2659 containing a single T-DNA, encoding, respectively, mikimopine (Davioud *et al.,* 1987), mannopine and cucumopine synthesis and strains A4, 1855 and HR1, with a split T-DNA, contain agropine and mannopine synthesis genes in their T_R-DNA. In *A. vitis*, the plasmids pTiS4 and pTiAB4 carry a vitopine (Canaday *et al.,* 1992) and nopaline synthesis gene, respectively, and pTiTm4 carries agrocinopine, octopine and cucumopine synthesis genes. Other opines have also been described (succinamopine, leucinopine, glutaminopine, etc.) but will not be discussed here.

The capacity of a given Agrobacterium strain to use its corresponding opine, which can not be used by most of the other soil organisms confers to Agrobacterium a competitive advantage in colonizing the plant tissues (Wilson *et al.,* 1995). These metabolites also play an

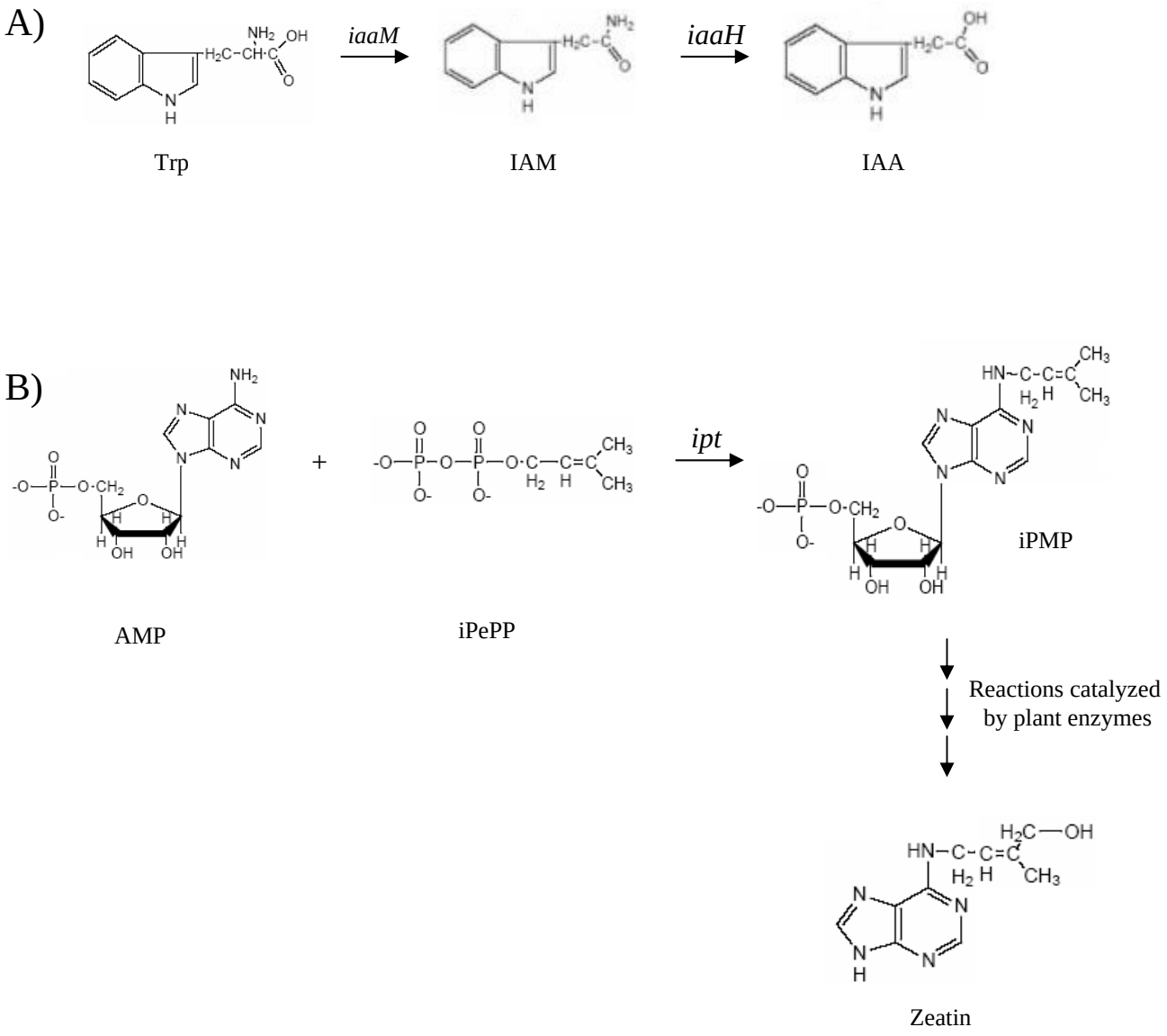

Figure 6. *A. tumefaciens* derived phytohormone biosynthesis pathways. A) Auxin biosynthesis catalyzed by the *iaaM* and *iaaH* oncogenes. B) Cytokinin biosynthesis catalyzed by the *ipt* oncogene (adapted from Escobar and Dandekar, 2003).

important role in regulating their own uptake and catabolism and in conjugal transfer of the Ti/Ri plasmids to other strains (Ellis *et al.,* 1982).

2. Hormone synthesis genes

2.1. *A. tumefaciens* and *A. vitis*

The T-DNAs of *A. tumefaciens* and *A. vitis* contain two loci called *tms* (tumor morphology shooty) and *tmr* (tumor morphology rooty) that are involved in auxin and cytokinin metabolism (Akiyoshi *et al.,* 1983). The *tms* locus is composed of two genes (*tms1/iaaM* and *tms2/iaaH*) that are expressed in tumor tissues and catalize the formation of the natural auxin, indole-3-acetic acid (IAA). *iaaM* and *iaaH* encode a tryptophan mono-oxygenase and indole-3-acetamide hydrolase, respectively, which catalyze the two-step conversion of tryptophan (Trp) to auxin (IAA) (Schröder *et al.,* 1984; Kemper *et al.,* 1985) (Figure 6A). The activity of the IaaM and IaaH proteins *in planta* leads to the accumulation of free IAA levels in crown gall tumors that are generally more than 10-fold greater than in surrounding tissues with highest auxin concentration at the tumor periphery (Weiler and Spanier, 1981; Veselov *et al.,* 2003). Likewise the level of free IAA in transgenic iaaM and iaaH plants is increased compared to control plants (Klee *et al.,* 1987; Eklöf *et al.,* 1996).

Recently Dunoyer and co-workers (2006) have proposed that IaaM and IaaH play a "secondary" role in tumorigenesis. They have shown that the T-DNA encoding genes are targeted by endogenous RNA silencing pathways. However, Agrobacterium has developed a mechanism to counteract RNA silencing-based plant defense, allowing high expression of targeted genes such as *iaaM* and *ags* (agropine synthase). They believe that the suppression of RNA silencing is caused by oncogene-mediated increase in auxin and/or cytokinin levels in transformed tissues and therefore the activity of these genes (and potentially other oncogenes) not only result in tumor formation but also play a role in circumventing plant-imposed barriers to tumor growth.

The second hormone synthesis locus on the T-DNA of Ti plasmid is *tmr* (*ipt*) which lies adjacent to *tms.* The Ipt protein is an isopentenyl transferase which catalyses the condensation of adenosine monophosphate (AMP) and isopentenyl pyrophosphate (iPePP) to produce isopentenyl adenosine monophosphate (iPMP) (Figure 6B). This is the rate-limiting step in cytokinin biosynthesis, and iPMP is rapidly converted to trans-zeatin by plant encoded enzymes (Escobar and Dandekar, 2003). The production of cytokinin by the *ipt* gene leads to

the accumulation of this hormone in both crown gall tumors (more than 100 fold compared to the surrounding tissues) (Weiler and spanier, 1981) and in *ipt* overexpressing plants (Estruch *et al.,* 1991a; Eklöf *et al.,* 1996).

2.2. *A. rhizogenes*

In *A. rhizogenes* the homologs of *iaaM* and *iaaH* are called the *aux1* and *aux2* genes respectively (auxin biosynthesis genes) (Binns and Costantino, 1998). These genes are found only in agropine-type Ri plasmids (with a split T-DNA) and are located on the T_R-DNA. The Ri plasmids containing a single T-DNA (mannopine, cucumopine and mikimopine strains), do not carry *aux* genes. Since these latter strains are still capable to induce a "hairy-root" phenotype, the presence of the T_R-DNA (including *aux* genes) is not indispensable to generate this phenotype. It has been demonstrated that the *aux* genes are required to reinforce the "hairy root" phenotype and to extend the host range of the bacterium (White *et al.,* 1985; Cardarelli *et al.,* l987; Hansen *et al.,* 1991; Sevon and Oksman-Caldentey, 2002).

No *ipt* gene homolog has been found on T-DNA of Ri plasmids (Binns and Costantino, 1998; Meyer *et al.,* 2000).

Concerning the origin of T-DNA hormone synthesis genes, they are most likely of prokaryotic origin. The main reason for this assumption is the presence of *iaa* homologs in the auxin-producing plant pathogens *Pseudomonas savastanoi* and *Erwinia* (*Pantoea*) *herbicola* and of the *ipt* gene in *A. tumefaciens* (*tzs*) and the lack of *iaaM*, *iaaH* and *ipt* homologous sequences in plant genomes (Yamada *et al.,* 1985; Manulis *et al.,* 1998).

3. The *rol* (root locus) genes

In addition to opine and hormone synthesis genes, the T-DNAs contain many other genes, with often very strong effects on growth, but for which no precise function is known. Among these genes are the four *rol* (root locus) genes, *rolA*, *rolB*, *rolC* and *rolD*. To identify the contribution of each T-DNA gene to the induction of hairy root disease, White and colleagues (1985) introduced a series of deletions and transposon insertions in the T-DNA regions of pRiA4. These mutations defined at least four genetic loci on the T_L-DNA that affected the root-inducing properties of *A. rhizogenes* on host plants and were designated by *rolA*, *rolB*, *rolC*, and *rolD* corresponding to *orf10*, *orf11*, *orf12* and *orf15* respectively (White *et al.,*

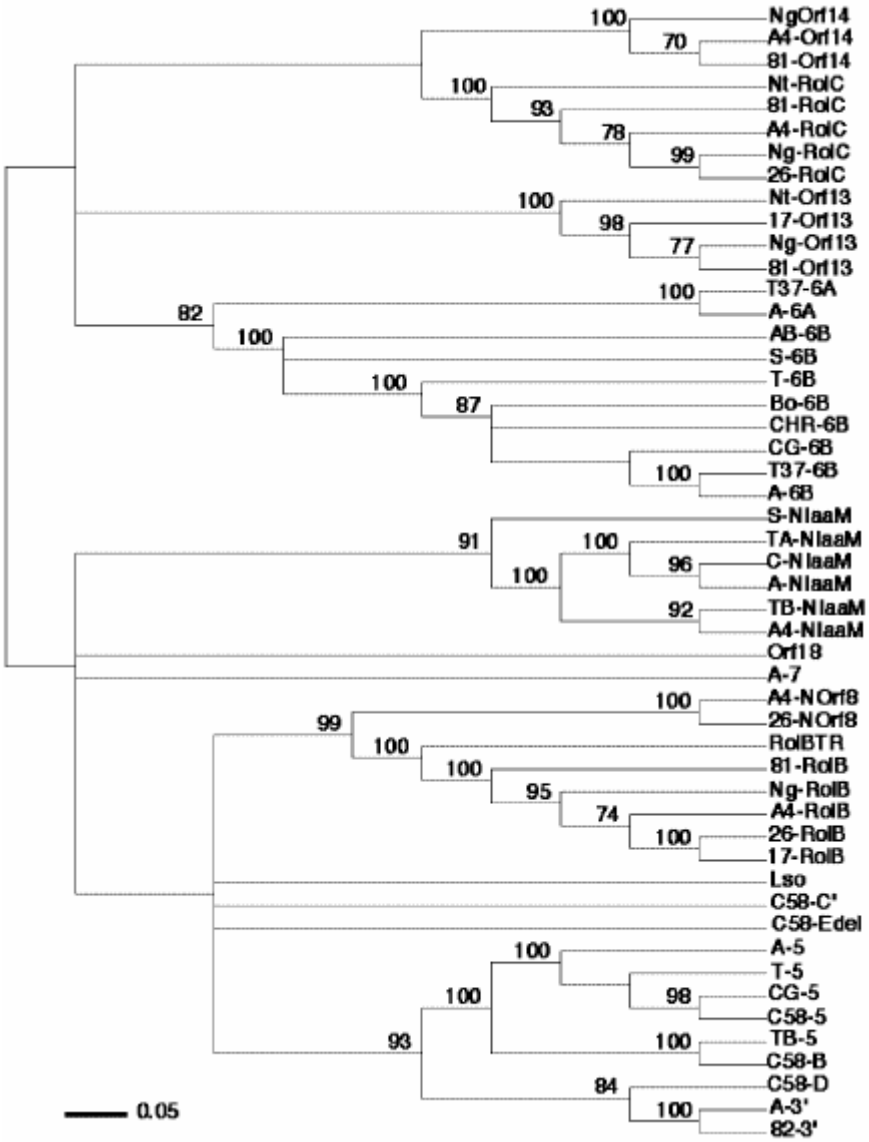

Figure 7. Phylogenetic tree of Plast proteins. The scale bar represents 5% sequence divergence (Helfer *et al.*, 2002).

1985).

Further studies demonstrated that the T-DNA portion encompassing the *rolA*, *rolB* and *rolC* genes is capable to induce adventitious roots on tobacco, kalanchoe and tomato leaves (Cardarelli *et al.,* 1987; Spena *et al.,* 1987; Vilaine *et al.,* 1987; Spanò *et al.,* 1988; van Altvorst *et al.,* 1992; Kiyokawa *et al.,* 1994) and plants carrying these genes are morphologically equivalent to those carrying the whole T_L-DNA (Spanò *et al.,* 1988).

Based on protein sequence homology, Tepfer and co-workers (1989) later classified many of the T-DNA genes in a group called the "*plast*" genes (for phenotypic plasticity) since they remarkably influence plant growth and morphology (Figure 7). They are highly diverged genes that encode proteins with about 20% homology. They include the genes *rolB, rolB^{TR}, rolC*, the 5' part of *orf8*, *orf13, orf14* located on pRi, genes *6a, 6b, lso, 5, 3', 7, b, c', d, e* on pTi and the 5' part of gene *aux1/iaaM* on both Ri and Ti plasmids.

Thus, *rolB* and *rolC* belong to the originally defined *rol* gene group, but were later found to be part of a much larger group, that of the *plast* genes. I will first discuss the *rolA*, *B*, *C* and *D* genes, with special emphasis on the two *plast* genes *rolB* and *rolC*, which are the subject of my Thesis. Since both *rolB* and *rolC* belong to the *plast* genes, I will then discuss the other *plast* genes insofar they have been studied. Interestingly, the fact that the *plast* genes are of common origin has been little exploited, possibly because the homologies are undetectable at the gene level and can only be seen on the protein level. Our group's working hypothesis is that inspite of their divergence the *plast* genes still share a common basic function. Proteins with different basic functions would for example be enzymes, (separated in various subgroups), transporters, transcriptional regulators, structural membrane proteins etc. We consider it highly unlikely that the various Plast proteins could belong to different functional groups. This means that once a basic function of one Plast protein would have been unambiguously demonstrated, the function of the other members might be more easily approached, especially by investigating the closest homologs to that protein. Here we will present the data for the different *plast* genes as reported in the literatur. From these data, no common basic function can yet be proposed, and several proposed functions seem completely unlinked. However, it should be born in mind that many of the data on *plast* genes have not yet been confirmed by other groups or by more detailed studies of the groups that initially reported these data, we therefore consider that many results are highly preliminary.

3.1. The *rolA* gene

The *rolA* gene is found on all Ri plasmids and encodes a small protein with a molecular mass of ~11 kDa (Nilsson and Olsson, 1997). *rolA* transgenic tobacco plants exhibit a bushy phenotype with wrinkled leaves, short internodes, reduced growth and abnormal flowers (Schmülling *et al.,* 1988; Carnerio and Vilaine, 1993; Trovato and Linhares, 1999). Reciprocal grafting experiments have shown that *rolA* expressing rootstocks or scions modify the phenotype of the untransformed part of the plant suggesting that the expression of this gene leads to the production of a factor capable of diffusing in the plant (Guivarc'h *et al.,* 1996).

Expression of this gene in tobacco causes a dramatic decrease in several classes of hormones, including auxin, cytokinin, gibberellic acid (GA) and abscisic acid. The amount of decrease depends on developmental stage of the plant and tissue type (Dehio *et al.,* 1993). Despite the low level of auxin in these plants, they demonstrated increased sensitivity to this hormone (Maurel *et al.,* 1991; Vansuyt *et al.,* 1992).

Dehio and co-workers (1993) reported that wild-type plants treated by gibberellin biosynthesis inhibitors demonstrated a phenotype similar to the phenotype of *rolA*-expressing plants. However when *rolA* transgenic plants were treated with GA, the phenotype was not completely restored (Dehio *et al.,* 1993). This indicates that the abnormalities induced by *rolA* expression are only partially related to GA and the reduced level of this hormone represents probably a secondary effect of the *rolA* expression.

rolA is transcribed in phloem cells, with stronger expression in the stem tissues and weaker expression in roots and leaves (Sinkar *et al.,* 1988a; Carneiro and Vilaine 1993). In 1994, Magrelli and colleagues discovered an intron in the 5' UTR sequence of *rolA* and showed that the mutations in the splice site abolish the *rolA* phenotype (Magrelli *et al.,* 1994). The intron is a bacterial promoter with initiation of transcription inside the intron (Pandolfini *et al.,* 2000) allowing the gene to be expressed both in *A. rhizogenes* and in the plant where the bacterial promoter is spliced out. Although *rolA* fused to GUS confers a plasma membrane localization in transgenic plants, no transmembrane motif has been identified in this protein, so it is thought to be a non-integral membrane associated protein (Vilaine *et al.,* 1998). In addition, as a RolA-GUS fusion protein enhances ß-glucuronidase activity up to 50-fold, it has been hypothesized that this protein may interfere with the protein degradation pathway (Barros *et al.,* 2003).

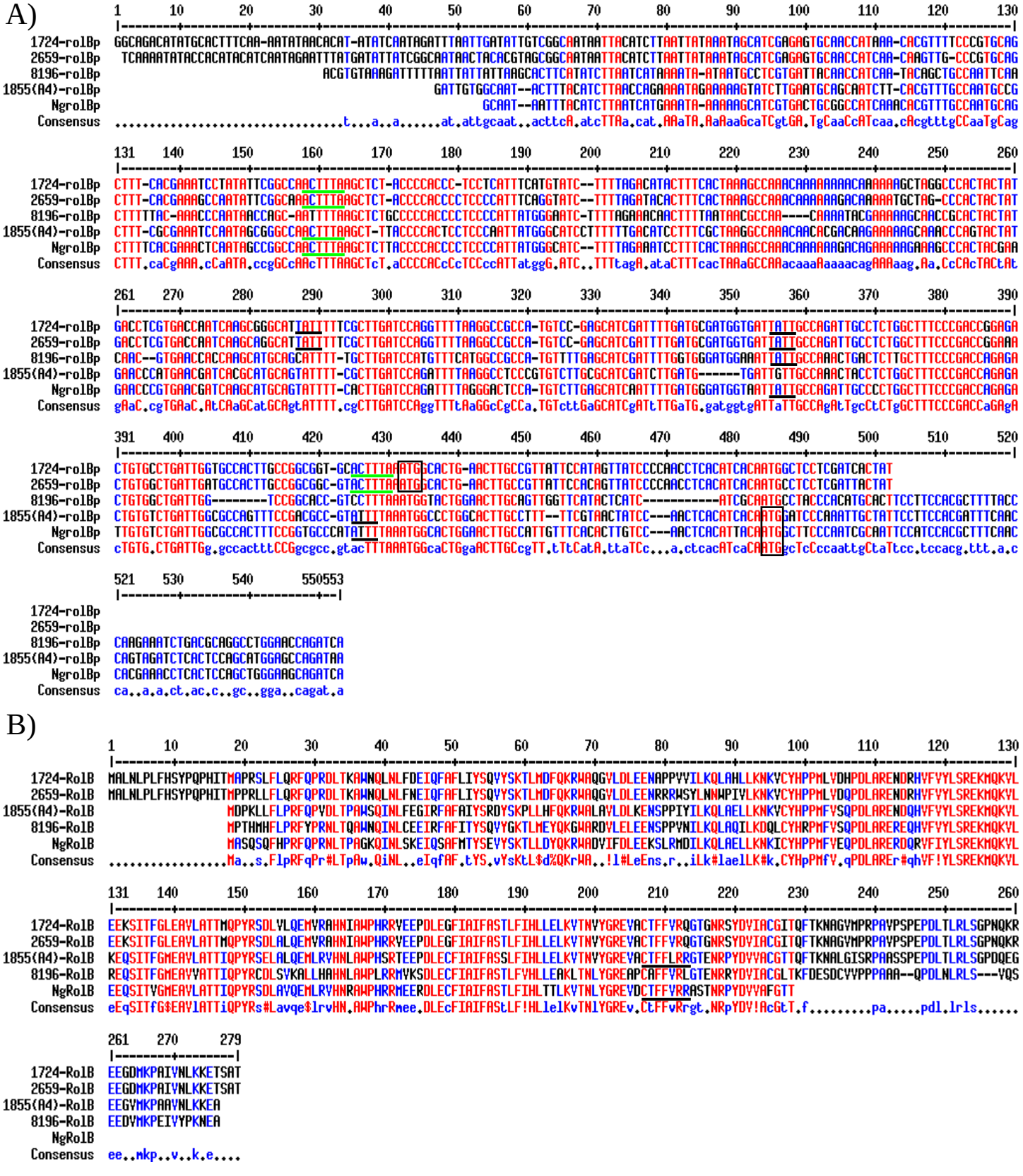

Figure 8. The alignment of A) part of the promoter region and B) the amino acid sequences of the 4 *rolB* genes (1724-*rolB*, 2659-*rolB*, 8196-*rolB* and 1855(A4)-*rolB*) and the homologous sequence in the genome of *N. glauca* (*NgrolB*). In the promoter sequences alignment, black lines and black rectangles represent the position of the possible TATA boxes and the start codon respectively. Green lines represent the position of the ACTTTA motif (see the 3.1.2.3). In the amino acid sequences alignment, the black line represents the position of the CX5R motif in 1855(A4)-RolB and NgRolB (see Chapter II, part 1.5). The sequences are from the NCBI DNA sequence database with accession nos. AB006689 (1724-*rolB*), AJ271050 (2659-*rolB*), M60490 (8196-*rolB*), K03313 (A4-*rolB*) and X03432 (*NgrolB*).

3.2. The *rolB* gene

The *rolB* gene is present in all Ri plasmids with approximately 60% identity between strains (Meyer *et al.,* 2000). Its coding region ranges from 765 (strain 8196) to 840 (strain 2659) bp in size, and encodes a protein of 254 to 279 amino acids. RolB proteins encoded by pRi1724 and pRi2659 have a 17 amino acid longer N-terminal stretch than the RolB encoded by pRi1855 (pRiA4), pRi8196 and NgRolB (the *Nicotiana glauca* homolog of the RolB protein that will be discussed later). Figure 8 shows the alignment of the promoter region and the amino acid sequences of 4 *rolB* genes (1724-*rolB*, 2659-*rolB*, 8196-*rolB* and 1855(A4)-*rolB*) and the homologous sequence in the genome of *N. glauca* (*NgrolB*).

Between 1985 and 2005, many efforts have been made to elucidate how the *rolB* gene functions, however, rather conflicting results were obtained. After 2005, most of the studies on *rolB* have been focused on its effects on the production of secondary metabolites and no new information concerning the basic mechanism of action of this gene has been reported.

3.2.1. *rolB* affects morphogenesis

Among the *rol* genes, *rolB* is the only one that, when inactivated in the pRiA4 context, totally suppresses root induction on Kalanchoe leaves by *A. rhizogenes* (White *et al.,* 1985). In addition, *rolB* is the only *rol* gene capable of inducing rooting nearly as efficiently as the wild type *A. rhizogenes* T-DNA on wounded tobacco stems (Cardarelli *et al.,* 1987) and tobacco leaves (Spena *et al.,* 1987) and of inducing the hairy root phenotype in a transgenic *rolB* plant (Cardarelli *et al.,* 1987; Bellincampi *et al.,* 1996; Altamura *et al.,* 1998; Binns and Costantino, 1998). It has been shown that *rolB* can induce rooting in apple rootstock (Zhu *et al.,* 2001). *rolB* under the control of its own promoter considerably improves the efficiency of rooting in transformed calli of quince (*Cydonia oblonga*) (Ražanskienė *et al.,* 2006).
A4-*rolB* transgenic tobacco plants in which *rolB* is under the control of 35S or its own promoter show growth abnormalities consisting in alterations of leaf morphology, increased stigma and flower size and increased formation of highly adventitious roots on the stem (Schmülling *et al.,* 1988).

In *N. tabacum* it has been demonstrated that 35S::A4-*rolB* transgenic calli are necrotic and impaired in shoot differentiation. Nevertheless, transgenic lines could be regenerated at a very low frequency displaying spontaneous necrosis (cellular death) in leaves of young plants (Schmülling *et al.,* 1988). In the case of *A. thaliana* too shoot regeneration from 35S::A4-*rolB* calli is difficult and the few regenerants show a pleiotropic phenotype including pronounced

growth retardation and early senescence (Dehio and Schell 1994). In ornamental carnation plant, *rolB* driven by the 35S promoter leads to early necrosis in leaves (Casanova *et al.,* 2005). In addition it has been reported that A4-*rolB* under the control of the 35S promoter is less efficient in root induction on tobacco leaf discs than the gene under the control of its own promoter (Spena *et al.,* 1987) suggesting that regulation of the *rolB* gene is important in maximizing root formation. Dehio and Schell showed in 1994 that in 35S::A4-*rolB A. thaliana* plants, *rolB* was expressed strongly and uniformly in seedlings, but in the course of further development, the gene was silenced. The silencing could be monitored by reversion of the RolB phenotype and a dramatic reduction of steady-state *rolB* transcripts.

Röder *et al.* generated transgenic *rolB* tobacco plants in which the gene was placed under control of a tetracycline-dependent promoter. In these plants the transgene did not interfere with the initial regeneration process and the function of the gene product could be analysed at defined time points during development. When the plants had four to five fully developed leaves induction of *rolB* with tetracycline using a hydroponic culture system resulted in extremely stunted plants with necrotic and wrinkled leaves unable to develop a floral meristem (Röder *et al.,* 1994).

3.2.2. *rolB* and auxin

The morphological abnormalities of *rolB* transgenic plants, root meristem neoformation on leaf discs and the growth pattern of these roots, characterized by fast growth, high branching and plagiotropism, initially led to the suggestion that the morphogenic effects of *rolB* are similar to auxin-mediated effects and involve changes in either the responsiveness to auxin or in auxin content (Cardarelli *et al.,* 1987; Shen *et al.,* 1988; Capone *et al.,* 1989b).

The first studies showed an increase of activity of the *rolB* promoter in tobacco mesophyll protoplasts treated with auxin (Maurel *et al.,* 1990; Capone *et al.,* 1991). Further studies demonstrated that *rolB* tobacco protoplasts exhibit increased sensitivity in their electrical response to auxin by a factor of up to 100,000, whereas in untransformed protoplasts the same auxin treatment induced a sensitivity that never exceeded 30- to 50-fold (Maurel *et al.,* 1994). It has been suggested earlier that auxin-induced hyperpolarization of the plasma membrane is due to excretion of protons via an H^+-ATPase protein pump located on the plasma membrane (Ephritikhine *et al.,* 1987; Keller and Van Volkenburgh 1998). This suggests that *rolB* could interfere with the proton pump.

It has been demonstrated that anti-ABP1 (auxin-binding protein) antibodies block the

polarization of the protoplast plasma membrane due to auxin, but in the case of *rolB* protoplasts a large amount of antibodies is necessary to block this polarization (Venis *et al.,* 1992).

Membrane preparations from *rolB* plant cells bind higher levels of auxin than untransformed cells and by using anti-RolB antibodies the additional auxin-binding activity is completely abolished (Filippini *et al.,* 1994).

Estruch *et al.,* (1991b) reported that the RolB protein, expressed in *E. coli*, exhibits a β-glucosidase activity able to hydrolyse indole-3-glucosides. On this basis, the authors made the assumption that RolB was able to increase the free IAA level in transformed cells by releasing the hormone from β-glucoside conjugates, as a result the intracellular auxin concentration would increase and cause the phenotypic alterations observed in *rolB* transgenic tissues. However this proposal has been invalidated by the results of independent laboratories that showed that neither the intracellular concentration nor the metabolism of auxin was changed by *rolB* expression in plant cells (Nilsson *et al.,* 1993; Schmülling *et al.,* 1993; Delbarre *et al.,* 1994). In addition, plants transgenic for auxin-synthesizing genes (Sitbon *et al.,* 1992) do not resemble 35S::A4-*rolB* transgenic plants (Schmülling *et al.,* 1988).
These data support the idea that the increased auxin sensitivity of *rolB*-transformed cells rather results from alterations in the reception/transduction of the auxin signal.

It has been suggested that some compounds have a negative influence on the interaction between auxin and *rolB*, such as α-1,4-oligogalacturonides (OG) with a specific degree of polymerization. Many of the developmental effects of oligogalacturonides appear to be due to their auxin antagonist activity (Branca *et al.,* 1988; Darvill *et al.,* 1992; Bellincampi *et al.,* 1993). It has been shown that oligogalacturonides are capable of inhibiting *rolB*-driven root morphogenesis in transgenic leaf explants, especially when this process requires exogenous auxin to induce *rolB* as in leaf mini-explants devoid of primary and secondary veins. In contrast, oligogalacturonides do not inhibit rhizogenesis when *rolB* transcriptional activation is made independent of auxin (tetracycline-induced *rolB* mini-explants). Moreover, once RolB is expressed in the mini-explants, oligogalacturonides have no effect on rhizogenesis (Bellincampi *et al.,* 1996). The effect of OGs on the auxin-dependent expression of *rolB* may be due to interference with processes along the transduction pathway leading from the perception of the auxin signal to the activation of the *rolB* promoter.

Auxins and auxin-transport inhibitors can be used to induce artificial development of parthenocarpic fruits in tomato. In transgenic tomato plants with the auxin synthesis gene

iaaH under the control of an ovary-specific promoter, ovary treatment with the auxin precursor (IAM) induces parthenocarpy (Szechtman *et al.,* 1997). Expression of *rolB* under the control of an ovary- and young-fruit-specific promoter also induced parthenocarpic fruit set and development (Carmi *et al.,* 2003) suggesting a close connection between auxin and *rolB*.

Auxin has been shown to play a key role in many aspects of flower development: floral meristem formation and subsequent formation of flower organ primordia (Oka *et al.,* 1999; Tobena-Santamaria *et al.,* 2002). Female organ development is regulated by auxin (Nemhauser *et al.,* 2000). Evidence has also been reported concerning the influence of auxin on stamen and anther formation (Okada *et al.*, 1991). It has been demonstrated that expression of *rolB* in tobacco anther cells reduces stamen elongation and delays the timing of anther dehiscence, suggesting the involvement of an auxin-like effect of *rolB* in these processes (Cecchetti *et al.,* 2004).

Although many authors insist on the root-inducing ability of *rolB*, it should be mentioned that the function of this gene is not restricted to roots because in tobacco thin cell layer (TCL) culture *in vitro*, *rolB* strongly promotes *de novo* formation of either root or flower primordia and this is thought to depend on cell competence and/or hormone balance (Altamura *et al.,* 1994). TCLs are small explants consisting of superficial stem tissues and able to produce organs *de novo*, depending on the hormonal balance of the culture medium, the developmental stage of the donor plant and the excision site on the donor (Tran Thanh Van *et al.,* 1974; Smulders *et al.,* 1990b). Since the formation of roots and flowers from non-transformed TCLs is mainly controlled by exogenous auxin (Smulders *et al.,* 1988; 1990a, b; Altamura, 1996), whereas shoot formation is mainly controlled by exogenous cytokinin (Tran Thanh Van *et al.,* 1974), it has been hypothesized that the meristem-promoting action of *rolB* is a consequence of the increased auxin sensitivity of *rolB*-transformed cells. However, additional experiments with TCLs and leaf explants cultured in different conditions (on hormone-free medium or with a wide range of concentrations of cytokinin alone) demonstrated that RolB also enhances the formation of adventitious shoot buds (Altamura *et al.,* 1998). It seems therefore that RolB has not only a positive effect on meristem formation in combination with auxin, but also in the presence of cytokinin.

Transformation of a facultative apomictic plant (*Hieracium piloselloides*) with *rolB* under the control of its own promoter or the 35S promoter induced the formation of ectopic meristems *in planta* that have the potential to differentiate into a range of plant organs, that is,

vegetative rosettes, capitula, roots and even embryos, suggesting that meristem induction is the primary effet of *rolB* and showing that *rolB*-induced meristems are initially indeterminate (Koltunow *et al.,* 2001).

3.2.3. *rolB* expression

Regulation of *rolB* expression has been extensively studied by means of the reporter gene *uidA* (GUS), mainly in tobacco (Schmülling *et al.,* 1989; Capone *et al.,* 1991; Chichiriccò *et al.,* 1992; Moriuchi *et al.,* 2004; Handayani *et al.,* 2005), carrot (Capone *et al.,* 1989a; Di Cola *et al.,* 1997), and hybrid aspen (Nilsson *et al.,* 1997). It has been shown that the intergenic region separating the *rolB* and *rolC* genes represents a bidirectional promoter. This bidirectional promoter regulates transcription for both genes in a similar fashion in aerial organs (in the leaves, phloem cells of stem and vascular tissues of anthers) of the tobacco plants, but in a distinct way in roots. The BGUS chimeric gene (the GUS gene under the control of the *rolB* promoter) is expressed mainly in the root cap and in the region of root cell division, and, at a much lower level, in the phloem, whereas CGUS (the GUS gene under the control of the *rolC* promoter) expression occurs mainly in the phloem (Schmülling *et al.,* 1989).

Histological analysis has shown that the expression of *rolB* is tissue-specific, limited to the meristems of roots, shoots and flowers, as well as to the phloem parenchyma pericycle, and the ray cells (Altamura *et al.,* 1991) and is developmentally regulated. In early stages of zygotic embryo development, the gene is inactive and then activated in all cells at the end of the globular stage (Chicchiriccò *et al.,* 1992). In the mature zygotic embryo, the central cylinder, as well as the shoot and root poles, and the procambial traces in the cotyledons, show expression (Altamura *et al.,* 1991). In the somatic embryo system the promoter of *rolB* is firstly activated in the central region of the globular embryos (Di Cola *et al.,* 1997). Lo Schiavo *et al.,* (1991) showed that this is the place where auxin synthesis occurs. Thus, in early somatic embryos, the *rolB* promoter is active only in those cells that, owing to the presence of auxin, will start to proliferate as either primary or secondary meristems.

In order to characterize the *rolB* upstream regulatory region, a GUS reporter gene was placed under the control of several fragments of the *rolB* promoter (Capone *et al.,* 1991; 1994). It was shown that a 1100 bp long promoter region is necessary for full expression. According to their results five regulatory domains (designated A-E) have been identified in the 5' non-coding region of *rolB* and different combinations of these domains direct the

expression of the gene in different cell types of the root apex.

Domain A is comprised between -623 and -341 (from the start codon), domain B between -341 and -306, domain C between -216 and -158, and domains D and E are comprised within two regions of about 70 and 80 bp centered around the CAAT and the TATA box respectively. The presence of all these domains together confers GUS activity to all cell populations of the root apex.

Domain A is necessary for expression in the non-meristematic cells of the root apex (i.e. protoderm cells). Domain B is a crucial regulatory domain and indispensable for expression in all tissues of the root apex, as deletion of this domain causes a total loss of activity of the promoter. This domain also controls expression in the shoot meristem and is necessary for the auxin responsiveness of the cells (Capone *et al.,* 1991). Domain C has both a positive and a negative regulatory role, as it is needed for expression in vascular meristematic cells, but seems to act as a negative regulatory element for expression in the protoderm in the absence of domain A. Domain D is required for the expression in outer meristematic cells (i.e. the dermatogen and cortex meristems). Domain E like domain B is indispensable for expression and its deletion totally suppresses expression in all tissues of the root apex.

The *rolB* promoter seems to be a mosaic of plant regulatory sequences, and the plant regulatory proteins that control *rolB* might also control endogenous plant genes, possibly involved in the same developmental events (e.g., meristem formation) (Binns and Costantino, 1998). In 1996, De Paolis and co-workers isolated a nuclear tobacco gene encoding a protein which binds to the ACTTTA motif within domain B of the *rolB* promoter via a single zinc finger of a Dof (DNA binding with one finger) type protein, they called it NtBBF1 (*N. tabacum rolB* domain B factor 1). The binding site is conserved in different *rolB* promoters except in the 8196-*rolB* promoter region (Figure 8) (Handayani *et al.,* 2005).

The Dof/BBF proteins are a large family of proteins present in phylogenetically distant plants such as tobacco (De Paolis *et al.,* 1996), Arabidopsis (Zhang *et al.,* 1995; De Paolis *et al.,* 1996), maize (Vicente-Carbajosa *et al.,* 1997), barley (Mena *et al.,* 1998), pumpkin (Kisu *et al.,* 1998) and snapdragon (Rengel *et al.,* 2001). In contrast, Dof proteins are not present in yeast or in animals (Yanagisawa and Sheen, 1998). The wide distribution of these proteins in plants only, suggests that they are involved in regulatory circuits specific to the plant kingdom. NtBBF1 is a highly hydrophilic protein with several potential phosphorylation sites (De Paolis *et al.,* 1996) both are expected characteristics for a regulatory protein. In the case of maize, Dof proteins are indeed transcription factors that contribute not only to binding to DNA but also to protein-protein interactions for the formation of complexes on DNA to

A)1724-RolB

MALNLPLFHSYPQPHITMAPRSLFLQRFQPRDLTKAWNQLNLFDEIQFAFLIYSQVYS
KTLMDFQKRWAQGVLDLEENAPPVVILKQLAHLLKNKVCYHPPMLVDHPDLAREN
DRHVFVYLSREKMQKVLEEKSITFGLEAVLATTMQPYRSDLVLQEMVRAHNIAWPH
RRVEEPDLEGFIAIFASTLFIHLLELKVTNVYGREVACTFFVRQGTGNRSYDVIACGIT
QFTKNAGVMPRPAVPSPEPDLTLRLSGPNQKREEGDMKPAIVNLKKETSAT

B)

RolB	**WT**	**L33F**	**F118L**	**M126T**	**V197G**	**C207R** ↓	**R212G**	**R218C**	**D221N**	**F230S**
Interaction	++	-	-	-/+	-	-	-	-	-	-
Nuclear localization	++	+/-	-	-/+	-	-	-	-	-	-
Root induction	++	+	+/-	+	+/-	+/-	+/-	+/-	+/-	+/-

Figure 9. Summary of the results obtained by Moriuchi *et al.*, (2004) for amino acid substitution mutants of 1724-RolB and their effects on the interaction of the protein with Nt14-3-3 ω II, its localization and its root induction capability. A) The amino acid sequence of wild type 1724-RolB protein. Amino acid residues in red represent the position of substitutions. B) Substitution mutations of amino acid residues of 1724-RolB impair interaction with Nt14-3-3 ω II protein, nuclear localization and root induction. The arrow indicates the C residue of the CX5R motif.

regulate gene expression (Yanagisawa and Sheen, 1998).

It has been shown that the ACTTTA target sequence of NtBBF1 protein is essential for tissue-specific expression of *rolB* as well as for auxin responsiveness of the *rolB* promoter. Moreover the expression pattern of *NtBBF1* is similar to that of *rolB,* that is, strongest in the apical meristems (Baumann *et al.,* 1999). These data provide evidence that the NtBBF1 protein acts as a trans-acting factor necessary for the control of *rolB* expression mediated by the ACTTTA cis element and can help in investigation of the mechanism of auxin induction of *rolB* and in the identification of other plant genes possibly involved in meristem formation and morphogenesis induced by *rolB*.

In transformed plant cells and tissues, *rolB* is induced by auxin with rather slow kinetics. The earliest detectable response to auxin (for example the increase in the sensitivity of the protoplast membrane to auxin) occurs after 6 to 8 hours of hormone treatment (Maurel *et al.,* 1990; 1994; Capone *et al.,* 1991). Thus, this gene does not belong to the class of early genes rapidly induced by auxin. The early genes play a role in the transcriptional regulation of late-responsive (secondary) genes (Abel and Theologis, 1996). *rolB* seems to belong to the secondary class of genes that determines a specific biological response as a long-term consequence of the auxin stimulus. For *rolB*, the long-term effect of auxin is on meristem formation later followed by organogenesis involving roots, shoots and flowers (Altamura *et al.,* 1994; 1996; 1998).

3.2.4. RolB localization

It has been reported that the A4-RolB protein overproduced in *E. coli* has tyrosine phosphatase activity and localizes to the plasma membrane in *rolB*-transformed carrot cells (Filippini *et al*., 1996).

Moriuchi and co-workers demonstrated in 2004 that the 1724-RolB protein localizes in the nucleus and interacts with a tobacco protein, Nt14-3-3 ωII. They showed that the majority of RolB mutants with a deletion or amino acid substitution are unable to interact with Nt14-3-3 ωII and have a weaker nuclear localization. In addition, these mutants showed decreased rooting activity indicating that the adventitious root induction of RolB is related to its interaction with Nt14-3-3 ωII or its nuclear localization. 14-3-3 protein family members usually bind to phosphoserine/threonine motifs (Yaffe *et al*., 1997), but no such motifs were found in RolB indicating that the binding mode is unrelated to the phosphoserine/threonine motif. Figure 9 summarizes the results obtained by Moriuchi *et al.,* (2004) for amino acid

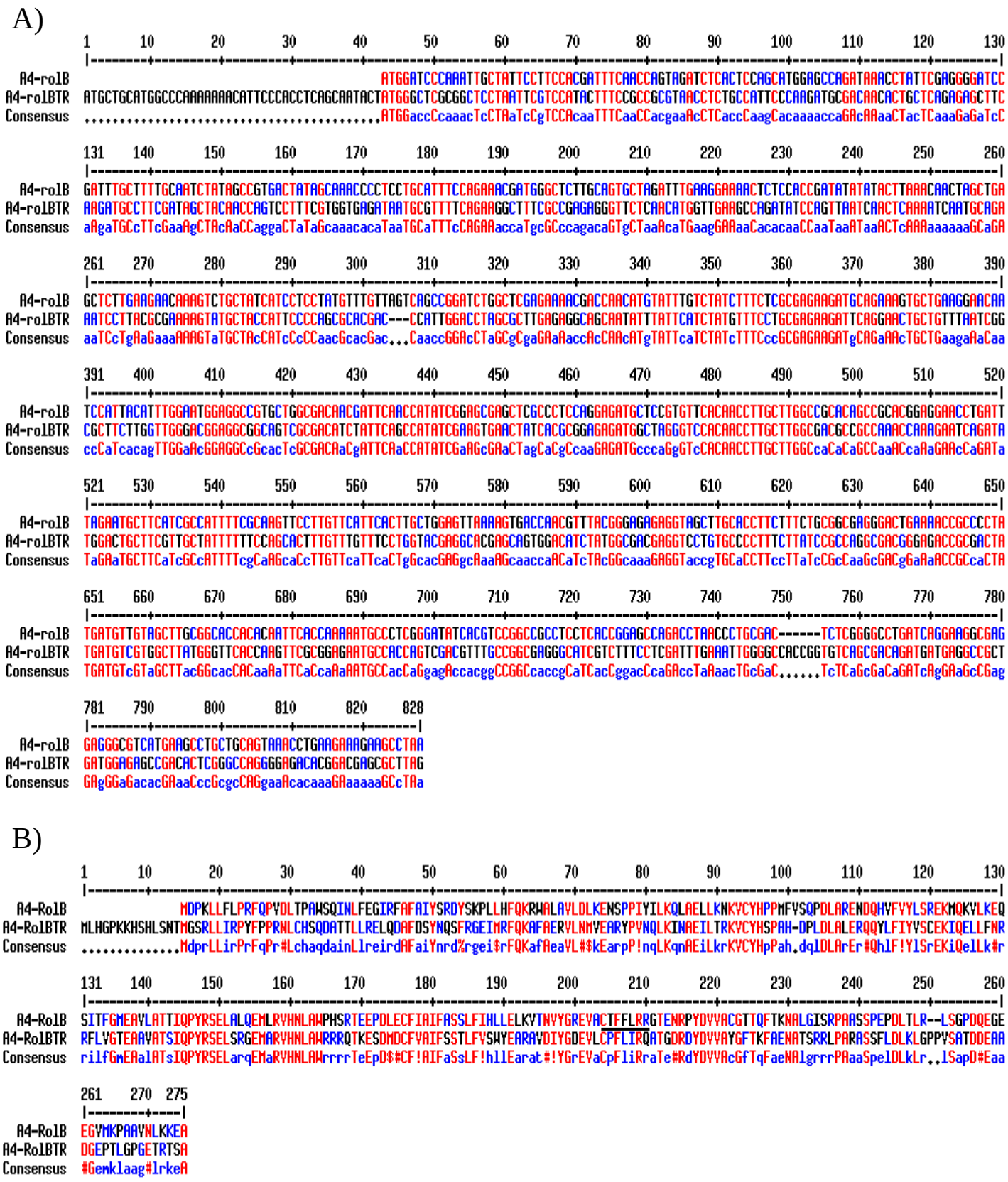

Figure 10. The alignment of the A) DNA sequences and B) amino acid sequences of A4-*rolB* and A4-*rolB*^{TR}. In the amino acid sequences alignment, the black line represents the position of the CX5R motif in A4-RolB. The accession number for *A4-rolB*^{TR} is X15952.

substitution mutants of RolB and the interaction of the mutated protein with Nt14-3-3 ωII, its localization and root induction capability. The pattern of expression of *Nt14-3-3 ωII* partly corresponds to that of 1724-*rolB* and the site of hairy root induction.

The interaction of RolB with Nt14-3-3 ωII and its nuclear localization support the notion that RolB may behave in a manner similar to that of the hepatitis C virus core protein, which interacts with 14-3-3ε and activates Raf (Rat fibrosarcoma)-1 kinase to contribute to hepatocyte growth regulation (Aoki *et al.,* 2000). Thus, once associated with Nt14-3-3 ωII protein and shuttled into the nucleus, RolB could modulate the expression of certain genes (i.e. auxin-responsive gene(s)), leading to *de novo* formation of root, shoot and flower meristems (Altamura *et al.,* 1994; 1996; 1998) or fertilization-independent fruit development, similarly to auxin-induced parthenocarpy (Carmi *et al.,* 2003; Shabtabi *et al.,* 2007).

3.2.5. A *rolB* gene on the T_R-DNA

Within the pRiA4 TR-DNA, an ORF homologous to the T_L-DNA *rolB* gene was found and named *rolB*^{TR} (Figure 10). It is 825 nucleotides long and codes for a protein with 274 amino acids. It carries at its 5' end a 42 bp extension compared to the A4-*rolB* gene (Figure 10A). The homology between *rolB* and *rolB*^{TR} is 52.6% for the nucleotide sequences and 40.6% for the protein sequence. No extended homologies were found in the 5' or 3' flanking regions. RolB^{TR} has also homology to the N-terminal part of the pRiA4 T_L-DNA A4-Orf8 protein (Bouchez *et al.,* 1990). The N-terminal RolB-like Orf8 fragment placed under 2x35S promoter control has biological activity; in transgenic tobacco plants it leads to strong accumulation of soluble sugars and starch, due to a block in sucrose transport (Umber *et al.*, 2002).

Despite the high degree of conservation in the coding region between A4-*rolB* and *rolB*^{TR}, *rolB*^{TR} is unable to induce rooting on tobacco leaf discs. Transgenic tobacco plants expressing 35S::*rolB*^{TR} have wrinkled leaves, a reduced size and form shoots at the base of the stem. This phenotype is different from that of *rolB* placed under the control of either its own or the 35S promoter (Lemcke and Schmülling, 1998a). Therefore, RolB^{TR} has morphogenic activity but is not a functional homolog of RolB. According to these authors, the two protein sequences have two significant differences. Firstly, the RolB^{TR} protein does not contain the CX5R motif that is characteristic of the tyrosine phosphatase superfamily and secondly, due to the 42 bp extension at the 5'end of the gene, the N-terminal part of RolB^{TR} contains 14 amino acids (Figure 10B) which are indispensable for its biological activity and are absent in A4-RolB.

In Chapter II, we will investigate the earlier reported tyrosine phosphatase activity of A4-RolB and test the functional importance of the tyrosine phosphatase CX5R motif in RolB proteins.

3.2.6. *rolB* and secondary metabolites

It has been reported that *A. rhizogenes rol* genes enhance the biosynthesis of certain groups of secondary metabolites in transformed plant cells (Palazon *et al.,* 1998; Bonhomme *et al.,* 2000; Bulgakov *et al.,* 2002; Shkryl *et al.,* 2007). Of these genes, *rolB* is apparently the most powerful inducer of secondary metabolism and at the same time, the most important inhibitor of callus growth. In *Rubia cordifolia* transformed calli or cell cultures, *rolB* expression positively correlated with increased expression of a key gene for anthraquinone biosynthesis, isochorismate synthase (ICS), leading to high levels of anthraquinone production and in parallel its expression inhibited growth of calli (Bulgakov *et al.,* 2002; Shkryl *et al.,* 2007). *rolB* has also been shown to stimulate more than 100-fold the biosynthesis of resveratrol in *Vitis amurensis* cell cultures (Kiselev *et al.,* 2007). Resveratrol is an important stilbene that is known to have antioxidant, anti-inflammatory, antibacterial, antiviral and antifungal activity. It is also a strong antitumoral agent effective against many types of cancer (Aggarwal *et al.,* 2004; Shankar *et al.,* 2007). Tyrosine phosphatase inhibitors suppress the *rolB*-gene-mediated stimulatory effect on resveratrol and anthraquinone production, thus indicating the involvement of tyrosine phosphorylation in the *rolB*-mediated secondary metabolism stimulation (Shkryl *et al.,* 2007; Kiselev *et al.,* 2007).

3.2.7. *rolB* effects on other genes

The mechanism by which RolB affects secondary metabolite production is not at all understood. Resveratrol is synthesized via the phenylpropanoid pathway (Langcake and Pryce, 1977) in which phenylalanine ammonia-lyase (PAL) and stilbene synthase (STS) are considered as key enzymes. It has been shown that in *rolB* transgenic *V. amurensis* cell cultures the expression of the *PAL* and *STS* genes is enhanced providing evidence that RolB could act as a transcription activator/ mediator for up regulation of these genes (Kiselev *et al.,* 2008). Another *plast* gene demonstrated to provoke the production of secondary metabolites is the T-*6b* gene of *A. vitis* (strain Tm4). T-*6b* expressing tobacco plants accumulate high levels of chlorogenic acid (CGA) in roots, however this phenylpropanoid accumulation does not play a role in the T-*6b*-induced phenotype and is only a secondary effect as could be

shown with a specific inhibitor of the PAL enzyme which abolishes CGA accumulation but does not affect the phenotype (Clément *et al*., 2007). This type of approach might also be used for tissues that express *rolB* in order to discriminate between primary and secondary effects.

It is known that calcium plays an important role in plant defense reactions such as phytoalexin biosynthesis (Lecourieux *et al*., 2006; Ramani and Chelliah, 2007) and is necessary for an increase in yield of secondary metabolite production (Dmitriev *et al.,* 1996, Lecourieux *et al.,* 2006; Ramani and Chelliah, 2007). Dubrovina *et al.,* (2009) investigated whether calcium ion fluxes also play a role in resveratrol production in *rolB* transgenic *V. amurensis* cell cultures. They showed that calcium channel blockers inhibit resveratrol biosynthesis in the *rolB* transgenic cultures. Since the main calcium sensors in plant cells are calcium-dependent protein kinases (CDPKs) (Lecourieux *et al*., 2006), Dubrovina and co-workers compared *CDPK* gene expression in *rolB*-expressing and control cultures. Their results showed an alteration in the expression of *CDPK* genes in *rolB* expressing cultures compared to controls. These results indicate that resveratrol biosynthesis in *rolB* transgenic cultures of *V. amurensis* is Ca^{2+} dependent and that *rolB* interferes with the *CDPK* transduction pathway to induce this biosynthesis.

Recently a gene has been isolated that is strongly overexpressed in *rolB* tobacco protoplasts: *rolB*-overexpressed1 (*ROX1*) (Cecchetti *et al.,* 2007). The amino acid sequence of ROX1 shares a conserved element with a number of plant proteins, such as TED3, which is involved in xylem development (Nishitani *et al.,* 2002). It has been shown that down-regulation of *ROX1* in antisense tobacco plants increases the length of the stamen by increasing the number of cells. The anthers show a delay in xylem differentiation. Conversely, overexpression of *ROX1* in anthers, as a consequence of anther-specific expression of *rolB* in tobacco plants, resulted in stamen filaments shorter than normal due to a reduced number of cells and accompanied by the precocious differentiation of anther xylem cells. These results show that *ROX1* plays a role in the balance between cell division and xylem differentiation during stamen development (Cecchetti *et al.,* 2007). These authors suggest that the auxin-like effect of *rolB* (i.e. cell division) could be related to the *ROX1* gene.

3.2.8 A model for *rolB*-mediated organogenesis

According to our knowledge about *rolB* gene expression and its relation with auxin, oligogalacturonides (OG), Nt 14-3-3 ωII, NtBBF1 and ROX1, and according to the models proposed already by (Baumann *et al.,* 1999; Altamura 2004) for the mechanism of action of

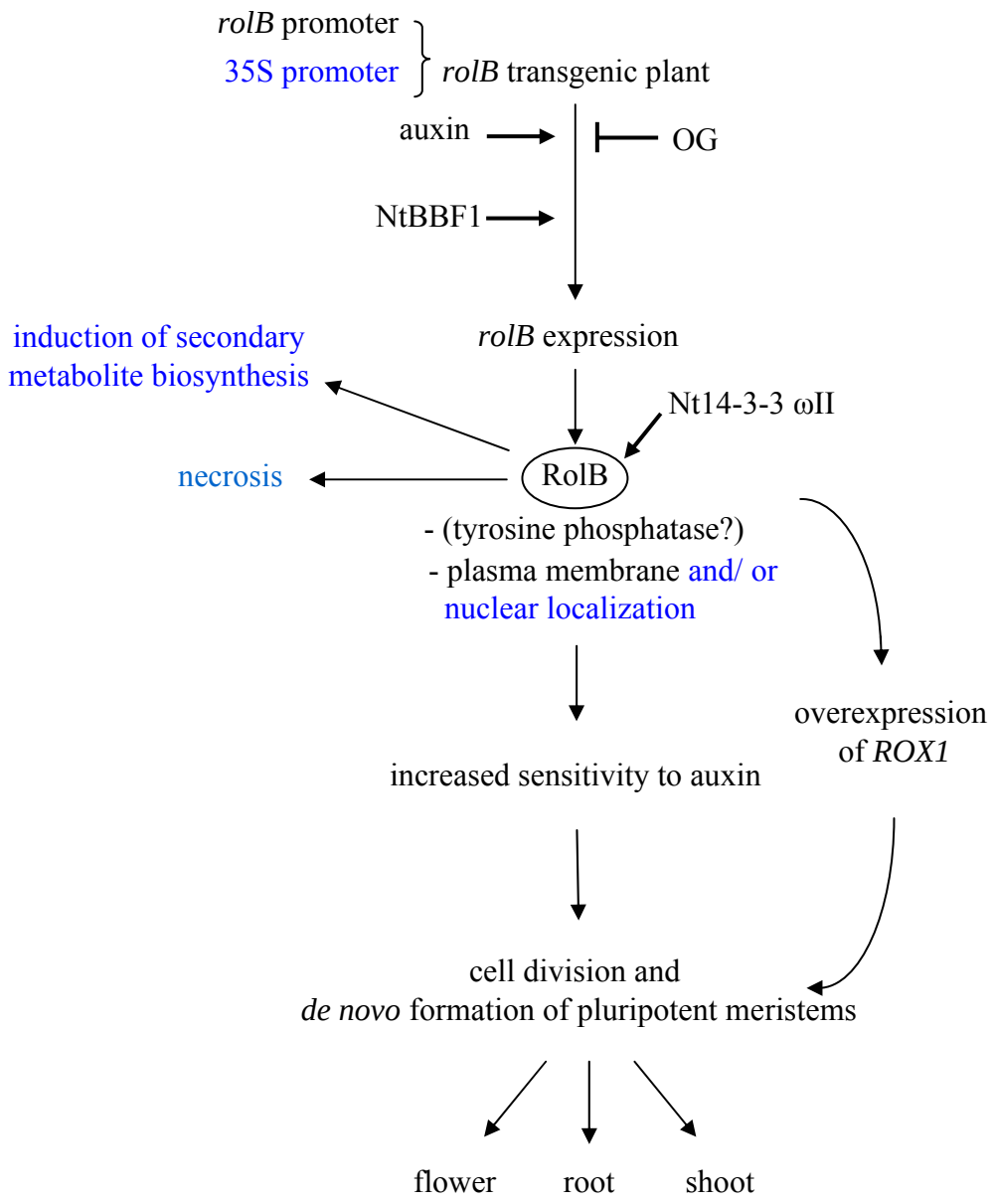

Figure 11. Model of *rolB*-mediated organogenesis. The parts indicated in blue have only been described for 35S-*rolB* constructs. The *rolB* promoter is induced by auxin and NtBBF1 positively regulates *rolB* expression. Oligogalacturonides (OG) have a negative effect on auxin-dependent *rolB* expression. RolB induces necrosis in 35S::*rolB* transgenic plants and up regulates the expression of *ROX1* and leads to *de novo* formation of root, shoot and flower meristems. The increased sensitivity of the cells to auxin is possibly also the trigger of the meristematic feature. The determination of a meristem towards a specific organ type depends on the hormonal balance, on the specific cell competence and on the developmental stage of the plant. The earlier reported tyrosine phosphatase activity of RolB protein will be investigated in chapter II (model modified and completed from Baumann *et al.*, 1999 and Altamura 2004).

rolB in plant morphogenesis, we can propose a combined tentative model (Figure 11) for *rolB*-mediated organogenesis.

According to this model, *rolB* is induced by auxin and the NtBBF1 protein acts as a trans-acting factor that positively regulates *rolB* expression. Oligogalacturonides (OG) have a negative effect on auxin-dependent *rolB* expression. RolB induces necrosis in 35S::*rolB* transgenic plants. Interaction of this protein with Nt14-3-3 ωII and its nuclear localization could modulate the expression of certain genes (such as auxin-responsive genes and *ROX1*) leading to *de novo* formation of root, shoot and flower meristems.

The increased sensitivity of the cells to auxin is also a possible trigger of the meristems. The determination of a meristem towards a specific organ type depends on the hormonal balance, on the specific cell competence and on the developmental stage of the plant (Altamura *et al.,* 1994; 1996; 1998).

Considering the regulation of the *rolB* promoter by plant regulatory proteins such as NtBBF1 and the possibility that the same proteins could also control the expression of endogenous plant genes with a specific morphogenic function, the phenotypes observed in *rolB* expressing plants and the different meristems induced by this gene may not only result from the activities of *rolB* but also from modified activities of the normal NtBBF1 target genes.

Unusual cell division and expansion has been observed in the root pericycle and vasculature of T-*6b* tobacco plants (Grémillon *et al.,* 2004). This could result from the fact that the roots of these plants show increased sucrose uptake and strong accumulation of sucrose, glucose and fructose (Clément *et al*., 2007). As shown in Chapter I, we have also demonstrated increased sucrose uptake for another *plast* gene, A4-*rolC*. In addition, overexpression of the *rolB*-like N-terminal part of *orf8* in tobacco leads to strong sucrose and starch accumulation. Sucrose absorption and accumulation have therefore emerged as possible common features of *plast* genes. Therefore, it would be interesting to study whether *rolB*-induced meristem induction also involves modified sugar uptake.

3.3. The *rolC* gene

The sequences of *rolC* genes from various Ri plasmids are similar in size ranging between 537 bp (strain 8196) to 543 bp (strain 2659, 1724, A4 and *NgrolC*) and encode proteins of 178 to 180 amino acids (approximately 20 kDa) that share more than 65% identity with each other (Meyer *et al.,* 2000). Figure 12 shows the alignment of the DNA and the amino acid

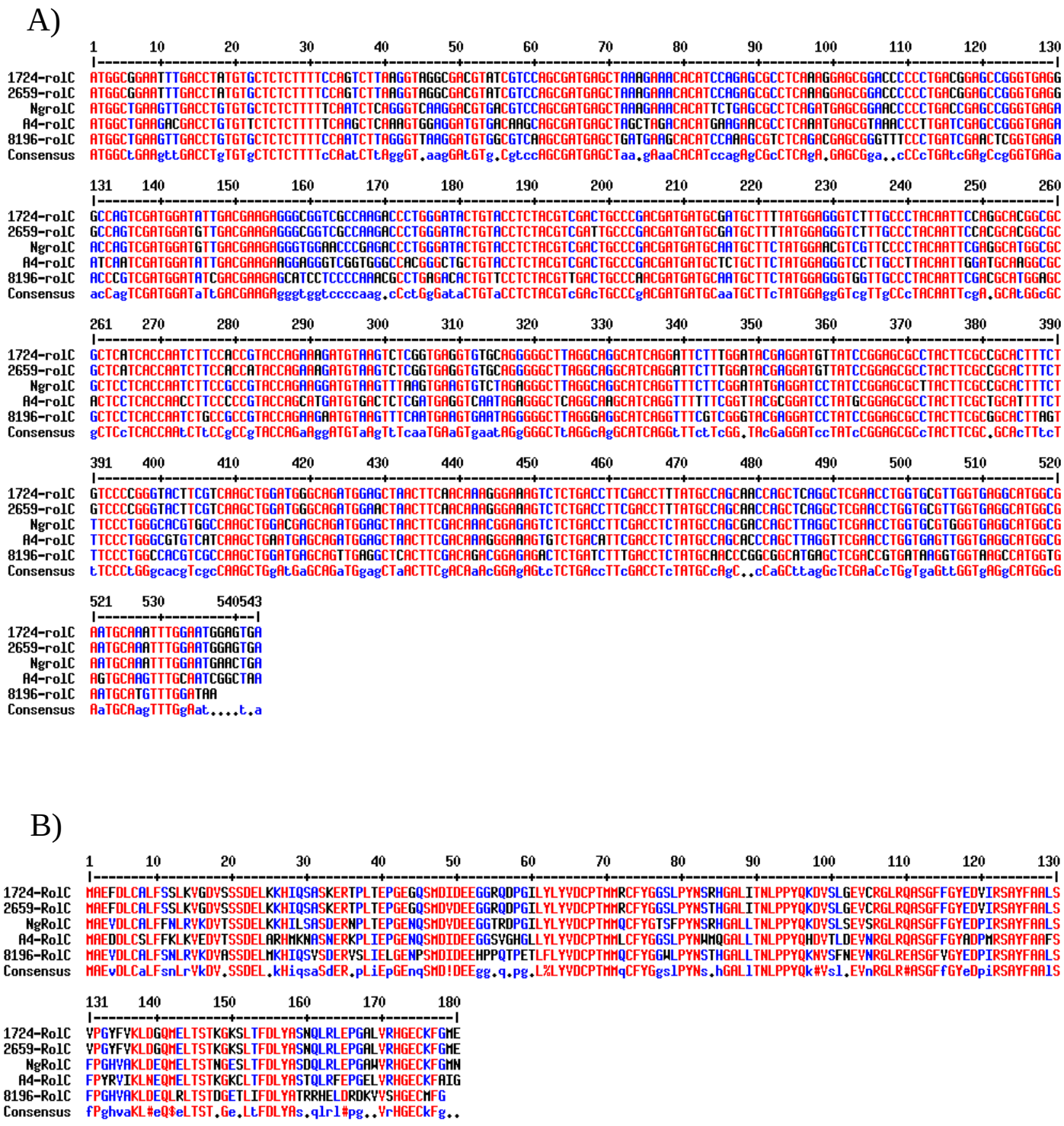

Figure 12. The alignment of the A) DNA and B) amino acid sequences of the 4 *rolC* genes (1724-*rolC*, 2659-*rolC*, A4-*rolC* and 8196-*rolC*) and the homologous sequence in the genome of *N. glauca* (*NgrolC*).

sequences of four *rolC*s (1724-*rolC*, 2659-*rolC*, A4-*rolC* and 8196-*rolC*) and the homologous sequence in the genome of *N. glauca* (*NgrolC*). Most studies of the *rolC* gene have been carried out with strain A4 of *A. rhizogenes*, we also used this gene in our experiments.

3. 3.1. *rolC* affects morphogenesis

The first *rolC* studies by White and co-workers (1985) showed that the inactivation of the *rolC* gene in *A. rhizogenes* (strain A4) leads to the attenuation of root-induction on Kalanchoe leaves. Further studies demonstrated that A4-*rolC* by itself, and under the control of the 35S promoter was able to generate roots on tobacco leaves that were more branched than *rolA*- or *rolB*-induced roots, but no such effect was found on Kalanchoe leaves (Spena *et al.,* 1987). To understand the effect of the gene in the plant, A4-*rolC* transgenic tobacco plants with *rolC* under control of its own promoter were generated. The phenotypic alterations of these plants consist of reduced apical dominance leading to increased branching, dwarfed plants with short internodes, lanceolate leaves, early flowering, reduced flower size and reduced pollen production (Schmülling *et al.,* 1988). Dwarfing was caused by reduced epidermal cell size in internodes (Oono *et al.,* 1990). In addition root production was increased compared to untransformed plants, but decreased compared to plants transformed with the complete set of *rol* genes (Palazòn *et al.,* 1998). Plants expressing 35S::*rolC* were male sterile and had a more pronounced phenotype including pale green leaves (Schmülling *et al.,* 1988). Similar alterations have been also reported for other A4-*rolC* transformed species, such as potato (*Solanum tuberosum*) (Fladung, 1990; Schmülling *et al.,* 1993), *Atropa belladonna*, where increased and advanced flowering was noteworthy (Kurioka *et al.,* 1992), and in woody species like hybrid aspen in which stem fasciation resulting from larger stem apices with increased numbers of small cells was also observed, a phenotype that has not been reported for *rolC* transformants elsewhere (Nilsson *et al.,* 1996a and b), and pear tree (Bell *et al.,* 1999). This gene has also been used to transform strawberry plants in which increased fruit weight and increased tolerance to infection by *Phytophthora cactorum* were observed (Landi *et al.,* 2009) and several ornamental flowering species (Casanova *et al.,* 2003; 2004; Christensen *et al.,* 2008) in order to obtain new morphological traits with relevance to ornamental value, like dwarf plants with bushy phenotypes.

3.3.2. *rolC* and plant hormones

3.3.2.1. Cytokinins

Some of the morphological phenotypes of *rolC*-transformed plants, such as reduced apical dominance and enhanced lateral shoot development, are suggestive of cytokinin activity (Schmülling *et al.,* 1988; Zuker *et al.,* 2001).

Early studies reported that the RolC protein (produced in *E. coli*) had a β-glucosidase activity capable of releasing active free forms of cytokinins from their inactive glucosidic conjugates (Estruch *et al.,* 1991a). However, this observation was based on an *in vitro* activity assay. Attempts to measure cytokinin levels *in planta* have produced conflicting results. In 35S::A4-*rolC* tobacco plants quantification of cytokinin content showed that not only the free cytokinin levels were not enhanced (Nilsson *et al.,* 1993; Schmülling *et al.,* 1993; Faiss *et al.,* 1996), but the concentration of some cytokinins, i.e. isopentenyladenosine (iPA, one of the first compounds in the cytokinin biosynthesis pathway) were even strongly reduced (Nilsson *et al.,* 1993). Moreover the concentration of cytokinin conjugates did not change (Nilsson *et al.,* 1996a; Faiss *et al.,* 1996). In hybrid aspen however, an increase in cytokinins of the Z-family has been reported (Nilsson *et al.,* 1996a).

It has been reported that the plant and bacterial glucosidases are high-molecular mass proteins (50–200 kDa) (Sinnott 1990). However, *rolC* genes encode proteins with a molecular mass of around 20 kDa (Slightom *et al.,* 1985). The studies of Bulgakov *et al.,* (2002) on β-glucosidase activity in *rolC* transformed callus culture from *P. ginseng*, using gel-permeation experiments showed that there is no β-glucosidase activity in the fractions containing proteins with molecular masses of around 20 kDa.

These results invalidate the hypothesis of a cytokinin glucosidase activity of the *rolC* gene product. In addition, it should be pointed out that despite the presence of some similarities between *rolC* and *ipt* transgenic tobacco plants, these plants exhibit several differences compared to each other specifically in their rooting system and the color of their leaves. The *ipt* transformants present an extremely reduced root system and dark green leaves, due to increased chlorophyll levels (Beinsberger *et al.,* 1991; Grossman *et al.,* 1991), whereas the transgenic 35S::*rolC* plants showed very strongly branching roots (Faiss *et al.,* 1996) and pale-green leaves, caused by a decrease in chlorophyll content (Schmülling *et al.,* 1988; Fladung, 1990). It has been reported that a cross between a dark-green 35S::*ipt* plant and a pale-green 35S::*rolC* plant produces hybrids with a general RolC phenotype, except for normally colored leaves. In other words, the pale-green color of *rolC* expressing leaves is

restored to the normal green color by cytokinins from the *ipt* plants, but in all other aspects the RolC phenotype is dominant over the Ipt phenotype. Thus, the pale-green color of *rolC* leaves is probably due to reduced cytokinin levels, but other aspects of the RolC phenotype are not directly caused by altered cytokinin pools (Nilsson *et al.,* 1993).

3.3.2.2. Auxins

The root stimulating action of *rolC* might be explained by an auxin-like effect of the gene (Schmülling *et al.,* 1988; Zuker *et al.,* 2001; Casanova *et al.,* 2003). The measurement of IAA levels in 35S::A4-*rolC* tobacco, potato and carnation plants showed no differences with respect to control plants (Nilsson *et al.,* 1993; Schmülling *et al.,* 1993, Casanova *et al.,* 2004) and there was even an IAA decrease in *rolC* hybrid aspen and chrysanthemum plants (Nilsson *et al.,* 1996a; Mitiouchkina and Dolgov, 2000). Thus, the auxin-like effect might be caused by an increase in auxin sensitivity. Indeed, the measurement of transmembrane hyperpolarization in response to auxin showed that *rolC* transgenic tobacco protoplasts are more sensitive to auxin than their wild-type counterparts (Maurel *et al.,* 1991).

3.3.2.3. Gibberellic acids

The most striking morphological alteration in *rolC* transgenic plants is the reduced size of the plant with shortened internodes. It is well known that the gibberellins increase the distance between the internodes (Graebe, 1987). Therefore the presence of short internodes in *rolC* plants could reflect a lower GA activity. Quantification of some GAs levels in A4-*rolC* and wild-type tobacco plants revealed that the general level of gibberellins, specifically GA1, was decreased in *rolC* tobacco plants (Schmülling *et al.,* 1993). The results obtained by Nilsson and co-workers (1993) showed an increase in GA19, indicating that RolC might block the activity of the GA19 oxidase, one of the most important enzymes in the gibberellin biosynthesis pathway, which converts GA19 to GA20. They also found a higher level of GA in leaves than in internodes. Application of GA3 on 35S::A4-*rolC* tobacco plants partially restored internode length but other morphological alterations were not reversed (Schmülling *et al.,* 1993). These results indicate that the developmental changes caused by A4-*rolC* can not be explained entirely by an alteration in the metabolism of gibberellins.

3.3.3. *rolC* expression pattern and RolC localization

Expression of *rolC* shows a complex pattern of regulation that varies depending upon the presence of other T-DNA sequences. In the absence of other T-DNA genes, the promoter of *rolC* confers root- and stem-specific expression and weak leaf-specific expression in tobacco plants (Spena *et al.,* 1987; Schmülling *et al.,* 1988; 1989). However, when expressed in the presence of the whole T-DNA, *rolC* is found to be also highly expressed in tobacco leaves (Durand-Tardif *et al.,* 1985; Leach and Aoyagi, 1991). In PC-GUS tobacco plants (carrying the *gus* gene under the control of the *rolC* promoter), promoter activity is detectable mainly in the phloem cells of roots, slightly less in the phloem tissues of the stem and in low quantity in the leaves. Phloem-specific expression of the *rolC* promoter is also reported in other plants such as transgenic PC-GUS rice (Matsuki *et al.,* 1989) and PC-GUS hybrid aspen plants (Nilsson *et al.,* 1996b). In the latter case, the activity of the *rolC* promoter was found in the companion cells of the phloem. Under control of the 35S promoter, *rolC* leads to severe developmental alterations in tobacco (Schmülling *et al.,* 1988). In 35S::A4-*rolC* tobacco cells, the RolC protein localizes in the cytosol (Estruch *et al.,* 1991c).

3.3.4. The 5' non-coding region of the *rolC* gene

In order to characterize the *rolC* upstream regulatory region, the GUS reporter gene was placed under the control of several fragments of the *rolC* promoter. The analysis of different deletion mutants demonstrated that a cis-acting DNA region required for phloem-specific expression in leaves and roots of *N. tabacum* localizes within the -153 region, and a minimum region needed for the expression in the seed embryo was located around position -120 (Sugaya and Uchimiya, 1992).

In transgenic PC-GUS carrot cell culture the activity of the *rolC* promoter increased during somatic embryogenesis (Fujii and Uchimiya, 1991). Subsequent analysis of the *rolC* promoter region revealed that the proximal −255 bp upstream region is involved in activation of the gene during somatic embryogenesis (Fujii *et al.,* 1994).

Although *rolC* expression is generally limited to phloem cells, the gene can be induced in any cell that has been placed on a medium with sucrose (Nilsson *et al.,* 1996). It has been shown that the sucrose responsive region of the *rolC* promoter is located between - 135 and - 94 bp, and is particularly rich in AT (Yokoyama *et al.,* 1994; Faiss *et al.,* 1996).

The locations of cis-acting elements for the phloem-specific expression and sucrose responsiveness overlap with each other indicating that the two are linked. In phloem cells,

high concentrations of sucrose are encountered owing to ongoing translocation of photosynthates from source to sink tissues; therefore, the sucrose inducibility of the promoter clearly makes sense.

3.3.5. *rolC* and meristem induction

The capacity of the *rolC* gene to induce root formation has been already mentioned for *rolC* transgenic tobacco leaves (Spena *et al.,* 1987; Schmülling *et al.,* 1988). In addition to tobacco, *rolC*-transformed *Atropa belladonna* leaves (Bonhomme *et al.,* 2000) and *rolC*-transformed carnation petals and leaves (Casanova *et al.,* 2004) show root induction. The *rolC* gene increases the rooting rate of trifoliate orange and Japanese persimmon cuttings, which, moreover, have potential as dwarfing rootstocks (Kaneyoshi and Kobayashi, 1999; Koshita *et al.,* 2002). In addition, adventitious shoot regeneration is also observed in *rolC*-transformed carnation petals and leaves (Casanova *et al.,* 2004) and in *rolC*-transformed *Panax ginseng* callus culture. In the last case, the expression of *rolC* induces also somatic embryogenesis (Gorpenchenko *et al.,* 2006). These results suggest that the *rolC* gene, similarly to *rolB*, has the potential to induce the formation of pluripotent meristematic cells.

Concerning the *rolC*-induced embryogenesis, it has been found that this induction is calcium-dependent and involves the CDPKs signalling pathway. Indeed, it has been shown that expression of *rolC* in transgenic callus cultures resulted in changes of expression of different *CDPK* genes (Kiselev *et al.,* 2008).

3.3.6. Cell-autonomous behavior of the *rolC* gene

It has been reported that the *rolC* gene product behaves in a cell-autonomous manner, indicating that the biological effects induced by this gene are not due to the *rolC*-mediated synthesis of a transported growth factor (Spena *et al.,* 1989). Three important types of evidence support this finding. First, *rolC*-induced roots, obtained via leaf disc inoculation, are restricted to transformed cells, and do not include neighbouring, untransformed cells (Oono *et al.,* 1987; Schmülling *et al.,* 1988). This indicates that, as far as rhizogenesis is concerned, the *rolC* gene product is neither diffusible nor transported. Second, in grafts, *rolC* transgenic shoots or root stocks do not alter the growth habit of normal root stocks or shoots, respectively (Estruch *et al.,* 1991c). Third, in 35S::*rolC* tobacco plants which carry a transposable element between the promoter and the *rolC* gene, spontaneous excision of the

transposon leads to pale green sectors in the leaves with sharp borders, that correspond to zones of expression. This indicates that with respect to leaf pigmentation the *rolC* gene product also behaves cell-autonomously (Spena *et al.,* 1989).

However, one finding contradicts the cell-autonomous behavour of the RolC protein presented above. It has been shown that in chimeric *rolC* tobacco plants in which the reproductive tissues were non-transgenic, the flowers were male sterile as is typical for 35S::*rolC* transgenic tobacco plants, indicating that expression of the *rolC* gene in transgenic parts of the plant has influenced the non-transgenic parts (Schmüllling and Schell, 1993).

3.3.7. *rolC* and secondary metabolites

Similarly to *rolB*, *rolC* can stimulate the production of high levels of secondary metabolites. In transformed plants and plant cell cultures, the *rolC* gene induces the production of tropane alkaloids (Bonhomme *et al.,* 2000), pyridine alkaloids, indole alkaloids (Palazón *et al.,* 1998), ginsenosides (Bulgakov *et al.,* 1998) and anthraquinone phytoalexins (Bulgakov *et al.,* 2002; Shkryl *et al.,* 2007). In the case of anthraquinone biosynthesis stimulation in *Rubia cordifolia* callus cultures, methyl jasmonate and salicylic acid have been shown to increase strongly anthraquinone accumulation in both transgenic and non-transgenic calluses, whereas ethylene, Ca^{2+} channel blockers, diphenylene iodonium (an inhibitor of NADPH oxidase) and staurosporine (a CDPK inhibitor) did not affect this biosynthesis either in transgenic or in non-transformed cultures. Treatment of the cultures by cantharidin, the protein phosphatase inhibitor, resulted in massive induction of anthraquinone accumulation in transgenic cultures only (Bulgakov *et al.,* 2004; 2005). These data indicate that the activator function of *rolC* in anthraquinone biosynthesis is independent of ethylene, jasmonic, and salicylic acid mediated and calcium-dependent NADPH oxidase and CDPKs pathways and suggest the involvement of a cantharidin-sensitive protein phosphorylation mechanism.

3.3.8. *rolC*-induced defense reactions

Induction of secondary metabolites biosynthesis by *rol* genes suggests that these genes might also be involved in plant defense reactions too. Kiselev and co-workers (2006) showed that overexpression of *rolC* in *P. ginseng* root and callus cultures resulted in induction of a member of the PR2 family of plant defense proteins, β-1,3-glucanase. It can be hypothesized that activation of defense reactions in transformed plants could provide advantages for *A.*

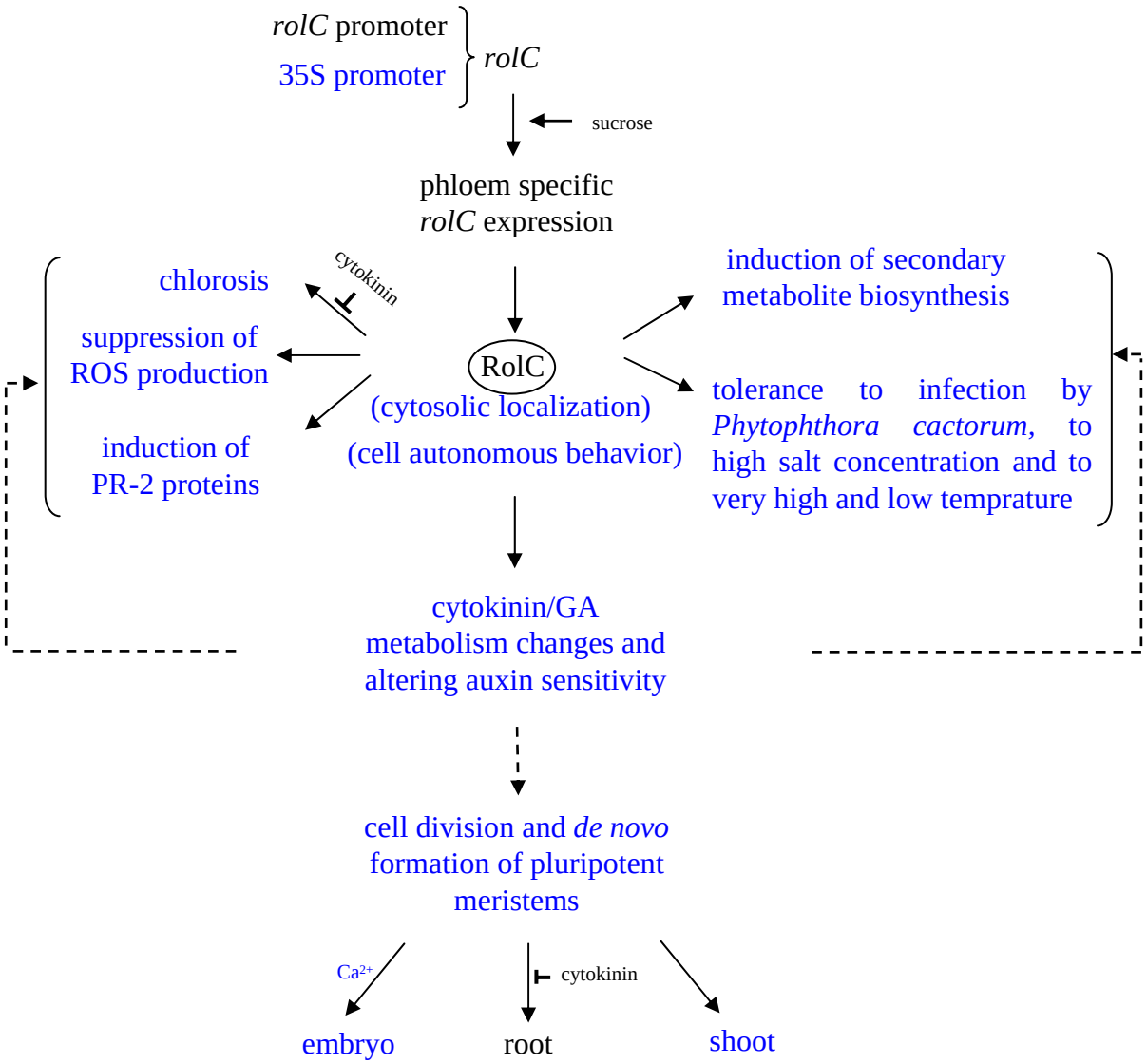

Figure 13. A model summarizing *rolC*-induced effects reported until now. The parts indicated in blue have only been described for 35S-*rolC* constructs. . The *rolC* promoter is induced by sucrose and confers phloem-specific expression. RolC localizes in the cytosol and behaves cell autonomously as far as root-induction and leaf chlorosis are concerned. These two phenomena are inhibited by cytokinin. RolC causes different responses in plants expressing this gene including chlorosis, suppression of ROS production, induction of secondary metabolites, induction of PR-2 proteins and tolerance to infection by *Phytophthora cactorum*, to high salt concentrations and to high and low temperature. In addition this gene causes changes in the metabolism of cytokinin and gibberellic acids (GA) and also alters auxin sensitivity. The changes in hormone balance could explain the appearance of such different responses. The meristems induced by *rolC* are pluripotent and capable to convert to root, shoot or somatic embryo. The effect of the gene on somatic embryogenesis is calcium-dependent.

rhizogenes by inhibiting competing soil borne microorganisms, such as fungi. It has been demonstrated that the expression of the *rolC* gene in genetically modified hybrid aspen plants could suppress mycorrhizal colonization of roots by four fungal species (Kaldorf *et al.,* 2002).

The production of Reactive Oxygen Species (ROS) is one of the defense reactions that is triggered by several stresses in animal and plant cells. Although the influence of the *rolC* gene on plant defense reactions has been demonstrated, it also has a strong inhibitory effect on ROS levels in transformed cells of *R. cordifolia* (Bulgakov *et al.,* 2008). The ROS-suppressing function of *rolC* is similar to that of NF-κB protein which represses apoptosis by ROS suppression, thereby ensuring survival of tumors (Sakon *et al.,* 2003).

rolC-expressing *R. cordifolia* calli have been found to be resistant to high salt concentrations and to high and low temperature conditions. Since it is known that ROS contribute to stress damage in the cells (Xiong *et al.,* 2002) and taking into account that *rolC* acts as a ROS suppressor in transformed cells, this gene may prevent oxidative damage and therefore permit the growth of the calli under the different stress conditions.

3.3.9. Biotechnological use of the *rolC* gene promoter

The phloem-specific expression of the *rolC* promoter makes it a useful tool in some biotechnological programs on pathogen resistance. A number of plant viruses, such as luteoviruses, reoviruses and most geminiviruses replicate exclusively in phloem-associated tissues and are transmitted by hemipteran vectors. Therefore the phloem-specific expression of an insecticidal gene that is toxic to hemipteran vectors can directly control the transmission of such viruses. It has been demonstrated that in potato plants, the expression of the coat protein gene from the phloem-limited Potato leafroll luteovirus under control of the *rolC* gene promoter confers virus-resistance to the plant (Graham *et al.,* 1997).

Similarly, in transgenic rice, tobacco and chickpea plants, the *ASAL* (*Allium sativum* leaf agglutinin) gene which encodes a plant lectin with insecticidal activity, placed under control of the *rolC* promoter, confers resistance against various hemipteran pests (Saha *et al.,* 2007).

3.3.10. A model for *rolC*-induced effects

Figure 13 presents a model summarizing the *rolC* gene characteristics and the various functions of its encoded protein that are reported until now.

3.3.11. *rolC* and CDPKs

The complex picture revealed by the various *rolC* gene studies (summarized in the model) raises the question as to how *rolC* could affect such different processes.

Because CDPKs are commonly accepted as molecules that mediate cross-talk between signaling pathways (Harper *et al.,* 2005) and on the other hand in *rolC* expressing *P. ginseng, V. amurensis* and *E. sericeum* cell cultures, changes in expression of different *CDPK* genes have been reported (Kiselev *et al.,* 2008; 2009; Bulgakov *et al.,* 2008), Bulgakov and co-workers (2008) proposed that the numerous apparently unrelated and largely unexplained effects seen in *rolC*-transformed cells could be explained by modulation of *CDPKs* expression and/or CDPKs activity. However, the possibility cannot be excluded that the physiological changes induced by the expression of the gene could also have an important role in the appearance of such different responses.

Many of these effects could be secondary, resulting from successive changes in structure and metabolism. In order to avoid such effects as much as possible, we have used inducible gene constructs allowing us to test *rolC* activity under normal growth conditions. In addition, we have tested the *rolC* gene of tobacco, *trolC* (Chapter I).

3.4. The *rolD* gene

rolD is found only in the T_L-DNA of agropine Ri plasmids, the other Ri plasmids carrying a single or split T-DNA do not contain this gene (Christey, 2001). The main phenotype of *rolD* expressing tobacco plants is early flowering and reduced rooting. Due to the early transition from vegetative to reproductive growth, some *rolD* expressing plants develop no vegetative buds (Mauro *et al.,* 1996). *rolD* encodes a cytosolic protein with ornithine cyclo-deaminase (OCD) activity, capable to convert ornithine to proline (Trovato *et al.,* 2001). Since high proline levels are found in flowers, some *rolD* phenotypes (early flowering) have been proposed to be related to proline production (Trovato *et al.,* 2001). Expression of *rolD* is not tissue specific, but is developmentally regulated. The promoter activity is seen in the elongating and differentiating tissues of each organ in adult plants but not in apical meristems. As the plant ages, expression decreases and ceases at senescence (Trovato *et al.,* 1997). Like *rolB*, *rolD* is considered as a late-auxin induced gene with a lag time of at least four hours. However, while *rolB* promoter activation strengthens with increasing auxin concentration, induction of the *rolD* promoter reaches a maximum at 1 μM IAA and decreases at higher

auxin concentrations. Similar to *rolB*, the *rolD* promoter contains a Dof-binding element involved in auxin induction. (Mauro *et al.,* 2002). About the possible role(s) of *rolD* on the meristems, Altumara (2004) suggested that it is involved in a later stage of the meristem formation than *rolB* and determines the fate of the meristem.

4. The other *plast* genes (with the exception of *rolB* and *rolC*)

4.1. The 5' part of the *orf8* gene

On the T-DNA of *A. rhizogenes,* the *orf8* gene has the longest sequence and is one of the most conserved genes between different strains (Ouartsi *et al.,* 2004). Different laboratories have transformed tobacco plants with a 35S::*orf8* construct; however the resulting plants did not exhibit similar phenotypes. Lemcke and colleagues (2000) reported that 35S::A4-*orf8* tobacco plants (*N. tabacum* cv. SRI) did not show morphological alterations, however an increase in IAM production was observed in these plants compared to untransformed tobaccos and the protein extracts from A4-*orf8* expressing *E. coli* bacteria showed tryptophan mono-oxygenase (IAM) activity. 35S::A4-*orf8* tobacco plants (*N. tabacum* cv. Samsun nn) from our group showed alterations such as stunted growth and rough mottled leaves and at later stages leaves became dark green with intercostal chlorosis (Umber *et al.,* 2005). Ouartsi and colleagues (2004) reported that in 35S::2659-*orf8* tobacco seedlings (*N. tabacum* cv. Xanthi XHFD8) the cotyledons showed thin and lanceolated shape but adult plants were not affected in their growth and development. It is possible that these phenotypic differences are due to the use of different plant cultivars and/or to the use of different *orf8* gene sequences derived from different Ri plasmids.

Similar to *rolB* and *rolD, orf8* is induced by auxin with a lag time of 6 hours. This indicates that *orf8* belongs to the category of the late-auxin response genes. However, no conserved Dof binding motif, involved in auxin responsiveness, has been found within the promoter. It has been demonstrated that *orf8* expressing tobacco seedlings are able to grow on media with concentrations of auxin that completely inhibit the growth of wild-type seedlings (Lemcke and Schmülling, 1998b; Ouartsi *et al.,* 2004).

The protein encoded by *orf8* has a particular structure and is probably a fusion protein. The N-terminal domain (NOrf8) shows homology with various Plast proteins such as RolB, RolC, and 6B. The C-terminal part (COrf8) is similar to the IaaM proteins of various other bacteria (Levesque *et al.,* 1988; Yamada *et al.,* 1985). Experiments in our laboratory using

transient leaf patch expression assays showed that neither the full length Orf8 protein, nor the C-terminal domain (COrf8) produced IAM. When the N-terminal domain (NOrf8) was expressed in tobacco under control of the 35S promoter, transgenic plants showed strong growth reduction, leaf chlorosis and high levels of sugars (glucose, fructose and sucrose) and starch (Otten and Helfer, 2001). Indeed, in these plants the sucrose transport from source to sink leaves is blocked and the plants resemble plants deficient in a sucrose transporter (NtSUT1). Grafting experiments demonstrated that sugar unloading was not impaired because 35S::N*orf8* shoots grafted onto wild-type rootstocks resumed growth due to sugar transport from the rootstock (Umber *et al.,* 2002). 35S::C*orf8* tobacco did not show any phenotypic alteration but accumulated less starch than untransformed plants (Umber *et al.,* 2002). Crosses between 35S::N*orf8* and 35S::C*orf8* tobacco lines generated plants in which sucrose and starch levels were significantly lower than in N*orf8* plants. This indicates that C*orf8* can reduce carbohydrate accumulation in both N*orf8* and wild-type background (Umber *et al.,* 2005).

4.2. Genes *orf13* and *orf14*

Like *orf8*, the *orf13* and *orf14* genes are also highly conserved between *A. rhizogenes* strains (Stieger *et al.,* 2004). It has been demonstrated that the inoculation of carrot discs with an Agrobacterium strain carrying A4-*rolABC* genes is insufficient to induce rooting and the presence of T_R-DNA *aux* genes or T_L-DNA *orf13* and *orf14* is necessary for rooting (Cardarelli *et al.,* 1987; Capone *et al.,* 1989b). In tobacco leaf discs *rolB* and *orf13* together were capable to elicit rooting nearly as well as the full length of T_L-DNA (Aoki and Syono, 1999b). These observations led to the idea that *orf13* and/or *orf14* may be involved in auxin biosynthesis. However, *orf13* and *orf14* show no homology with auxin biosynthetic genes. Furthermore, unlike the auxin biosynthesis genes (Camilleri and Jouanin, 1991), *orf13* and *orf14* cannot induce roots on tobacco leaf discs (Cardarelli *et al.,* 1987).

4.2.1. The *orf13* gene

It has been demonstrated that the *orf13* gene is capable to induce cell proliferation (green callus) on carrot and tobacco leaf discs (Hansen *et al.,* 1993; Fründt *et al.,* 1998). In transgenic tobacco plants, expression of *orf13* under the control of the 35S promoter provokes morphological alterations including growth reduction, short internodes, poorly developed roots, reduced apical dominance, wrinkled dark green leaves and asymmetric flowers (Hansen

et al., 1993; Lemcke and Schmülling, 1998). The growth reduction results from the inhibition of cell division in the apical meristems (Lemcke and Schmüling, 1998). Similar morphological changes have been observed in plants with elevated cytokinin levels (Medford *et al.,* 1989), however, no increase in cytokinin production was observed in *orf13*-expressing tobacco plants (Lemcke and Schmülling, 1998). Early grafting experiments showed that the *orf13* phenotype is transmissible from transgenic scions to the offshoots of wild-type root-stocks, therefore *orf13* expression must generate a diffusible substance (Hansen *et al.,* 1993). However, transmission by grafting could not be confirmed by others (Lemcke and Schmülling, 1998). The *orf13* promoter is strongly wound-inducible in most tissues and the addition of auxin, but not cytokinin, after wounding increases the activity of the promoter. This wound-induced expression is seen only in tissues immediately surrounding the wound and is not systemic. Repeats of an 11 bp motif have been identified in the promoter that may be responsible for wound inducibility of the promoter (Hansen *et al.,* 1997).

4.2.2 The *orf14* gene

Only few experiments have been done to characterize *orf14*. As mentioned above this gene acts synergistically with the *rol* genes and *orf13* to improve root induction (Capone *et al.,* 1989; Aoki and Syono, 1999b). It is not capable of inducing cell proliferation on tobacco leaf discs and its overexpression under control of the 35S promoter does not produce any morphological alteration (Lemcke and Schmülling, 1998b). Thus it does not seem to have oncogenic properties by itself.

4.3. Gene *6a*

6a genes are localized on the T-DNA of *A. tumefaciens* and *A. vitis* (Garfinkel *et al.,* 1981; Hooykaas *et al.,* 1988). It has been reported that this gene favours opine secretion and has no direct role in tumorigenesis (Messens *et al.,* 1985).

4.4. Gene *6b*

6b genes are localized on the T-DNA of *A. tumefaciens* and *A. vitis* (Garfinkel *et al.,* 1981; Hooykaas *et al.,* 1988) and considered as oncogenes capable of inducing tumors on several plant species such as *N. glauca* and *Kalanchoe tubiflora* (Hooykaas *et al.,* 1988; Tinland *et al.,* 1989), grapevine (Huss *et al.,* 1990), and *N. rustica* (Paulus *et al.,* 1991) and producing

morphological abnormalities in transgenic *N. rustica* (Tinland *et al.,* 1992), *N. tabacum* (Helfer *et al.,* 2003; Grémillon *et al.,* 2004; Clément *et al.,* 2006; Terakura *et al.,* 2006) and *A. thaliana* (Helfer *et al.,* 2003; Terakura *et al.,* 2006). The oncogenic properties between *6b* variants are different. This could be due to differences in their non-coding regions, coding regions or both. According to Helfer and colleagues (2002) the tumorigenic capacity of C58-*6b* (*A. tumefaciens*) and CG474-*6b* (*A. vitis*) is relatively weak, that of Ach5-*6b* (*A. tumefaciens*) and AB4-*6b* (*A. vitis*) is intermediate and the S4-*6b* (*A. vitis*) and Tm4-*6b* (*A. vitis*) genes have the strongest oncogenic activity. Using a hybrid structure between Ach5-*6b* and Tm4-*6b*, the N-terminal region of the Tm4-6B protein and especially the residues 58T and 59G have been identified as major oncogenic determinants (Helfer *et al.,* 2003).

The *6b* promoter from strain Bo542 is induced by wounding and auxins, not by cytokinins and its activity has been detected primarily in roots (Bagyan *et al.,* 1995). AKE10-*6b* is strongly expressed in small shoots, but not in mature plants (Wabiko and Minemura, 1996) and wounding, auxins or cytokinin treatments have no effect on its expression. C58-*6b* induction needs a long-term cytokinin treatment (Gális *et al.,* 1999).

Tobacco plants expressing the C58-*6b* gene were phenotypically normal (Gális *et al.,* 1999). *N. rustica* plants carrying a heat shock-inducible-Tm4-*6b* gene formed tubular leaves, adventitious shoots on hypocotyls and thick roots (Tinland *et al.,* 1992). In dexamethasone inducible-Tm4-*6b* tobacco plants, induction of young plants led to enations (leaf blade outgrowths on the abaxial side) and strong radial root growth (Grémillon *et al.,* 2004). The AKE10-*6b* gene led to the formation of asymmetric leaves and enations in tobacco plants (Wabiko and Minemura, 1996) and 2x35S::AB4-*6b* tobacco and Arabidopsis plants showed morphological modifications such as enations and catacorollas (a special type of double flower) in tobacco and long tubular structures on the abaxial leaf surface (equivalent to enations in tobacco leaves) in Arabidopsis leaves (Helfer *et al.,* 2003).

The enations and strong radial root growth result from additional cell division on the lower leaf side (Helfer *et al.,* 2003; Terakura *et al.,* 2006) and in the root pericycle and vasculature, respectively (Grémillon *et al.,* 2004). It has been demonstrated that the expression of several genes that are related to cell division and meristem functions such as the *KNOX* (KNOTTED1-like homeobox) family of genes, cell cycle regulating genes (*CYCLINB, CYCD3,1*) and M phase-specific genes, was up-regulated in *6b* expressing plants (Terakura *et al.,* 2006; 2007).

In tobacco, the 6B protein localizes in the nucleus and interacts with the putative

transcription factors NtSIP1 (*N. tabacum* Six-b Interacting Protein1) and NtSIP2 (Kitakura *et al.,* 2002; 2008) and histone H3 (Terakura *et al.,* 2007). Therefore it is plausible that it functions as a transcriptional co-activator/mediator to activate transcription of several genes or acts as a histone chaperone capable of remodeling the chromatin structure making it more accessible to the transcription machinery and thus leading to up-regulation of some genes.

Grafting experiments showed that the *6b*-induced phenotype can be transmitted from *6b* plants to non-transformed rootstocks or scions and in leaf patch assays, the 6B protein was found to move through leaves and enter the vascular system (Helfer *et al.,* 2003).

In addition to stimulation of cell division, 6B induces also cell expansion by increasing sugar concentrations (Clément *et al.,* 2006), abnormal accumulation of phenolics has been demonstrated to be a secondary effect (Clément *et al.,* 2007).

6b-induced growth modifications have been compared to phytohormone effects. Spanier *et al.,* (1989) reported that Ach5-*6b* reduced the sensitivity of plant cells to cytokinin and modulated the inductive effects of this hormone on shoot development. Bonnard and colleagues (1989) demonstrated that the Tm4-*6b* gene, similar to auxin synthesis genes, could inhibit *ipt*-induced shoot formation on tobacco stems, suggesting that the growth-modifying effects of *6b* may result from an increase in auxin levels. However, this gene could not replace exogenous auxins in root induction assay (Tinland *et al.,* 1990; Wabiko and Minemura, 1996). Moreover, the morphology of *6b* plants was not typical of an auxin phenotype (Grémillon *et al.,* 2004; Helfer *et al.,* 2003; Tinland *et al.,* 1992). Gális and colleagues (2002) reported an increase in IAA levels in C58-*6b* transgenic tobacco plants and attributed this to the accumulation of flavonoids that protect IAA against degradation. In contrast, Clément and co-workers did not found any difference in IAA level nor in the expression of IAA-induced genes in Tm4-*6b* tobacco plants (Clément *et al.,* 2006). These observations suggest that *6b* genes induce only part of the auxin response repertoire.

4.5. Gene *5*

The Ach5-*5* gene is carried on the T_L-DNA of octopine type plasmid pTiAch5 and encodes a protein of 26 kDa. Expressed in *E. coli*, the protein shows enzymatic activity which catalyzes the synthesis of indole-3-lactate (ILA) from tryptophan (Körber *et al.,* 1991). ILA is similar to IAA, it has a low auxin activity (Scott, 1984) and competes with IAA in binding to receptors of auxin. Addition of ILA to callus culture facilitates shoot differentiation. Tobacco

plants expressing Ach5-*5* under control of the 35S promoter showed growth reduction and contained a lower IAA and a higher ILA level compared to control plants. The high level of ILA might reduce the toxicity of auxin as suggested by the fact that the seeds of these plants are resistant to exogenously supplied IAA (Körber *et al.,* 1991). The expression of the Ach5-*5* gene is localized mainly in the calli, tumors and stems (Koncz and Schell, 1986). The gene is induced by auxin but this induction is repressed by ILA suggesting that it is regulated by a negative feedback loop (Körber *et al.,* 1991). Thus it appears that the Ach5-*5* gene can negatively control auxin responsiveness, thereby increasing the cytokinin:auxin ratio in tumors and increasing shoot development.
Although these results suggest that the Ach5-*5* gene product has enzymatic activity, these results have not been confirmed by other groups.

4.6. Gene *lso*

The limited host range *A. tumefaciens* strain AB2/73 is capable of inducing tumors on only a few plant species including *Lippia canescens, Kalanchoe tubiflora* and *N. glauca* (Otten and Schmidt, 1998). The single and very small T-DNA of pTiAB2/73 contains two ORFs called *lsn* (Lippia strain nopaline synthase-like) and *lso* (Lippia strain oncogene). *A. tumefaciens* strains carrying only *lso* induce small tumors on *Kalanchoe tubiflora* indicating that this gene does act as an oncogene, at least in some plant species (Otten and Schmidt, 1998).

4.7. Gene *e*

Gene *e* is present on the T-DNA of some nopaline strains of *A. tumefaciens*. It has been demonstrated that mutation of C58-*e* led to the formation of smaller or no tumors (depending on the host) suggesting that this gene possesses oncogenic activity.

4.8. Gene *c'*

Deletion of the left side of the T-DNA of pTiC58 (comprising the genes *a, b, c, c', d, e, f* and *acs*) induces tumours with very few shoots on *K. tubiflora* stems. It has been demonstrated that a fragment containing genes *c* and *c'* can partially restore shoot induction when co-infected with the deleted T-DNA (Otten *et al.,* 1999).

4.9. Gene *3'*

Gene *3'* is present on the T-DNA of pTiAB4 and the T_R-DNA of pTiAch5. Ach5-*3'* is capable of inducing small tumors on the stem of *K. tubiflora* (Otten *et al.,* 1999) and thus possesses a low oncogenic activity.

5. Remaining T-DNA genes

5.1. The *orf13a* gene

When Hansen and colleagues (1991) sequenced pRi8196, they identified another ORF between *orf13* and *orf14* on the opposite strand and called it *orf13a.* Subsequent analyses demonstrated that shorter homologs also exist in the same region of pRiA4 and pRi2659. Transcription of *orf13a* is tissue specific with a high expression level in leaf vascular tissues (Hansen *et al.,* 1994). Because the *orf13a* encoded protein contains motifs common to phosphorylated gene regulation proteins Hansen and colleagues (1994) hypothesized that this protein can interact with DNA.

5.2. The *orf3n* gene

The *orf3n* gene is present on the T_L-DNA of agropine-type Ri plasmids such as pRiA4 and pRiHRI. It does not belong to the *plast* gene family and does not have homology to other proteins. However since it modifies plant morphology, it will be described here. Expression of 35S::HRI-*orf3n* in transgenic tobacco plants caused alteration in internode length, leaf morphology and growth (Lemcke and Schmülling, 1998b). The leaves had long petioles, the internodes were shortened at the base of the plant and elongated towards the apex, and the tips of upper leaves were necrotic. Onset of flowering was delayed and the inflorescences were less dense than in wild-type.

Shoot formation from *orf3n* callus was inhibited on media containing cytokinins and auxins. Plantlets also showed decreased sensitivity to auxin and cytokinin, remaining small and forming less callus than controls (Lemcke and Schmülling, 1998b). These observations suggest that HRI-*orf3n* gene expression leads to a reduction of cell differentiation in the plant.

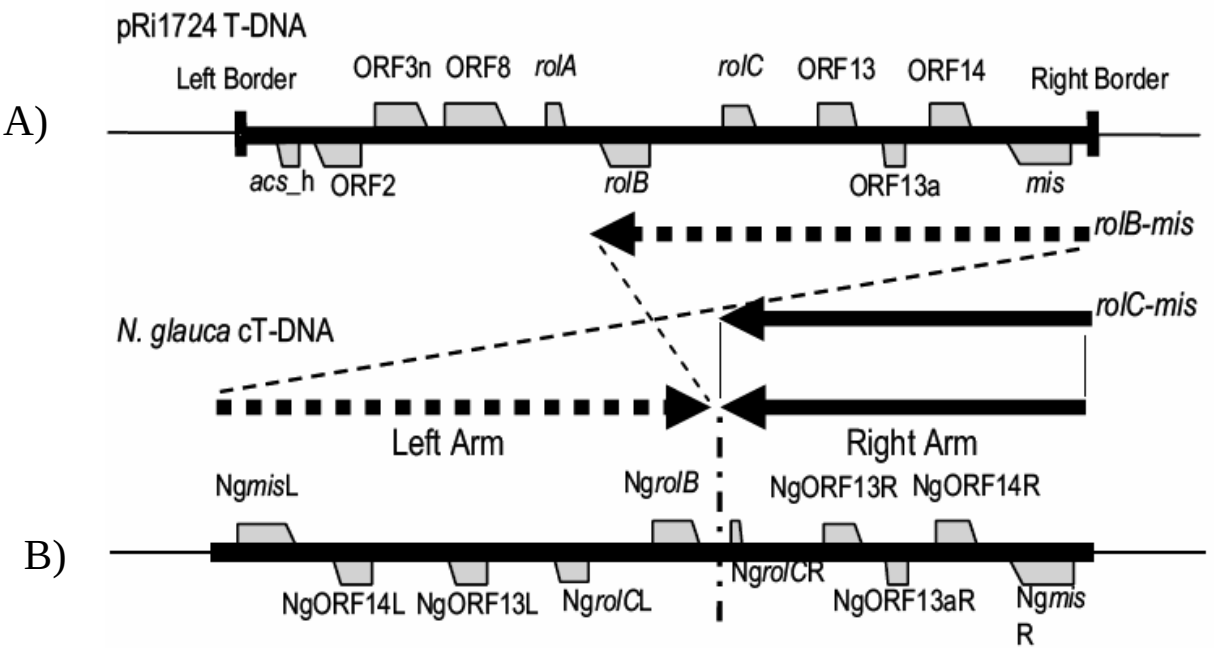

Figure 14. Structure of the cT-DNA in the *N. glauca* genome.
A) The T-DNA of pRi1724 and its flanking region are illustrated. A line and a broken line with an arrowhead indicate the different regions found in the cT-DNA. B) *N. glauca* cT-DNA and its flanking region is demonstrated. The lines and the broken line with an arrowhead indicate the imperfect inverted repeat (Adapted from Suzuki *et al.*, 2002).

III. Horizontal gene transfer from Agrobacterium to plant

1. Discovery of a cellular T-DNA (cT-DNA)

In early investigations of plant transformation by Agrobacterium, most researchers believed that there was no significant homology between T-DNA sequences and plant genomes. However, later studies revealed that the genome of some plants contain sequences similar to the Agrobacterium T-DNA genes.

White and co-workers (1982) detected a region homologous to the T_L-DNA of *A. rhizogenes* in the genome of *N. glauca* and called this DNA the cellular T-DNA (cT-DNA). Furner *et al.,* (1986) confirmed this discovery by analyzing different varieties of *N. glauca* collected worldwide. The same group found that the *N. glauca* cT-DNA is organized as an imperfect inverted repeat with a left and right arm. They determined the sequence of the *rolB-C* region and identified two *rol* genes, called *NgrolB* and *NgrolC* (*Ng* for ***N. glauca***), in this region. *NgrolC* was found on both the right and left arm (*NgrolC*R and *NgrolC*L), whereas *NgrolB* was only present on the left arm. They suggested that the cT-DNA resulted from infection by *Agrobacterium* early in the evolution of the genus Nicotiana.

The presence of such homologous sequences raised many questions: Do other T-DNA like sequences exist in the *N. glauca* genome? Are cT-DNA sequences present in other plant species? Are they involved in genetic tumor formation? Are they functional sequences? Why do Nicotiana plants fail to exhibit the characteristics of hairy root syndrome, despite containing such genes? In the following sections I will present some papers relative to these questions.

1.1. The *N. glauca* cT-DNA

The cT-DNA structure of *N. glauca* has been gradually completed. Two other ORFs were identified that corresponded to the *orf13* and *orf14* genes of pRi T_L-DNA and were named *Ngorf13* and *Ngorf14* (Aoki *et al.,* 1994). (These genes were subsequently called *Ngorf13*L and *Ngorf14*L because of their location on the left arm) (Figure 14B). The coding regions of *Ngorf13* and *Ngorf14* are intact and are preceded by putative transcriptional elements similar to eukaryotic promoter elements, namely the TATA box and the CAAT box, and contain a putative polyA addition signal, downstream of the termination codon.

Suzuki and colleagues (2002) sequenced the right arm of the cT-DNA region and found sequences similar to *Ngorf13* and *Ngorf14* in this region (called *Ngorf13*R and *Ngorf14*R*)*. In

Genus	Subgenus	Section	Species
Nicotiana	Rustica	Paniculatae	**glauca* *paniculata* *knightiana* **benavidesii* **cordifolia*
		Rusticae	*rustica*
	Tabacum	Tomentosae	**tomentosiformis* **otophora* **setchellii* *glutinosa* **tabacum*
	Petunioides	Undulatae	*undulata* **arentsii* *sylvestris* *langsdorfii*
		Alatae	*alata*
		Noctiflorae	*noctiflora* *petunioides*
		Acuminatae	**acuminata* *attenuata* **miersii*
		Bigelovianae	**bigelovii* *clevelandii*
		Suaveolens	**debneyi* **gossei* **suaveolens* **exigua*

Table I. Presentation of genus Nicotiana.
Stars (*) represent the species containing cT-DNA (adapted from Intrieri and Buiatti, 2001).

addition, they found a *Ngorf13a* gene, between *Ngorf13*R and *Ngorf14*R that was absent in the left arm (Figure 14B). Using inverse (i)PCR, DNA fragments external to *Ngorf14* from the right and left arms were isolated. These fragments contained mikimopine synthase (*mis*) genes similar to that of the pRi1724 T-DNA. The authors proposed that the cT-DNA in the genome of wild type *N. glauca* originated from a mikimopine-type Ri plasmid.

A comparison of cT-DNA sequences of the left and right arm demonstrated that they have 96.9% homology up to the right border sequences. The very high DNA homology indicates that each arm originated from the same T-DNA. On the other hand the CG content in the cT-DNA was 44.7 %, whereas that outside the left and the right arm was 35.7 and 39.3 % respectively; this difference in the nature of DNA sequence inside and outside the cT-DNA confirms that the cT-DNA is of a different origin as the genomic DNA of *N. glauca*.

1.2. cT-DNA sequences in other species

The genus Nicotiana contains approximately 70 species, grouped into three subgenera, Rustica, Tabacum, and Petunioides (Goodspeed, 1954) (Table I). Furner and co-workers (1986) reported that at least 4 species, *N. otophora, N. tomentosiformis, N. tomentosa* and *N. benavidesii* share homology with pRi T-DNA and *N. tabacum* showed significant homology, though the large region, corresponding to the *rolB* and *rolC* loci, seemed absent. Ten years later, Meyer and colleagues (1995) could amplify from the genome of *N. tabacum* (cv. Havana 425), sequences homologous to the pRiA4 *rolB*, *rolC* and *orf13* genes (named *trolB*, *trolC* and *torf13* respectively). They also amplified the intergenic regions of these genes and suggested that the tobacco genome contains at least one copy of the *rolB*, *rolC*, and *orf13* loci. They studied the 5' and 3' untranslated regions (UTR) of *trolC* and identified several features of plant genes including two TATA binding motifs and a CAAT-like box upstream of the start codon and a putative polyadenylation (polyA) signal downstream of the stop codon.

Tobacco is an amphidiploid species derived from ancestors most closely related to the present-day species *N. sylvestris* and *N. tomentosiformis* (Gerstel, 1976). Southern analyses showed that *trolC* is derived from the tomentosiformis ancestor (Meyer *et al.,* 1995). This suggests that the cT-DNA resulted from an ancient transfer of DNA between *A. rhizogenes* and a progenitor of modern tobacco.

Fründt and colleagues (1998) found in the genome of *N. tabacum* (cv. Havana 425) a second copy of *torf13* and thus the *torf13* genes were denominated *torf13-1* (the previous) and *torf13-2* (the new) (Fründt *et al.,* 1998). *torf13-2* differed in nucleotide sequence from *torf13-*

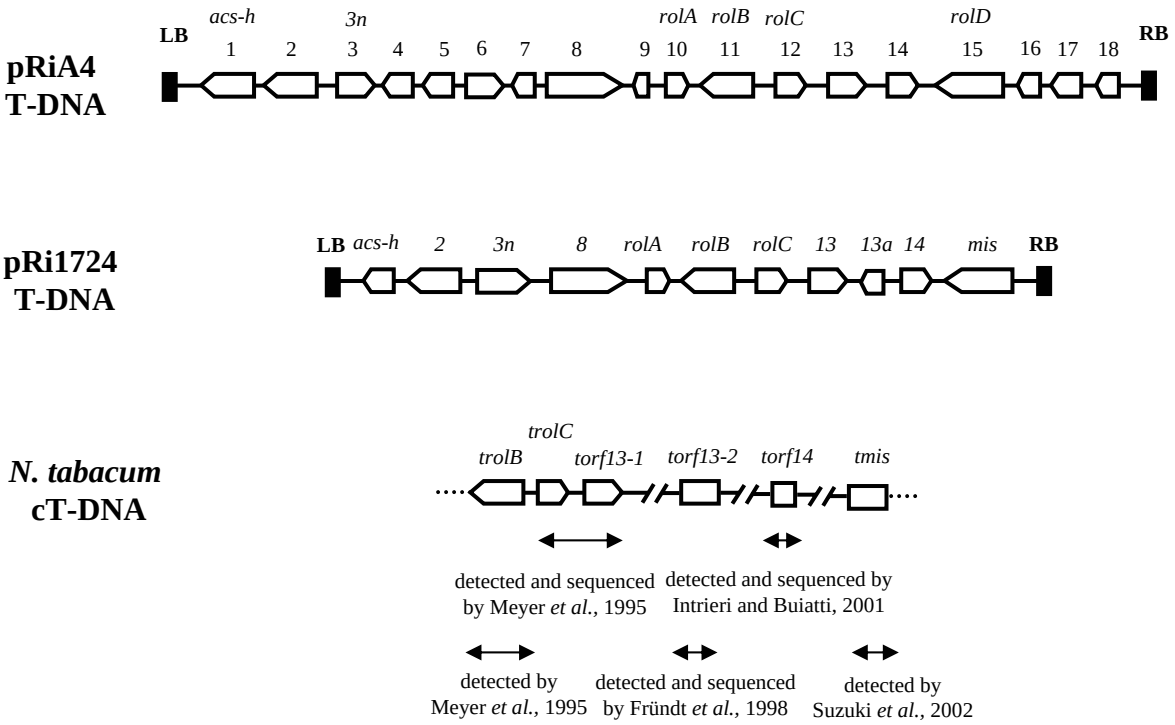

Figure 15. Structure of cT-DNA in *N. tabacum* (as reported before the start of our work).
The T-DNAs of pRiA4 and pRi1724 are shown for comparison. ORFs are presented as arrow boxes (orientation 5' to 3') and the numbers/names of the ORFs are presented above the boxes. *torf13-2*, *torf14* and *tmis* are connected to each other using oblique lines because their position to each other and to the *trolB-torf13-1* region has not been determined so far. Dotted lines represent plant sequences. *mis*: mikiomopine synthase gene; *acs-h*: agrocinopine synthase gene.

1 at 4% of the positions and contained an insertion of 15 bp at position 254. The 5' and 3' UTR of *torf13-1* showed the same motifs as *trolC* and Southern hybridization indicated that *torf13-1* and *torf13-2*, like *trolC,* are of *N. tomentosiformis* origin (Fründt *et al.,* 1998).

Using DNA gel blot analysis, *mis* homologs were detected in Nicotiana species *N. tomentosa, N. tomentosiformis* and *N. tabacum* but not in *N. otophora* and *N. benavidesii* (Suzuki *et al.,* 2002) and using PCR analysis *orf14* homologs have been amplified from *N. tabacum, N. tomentosiformis, N. cordifolia* and *N. otophora* (Intrieri and Buiatti, 2001). These results demonstrate that the cT-DNAs are not limited to *N. glauca*.

According to the results obtained until our laboratory started its investigation on the *N. tabacum* cT-DNA, the structure of this molecule can be summarized as in Figure 15.

1.3. cT-DNA sequences outside the genus Nicotiana

The existence of T-DNA like sequence in plants outside the genus Nicotiana has been reported by several groups. Southern analysis indicated that carrot (*Daucus carota*) contains sequences homologous to the pRi1855 T-DNA (Spanò *et al.,* 1982). The genomes of field bindweed (*Convolvulus arvensis*) and carpet bugleweed (*Ajuga reptans*) also seem to have sequence homology to the T-DNAs of pRi8196 and pRi1724, respectively (Tepfer, 1982; Tanaka *et al.,* 1998). Apple contains sequences homologous to T_L- and T_R-DNA of pRiA4 (Lambert and Tepfer, 1992). However, there are no conclusive data for the existence of T-DNA like sequences outside of the genus Nicotiana and more experiments are necessary to confirm it. Very recently, cT-DNA sequences were reported for a member of the Scrophulariacceae, *Linaria vulgaris* (6.7 Kb, GenBank: EU735069.1).

1.4. Expression of cT-DNA genes in normal tissues and in genetic tumors

Genetic tumors are spontaneous outgrowths in a number of plants species that are formed in the absence of any obvious external cause. It is thought that they result from the mutation of genes or their aberrant regulation or from changes in the hormonal balance in the plant. The most often reported examples of genetic tumors have been those that arise from combinations of species of the genus Nicotiana, e.g. genetic tumors derived from hybrids of *N. glauca* x *N. langsdorffii* (Kostoff, 1943; Kung, 1989; Ichikawa and Syono, 1991; Sekine *et al.,* 1993). When cT-DNA sequences were detected in *N. glauca,* they were rapidly proposed to play a role in the genetic tumors formation in the Nicotiana genus. Several groups studied the

expression of cT-DNA gene in the genetic tumors of *N. glauca* x *N. langsdorffii* and found that *NgrolB*, *NgrolC, Ngorf13* and *Ngorf14* are highly expressed in the tumors (Ichikawa *et al.,* 1990; Aoki *et al.,* 1994).

However, further experiments demonstrated that the cT-DNA genes expression was not limited to the tumors and included also normal tissues. In wild-type *N. glauca* plants transcripts of *NgrolB* and *NgrolC* were detected in stem tissue and callus (Aoki and Syono, 1999c) and those of *Ngmis*L and *Ngmis*R in the stem, leaves and flowers (Suzuki *et al.,* 2002).

In transgenic hybrid plants harboring a β-glucuronidase (GUS) gene under control of *NgrolB, NgrolC* or *Ngorf13* promoters, the analysis of the promoter activity revealed that the *NgrolB* expression was detectable in meristematic zones (root and shoot apex) (Nagata *et al.,* 1996), *NgrolC* expression was seen mainly in vascular systems of the roots and the stem tissue (Nagata *et al.,* 1996) and *Ngorf13* expression was detected in the vascular bundles and parenchymatous tissue, and moreover, was decreased by ageing (Udagawa *et al.,* 2004).

In the case of *N. tabacum* plants, the *trolC* and *torf13* genes showed a similar pattern of expression and their transcripts accumulated in shoot tips and in upper and middle leaves of the young plant (Meyer *et al.,* 1995; Fründt *et al.,* 1998).

When the effect of hormones on the expression of cT-DNA genes was investigated, it was found that treatment by auxin promoted the *NgrolB* promoter activity in *NgrolB*::GUS transgenic genetic tumors (Nagata *et al.,* 1995). Using tobacco callus culture it has been demonstrated that *trolC* expression is down-regulated by auxin and induced by cytokinin (Meyer *et al.,* 1995) and tobacco leaf disc treatment with auxin or cytokinin led to a decrease in the level of *torf13* transcripts (Fründt *et al.,* 1998).

These observations suggest that the expression of cT-DNA genes is not only regulated developmentally but also by phytohormones and imply that these genes can have a function(s) in plant development. However, only a few studies have been carried out in this area and many questions remain to be solved.

1. 5. Function of cT-DNA genes

Measurement of *NgrolB* and *NgrolC* promoter activity during the development of transgenic genetic tumors showed that the pattern of expression of the *NgrolB* promoter coincided with cell division and that of the *NgrolC* promoter with the initiation of differentiation of organs in tumors (Nagata *et al.,* 1996). The maintenance of the cells in a meristematic state (cell division) or the differentiation into organs is known to be controlled

by the ratio of auxin and cytokinin. Based on these data Nagata and co-workers proposed that the expression of *NgrolB* and *NgrolC* changes the sensitivity to or synthesis of hormones (as reported for the bacterial homologous genes) and leads to the formation of tumors with rudimentary buds.

Aoki and colleagues showed that *Ngorf13* and *Ngorf14* promote Ri*rolB*-mediated adventitious root induction on tobacco leaf discs with the same efficiency as the bacterial genes, Ri*orf13* and Ri*orf14* (Aoki and Syono, 1999b). In the case of tobacco cT-DNA genes, it has been demonstrated that the *torf13-1* gene, like Ri*orf13*, can induce the formation of dense green callus on the surface of carrot discs suggesting the involvement of this gene in cell proliferation (Fründt *et al.,* 1998).

The morphogenetic abnormalities of transgenic plants expressing *Ngorf13* (dark green round leaves, reduced growth, and irregularly shaped flowers, Aoki and Syono, 1999a) and *NgrolC* (dwarfed phenotype, lanceolate and pale green leaves and slender and small floral organs, Aoki and Syono, 1999b) are similar to those of Ri*orf13* (Hansen *et al.,* 1993) and Ri*rolC* expressing plants (Schmülling *et al.,* 1988, Oono *et al.,* 1993).

Despite the expression of *Ngmis* genes in *N. glauca*, mikimopine was not detectable in the plant. However, when the *NgmisR* gene was expressed in *E. coli,* the purified protein was able to synthesize mikimopine from the substrates, L-histidine and α–ketoglutaric acid and the synthesized mikimopine was completely degraded by a mikimopine-type *A. rhizogenes* (Suzuki *et al.,* 2001; Suzuki *et al.,* 2002).

All of the observations mentioned above show that several cT-DNA genes have retained their function after an ancient infection by bacteria.

The features typical of regenerated *A. rhizogenes* transformants, such as reduced stature, altered flower and leaf development and increased rootiness (Ackermann 1977; Tepfer 1984), could have led to increased reproductive isolation, increased fitness, and eventually speciation. If, as proposed, expression of these genes has an adaptive significance, then altering their expression by transformation with sense and antisense constructs might provide a fruitful approach for investigating their biological effects.

1.6. Mutation of cT-DNA gene *NgrolB* and restauration of its activity

Among the *rol* genes, *rolB* has been reported to be the most important gene for hairy root induction, because introduction of this gene alone can induce root formation on tobacco leaf discs and its inactivation totally suppresses the root induction capability of the bacterium.

rolC

Species	*N. gla.*	*N. cor.*	*N. tom.*	*N. oto.*	*N. tab.*	*N. deb.*	*A. rhi. 1*	*A. rhi. 2*	*A. rhi. 3*
N. glauca		93.5	68.3	68.3	68.6	93.4	86.4	80.1	84.2
N. cordifolia			66.3	66.3	66.6	89.5	82.9	76.4	81.7
N. tomentosiformis				97.5	98.7	67.3	66.6	71.9	63.8
N. otophora					98.3	67.0	66.8	72.3	63.3
N. tabacum						67.3	67.0	72.1	64.0
N. debneyi							87.1	78.4	84.8
A. rhizogenes 1								77.4	79.9
A. rhizogenes 2									74.1
A. rhizogenes 3									

Orf13

	N. gla.	*N. cor.*	*N. tom.*	*N. oto.*	*N. tab.*	*A. rhi. 1*	*A. rhi. 2*	*A. rhi. 3*
N. glauca		98.5	82.9	70.4	81.4	85.3	88.0	88.2
N. cordifolia			82.8	70.3	81.4	85.0	87.7	87.8
N. tomentosiformis				79.8	97.8	77.7	79.2	79.4
N. otophora					81.0	66.9	69.2	69.4
N. tabacum						77.0	78.6	78.8
A. rhizogenes 1							89.7	89.8
A. rhizogenes 2								98.2
A. rhizogenes 3								

Orf14

	N. gla.	*N. cor.*	*N. tom.*	*N. oto.*	*N. tab.*	*A. rhi. 1*	*A. rhi. 2*	*A. rhi. 3*
N. glauca		98.8	93.8	97.1	95.0	82.7	84.6	83.5
N. cordifolia			94.6	97.8	95.3	83.3	85.0	83.8
N. tomentosiformis				95.9	96.9	81.6	83.5	82.3
N. otophora					96.3	84.3	86.4	85.2
N. tabacum						81.9	83.9	82.7
A. rhizogenes 1							86.2	84.5
A. rhizogenes 2								90.6
A. rhizogenes 3								

Table II. Nucleotide sequence homology between Ri-*rolC*, -*orf13*, and -*orf14* and their plant homologues. Numbers represent the value of homology in percentage. *A. rhizogenes* 1: strain 1724; 2: strain A4; 3: strain 8196 (Intrieri and Buiatti, 2001).

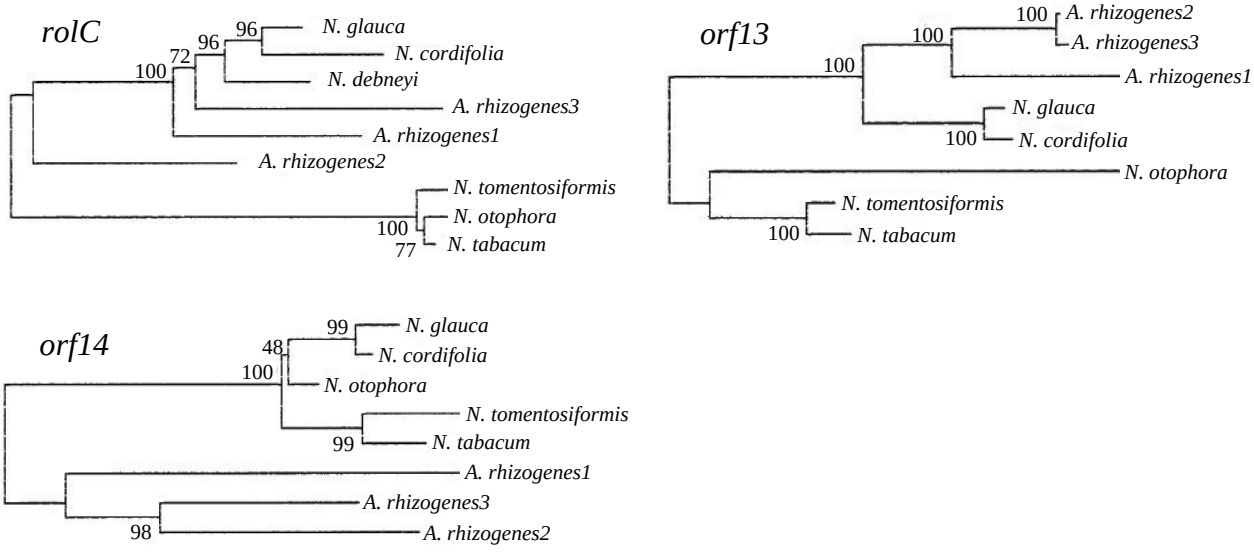

Figure 16. Phylogenetic analysis of *rolC*, *orf13*, and *orf14* and their plant homologues. *A. rhizogenes* 1: strain 1724; 2: strain A4; 3: strain 8196 (Intrieri and Buiatti, 2001).

However, the *NgrolB* gene alone or in combination with *NgrolC, Ngorf13*, and *Ngorf14* did not generate adventitious roots (Aoki and Syono, 1999a; b) and transgenic tobacco plants expressing *NgrolB* under control of the 35S promoter did not exhibit any alteration. Comparison of Ri*rolB* and *NgrolB* sequences revealed that *NgRolB* contains two point mutations generating two early-termination codons. The first termination codon reduces the protein length by 48 aa. Single-base site-directed mutagenesis at two positions led to the production of the full-length NgRolB protein (NgRolB*) with the capability for root formation on leaf discs and induction of morphological abnormalities in 35S::*NgrolB** transgenic tobacco plants (Aoki and Syono, 1999b). Therefore the authors suggested that the lack of phenotypic alteration in *N. glauca* plants may be due to the presence of a truncated and thus biologically inactive NgRolB protein in these plants.

1.7. Phylogenetic analysis of cT-DNA genes in the genus Nicotiana and their evolution

At least 15 species in 7 sections in all three subgenera of the genus Nicotiana seem to contain cT-DNAs in their genomes (Furner *et al.,* 1986; Intrieri and Buiatti, 2001) (Table I). Although other species in the genus Nicotiana may also contain cT-DNAs, no further reports have yet appeared. Thus, not every Nicotiana species contains cT-DNAs, suggesting that only a subset of Nicotiana species were transformed or that an initially common cT-DNA was deleted during the long evolutionary process in some lines. The examination of the homology levels between each of the Nicotiana species, and homologies with the present-day pRi T-DNA open the way for elucidating the evolution of the genus Nicotiana. Intrieri and Buiatti (2001) performed a phylogenetic analysis to compare the nucleotide sequences of *rolC*, *orf13*, and *orf14* from 6 different Nicotiana species (*N. glauca*, *N. ordifolia, N. tomentosiformis, N. otophora, N. tabacum* and *N. debneyi*) with 3 different strains of *A. rhizogenes* (strain 1724, A4 and 8196) (Table II, Figure 16) .

According to homologies found, the authors concluded that the evolution of T-DNA genes was different in Agrobacteria and in plants. Nicotiana cT-DNA genes followed Nicotiana species evolution, being clustered into two groups; one included *N. glauca* and *N. cordifolia* (subgenus Rustica), the second was limited to species belonging to the subgenus *Tabacum* (Figure 16) and the pRi T-DNA genes (*rolC, orf13* and *orf14*) were clustered with each other. Therefore it was impossible to find which pRi(s) was eventally the origin of the cT-DNA genes. In the phylogenetic analysis of the *rolC* gene however, a surprising result appeared: the

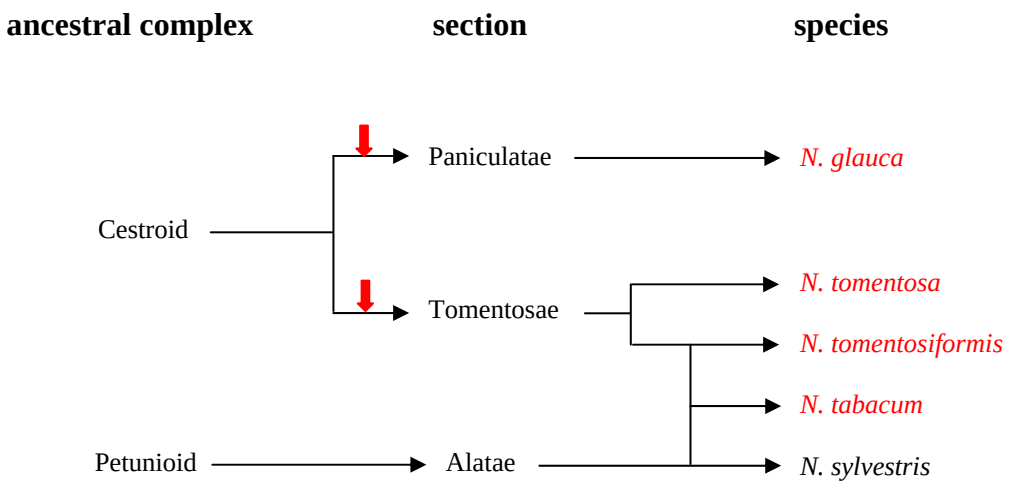

Figure 17. Independent insertion events in the ancestors of *N. glauca* and *N. tabacum*.
The different T-DNA insertion sites in the genome of *N. glauca* and *N. tabacum* indicate that at least two independent transformation events (red arrows) have occurred in the ancestors of these two plants. The tree shows only the divergence pattern but not the evolutionary distance. The species containing cT-DNA sequences are presented in red.

rolC gene of *N. debneyi* was clustered with *NgrolC* of *N. glauca*; although these two species are at a remote distance in Nicotiana evolution (Goodspeed, 1954). This suggests that independent infections by *A. rhizogenes* have occurred in several ancient Nicotiana plants.
It should be mentioned that the elucidation of the evolution of the genus Nicotiana is even more complicated due to the presence of interspecific hybrids in this genus.

N. glauca is a member of the section Paniculatae. The tomentosiformis ancestor of tobacco is a member of the section Tomentosae (Goodspeed, 1954; Gerstel, 1976). Both sections are thought to be descended from the same Cestroid ancestral complex (Goodspeed 1954; Gerstel, 1976). On the other hand, the sylvestris ancestor of tobacco in section Alatae is descended from the Petunioid ancestral complex (Figure 17). Southern analyses (Furner *et al.,* 1986; Meyer *et al.,* 1995; Fründt *et al.,* 1998) have shown that cT-DNA sequences are only found in species derived from the Cestroid ancestral complex that gave rise to *N. glauca* and *N. tomentosiformis*. Thus, the cT-DNA found in tobacco and *N. glauca* could have arisen either from a single transformation event in a Cestroid common ancestor, or from independent transformation events that occurred after the Tomentosae and Paniculatae sections diverged. Suzuki and colleagues (2002) reported that the site of insertion of T-DNA in *N. tabacum, N. tomentosiformis* and *N. tomentosa* differed from that in *N. glauca.* This indicates that *A. rhizogenes* independently infected ancestors of these two groups (section Tomentosae and section Paniculatae) (Figure 17).

Exactly when each infection event occurred is still unknown; however, Suzuki and co-workers (2002) estimate that the insertion of T-DNA into *N. tomentosiformis* occurred before the formation of *N. tabacum*, which was less than 6 million years ago (Okamuro and Goldberg, 1985). As mentioned previously, only a few studies reported the existence of cT-DNA like sequences in other plant genera, suggesting that the genus Nicotiana is particularly susceptible to natural transformation by *A. rhizogenes* and regeneration (Spanò *et al.,* 1982; Tepfer, 1982; Tanaka *et al.,* 1998).

IV. Presentation of my PhD work

When I arrived in the laboratory, the activity of some *plast* genes (including *lso*, *6b*, *orf8*) had already been studied in detail in our group, and new *6b* gene properties had been revealed using a T-*6b* gene under control of a dexamethasone-inducible promoter in tobacco. By searching for common characteristics of *plast* genes and thereby hoping to approach their basic function, we decided to extend these studies to two other *plast* genes, namely A4-*rolC* and A4-*rolB* and their homologs in tobacco. I will present the results of my PhD work in two parts: in Chapter I, I will describe the biological activities of A4-*rolC* and the tobacco *trolC* gene, and their relation to the other *plast* genes, in Chapter II, I will present results obtained for A4-*rolB*.

RESULTS

Chapter I

The biological activities of A4-*rolC* and the tobacco homolog *trolC*

I. Introduction

The three *rol* genes (*rolA, rolB* and *rolC*) of *A. rhizogenes* are considered as the essential genes in the induction of the hairy root phenotype. Despite much research over the years, the biochemical and molecular functions of these genes in modifying plant development remain poorly understood, reflecting the complexity of the system. Therefore it would be important to study their characteristics more in detail in order to clarify their mode of action. Although several biological effects of the *rolC* gene have been determined previously, we have tried to learn more about its characteristics using inducible gene constructs, in order to avoid secondary effects due to accumulation of various abnormalities. In this Chapter, I will describe the detailed results that we obtained for this gene and its homologous gene in tobacco, *trolC*. In addition I will present the relation of these two genes with other *plast* genes.

As mentioned in the Introduction the expression of the *rolC* promoter is regulated by sucrose (Nilsson *et al.,* 1996). Fladung and Gieffers (1993) showed that the leaves of 35S::A4-*rolC* transgenic potato contain high levels of fructose and glucose compared to normal leaves. Based on these observations, it has been suggested that *rolC* could play a role in sucrose metabolism and/or transport (Nilsson and Olsson, 1997). In this chapter we will present strong evidence for this hypothesis (see: results in publication).

To extend our knowledge about the mechanism of action of *(t)rolC*, we decided to search for possible cellular partners using the yeast two hybrid system.The results will also be presented in this chapter (see: results non-submitted to publication).

II. Results

1. Results accepted in the journal of "Molecular Plant-Microbe Interactions"

Biological activity of the *Agrobacterium rhizogenes*-derived *trolC* gene of *Nicotiana tabacum* and its functional relation to other *plast* genes

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ABSTRACT

Agrobacterium rhizogenes induces hairy roots through the activity of three essential T-DNA genes*, rolA, rolB* and *rolC*, whereas the *orf13* gene acts as an accessory root-inducing gene. *rolB, rolC* and *orf13* belong to the highly diverged plast gene family with remotely related representatives in the endomycorrhizal basidiomycete *Laccaria bicolor*. *Nicotiana glauca* and *Nicotiana tabacum* contain *A. rhizogenes*-derived T-DNAs with active *plast* genes. Here we report on the properties of a *rolC* homolog in *Nicotiana tabacum*, *trolC*. Dexamethasone-inducible *trolC* and A4-*rolC* genes from *A. rhizogenes* strain A4 induce comparable, strong growth effects affecting all parts of the plants. Several have not been described earlier and were found to be very similar to the effects of the distantly related *plast* gene *6b*. They include leaf chlorosis and starch accumulation, enations, increase of sucrose-dependent leaf disk expansion, growth of isolated roots on low sucrose media and stimulation of sucrose uptake by small root fragments. Collectively, our findings indicate that enhancement of sucrose uptake plays an important role in generating the complex *6b* and *rolC* phenotypes and might be an ancestral property of the plast genes.

INTRODUCTION

A. rhizogenes is well-known for its capacity to induce hairy roots on a large number of plant species. Different strains harbor root-inducing (pRi) plasmids with different T-DNA structures, but all have a conserved central portion carrying the root locus (*rol*) genes *rolA*, *rolB* and *rolC*. These three genes are both necessary and sufficient for hairy root induction, although pRi8196 does not require *rolA* (Hansen *et al.,* 1991). *orf13* and *orf14* enhance *rolABC*-induced root growth and are considered to be accessory (Spena *et al.,* 1987, Capone *et al.,* 1989, Costantino *et al.,* 1994, Aoki and Syono 1999b, Meyer *et al.,* 2000). *rolA*, *rolB*, *rolC* and *orf13* modify growth when expressed individually, whereas *orf14* does not (Lemcke and Schmülling, 1998).

Among the *A. rhizogenes* T-DNA genes, *rolB, rolC*, *orf13*, *orf14* and the 5' part of *orf8* belong to a family of highly diverged genes without detectable DNA similarity but with statistically significant similarity at the protein level. They have been called *plast* genes (for phenotypic plasticity) (Levesque *et al.,* 1988), *plast*-like genes also occur on the T-DNAs of *A. tumefaciens* and *A. vitis* (*b*, *c'*, *d*, *e*, *3'*, *5*, *6a*, *6b*, *7*, *lso* and the 5' part of *iaaM*, Helfer *et al.,* 2002), but up to now, none were reported outside a T-DNA context. The mechanism of action

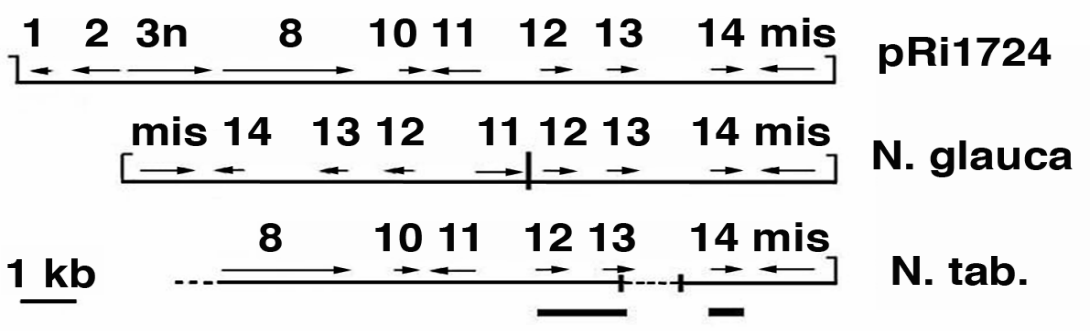


Figure 1. T-DNA and cT-DNA maps.
pRi1724: T_L-DNA of *A. rhizogenes* strain 1724 (NC_002575.1). *N. glauca*: cT-DNA of *N. glauca* (Suzuki *et al.*, 2002). N. tabacum: cT-DNA fragments of *N. tabacum* (this work). Arrows: open reading frames. 10, 11 and 12: *rolA*, *rolB* and *rolC*. mis: mikimopine synthase gene. Thick horizontal lines: earlier published sequences from *N. tabacum.*

Table I. Overview of *N. tabacum* cT-DNA sequences.

cT-DNA region	Tobacco cv.	accession	authors	DNA type
torf8	Petit Havana	EH664027	Millar et al. unpublished	c-DNA
torf8	Hicks Broadleaf	FH900730	Opperman et al. unpublished	Genomic DNA
torf8	Hicks Broadleaf	FH901074	Opperman et al. unpublished	Genomic DNA
torf8	Samsun nn	FN667969	this paper	PCR
trolA	Samsun nn	FN667969	this paper	PCR
trolB	Petit Havana	EH664294	Millar et al. unpublished	c-DNA
trolB	Hicks Broadleaf	FH925392	Opperman et al. unpublished	Genomic DNA
trolB	Samsun nn	FN667969	this paper	PCR
trolC	Havana 425	X91881	Meyer et al. 1995	PCR
trolC	Samsun nn	FN667969	this paper	PCR
trolC-torf13	Havana 425	AJ010794, AJ007621, AJ007622	Fründt et al. 1998	PCR
torf13	not specified	AF281242	Intrieri and Buiatti 2001	PCR
torf13	Samsun nn	FN667969	this paper	PCR
torf14	not specified	AF281246	Intrieri and Buiatti 2001	PCR
torf14	Samsun nn	FN667970	this paper	PCR
tmis	Samsun nn	FN667970	this paper	PCR

of *plast* genes remains controversial, especially since the earlier proposed glucosidase activities of *rolB* and *rolC* have been questioned (Nilsson *et al.,* 1993; Faiss *et al.,* 1996; Nilsson and Olsson 1997; Meyer *et al.,* 2000).

T-DNAs (called cellular T-DNAs or cT-DNAs) also occur in *N. glauca* and *N. tabacum* and result from ancient transformation events (White *et al.,* 1983; Furner *et al.,* 1986; Meyer *et al.,* 1995; Intrieri and Buiatti, 2001; Aoki, 2004). The *N. glauca* cT-DNA (Furner *et al.,* 1986; Aoki *et al.,* 1994; Aoki, 2004) has been fully sequenced and consists of two inverted, incomplete T-DNA fragments of a pRi1724-like cT-DNA (Figure 1). *NgrolC* (Nagata *et al.,* 1995; Aoki and Syono 1999a) and *Ngorf13* (Aoki and Syono 1999c) are transcribed and have morphogenetic activity, whereas *NgrolB* is mutated. In the case of *N. tabacum* a *trolC* (Meyer *et al.,* 1995), a complete and a partial *torf13* (Fründt *et al.,* 1998), and a *torf14* (Intrieri and Buiatti, 2001) sequence have been reported. *mis* (mikimopine synthase)-like sequences were identified by Southern analysis (Suzuki *et al.,* 2002). *torf13* of cultivar Havana 425 induces callus on carrot disks (Fründt *et al.,* 1998) but the activity of *trolC* has not been tested. Here we show that *N. tabacum* cultivar Samsun nn has a mutated *torf13* gene and contains only one intact *rol* gene, *trolC*. Its growth-modifying properties are very similar to those of the *A. rhizogenes* A4 *rolC* gene and partially similar to those of the distantly related *6b plast* gene, suggesting conservation of an ancestral function.

RESULTS

Nicotiana tabacum cT-DNA contains the three essential *rol* genes *rolABC.*

Since the publication of the tobacco *trolC*, *torf13* and *torf14* sequences a large number of tobacco expressed sequence tags (EST) and genomic survey sequences (GSS) have been reported. Among these we found three *torf8* and two *trolB* fragments (Table I). The new tobacco sequences and the *N. glauca* cT-DNA were used to design primers allowing PCR amplification of two cT-DNA fragments from *N. tabacum* cv. Samsun nn. The first fragment carries *torf8*, *trolA*, *trolB*, *trolC* and *torf13*, the second *torf14* and *tmis* (Figure 1, accession numbers FN667969 and FN667970 respectively). The two sequences align with the *A. rhizogenes* T_L-DNA of pRi1724, but no fragments could be amplified between *torf13* and *torf14*. The *torf8*-*torf13* region is most closely related to sequences from A4 and *N. glauca* (70% and 80% homology respectively), whereas *torf14*-*tmis* is 96% homologous to the *N. glauca* sequence up to the right border, but homology does not extend beyond, confirming

that *torf14-tmis* is not inserted in the same region as *Ngorf14-Ngmis* in *N. glauca*, as earlier deduced from Southern analysis (Suzuki *et al.,* 2002). Contrary to *N. glauca* which lacks *rolA*, *N. tabacum* contains all three essential *rolABC* genes. However, *trolA* has an internal stop codon at position 112 (normal open reading frame: 270 nt), and *trolB* carries a 40 nt direct repeat at position 24 (TCAACGCTT-(T/A)-CACCCAAGAAATCTCACTCGAGCATTG AGC) with one mismatch, thereby causing an early frameshift (normal ORF size 783 nt), only *trolC* is intact. Interestingly, cv. Samsun nn *torf13* (*S-torf13*) differs from cv. Havana 425 *torf13-1 (H-torf13-1)* (AJ007621). *H-torf13-1* is intact, but *S-torf13* is truncated beyond nucleotide 400 as shown by PCR experiments with *H-torf13-1* primers downstream position 400 (not shown). The *torf13* genes of *N. tabacum* cv. Wisconsin 38 and *N. tomentosiformis* are intact, whereas those of *N. tabacum* cv. Basma Drama 2, Samsoun and Xanthi are truncated (results not shown). *torf8* has a stop codon at position 1873 (normal ORF size 2286 nt). *torf14* is intact, and *tmis* has a stop codon at position 199 (normal ORF size 987 nt). Thus, *trolC* is the only potentially growth-modifying cT-DNA gene in cv. Samsun nn. This gene is expressed as shown by RT-qPCR analysis. In medium-sized leaves of *N. tabacum* Samsun nn plants (height 20 cm) the *trolC/EF2* expression ratio (tested with appropriate primers, see Matarials and Methods) is 1.0x10-4 (SD: 0.3x10-4).

Inducible expression of dex-*trolC*-HAHIS and dex-A4-*rolC*-HAHIS in tobacco.

Earlier studies showed that *trolC* is transcribed (Meyer *et al.,* 1995), but its biological activity has not been investigated. The *A. rhizogenes* A4-*rolC* gene causes dwarfing in tobacco and pale-green lanceolated leaves (Schmülling *et al.,* 1988). We therefore used A4-*rolC* as a standard to test *trolC*. In order to detect the proteins, we added an HAHIS tag (Thomas et al. 2006) at the 3' end of both genes. Experiments in our laboratory with other inducible HAHIS constructs showed that this construct does not modify tobacco growth even at high expression levels as detected with the anti-HAHIS antibody (results not shown). We therefore felt confident that this tag would not disturb plant growth. The tagged constructs were placed under inducible promoter control, using the pTA7002 vector (Aoyama and Chua 1997) as earlier done for the T-6b plast gene (Grémillon et al. 2004). The pTA7002 vector allows controlled gene induction by addition of dexamethasone (dex) to the medium or by spraying plants with a dex solution. *N. tabacum* plants carrying a dex-GFP gene in the same pTA7002 vector (Shen, 2001) and induced to high GFP levels did not show any particular growth effects under the conditions used in this work (results not shown). Dexamethasone (dex)-inducible dex-*trolC-HAHIS* and dex-A4-*rolC-HAHIS* genes were introduced in *N.*

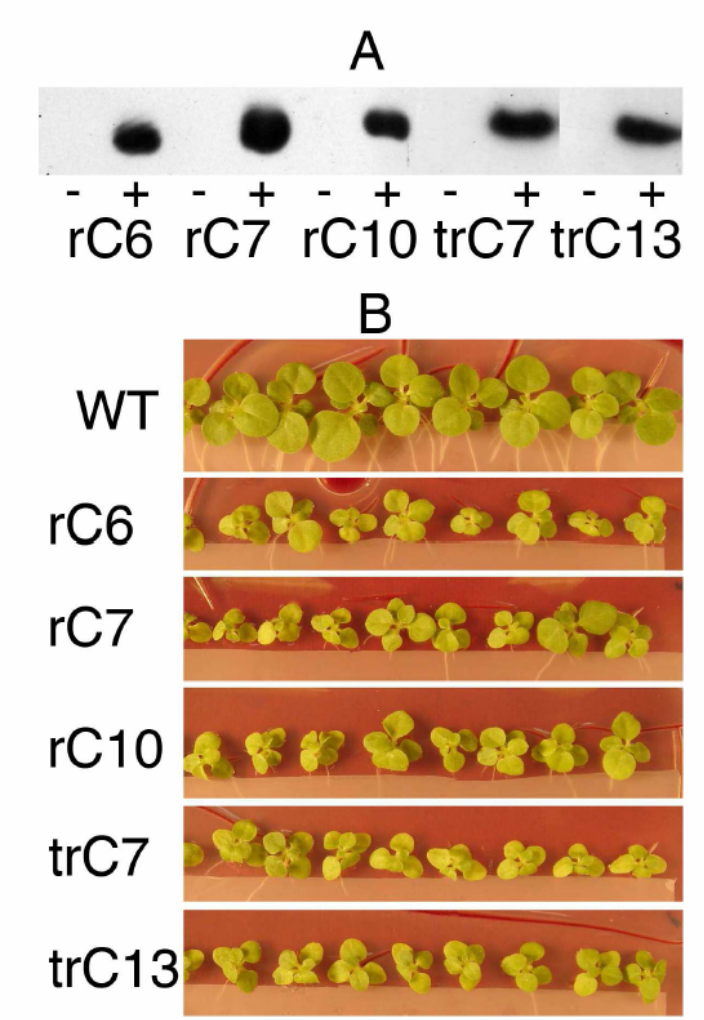


Figure 2. Initial characterization of transformed homozygous lines.
A. Expression of transgene-encoded proteins 24 hrs after leaf infiltration with 10 mM MgSO4 (-) or with 3 µM dex in 10 mM MgSO4 (+). Each sample contains the same total amount of protein. B. Phenotypes of induced seedlings 8 days after transfer to medium with 3 µM dex. Growth of induced *trolC* and A4-*rolC* lines has slowed down. WT, rC6, rC7, rC10, trC7 and trC13: wild-type, dex-A4-*rolC-HAHIS-6*, dex-A4-*rolC-HAHIS-7*, dex-A4-*rolC-HAHIS-10*, dex-*trolC-HAHIS*-7 and *dex-trolCHAHIS-13* respectively.

tabacum cv. Samsun nn (Materials and Methods). Several independent transgenic lines showed high levels of tRolC-HAHIS or A4-RolC-HAHIS protein 24 h after leaf infiltration with dex. Five homozygous single-locus F2 lines (dex-*trolC-HAHIS-7, -13* and dex-A4-*rolC-HAHIS-6, -7 and -10*) with high expression levels (Fig. 2A) were chosen for further studies. When seedlings of these lines were grown on inducing medium with 3 µM dex, they showed a comparable growth reduction, reproducing the earlier reported *rolC* dwarfing phenotype (Fig. 2B). The dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* lines were selected for further detailed studies. The phenotypic range for *trolC* and A4-*rolC* was determined with different inducer concentrations. Seedlings were grown on nylon filters on vertical plates containing media with dex concentrations ranging from 0 to 3 µM. Results are shown in Fig. 3. tRolC-HAHIS and A4-RolCHAHIS proteins reached maximal levels at 0.3 µM dex (Fig. 3A). Above 0.03 µM dex cotyledons and leaves were smaller and pale-green (Fig. 3B). Root hairs of dex-*trolCHAHIS-7* and dex-A4-*rolC-HAHIS-7* seedlings became shorter at 0.01 µM dex and disappeared at 0.3 µM (Fig. 3C), whereas root growth slowed down (Fig. 3D) from 0.1 µM dex onwards. At later stages, cotyledons and first true leaves became chlorotic, thick and epinastic. However, subsequent leaves and lateral roots of dex-*trolC-HAHIS-7*seedlings normalized contrary to those of dex-A4-*rolC-HAHIS-7* seedlings (Fig. 3E and 3F, see also below).

In order to compare *trolC* and A4-*rolC* effects on plants growing under greenhouse conditions, dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* seeds were germinated in soil and sprayed with 3 µM dex at the two-cotyledon stage. Dex-sprayed *trolC* and A4-*rolC* plants showed considerable growth reduction compared to wild-type plants (Figure 4A). At later stages, side shoots replaced the main shoots which appeared to be blocked. Surprisingly, several plants showed enations along the central leaf veins (Figure 4B). Enations are a rare type of plant growth modification, found in some viral diseases and in tobacco plants that express the 2x35S-AB-*6b* (Helfer *et al.,* 2003) or dex-T-*6b* (Grémillon *et al.,* 2004constructs, derived from two *6b* genes from the *A. vitis* nopaline strain AB4 and the *A. vitis* octopine/cucumopine strain Tm4 respectively. The induction of enations reveals an unsuspected, highly specific relation between *rolC* and *6b plast* genes. When dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* plants with a height of 20 cm were sprayed with 3 µM dex, narrow leaves with irregular chlorosis patterns developed (Figure 4C), demonstrating that *trolC* and A4-*rolC* not only interfere with early development, but also induce changes in older plants. Chlorosis could be induced locally by placing small 10 µM dex-containing lanolin

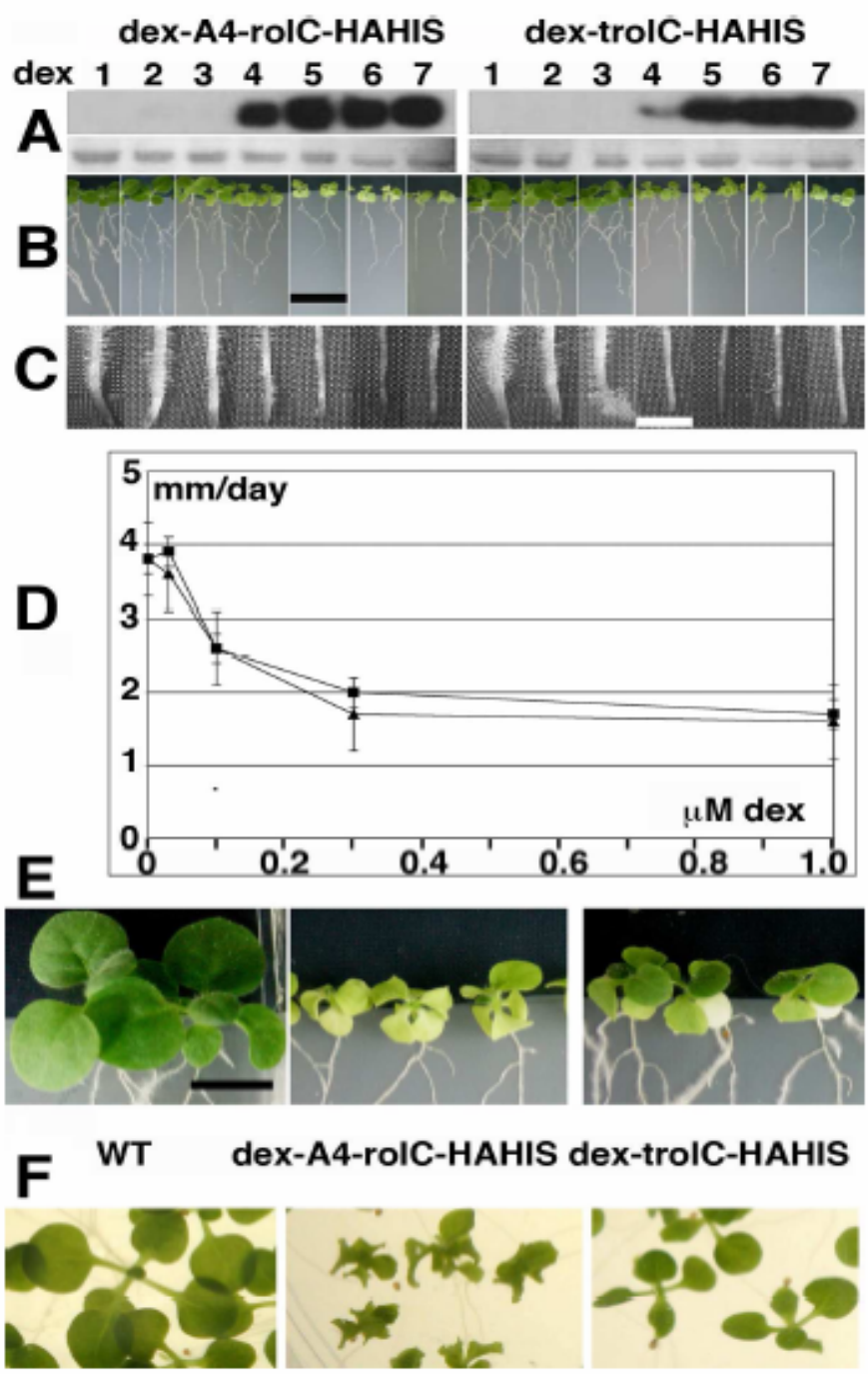


Figure 3. Control of dex-A4-*rolC-HAHIS* and dex-*trolC-HAHIS* expression by different dex concentrations and associated phenotypes.

dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* seedlings were induced on media with 0, 0.01, 0.03, 0.1, 0.3, 1 and 3 µM dex (lanes 1 to 7 respectively). A. Western analysis of A4-RolC-HAHIS and tRolC-HAHIS proteins with an anti-HA antibody, 24 hrs after induction. Top: A4-RolC-HAHIS and tRolC-HAHIS proteins, bottom: loading control. A4-RolC and tRolC proteins are detectable from 0.1 µM dex onwards. B. Seedlings 8 days after induction, shoots are chlorotic and smaller from 0.1 µM dex onwards. Bar: 2 cm. C. Roots 8 days after induction, root hairs are reduced from 0.03 µM dex onwards. Bar: 0.25 cm. D. Root growth (mm/day) of seedlings decreases with increasing dex concentration. Squares: dex-*trolC-HAHIS-7*, triangles: dex-A4-*rolC-HAHIS-7*. Valuesare means +/- standard deviation. For each time point, 24 seedlings were measured. Experiments were repeated three times. Experimental errors were less than 3%. E. Wild-type, dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* seedlings 18 days after induction. dex-A4-*rolC-HAHIS-7* seedlings remain highly abnormal and yellow but dex-*trolC-HAHIS-7* seedlings normalize: new leaves are normal and dark green and new lateral roots have normal root hairs. Bar: 1 cm. F. Wild-type, dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* seedlings 28 days after induction. dex-A4-*rolC-HAHIS-7* seedlings continue to generate abnormal leaves, whereas dex- *rolC-HAHIS-7* seedlings produce normal leaves. Bar: 1 cm.

spheres on well developed leaves, the induced areas not only became chlorotic but also accumulated high levels of starch as revealed by iodine staining (Figure 4D), showing that *trolC* and A4-*rolC* can act directly on mature leaf tissues. The chlorotic areas of uniformly sprayed leaves also correspond to high starch zones (Figure 4E). Starch accumulation has also been reported for *6b* (Helfer *et al.,* 2003, Clément *et al.,* 2006) and for the *rolB*-like 5' part of *orf8* (Umber *et al.,* 2002). The local induction of starch accumulation by lanolin spheres might suggest that *trolC* and A4-*rolC* directly affect leaf-specific processes (like photosynthesis). The root changes observed in seedlings could then be an indirect result of these leaf changes. Alternatively, *trolC* and A4-*rolC* act on processes that are found in both leaves and roots, which would indicate a more general cellular target. As a next step we therefore tested the effects of *trolC* and A4-*rolC* on isolated shoots and roots by using leaf disks and isolated roots.

***trolC* and A4-*rolC* induce changes in leaf disks.**

Induction of dex-*trolC-HAHIS* and dex-A4-*rolC-HAHIS* seedlings strongly reduces shoot and root growth. When roots were removed before induction, isolated induced shoots still slowed down their growth and became chlorotic, confirming that the action of *trolC* and A4-*rolC* on leaves is direct and does not require previousmodification of roots. Local starch accumulation in leaves is most likely due to sucrose accumulation, this was analyzed using a sucrose-dependent leaf disk expansion assay. Tobacco leaf disks from immature leaves floated on a simple medium (Keller and Van Volkenburgh 1997) will expand to a limited extent on medium without sucrose. When 3% sucrose is added, expansion increases by about 60%. The expansion of dex-*trolCHAHIS-7* and dex-A4-*rolC-HAHIS-7* leaf disks in 3% sucrose was further increased upon induction with dexamethasone, whereas no increase occurred when the inducing medium contained no sucrose (Fig. 5). It can therefore be hypothesized that A4-*rolC* and *trolC* stimulate the uptake of sucrose from the medium which then leads to increased expansion. Since T-*6b* also stimulates tobacco leaf disk expansion in the presence of sucrose (Clément *et al.,* 2006), this further confirms the functional relation between *rolC* and *6b* (see above).

***trolC* and A4-*rolC* induce changes in isolated roots.**

Isolated tobacco roots do not grow on medium with 1% sucrose. However, on the same medium isolated dex-induced *trolC* and A4-*rolC* roots continued to grow, although they lost

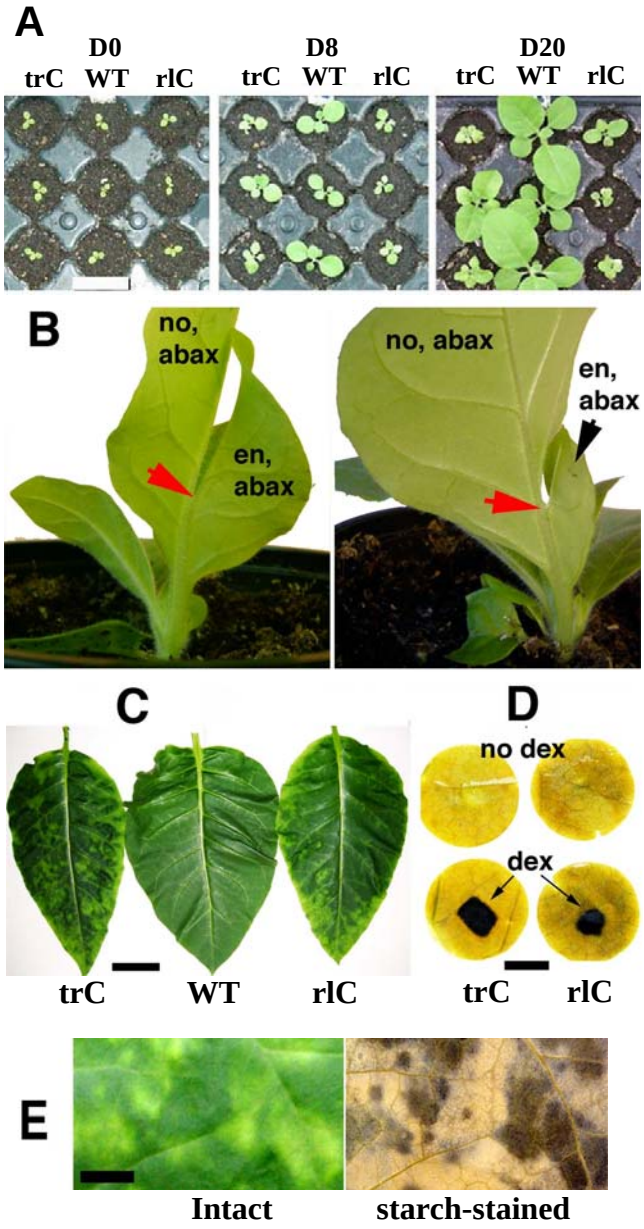


Figure 4. *trolC* and A4-*rolC* modify growth of tobacco plants in soil.
Wild-type (WT), dex-*trolC-HAHIS-7* (trC) and dex-A4-*rolC-HAHIS-7* (rC) plants were sprayed with a 3 µM dex solution. A. Seedlings after induction at the 2-cotyledon stage on day 0, 8 and 20. Wild type plants develop normally, dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* plants show reduced growth. Bar: 3 cm. B. Enations (black arrows) on dex-*trolC-HAHIS-7* (trC) and dex-A4-*rolC-HAHIS-7* (rC) plants 40 days after induction at the 2-cotyledon stage. no, abax: normal leaf, abaxial side. en, abax: enation, abaxial side. Enations are abnormal, extra leaf blades that are partially fused at their basal side to a normal leaf along the central leaf veins, and oriented in a mirror-wise fashion. Red arrows: points from where on normal leaves and enations are fused. Bar: 2 cm. C. Leaves of plants induced at an intermediate, non-flowering stage, 7 days after induction. dex-*trolC-HAHIS-7* (trC) and dex-A4-*rolC-HAHIS-7* (rC) leaves show irregular chlorosis patterns and are reduced in width compared to a wild-type leaf (WT). Bar: 2 cm. D. Iodine staining revealing starch (black areas) in dex-*trolC-HAHIS-7* (trC) and dex-A4-*rolC-HAHIS-7* (rC) leaves, 7 days after local induction with a dex-containing lanolin sphere placed at the center. Upper two disks: treated with lanolin spheres without dex, lower two disks: treated with lanolin spheres with 10 µM dex. Starch accumulates within a small zone around the inducing lanolin sphere. Bar: 1 cm, small black dot: size of the lanolin sphere. E. Starch accumulation in a dex-sprayed dex-A4-*rolC-HAHIS-7* leaf. Left: Irregular chlorosis as in C, right: same area, cleared with hot 70% ethanol and stained for starch with iodine, starch appears black. Chlorotic and starch-containing areas coincide.

their root hairs as in the case of intact seedlings. Sensitivity to sucrose was tested by placing dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* roots on media with different sucrose concentrations (from 0 to 2%) with or without 3 μM dex. Growth did not occur on medium without sucrose, but was already visible at 0.1%; both lines showed similar sensitivity (Figure 6A). Interestingly, dex-*trolC-HAHIS-7* roots grew initially as fast as dex-A4-*rolC-HAHIS-7* roots but ceased growth at day 5, whereas dex-A4-*rolC-HAHIS-7* roots continued their growth for over ten days, an example (3 μM dex and 1% sucrose) is shown in Figure 6B. Growth of isolated roots on low sucrose has also been demonstrated for dex-T-*6b* roots, and absorption of radioactively marked sucrose by these roots increased significantly upon induction (Clément *et al.,* 2007). Sucrose uptake by dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* root fragments was measured in the same way, using wild-type tobacco and the dex-T-*6b* line D6Nt17 (Clément *et al.,* 2007) as negative and positive controls respectively. Sucrose uptake of wild-type tobacco root fragments was not modified by dex treatment, whereas root fragments of induced dex-T-*6b*, dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* seedlings showed a clear increase in sucrose uptake (Figure 6C).

Expression of dex-*trolC-HAHIS* is affected by silencing.

In the course of our induction studies some dex-*trolC-HAHIS*-induced growth changes rapidly reversed to normal in spite of the continued presence of inducer; shoots and lateral roots normalized (Figure 3E and 3F), whereas isolated roots stopped their growth on 1% sucrose (Figure 6B), dex-A4-*rolC-HAHIS* changes on the contrary appeared stable. This difference in phenotype stability might be due to differences in tRolC and A4-RolC protein stability. Protein levels were compared for different induction times. Whereas A4-RolC-HAHIS protein levels remained stable for two weeks, tRolC-HAHIS protein showed a decrease starting after day 1 and resulting in very low levels after two weeks (Figure 7A). Transgene transcripts measured by RT-qPCR were found to decrease in dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS-7* seedlings, but more rapidly and to lower levels in dex-*trolC-HAHIS-7* (Figure 7B). dex-*trolC-HAHIS-7* plants showed increasing amounts of a 21 nt siRNA, demonstrating post-transcriptional gene silencing, dex-A4-*rolC-HAHIS-7* plants did not (Figure 7C).

Expression of *trolC* and A4-*rolC* in *Arabidopsis thaliana.*

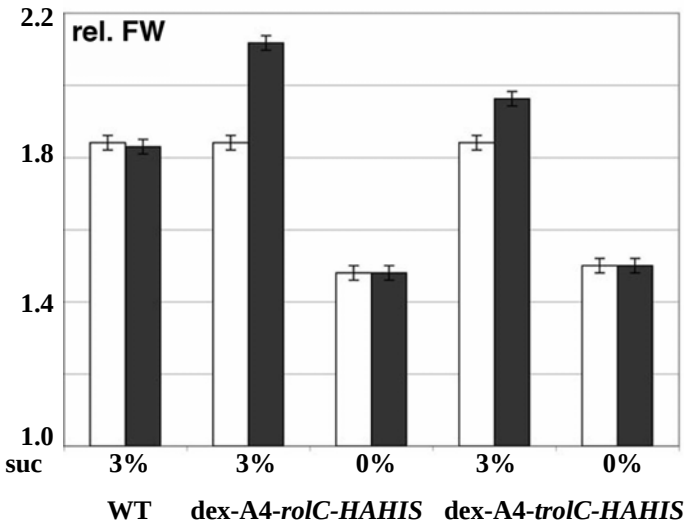


Figure 5. Increase of leaf disk expansion by *trolC* and A4-*rolC* and dependence on sucrose.
Disks of wild-type, dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* lines were placed on liquid medium and expansion after 5 days was expressed as the ratio of (fresh weight at day 5/fresh weight at day 0), indicated as relative fresh weight. White bars: no induction, black bars: induction by 3 µM dex. Induced dex-A4-*rolC-HAHIS* and dex-*trolC-HAHIS* disks show increased expansion but only on sucrose-containing media. Values are means +/- standard deviation. For each medium, 12 disks were measured per line. Experiments were repeated twice. Experimental errors were less than 1%.

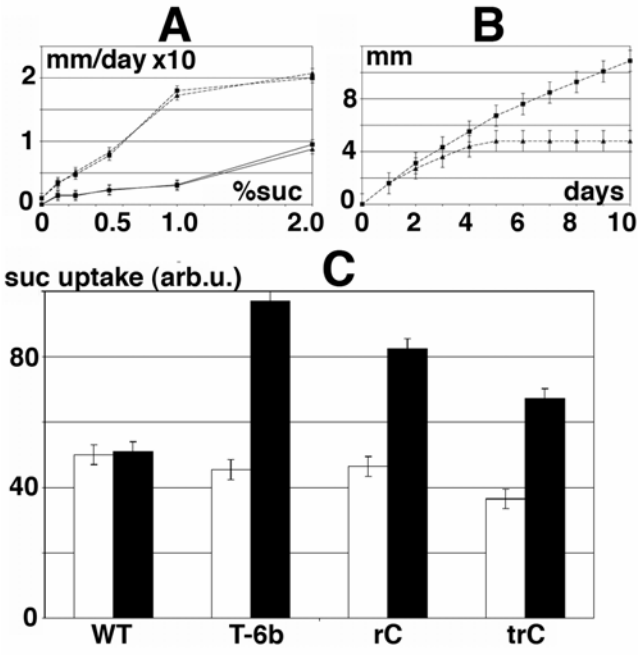


Figure 6. Influence of *rolC* and *trolC* on root growth and sucrose uptake.
A. dex-A4-*rolC-HAHIS-7* (squares) and dex-*trolC-HAHIS-7* roots (triangles) were grown on different sucrose concentrations. Growth in mm/day over a 5-day period. Continuous lines: non-inducing medium, Broken lines: inducing medium. B. Length increase (in mm) of dex-A4-*rolC-HAHIS-7* (squares) and dex-*trolC-HAHIS-7* roots (triangles) on medium with 1% sucrose and 3 µM dex, during a 10-day induction period. dex-A4-*rolC-HAHIS-7* roots show regular growth, dex-*trolC-HAHIS-7* roots slow down and stop growth at day 5. For A) and B), values are means +/- standard deviation. For each condition, 10 roots were measured per line. Experiments were repeated three times. Experimental errors were less than 3%.C. Sucrose uptake by subapical 3 mm long root fragments of wild-type (WT), dex-T-*6b* (T-6b), dex-A4-*rolC-HAHIS-7* (rC) and dex-*trolC-HAHIS-7* (trC) lines, without induction (white bars) or after induction with 3 µM dex (black bars). dex-T-*6b*, dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* lines show a clear increase in sucrose uptake, the WT control does not. Data are expressed as percentage of sucrose taken up after 24 hours. Values are means +/- standard error. Root fragments were pooled in groups of five, ten groups were used per line. Experiments were repeated two times. Experimental errors were less than 10%.

Our results show that dex-*trolC-HAHIS* expression is rapidly silenced in tobacco. Expression of exogenous *trolC* and A4-*rolC* genes in tobacco might therefore lead to silencing of the endogenous tobacco *trolC* gene and make it difficult to distinguish between the effects of exogenous and endogenous *rolC* genes. In order to confirm *trolC* activity in a plant species lacking *rolC*-like genes, we transformed *A. thaliana* ecotype Col-0 with 2x35S-*trolC,* 2x35S-A4-*rolC,* 2x35S-*trolC-HAHIS*, 2x35S-A4-*rolC-HAHIS* and the pBI121.2 vector control. Plants of the T3 generation expressing *trolC* or A4-*rolC* (with or without HAHIS tag) showed similar phenotypes. Root growth was strongly reduced (Figure 8A), flowering occurred early, both *in vitro* (Figure 8B) and *in vivo* (Figure 8C) and plants were slightly less green than control plants. Thus, *trolC* and A4-*rolC* are biologically active in a plant species without an endogenous *rolC* gene.

Plast-like proteins in another eukaryote, *Laccaria bicolor*.

Sofar, *plast* genes have only been reported in *Agrobacterium* and a few *Nicotiana* species, in the latter group they are clearly derived from *Agrobacterium* by horizontal transfer. The Plast proteins similarly constitute an isolated group of unknown origin (Studholme *et al.,* 2005). However, a recent databank search identified 5 *Laccaria bicolor* S238N-H82 proteins with unknown function as possible Plast-like protein homologs (between brackets: NCBI code): EDR08145.1 (XP_001881215.1) (491 aa), EDR04342.1 (XP_001884861.1) (451 aa), EDR04443.1 (XP_001884962.1) (273 aa), EDR04444.1 (XP_001884963.1) (284 aa) and EDR04445.1 (XP_001884964.1) (409 aa) (Martin *et al.,* 2008). Four of the five proteins (XP_001884861.1, XP_001884962.1, XP_001884963.1 and XP_001884964.1) are coded by genes clustered within a 33 kb region. The common region of the 5 *Laccaria* proteins is about 250 amino acids in size, corresponding to the average size of the *Agrobacterium* Plast proteins (Figure 9A), and similarity values between the common regions range from 56-84%. XP_001884962.1 aligns with almost all Plast proteins above statistically significant levels (not shown), whereas the four others only show significant homology to Plast proteins C', D and 5. Similarity values between Plast and *Laccaria* proteins are in the range of inter-Plast similarity values (about 20%). An alignment of XP_001884962.1 and the *Agrobacterium* Plast protein with the highest similarity score (protein 5) is shown in Figure 9B.

DISCUSSION

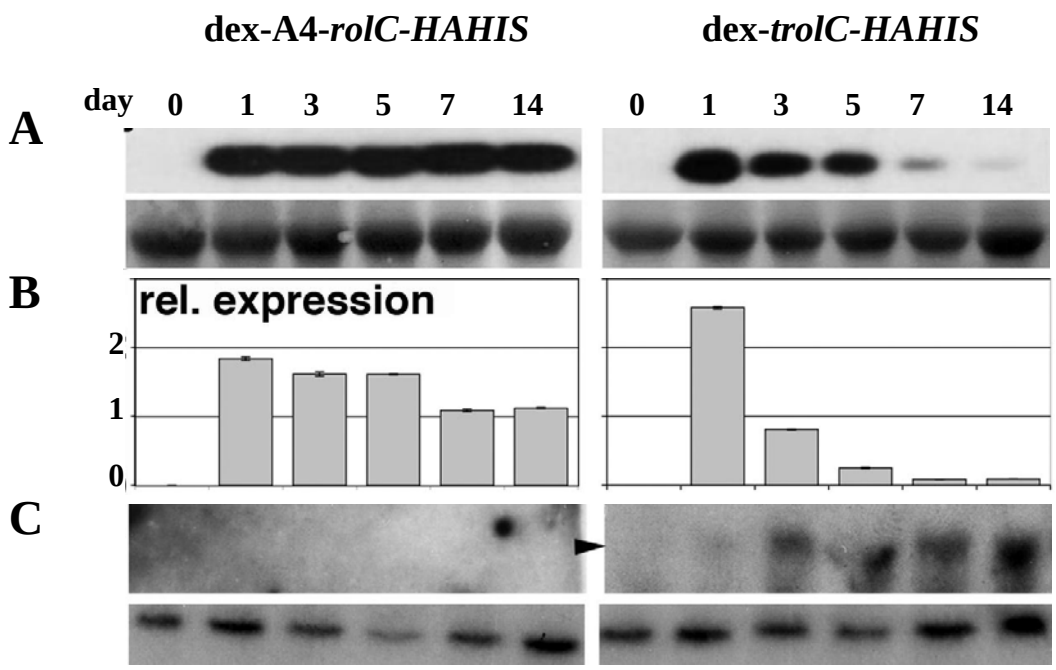


Figure 7. Progressive silencing of dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* after induction.
Seedlings were placed on medium with 3 µM dex and analyzed 0, 1, 3, 5, 7 and 14 days later. A. Western analysis: top: A4-RolC-HAHIS and tRolC-HAHIS proteins, bottom: loading control. Whereas A4-RolC-HAHIS amounts remain stable, tRolC-HAHIS levels rapidly decline. B. RT-qPCR analysis. The same samples as in A. were analyzed for mRNA levels by RT-qPCR. Units: relative expression levels x 0.0001 (calculated with respect to values at day 0, set at 1). dex-A4-*rolC-HAHIS* mRNA levels decline slowly, whereas dex-*trolC-HAHIS* mRNA levels decline more rapidly. C. siRNA analysis. Top: dex-A4-*rolC-HAHIS* samples show no signal, whereas dex-*trolC-HAHIS* samples show increasing quantities of a 21 nt siRNA (arrow). Bottom: RNA loading control obtained by hybridizing the filter to a *U6* RNA probe (Gy *et al.*, 2007).

The *Nicotiana* cT-DNAs constitute the only known examples of naturally occurring DNA transfer in plants. Among the many unsolved questions regarding this phenomenon are those concerning the relation between the *N. tabacum* and *N. glauca* cT-DNAs, the present-day role of the cT-DNA genes, and the survival of the initial regenerants. The relation between the *Nicotiana tabacum* cT-DNAs and those of *N. glauca* are more complex than suspected. Two non-adjacent sequences occur in *N. tabacum*: the first carries *torf8* to *torf13* (with possibly an extension to the left) and is moderately related (70-80% homology) to the corresponding sequences from *A. rhizogenes* A4 and *N. glauca*. The second one carries *torf14* and *tmis* and extends up to the right border, this sequence is practically identical (96% homology) to the *N. glauca Ngorf14-Ngmis* region but inserted in a different region. It should be noted that the volution of *Nicotiana* species is extremely complex because of the occurrence of interspecific hybrids. Trees for different *Nicotiana* genes do not always coincide, so that no consensus has yet emerged for trees that reflect the evolution of the entire species (Kelly et al. 2010). Therefore, the tree that we present in Fig. 10A only reflects the evolution of the cT-DNA regions in the different species and does not pretend to represent species evolution.

To account for the present data we propose the following evolutionary scenario (Figure 10A). First, the two arms of the inverted repeat of the cT-DNA of *N. glauca* are clearly derived from a single transformation event (Figure 10A, arrow 1, the inverted repeat structure is indicated by a) and therefore had initially the same sequence. The 5% divergence between the two arms (Furner *et al.,* 1986) is due to divergent evolution between the two arms. The *torf14-tmis* sequence of *N. tabacum* shows 4% divergence with respect to the two *Ngorf14-Ngmis* sequences but is located in another site. The easiest assumption is that the *torf14-tmis* fragment (Figure 10A, fragment b) derives from the same transformation event and T-DNA as fragment a, but was inserted elsewhere. Later, fragment a and b separated and are now found in two different *Nicotiana* species. Secondly, since *trolB-torf13* (Figure 10A, fragment c) and *NgrolB-Ngorf13* differ by about 20%, we propose that *trolB-torf13* (fragment c) originates from another T-DNA than *NgrolB-Ngorf13*, and therefore from another transformation event (Figure 10A, arrow 2). The order of entry (1>2 or 2>1) is unknown, and both fragments might have been combined by crosses rather than by a secondary transformation. Thirdly, the *torf8-torf13* fragment underwent a modification at the *torf13* end (Figure 10A, c to c', modification indicated by arrow 3). The tobacco cultivars with a truncated *torf13* gene belong to the oriental tobacco group. It would be interesting to test the influence of the wild-type *torf13* gene from Havana 425 on cv. Samsun nn and other oriental cultivars as this may provide

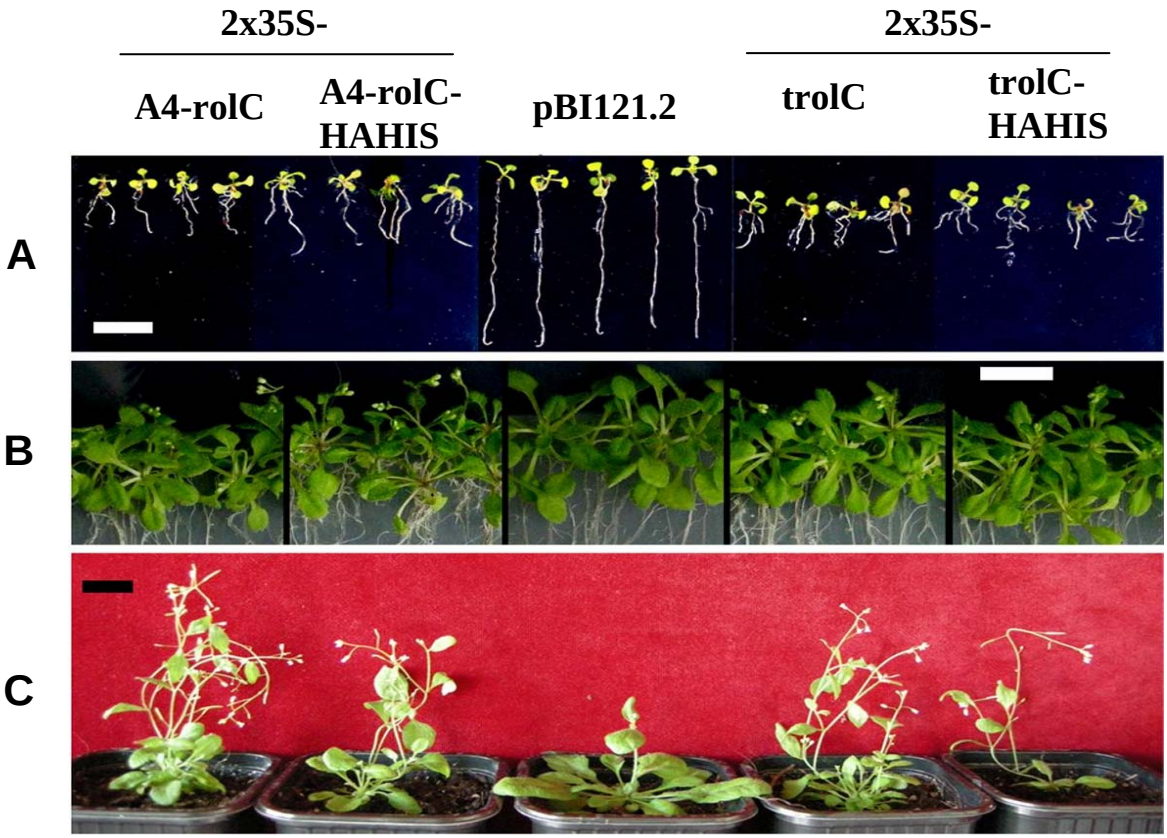


Figure 8. Expression of A4-*rolC* and *trolC* in *Arabidopsis thaliana*.
Tagged and non-tagged version of A4-*rolC* and *trolC* under 2x35S promoter control and the empty pBI121.2 vector construct were used to transform *Arabidopsis thaliana* ecotype Col-0. Shown are T3 plants. A. 10 days after germination *in vitro*. Both the tagged and non-tagged A4-*rolC* and *trolC* versions cause strong root growth inhibition, pBI121.2 vector plants are normal. B. 21 days after germination *in vitro*. Early flowering of plants with tagged and non-tagged versions of A4-*rolC* and *trolC*. C. 32 days after germination in soil. Early flowering of plants with tagged and non-tagged versions of A4-*rolC* and *trolC*. Bars (A and B): 1 cm, (C): 2 cm.

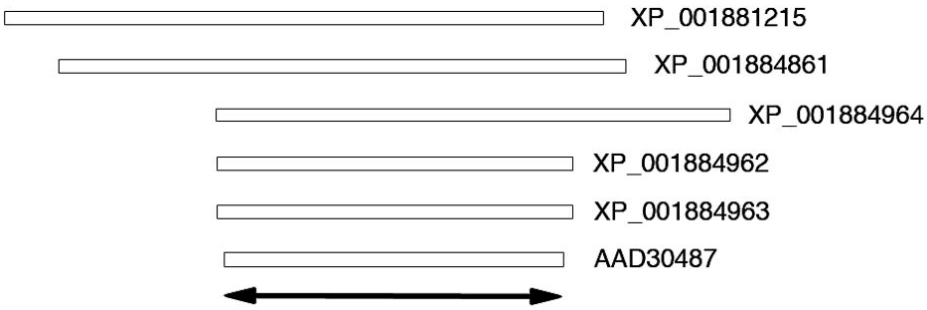
some ideas on the consequences of the loss of *torf13*. We believe our evolutionary model can provide a framework for further studies, especially with regard to cT-DNAs from other *Nicotiana* species.

N. tabacum carries the three essential hairy root genes *rolA, rolB* and *rolC*, but *trolA* and *trolB* are mutated. *trolC* has strong morphogenetic activity, very similar to that of A4-*rolC* from *A. rhizogenes*. Functional comparison was facilitated by the use of dex-inducible constructs avoiding the accumulation of growth abnormalities as in the case of constitutive genes.

In dex-*trolC-HAHIS*, but not in dex-A4-*rolC-HAHIS* plants, post-transcriptional gene silencing led to a rapid and strong decrease in transgene mRNA and protein levels, with concomitant reversion to normal phenotypes. The strong *trolC-HAHIS* silencing merits further investigation as it might also affect endogenous *trolC* expression. At the protein level, tRolC-HAHIS might compete with putative tRolC partners for binding or other types of interaction, thereby producing dominant negative effects. In addition, it is possible that the various abnormalities induced by strong dex-*trolC-HAHIS* expression produce indirect changes in expression of the endogenous *trolC* gene. These possibilities remain to be studied. However, the data from Arabidopsis show that the *trolC-HAHIS* gene construct does not need to interact with an endogenous *trolC* gene to produce a strong biological effect.

In this work, we have used several new *rolC* assays monitoring growth changes after induction of seedlings, intact plants, leaf disks or isolated roots. These assays have revealed hitherto unsuspected functional similarities between *rolC* and *6b*. In spite of the fact that 6B and RolC proteins show only 23% similarity, AB-*6b*, T-*6b*, *trolC* and *rolC* all induce enations. Enations are secondary leaf blades arising in a mirror-wise fashion along the veins of the abaxial leaf side. Since these growth modifications are very unusual, we believe this provides a strong indication that *rolC* and *6b* impact similar pathways. *rolC* and *6b* also induce leaf chlorosis and starch accumulation. They stimulate leaf disk expansion on sucrose-containing media, reduce root hair development and root growth and stimulate sucrose uptake by root fragments. In view of these results, we propose that the various *rolC* and *6b* effects are caused by enhanced sucrose absorption and retention. RolC is cytosolic (Estruch *et al.,* 1991; Oono *et al.,* 1991) and therefore probably not a transporter itself. The regulation of *rolC* transcription by sucrose and increased hexose levels in *rolC* transgenic potato has earlier led to the suggestion that *rolC* plays a role in sucrose metabolism and/or transport (Nilsson and Olsson 1997). Our studies provide strong experimental support for this idea and indicate a role in

A



B

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6    YLLPQAFTPSDCSGITNPTELEAMLKYALSRYKLHI-ERTYKSQVAWAALLLPPFLNEPA    64
     Y     F   D S I +  EL+ +L++    Y+    E    +Q +W   ++    + P
2    YHSRPIFNIIDSSNIQDRRELKLVLRHTEIAYRSFAQEDLIPAQRSWMNSIINT--DVPI    59

65   DAPTLTMAQRIIEKRELMPHYQPFGEKHYEPGQPQDDIHEIRDTILIDAQPLFTYSCADR   124
     D P +     ++++         F E    PG        I   I+++    + Y
60   D-PAI---DEVVKR---------FCEVACLPGP-----AGIPLNIILNDSLTYVYCSFQA   101

125  VEQQVTHHGLDISGGAECIYQHQQSSFPVGAYGITIPPYNQDISQAAMRSFMQTPIQT--   182
     + +             +  Y         G    T+PPY + I++  MRS+     Q
102  MRKY----------AHKRFYD---GVSDEGVVISTVPPYAEGITKETMRSWHNNVCQNTS   148

183  ------NHWIMFIPTTGFSLKSQLIFSHPNSGFKEVFNHYGLATPHLSPGTYLPY--DIV   234
           + +I F+PT+         FSH   G              L+P    P+  +I+
149  NETHDLDAYIAFLPTS----LQNPSFSHMKIG----------CDSFLAPSRVDPFCVEII   194

235  AIGDTL  240
     A+G  L
195  AVGKAL  200
```

Figure 9. Plast-like protein homologs in *Laccaria bicolor*.
A. Schematic alignment of 5 *Laccaria bicolor* Plast-like proteins with the *Agrobacterium* Plast protein AAD30487 (gene *5* protein from *A. tumefaciens* strain C58). Double-headed arrow: common part of Plast proteins, about 250 amino acids. B. Alignment of protein XP_001884962.1 from *Laccaria bicolor* (top line) with gene *5* protein (bottom line). In bold: residues conserved in the *Agrobacterium* Plast family.

sucrose uptake. In natural *A. rhizogenes* infections, sucrose accumulation may stimulate initiation and outgrowth of hairy roots. Since the tobacco *trolC* gene is expressed (Meyer *et al.,* 1995) and functionally active in tobacco (this study), it could play a significant role in tobacco growth and physiology.

In spite of their similarities, *6b* and *rolC* plants also show characteristic differences. *6b* plants have dramatically expanded roots and show ectopic divisions in the root stele, around leaf veins and on seedling stems (Helfer *et al.,* 2003; Grémillon *et al.,* 2004), contrary to this, *trolC* or A4-*rolC* plants do not. Instead, *trolC* and A4-*rolC* leaves are pale-green and narrow, show wrinkled edges and irregular chlorosis and are epinastic in seedlings. Possibly, these differences are due to differences in Plast protein mobility. The *6b* phenotype is graft-transmissible (Helfer *et al.,* 2003) and the 6B protein moves in leaves (Grémillon *et al.,* 2004) whereas *rolC* effects are cell-autonomous (Spena *et al.,* 1989; Fladung and Ahuja, 1997; Gidoni *et al.,* 2001; Fladung *et al.,* 2004). 6B has also been shown to interact with the NtSIP1 protein (Kitakura et al. 2002) and to enter the nucleus (where it acts as a histone H3 chaperone protein, Terakura et al. 2007). In view of the partial functional similarities between the RolC/tRolC and 6B proteins it will be interesting to test the interaction of RolC/tRolC with NtSIP1 and H3 and their possible localization in the nucleus. 6B has also been shown to enter the nucleus (where it interacts with histone H3, Terakura *et al.,* 2007). Whether other *plast* genes share activities with *rolC* and *6b* remains to be tested. Interestingly, the *rolB*-like 5' part of *orf8* expressed under 2x35S promoter control causes dwarfing, irregular leaf chlorosis and starch accumulation in tobacco with a strong reduction of sucrose export (Otten and Helfer, 2001; Umber *et al.,* 2002).

Our studies confirm that *rolC* accelerates flowering as noted in tobacco and in other plant species (Faiss *et al.,* 1996; Casanova *et al.,* 2005). Possibly, this *rolC* property led to rapid speciation of the initial *Nicotiana* transformants.

The occurrence of *plast*-like genes in the ectomycorrhizal basidiomycete *Laccaria bicolor* is of considerable interest. The close association of *Agrobacterium* and *Laccaria* with plant roots and their common interest in plant-derived metabolites (López *et al.,* 2008) might have favored horizontal DNA transfer from the fungus to the bacterium (Figure 10B, arrow 1). A long period of divergence then generated the many different *Agrobacterium plast* genes. Although a transfer from the bacterium to the fungus cannot be excluded, the lack of other T-DNA genes like opine synthesis genes (often found close to the right T-DNA borders and therefore likely to be cotransferred) and hormone synthesis genes in the two chromosomal

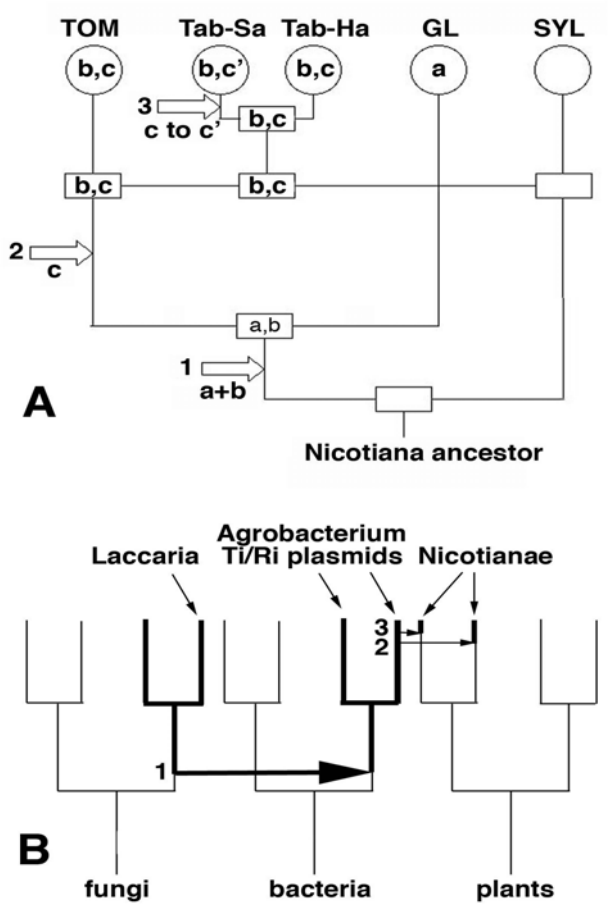

Figure 10. Evolutionary models.
A. Origin of *Nicotiana* cT-DNAs. TOM: *N. tomentosiformis*, TAB-Sa: *N. tabacum* cv. Samsun, TAB-Ha: *N. tabacum* cv. Havana, GL: *N. glauca*, SYL: *N. sylvestris*. Circles indicate present-day species, squares ancient species. Arrow 1: transformation by a 1724-like mikimopine strain, insertion of an inverted repeat inherited by GL (fragment a) and an *orf14-mis* fragment inherited by TOM and TAB (fragment b). *N. tabacum* is a hybrid of *N. tomentosiformis* and *N. sylvestris*. Arrow 2: Insertion of a second cT-DNA with *orf8-orf13* genes by an unidentified *A. rhizogenes* strain, and inherited by TOM and TAB (fragment c). Arrow 3: Partial deletion of *torf13* in one group of TAB cultivars, inherited by TAB-Sa and related tobaccos (c to c').
B. Origin of *plast* genes. Arrow 1: Hypothetical transfer of *plast* genes from a fungus to *Agrobacterium* by horizontal gene transfer. Subsequent divergence of *plast* genes between *Agrobacterium* and fungi and within *Agrobacterium* reduced DNA homology to undetectable levels and left only weak protein homology. Arrows 2 and 3: Recent horizontal transfer of Agrobacterium *plast* genes to Nicotiana, DNA and protein are still significantly homologous. Thick lines: species with *plast* or *plast*-like genes.

Laccaria regions that contain *plast*-like genes makes this less likely. More recently, at least two transfers occurred from *Agrobacterium* to *Nicotiana* (Figure 10B, arrow 2 and 3). The distribution of *plast*-like genes in other fungi, their role in *Laccaria* and their effects on plants should constitute topics for further studies.

MATERIALS AND METHODS

A4-*rolC* and *trolC* constructs.

For constitutive expression, A4-*rolC* and *trolC* coding regions were cloned in a 2x35S promoter cassette by PCR and subcloned into the pBI121.2 binary vector (Jefferson *et al.,* 1987) as described for other constructs (Otten and Helfer, 2001). Dexamethasone (dex)-inducible constructs were prepared as for dex-T-*6b* (Grémillon *et al.,* 2004), using the pTA7002 binary vector (Aoyama and Chua, 1997). Where indicated, a HAHIS tag from pNTL2104 (Thomas *et al.,* 2006) was added at the 3' end to permit protein detection using anti-HA antibodies. All constructs were checked by sequencing before subcloning. Binary vectors with constructs were introduced in the disarmed *Agrobacterium* helper strain LBA4404 (Hoekema *et al.,* 1983).

Transformation and regeneration.

Tobacco transformation and regeneration was as described (Otten and Helfer, 2001). Arabidopsis thaliana ecotype Colombia (Col-0) plants were transformed by the floral dip method (Clough and Bent, 1998).

Induction by dexamethasone.

Plants were induced with dexamethasone in different ways. Mature leaves were infiltrated with a dex solution in 10 mM $MgSO_4$ using a 1 ml syringe without a needle. Seedlings were germinated on MS255 (Duchefa) agar plates with 1% sucrose, 10 days later placed on nylon filters on vertical plates with the same medium, and transferred to inducing vertical plates 3 days later. Leaf disks were induced by floating them upside down on a solution of 10 mM KCl, 0.5 mM HEPES buffer pH 6.0, with varying concentrations of sucrose and weighed at one-day intervals (Keller and Van Volkenburgh, 1997; Clément *et al.,* 2006). Intact small or medium-sized plants in soil were sprayed on two consecutive days with 3 μM dex and 0.05% Tween-20 in water. Leaves of different sizes were locally induced by placing lanolin spheres (1 mm diameter) containing 10 μM dex on the upper leaf surface. One week later, disks were removed and stained for starch with iodine.

Sucrose absorption.

Sucrose absorption was tested with 3 mm long root fragments situated originally at 9-12 mm from the root apex. These fragments were obtained from intact seedlings grown for 3 days on inducing medium on vertical plates. Five fragments were floated on a 15 µl drop of MS255 medium with 1 mM ^{14}C-sucrose in microtiter plates (Clément *et al.,* 2007). Radioactivity in the medium was measured at the start of the experiment and after 24 hours, all experiments were carried out in triplicate.

***Arabidopsis thaliana* seed germination.**

Arabidopsis thaliana seeds (generation T3) were germinated on MS255 (Duchefa) medium in the presence of 50 mg/l kanamycine and 1% sucrose for 8 days and then transferred to soil or to nylon filters on vertical agar plates containing the same medium.

RNA extraction and siRNA gel blotting.

RNA was extracted from leaves of tobacco seedlings using Tri-Reagent (Sigma). Leaves of fifteen plants (two leaves per plant) were ground in liquid nitrogen. The resulting powder was homogenized in 1 ml Trizol and 200 µl chloroform was added. The upper phase was precipitated with 1 volume isopropanol. After washing with 70% ethanol (1 ml), RNAs were taken up in RNAse-free H_2O. RNA gel blot analysis of low molecular weight RNA was conducted on 25 µg of total RNA as described (Akbergenov *et al.,* 2006). The A4-*rolC* coding sequence and the 183-395 fragment of the *trolC* coding sequence were used as probes. Blots were stripped and rehybridized with a probe complementary to *U6* RNA as a loading control (Gy *et al.,* 2007).

RNA extraction and RT-quantitative PCR.

RNA was extracted as described above. After treating with RNase-free DNase (Qiagen kit) cDNA was synthesized with 5 µg total RNA, using Superscript III Reverse Transcription kit (Invitrogen). Real-time quantitative PCR reactions (10 µl) were performed in 384-well optical plates on a BioRad i-cycler apparatus using PCR master 2x mix (Roche) containing 480 SYBER-Green I fluorescein reporter with gene specific primers. Quantification was performed in triplicate. *Nicotiana tabacum* translation elongation factor gene (*EF2*, GenBank:

AJ299248.1) was used as an internal standard for equalization of RNA levels. Quantifications were then normalized to non-induced tobacco plantlets. Error bars represent the standard deviation from three replicates. Experiments were repeated three times in a biologically independent manner.

RT-qPCR primers.

EF2-Forward: 5'-ctgaaccagaagcgtggaca-3', *EF2*-Reverse: 5'-ccagatgtagcagccctcaag-3', *trolC*-Forward: 5'-ctgaaccagaagcgtggaca-3', *trolC*-Reverse: 5'-ccctgacattcagtctttatgcac-3', *rolC*-Forward: 5'-gggcagtcgacgtagagg -3', *rolC*-Reverse: 5'-cgatggatattgacgaagaagg -3'.

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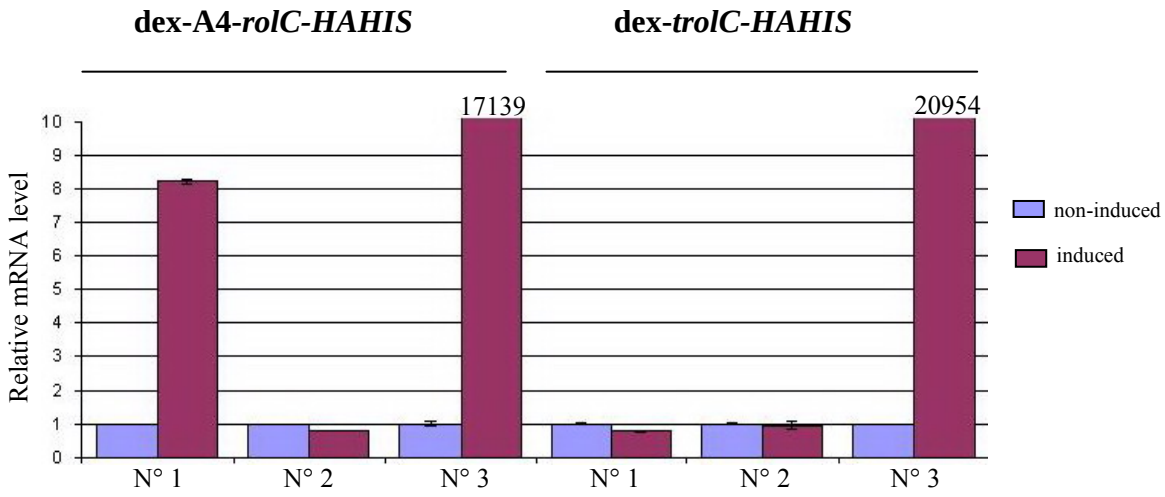


Figure 11. Stability of silencing in dex-A4-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* plants after re-induction assay. Plants N° 1 and N° 2 were first induced by 3 µM dex in the cotyledon stage, plant N° 3 was not. After two months the leaf discs of these plants were re-induced and the expression of *trolC* and A4-*rolC* was analyzed after 24 h by RT-qPCR. Upon induction, a very small increase in mRNA is found in dex-A4-*rolC-HAHIS-7* plant N° 1, no increase is seen in dex-A4-*rolC-HAHIS-7* plant N° 2, and dex-*trolC-HAHIS-7* plants N° 1 and 2. The control plants (N° 3) show a 20.000 fold induction. Numbers on top of the bars represent the relative values with respect to the non-induced value, set at 1.

2. Results non-submitted to publication

2.1. *(t)rolC* silencing is stable during plant growth

As described previously, the induction of transgenic dex-*trolC-HAHIS-7* plants led to considerable reduction of the tRolC protein and *trolC* transcript levels over a period of 15 days in the presence of dexamethasone, in dex-A4-*rolC-HAHIS* plants no changes in A4-RolC protein levels and only a low reduction in A4-*rolC* transcripts were observed (Figure 7). In order to test whether these genes remained non-inducible over a longer period, we analyzed transcript levels after dexamethasone treatment 2 months after the initial induction.

Two plants of each line (N° 1 and N° 2) were chosen at the two cotyledon-stage and induced by spraying with 3 µM dex. One plant of each (N° 3) was kept as a non-treated control. After 2 months leaf discs of these six plants were floated on a liquid medium containing 3 µM dex. Leaf discs on the medium without dex served as negative controls. After 24 h, the expression of the genes was determined by RT-qPCR. As shown in Figure 11 no increase in transcript levels occurred in the earlier induced dex-*trolC-HAHIS-7* plants, whereas the control plant that was not treated at an early stage showed a 17.000-fold level of induction. In the dex-A4-*rolC-HAHIS* leaf discs, plant N° 1 showed a low, ~8 fold increase in transcripts, plant N° 2 showed no induction and the control plant N° 3 showed a 20.000-fold induction level. These results demonstrate that dex-*trolC-HAHIS-7* and dex-A4-*rolC-HAHIS* plants can no longer be induced after initial induction and silencing.

The stability of silencing after 2 months in leaves that were not yet present at the time of the first induction suggests that the siRNA molecules resulting from the degradation of *trolC* and A4-*rolC* transcripts during the first induction have spread systemically, probably providing a rapid and efficient silencing system upon renewed induction in all parts of the plants.

2.2. Interaction of (t)RolC with cellular protein(s)

One of the methods for studying the mechanism of action of proteins is looking for cellular partners in the host plant. As reported in the first part of this chapter, plants expressing *rolC* and *trolC* show several common characteristics such as reduction in general growth, the presence of small and pale-green leaves, early flowering and the accumulation of sucrose. These similarities indicate that both genes influence the same signalling pathways

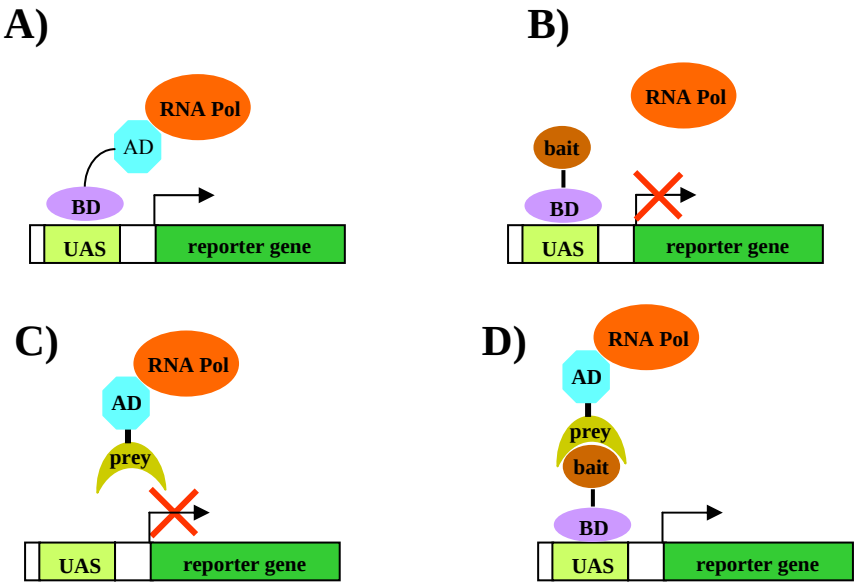


Figure 12. Principle of yeast two-hybrid assay. This technique is used to check the interaction of two proteins, indicated as *Bait* and *Prey*. A) The *Gal4* transcription factor gene encodes a protein with two domains (BD, DNA Binding Domain binding to an upstream activating sequence (UAS) and AD, transcription Activating Domain interacting with the RNA polymerase, RNA Pol). Both are essential for transcription of the reporter gene. B,C) Two fusion proteins are prepared: *Gal4BD+Bait* and *Gal4AD+Prey*. None of them is sufficient to initiate the transcription of the reporter gene alone. D) In the case of interaction between bait and prey the complex can bind onto UAS and transcription of the reporter gene occurs.

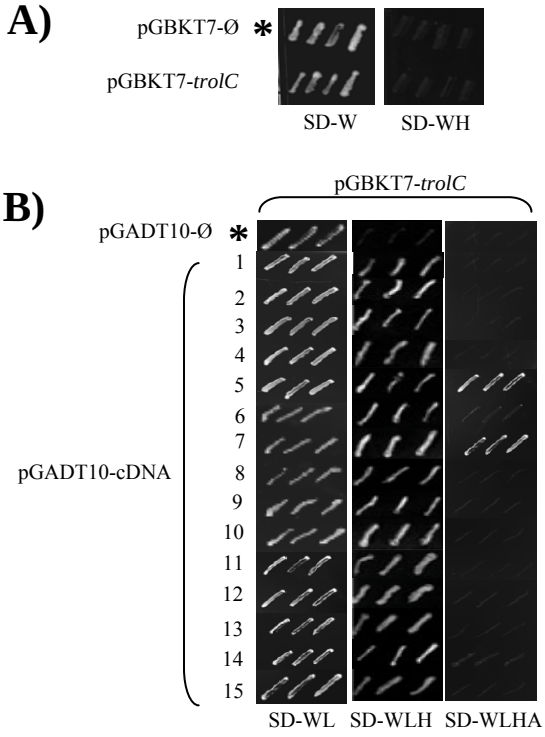


Figure 13. Yeast two-hybrid screen with an Arabidopsis cDNA library to detect the partners of the tRolC protein. A) Autoactivity test for tRolC on transcription of the *HIS* reporter gene. No growth of pGBKT7-*trolC* transformed yeast cells was observed on selective medium (SD-WH) meaning that tRolC does not function as a protein containing an AD. B) The result of the screen : 15 cellular partners were found to interact with tRolC and among them two (N° 5 and 7) interact strongly. Triple replicates of double transformant clones were subcultured. SD-WL is non-selective medium and SD-WLH and SD-WLHA are selective media for interaction.
pGBKT7-Ø and pGADT10-Ø represent empty vectors. The stars (*) represent the negative controls in the experience.

and/or may interact with the same partners in the plant to alter plant morphology and development. Therefore we attempted to find protein partner(s) that would interact with both tRolC and RolC. To this end, we first carried out a yeast two-hybrid screen with an Arabidopsis cDNA library (Arabidopsis Biological Resource Center CD4-22, CD4-30) to find the cellular partners of tRolC and in a second step tested the interaction of these partners with RolC.

The principle of the yeast two-hybrid system is shown in Figure 12.

trolC was cloned into pGBKT7 (CLONTECH) carrying the GAL4 binding domain (BD) and the *TRP1* nutritional marker gene (Tryptophan synthesis gene) and used in combination with a cDNA library of Arabidopsis cloned in pGADT10 (CLONTECH) carrying the GAL4 activation domain (AD) and the *LEU1* nutritional marker gene (Leucine synthesis gene). Cells of *Saccharomyces cerevisiae* strain AH109 (CLONTECH) carrying the Histidine (*HIS)* and Adenine (*A*) synthesis reporter genes were transformed with pGBKT7-*trolC* and cultured on selective "synthetic defined" (SD) medium without Tryptophan (SD-W; SD is a medium without amino acids that is complemented with amino acids and used for growing most *S. cerevisiae* strains). To test whether tRolC can activate the reporter gene by itself, pGBKT7-*trolC* yeast colonies were spread on medium without Histidine and Tryptophan (SD-HW). None of the transformants grew on this medium showing that tRolC has no AD activity (autoactivity) (Figure 13A). Subsequently we transformed pGBKT7-*trolC* yeast cells with pGADT10-cDNA (CLONTECH). Fifteen of the 5×10^6 double transformants grew on the selective medium without H, W and L (SD-HWL) (Figure 13B) and among them two (clone N° 5 and 7) grew under strong selective conditions, in the absence of the four amino acids in the medium (SD-AHWL). The plasmids of fifteen clones were extracted, sequenced and analyzed by BLAST (http://www.ncbi.nlm.nih.gov/ BLAST/) against the complete Arabidopsis genome sequence. Table I presents the results of the DNA sequence analysis. Among the 15 clones eight sequences (clones N° 5, 7, 9, 11, 12, 13, 14 and 15) were in the correct translation frame. Clones N° 5 and 7 had the same cDNA, corresponding to a member of the TCP family of genes, *TCP13* (At3g02150.2). These are transcription factors controlling multiple developmental traits in plants (for more details see discussion and perspectives).

In order to test whether RolC is capable to interact with the same partners as tRolC, we firstly analysed the autoactivity of RolC on reporter gene transcription (figure 14A). Yeast cells were transformed with pGBKT7-*rolC* and cultured on SD-W medium. The resulting colonies were spread on SD-WH medium. The yeast cells grew on this medium meaning that

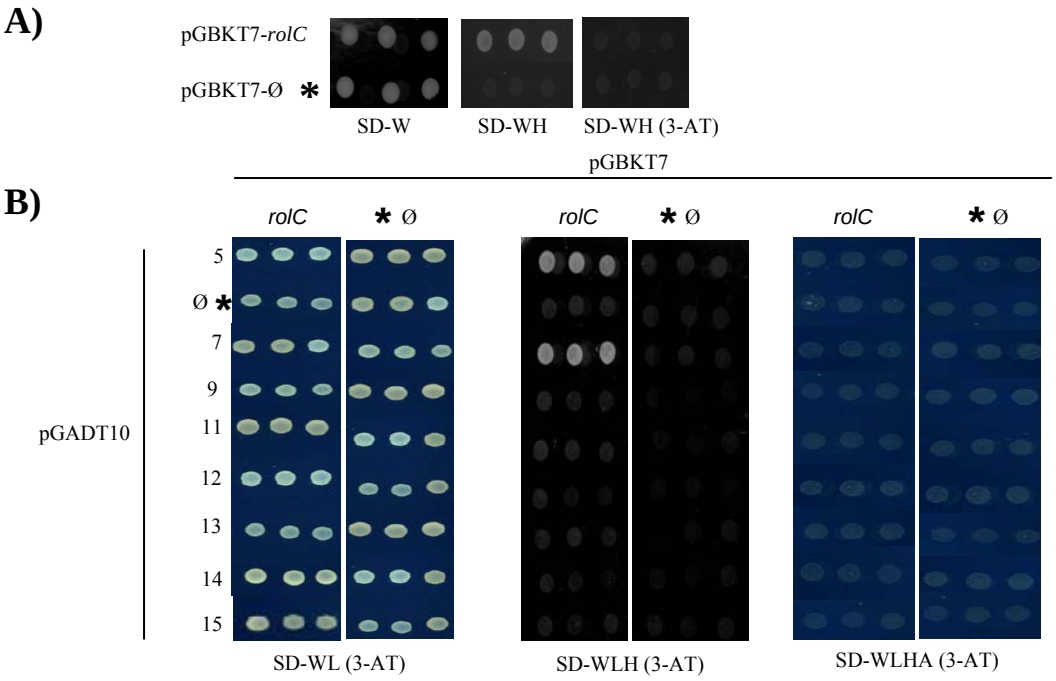


Figure 14. Interaction of RolC with the partner proteins of tRolC.
A) Autoactivity test of RolC on transcription of *HIS* reporter gene. The pGBKT7-*rolC* transformed yeast cells grow on selective medium (SD-WH) meaning that RolC functions as an activation domain. The presence of 3-AT inhibitor in the medium suppresses the growth of false positives. B) Interaction of RolC with proteins from Arabidopsis that interact with tRolC.
Triple replicates of double transformant clones were subcultured. SD-WL is non-selective medium and SD-WLH and SD-WLHA are selective media for interaction.
pGBKT7-Ø and pGADT10-Ø represent empty vectors. The stars (*) indicate the negative controls in the experience.

RolC has autoactivity with respect to Histidine synthesis reporter gene transcription. To suppress background colony growth 3 mM 3-aminotriazole (3-AT), a competitive inhibitor of the product of the *HIS* gene, was added to the SD-WH medium. For the further experiments cited below we used this more selective medium.

The pGBKT7-*rolC* yeast cells were transformed with the eight pGADT10-cDNAs clones mentioned above, cultured on SD-WL medium and resulting colonies were spread on SD-WLH and SD-WLHA media. Only cells harbouring pGBKT7-*rolC* and pGADT10-cDNA (clones N° 5 and 7) grew on SD-WLH medium and no colonies were observed on the more stringent medium (SD-WLHA) (Figure 14B). The yeast cells transformed with pGADT10-cDNAs and pGBKT7-Ø (empty vector) did not grow on SD-WLH medium showing that the encoded protein does not function as a protein containing a BD (Figure 14B).

We conclude that RolC and tRolC interact with the same plant protein although the interaction of tRolC with this host protein is stronger than that of RolC. This interaction could provoke the similar morphological alterations in *(t)rolC* transgenis plants.

Clone number	Accession number	Description
5	AT3G02150.2	Plastid Transcription Factor 1 (PTF1); **T**eosinte branched 1, **C**ycloidea and **P**CF Transcription factor 13 (TCP13); A chloroplast trans-acting factor of the psbD light-responsive promoter; The *TCP* class of genes are involved in control of leaf differentiation
7	AT3G02150.2	Plastid Transcription Factor 1 (PTF1); **T**eosinte branched 1, **C**ycloidea and **P**CF Transcription factor 13 (TCP13); A chloroplast trans-acting factor of the psbD light-responsive promoter; The *TCP* class of genes are involved in control of leaf differentiation
9	AT2G40316.1	Unknown protein; Functions in : molecular_function unknown; Located in : endomembrane system
11	AT2G05070.1	The light-harvesting chlorophyll a/b-binding (LHC) proteins that constitute the antenna system of the photosynthetic apparatus; It Belongs to the *lhc* (light harvesting complex) super-gene family
12	AT3G01540.2	*A. thaliana* Dead Box RNA helicase (ATDRH)
13	AT4G35100.1	A member of the plasma membrane intrinsic proteins (PIP); it functions as aquaporin
14	AT5G58020.1	unknown protein
15	AT3G15820.1	Phosphatidic acid phosphatase (PAP)-related protein

Table I. Results of DNA sequence analysis of tRolC-cellular partners by BLAST against the complete Arabidopsis genome sequence.

III. Conclusions

In this Chapter we have obtained the following results:

- The cT-DNA sequences of *N. tabacum* (cv. Samsun nn) differ in structure and sequence from the *N. glauca* cT-DNA. They are composed of two non-adjacent sequences: the first carries *torf8* to *torf13* (*torf8, trolA, trolB, trolC* and *torf13*) and the second contains the *torf14-tmis* region as well as the right border. Among the three essential *rolABC* hairy root genes, *trolA* and *trolB* are mutated but *trolC* is intact. *trolC* causes morphogenetic abnormalities very similar to those of its bacterial homolog, A4-*rolC,* and partially similar to those of another *plast* gene, *6b,* suggesting that A4-*rolC, trolC* and *6b* function via a similar basic mechanism. According to our results, this could be based on an increase in sucrose uptake and its retention in the cells.

- We have demonstrated that the induction of dex-*rolC-HAHIS-7* and dex-*trolC-HAHIS-7* plants leads to the silencing of *trolC* and *rolC,* although this silencing begins later in dex-*rolC-HAHIS-7* plants. Due to the silencing the plants are no longer inducible by dexamethasone.

- RolC and tRolC both interact with a single plant protein, TCP13, that plays an essential role in plant development. However, this interaction still has to be confirmed.

- We have identified 5 proteins encoded by the ectomycorrhizal basidiomycete *Laccaria bicolor,* which share homology with Plast proteins. This finding suggests that the *plast* genes whose origins have not been identified sofar originate from fungi and were introduced into Agrobacterium by horizontal gene transfer.

Chapter II

The RolB protein and its characteristics

I. Introduction

rolB seems to be the most important *rol* gene for hairy root induction, consequently many groups have tried to elucidate its mechanism of action; however, the findings from some studies are rather conflicting and therefore the role of this gene in hairy root induction is still not understood. This fact is not entirely surprising, since *rolB* has no homology to other genes, except to *plast* genes, and causes multiple morphological, biochemical and physiological alterations in transformed plants. After having obtained interesting new results for A4-*rolC,* showing an unexpected functional relation to the earlier studied T-*6b plast* gene providing a first hint at a common basis for *plast* gene function, we decided to investigate the A4-*rolB* gene in order to enlarge these *plast* gene studies. In parallel, we tried to answer the following questions: what is the nature of the striking *rolB*-induced necrosis? Is it similar to a classic hypersensitive response (HR) or to the senescence phenomenon? Are cytokinins capable to inhibit *rolB*-induced necrosis as they do in leaf senescence? What is the importance of the CX5R tyrosine phosphatase motif in RolB? Can we confirm that RolB has tyrosine phosphatase activity and what are the targets of this activity?

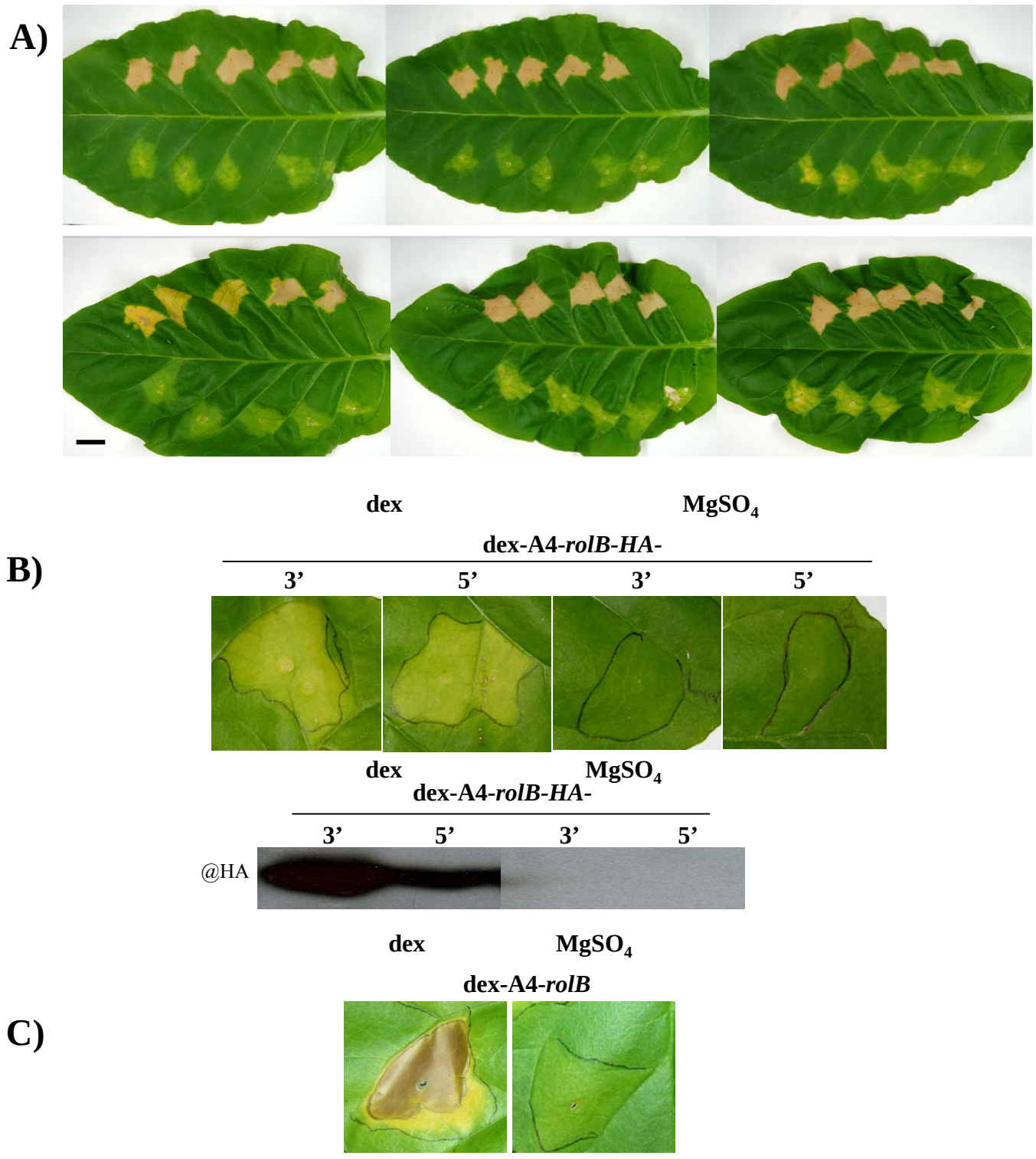


Figure 15. Transient expression in tobacco of different inducible A4-*rolB* constructs. A) Leaves of a Wisconsin 38 tobacco plant (top) and a Samsun nn tobacco plant (bottom), infiltrated with a 2x35S-A4-*rolB* construct (A846) (upper part of leaves) or a dex-A4-*rolB*-HAHIS construct (A987). In order to determine the efficiency of the constructs, different bacterial concentrations were used. From left to right on each leaf: bacterial concentrations of 0.05, 0.1, 0.3, 0.4, 0.5 OD600 equivalent. Leaves are ordered from oldest to youngest (left to right). 24 h after infiltration, leaves were re-infiltrated with 3 µM dex. Reactions in the A987 patches are much weaker than in the A846 patches, indicating that the dex-A4-*rolB*-HAHIS construct has only low biological activity (Bar: 25 mm). B) Leaves of a *N. tabacum* plant were infiltrated firstly by the bacterium carrying a dex-A4-*rolB*-HA construct (HA tag at 3’- or 5’-end of *rolB*) and after 24 h with 3 µM dexamethasone or with 10 mM $MgSO_4$ as a negative control. Proteins were extracted from the infiltrated zones 48 h after induction and submitted to protein gel blotting with @HA antibodies. C) *N. tabacum* leaves infiltrated by a bacterium carrying a dex-A4-*rolB* construct and after 24 h with 3 µM dexamethasone or with 10 mM $MgSO_4$. Pictures were taken 5 days after induction by dex.

II. Results

1.1. Construction of several dexamethasone-inducible A4-*rolB* genes

In order to facilitate the study of A4-*rolB* we decided to use the same approach as applied earlier with success for T-*6b* and A4-*rolC*, namely expression of a 3'-HAHIS-tagged version of the gene under control of a dexamethasone (dex)-inducible promoter, in the same hosts as used before (*N. tabacum* cv. Samsun nn and *A. thaliana*). The HAHIS epitope serves for easy detection of the fusion protein since it can be detected with commercially available @HA antibodies. We prepared an A4-*rolB* construct, tagged it with an HAHIS epitope at the 3' end and introduced it in the pTA7002 binary vector behind the dexamethasone-inducible promoter carried by this vector (Aoyama and Chua, 1997). The resulting construct was transformed into *Agrobacterium* strain LBA4404, yielding strain A987. Tobacco leaves (cv. Wisconsin and cv. Samsun nn) were infiltrated with A987 at different bacterial concentrations and with an earlier obtained 2x35S-A4-*rolB* gene carrying strain (A846) as a positive control, the results showed that the tagged, inducible construct was much less efficient as the 2x35S-A4-*rolB* gene (Figure 15A) inspite of producing high levels of A4-RolB-HAHIS protein (not shown). This suggests that the tagged protein does not have a normal RolB activity. We therefore prepared two new dex-A4-*rolB* constructs with a 5'-HA and 3'-HA tag, without the HIS part, yielding A1031 and A1032 respectively. Biological activity and RolB protein were measured by infiltration of leaves of young *N. benthamiana* and *N. tabacum* plants. After 24 h leaves were infiltrated with 3 μM dexamethasone to induce the *rolB* genes or with $MgSO_4$ (10 mM) as a negative control. The proteins of the infiltrated zones were extracted 48 h after induction. Using western blotting the A4-RolB-HA proteins were easily detected by @HA antibodies. No protein was detected in the case of non-induced leaves indicating that the promoter is not leaky. The signal detected for A4-RolB with 3'-HA was stronger than that of A4-RolB with 5'-HA. Chlorosis was observed after 3 days but was never followed by necrosis (Figure 15B). It means that the presence of the HA tag at either end of the A4-RolB protein interferes with its biological activity. We thus decided to prepare a dex-A4-*rolB* construct without HA tag and prepare @RolB antibodies. Infiltration of tobacco leaves with the bacteria containing this construct produced necrotic symptoms after 5 days (Figure 15C). Unfortunately, and in spite of several attempts, no RolB antibodies of sufficient sensitivity could be produced sofar (L. Otten, unpublished).

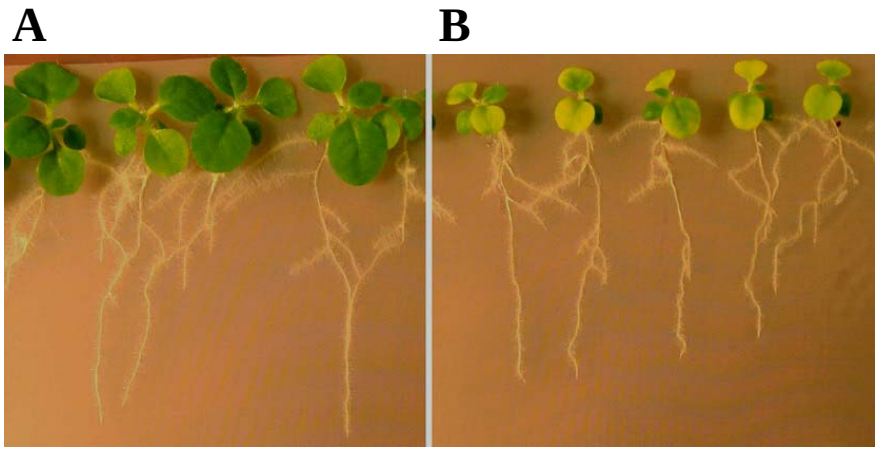


Figure 16. Growth of seedlings of A) W38tetR and B) B5-7 seedlings one week after transfer to the medium with 4 μM tetracycline. Although B5-7seedlings are strongly modified, root growth is fairly normal.

The various problems that we encountered, such as weak activity of the HAHIS or HA tagged-A4-*rolB* constructs and weak antigenic activity of the @RolB antibodies, persuaded us to use an already available transgenic *N. tabacum* cv. Wisconsin 38 line with a tetracycline-inducible A4-*rolB* gene (B5-7), kindly provided by Dr. Thomas Schmülling, for further experiments (see below).

1.2. A4-*rolB* effects on seedlings

The B5-7 transgenic plants contain a tetracyclin-inducible A4-*rolB* gene. These plants produce tetracycline repressor (TetR) molecules from a constitutively expressed *tetR* gene. They also carry an A4-*rolB* gene under control of a modified version of the CaMV 35S promoter. Three tetracycline (tet) operators are placed in the vicinity of the promoter's TATA box. Owing to the efficient repression of the modified 35S promoter by TetR molecules, these plants are phenotypically indistinguishable from wild type. Addition of the tetracycline (tet) inducer molecules prevents the repressor from binding to its operator and leads to full derepression of the promoter and to A4-*rolB* expression (Röder *et al.,* 1994). A transgenic tobacco line called W38tetR contains the *tetR* gene without A4-*rolB* and shows no alteration in phenotype, even after treatment with tetracyclin (Röder *et al.,* 1994), this line served as a negative control for our experiments.

As mentioned previously, the *plast* genes T-*6b* (Clément *et al*., 2007) and A4-*rolC* (Chapter I) inhibit root growth at the seedling stage but stimulate growth of cut roots on low sucrose medium. We therefore studied whether A4-*rolB* could influence root growth in a similar way. Seeds of B5-7 and W38tetR plants were grown on MS222 medium in the presence of 1% sucrose and 10-days old seedlings were transferred to nylon filters on vertical plates containing the same medium. After 2 days, seedlings were transferred to induction medium containing 4 µM tetracycline. Contrary to A4-*rolC* and T-*6b* seedlings which show a dramatic decrease in root growth and root hair density upon induction, the growth rate of roots and root hairs in A4-*rolB*-induced B5-7 roots was similar to W38tetR control roots or to non-induced controls, but leaf growth was strongly inhibited and leaves became pale green to yellow (Figure 16). This is remarkable, since *rolB* gene might be expected to modify root growth.

Another common property for T-*6b* and A4-*rolC* plants is strong sucrose accumulation. It has been shown that tobacco leaf discs expressing T-*6b* accumulate glucose, fructose and

sucrose (Clément *et al.,* 2006) and the root fragments of these plants show strongly enhanced sucrose uptake from the medium (Clément *et al.,* 2007). A4-*rolC* and *trolC* have very much the same properties (Chapter I). It was therefore of interest to test this property for the A4-*rolB* gene. We measured the level of glucose, fructose and sucrose in leaf discs of transgenic B5-7 plants infiltrated or not with 4 µM tetracycline, after 4 days. The results of these experiments (not shown) were highly variable. We noticed that the cv. Wisconsin 38 control plants did not behave in the same way as the untransformed cv. Samsun plants (used as control in the T-*6b*, A4-*rolC* and *trolC* experiments) in these assays. In some cases, an increase in sugars could be measured, in others, no increase was seen. Since we used greenhouse-grown plants for these experiments, it is possible that various conditions (time of the day, water status, physiological age of the plant, greenhouse temperature, or fluctuating success of biological control in the greenhouse) influenced the outcome of our experiments in an uncontrolled way and that the Wisconsin 38 cultivar is specially sensitive to such variations. It will be necessary to repeat these experiments under more strictly controlled conditions.

1.3. A4-*rolB*-induced necrosis and HR

When A4-*rolB* was induced by infiltration of B5-7 leaves with 4 µM tetracycline, cellular death readily appeared in the infiltrated zones. Older leaves were more sensitive than younger leaves and necrosis appeared sooner. Treatment with tetracycline had no effect on W38tetR plants. The induction of a necrotic response is reminiscent of an HR reaction. HR is one of the plant defense strategies by which plants counter-attack pathogens by rapidly developing cell and tissue necrosis around the initial site of infection (Dangl *et al.,* 1996; Richberg *et al.* 1998). This inducible response serves to restrict pathogen multiplication and systemic spread by confining the pathogen to tissues in the immediate vicinity of the initially infected cell. A local HR is often associated with the onset of systemic acquired resistance in distal plant tissues, elicited by a pathogen-encoded avirulence determinant that directly or indirectly interacts with the product of a plant resistance gene (Dangl 1998; Ryals *et al.,* 1996). Such a response is clearly advantageous to the plant as it limits the pathogenic effects of infection, although pathogens have developed countermeasures to overcome such defense mechanisms. One of the most important characteristics of the HR is the induction of PR (Pathogenesis-Related) proteins (Linthorst 1991; Tornero *et al.,* 1997; Elvira *et al.,* 2008). These are a group of plant proteins induced by different stress stimuli with an important role in plant defense

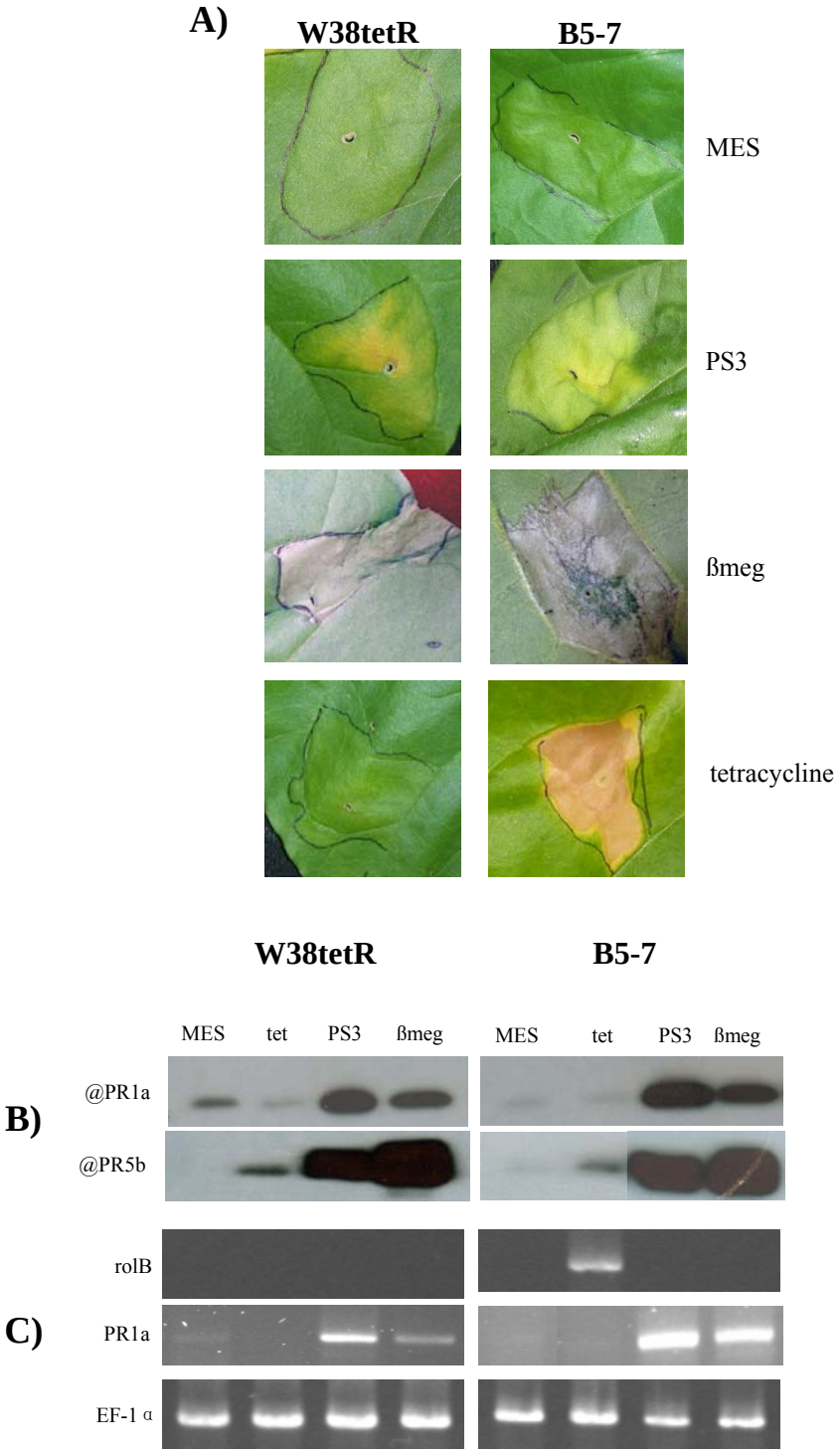


Figure 17. Leaves of W38tetR or B5-7 tobacco plants were infiltrated with MES buffer (2 mM), PS3 (200 µg/mL), ß-megaspermin (ßmeg)(50 nM) and tetracycline (tet) (4 µM) A) MES buffer does not provoke symptoms, PS3 induces chlorosis and ßmeg induces necrosis on W38tetR and B5-7 leaves. Induction of *rolB* due to tetracycline treatment in B5-7 leaves leads to necrotic lesions B) Expression of PR proteins. Proteins were extracted 3 d after treatments and submitted to protein gel blotting with antibodies @PR1a or @PR5b. C) Expression of *PR1a* and *rolB* transcripts. Total RNA was extracted 24 h after treatments and used for semi-quantitative RT-PCR analysis using *PR1a* and *rolB* specific primers. Equal loading was monitored by amplification of EF1 α cDNA.

against pathogens and in general adaptation to stressful environments (Aglika, 2005).

Sulfated laminarin (ß-1,3 glucan sulfate, also called PS3) is known to induce the accumulation of phytoalexins and the expression of a set of PR proteins. Tobacco leaves infiltrated with this molecule develop a slight chlorosis after 2 days and under UV light reveal a blue fluorescence limited to the infiltrated tissues as a result of scopoletin (a phytoalexin) accumulation (Costet *et al.,* 2002; Menard *et al*., 2004).

ß-megaspermin, an elicitin secreted by *Phytophthora megasperma* H20, has been demonstrated to be one of the most powerful molecules in inducing HR with features of programmed cell death, production of ethylene, and expression of typical defense responses such as phytoalexins and PR proteins. A narrow region of living cells surrounding the necrotic tissue shows under UV light a blue fluorescence as in the case of PS3 treatment (Baillieul *et al.,* 2003). Since the group of Serge Kaufmann at the IBMP has been interested in HR for many years and developed several useful tools to study this phenomenon, we decided to examine whether A4-*rolB* induced necrosis is similar to a classic HR and associated with the expression of PR proteins.

We infiltrated leaves of B5-7 and W38tetR tobacco plants with 4 µM tetracycline, MES buffer (2 mM), PS3 (200 µg/mL) and ß-megaspermin (50 nM). MES buffer served as a negative control and PS3 and ß-megaspermin were used as positive controls for the induction of PR protein expression. ß-megaspermin was also used as a positive control for induction of necrosis. All solutions (tetracycline, PS3 and ß-megaspermin) were prepared with 2 mM MES buffer.

MES buffer had no effect on B5-7 or W38tetR leaves and no lesions appeared. PS3 induced chlorosis in infiltrated zones on both types of leaves after 2 days. In the case of infiltration with ß-megaspermin, necrotic lesions started to appear on both B5-7 and W38tetR leaves after 1 day and after 4 days the entire infiltrated zone was necrotic. Tetracycline treatment induced chlorosis on the B5-7 leaves after 2-3 days, followed by necrosis after 5-6 days whereas it had no effect on W38tetR leaves (Figure 17A). Using protein gel blotting we monitored the expression of acidic PR1 (PR1a) and basic PR5 (PR5b) proteins in infiltrated zones after 3 days. High levels of expression of these proteins occurred in tissues infiltrated by PS3 and ß-megaspermin whereas no significant levels of PR proteins were induced in the case of A4-*rolB* expression (Figure 17B). RT-PCR analysis confirmed the expression of A4-*rolB* transcripts in tetracycline-infiltrated zone of B5-7 plants and detected a high level of expression of *PR1a* transcripts in PS3 and ß-megaspermin infiltrated zones of both B5-7 and W38tetR (Figure 17C). These results showed that A4-*rolB*-induced necrosis does not have the

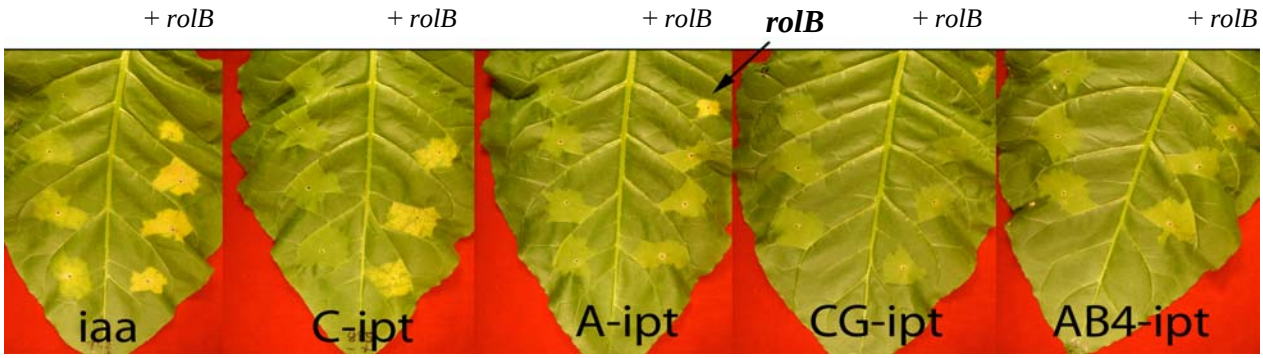


Figure 18. Inhibition of 2x35S::A4-*rolB*-induced necrosis by different *ipt* genes. On the left of each leaf: *iaa* or *ipt* strain mixed 1:1 with empty vector strain. On the right sides: 1:1 mixtures of 2x35S::A4-*rolB* strain with *iaa* or *ipt* strain. Arrow indicates a 1:1 mixture of the 2x35S::A4-*rolB* strain with the empty vector strain. C-*ipt* does not completely suppress the necrotic phenotype. Pictures were taken 10 days after infiltration.

Table II. References of genes of different strains used in co-inoculation experiments.

Gene(s)	Agrobacterium strain	reference
iaa	Ach5	Huss *et al*., 1989
C-*ipt*	C58	Otten, unpublished
A-*ipt*	Ach5	Tinland *et al.*, 1989
CG-*ipt*	CG474	Otten, unpublished
AB4-*ipt*	AB4	Otten and De Ruffray, 1994

characteristic of a classic HR reaction. We therefore became interested in testing the possible connection between A4-*rolB*-induced necrosis and senescence.

1.4. A4-*rolB*-induced necrosis and the effect of cytokinins

Senescence is a key developmental step in the life of each plant which is genetically controlled and characterized by progressive yellowing of the leaves due to chlorophyll and photosynthetic protein degradation, it is defined as a sequence of biochemical and physiological events comprising the final stage of development until cell death (Smart, 1994; Buchanan-Wollaston *et al.,* 2003). Developmental signals, ageing or stress can induce leaf senescence. During this phase, chloroplast proteins (mainly Rubisco) are degraded (Mae *et al.,* 1983). Several proteases, in particular cysteine proteases such as SAG12, have been demonstrated to be specifically expressed and involved in the degradation of proteins during plant senescence (Page *et al.,* 2001). The expression of genes involved in nitrogen-assimilation such as nitrate reductase and glutamine synthetase considerably decreases during senescence and these genes are therefore used as senescence markers (Masclaux *et al.,* 2000).

Cytokinins are known to inhibit senescence. It has been reported that in transgenic tobacco plants carrying the *ipt* (isopentenyl transferase) gene placed under control of a heat shock-inducible promoter leaf senescence can be delayed (Schmülling *et al.,* 1989; Smart *et al.,* 1991) and an *ipt* gene under control of a chalcone synthase promoter increased chlorophyll levels in leaves (Wang *et al.,* 1997). Likewise, introduction of the *ipt* gene under control of the senescence-associated SAG promoter from Arabidopsis in Petunia, was able to inhibit leaf senescence, a result of considerable practical horticultural importance (Jandrew and Clark, 2001). One of the morphological characteristics of *ipt* transformants is the presence of dark-green leaves caused by an increase in the chlorophyll content (Medford *et al.*, 1989; Beinsberger *et al.,* 1991; Grossman *et al.,* 1991; Hewelt *et al.,* 1994).

If indeed *rolB*-induced necrosis is similar to senescence, its effects might be abolished by cytokinins. Different *ipt* gene constructs from different *Agrobacterium* strains are available in our group. These genes are under control of their own promoter and induce the synthesis of cytokinins. As a control we used an auxin-inducing strain, carrying the *iaaM* and *iaaH* genes of strain Ach5, which induce auxin synthesis (Table II). Leaves of *N. tabacum* cv. Samsun nn were infiltrated with 1:1 mixtures of the 2x35S::A4-*rolB* strain A846 and one of the *ipt*- or *iaa*-carrying strains (Figure 18 right side of the leaves), as controls the *ipt*, *iaa* and 2x35S::A4-*rolB* strains were mixed 1:1 with an empty vector strain, LBA4404 (pBI121.2), in

order to simulate the dilution effect of mixed infections (Figure 18 left side of the leaves). Whereas the 2x35S::A4-*rolB* strain (mixed 1:1 with the empty vector strain) induced necrosis as expected (arrow in Figure 18), the individual *ipt* and *iaa* genes (mixed 1:1 with the empty vector strain) induced little or no effect by themselves. Remarkably, mixtures between the 2x35S::A4-*rolB* strain and different *ipt* genes diminished (C-*ipt*, AB4-*ipt*) or fully abolished (A-*ipt*, CG-*ipt*) the necrotic A4-*rolB* effect. The *iaa* genes did not diminish A4-*rolB*-induced necrosis. These results show that the necrotic effect can be prevented by cytokinins and in this respect resembles leaf senescence.

1. 5. Is RolB a tyrosine phosphatase?

It was reported that RolB has tyrosine phosphatase activity (Filippini *et al.,* 1996), but no further reports have appeared on its characterization (Km and Vmax values, possible protein targets, inhibitors, biological effects) since 1996. Tyrosine phosphatases play important roles in cellular signaling processes such as regulating activity of MAP kinases, transcription factors and ROS signaling (Laloi *et al.,* 2004; Gupta *et al.,* 2003). Before attempting to study the possible consequences of RolB tyrosine phosphatase activity, we thought it would be important to try to first confirm the earlier findings. Three approaches were used for this: a) sequence comparison, using the latest information on tyrosine phosphatases, b) mutation of the A4-RolB CX5R motif thought to play a role in the RolB tyrosine phosphatase activity (Lemcke and Schmülling, 1998a) and c) expression of A4-RolB in *E. coli* followed by enzyme tests.

a) Sequence comparisons

It has been reported that A4-RolB contains a CX5R motif typical for tyrosine phosphatases (Lemcke and Schmülling, 1998). Protein tyrosine phosphatases (or PTPases) are divided into three subgroups: receptor-like PTPases, intracellular PTPases, and dual-specificity PTPases or dsPTPs (Stone and Dixon, 1994). The common structural features of the receptor-like PTPases include an extracellular domain, a single membrane-spanning region, and one or two cytoplasmic catalytic domains. The intracellular PTPases contain a single catalytic domain and various N- and C-terminal extensions (Mauro and Dixon, 1993). Dual-specificity PTPases constitute a special class of intracellular PTPases that are unique in their ability to hydrolyze both phosphoserine/threonine and phosphotyrosine residues. The PTP domains (240-250 amino acids), common to all PTPases, always contain the conserved

Position in 1724-RolB	205	206	207	208	209	210	211	212	213	214	215
General PTPase motif	**I/V**	**H**	**C**	X	A	**G**	X	X	**R**	**S/T**	G
A4-RolB	V	A	C	T	F	F	L	R	R	G	T
1724-, 2659- RolB	V	A	C	T	F	F	V	R	Q	G	T
8196-RolB	A	P	C	A	F	F	V	R	L	G	T
NgRolB	V	D	C	T	F	F	V	R	R	A	S
A4-RolBTR	V	L	C	P	F	L	I	R	Q	A	T
mutated A4-RolB	V	A	A/S	T	F	F	L	R	K/A/ L/Q	G	T

Table III. Comparison of the amino acid sequences of the 4 RolB proteins (A4-RolB, 1724-RolB, 2659-RolB, 8196-RolB) and A4-RolB^{TR} and the homologous sequence from *N. glauca* (NgRolB) with the general PTPase motif. The amino acids in red represent the mutated residues.

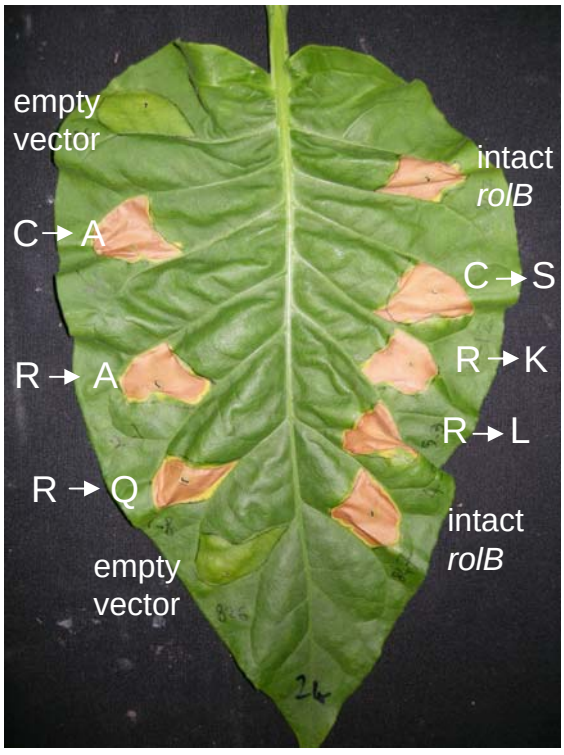


Figure 19. Infiltration of a wild-type tobacco leaf (*N. tabacum* cv. Samsun nn) with Agrobacterium strains containing six different mutated *rolB* genes, intact *rolB* and empty vector. After 4-5 days, the necrotic phenotype appears in the leaf patches infiltrated by all these mutated strains as in the case of the intact *rolB* gene, but not in the zones infiltrated with the empty vector.

motif (I/V)HCXAGXXR(S/T)G which corresponds to the catalytic site, this motif carries a conserved essential cysteinyl (C) residue, that is involved in the formation of a phosphoenzyme intermediate (Guan and Dixon, 1990; Denü *et al.,* 1996; Xu *et al.,* 1998). AtPTP1, the first plant tyrosine-specific phosphatase to be cloned and studied in detail, is of the dual-specificity type (Xu *et al.,* 1998).

If tyrosine phosphatase activity is crucial to RolB function, the conserved PTPase motif should be present in all RolB proteins. When the RolB amino acid sequences were compared to the general PTPase motif (Table III) the first residue (I/V) was indeed found in most RolB's, except in 8196-RolB, the second residue (H) is lacking in all RolBs, the third (C) is present in all, the fifth and sixth (AG) are absent from all RolBs, the ninth (R) is only found in A4-RolB, and the tenth and eleventh (S/T and G) are lacking in all RolB proteins. On the basis of these comparisons, it seems highly unlikely that RolBs are tyrosine phosphatases since they lack most or all of the conserved motif elements.

b) Mutations in the RolB CX5R motif

According to Lemcke and Schmülling 1998, the CX5R motif in A4-RolB is the main PTPase motif. To test its importance in A4-RolB activity, we introduced different point mutations in this motif using PCR primers (C was converted to S or A, and R converted to A, K, Q or L). We placed the mutated A4-*rolB* genes under control of the 2x35S promoter in the binary vector pBI121.2 and introduced them in *Agrobacterium* strain LBA4404 carrying the helper plasmid pAL4404. *Agrobacterium* cells containing empty vector (A826) or the intact 2x35S::A4-*rolB* gene in the pBI121.2vector (A846) were used as negative and positive controls respectively. Leaves of wild-type tobacco plants (*N. tabacum* cv. Samsun nn) were infiltrated with the different *Agrobacterium* strains. After 4-5 days, the necrotic phenotype appeared on the leaf patches infiltrated by strains carrying either the intact A4-*rolB* gene or one of the mutated A4-*rolB* genes, but not in the zones infiltrated with the strain containing the empty vector (Figure 19). Clearly, the CX5R motif plays no role in generating the necrotic phenotype, the involvement of a tyrosine phosphatase activity in necrosis is therefore highly unlikely.

c) Expression of A4-RolB in *E. coli* and enzyme tests

In order to test the possible enzyme activity of A4-RolB more directly, we expressed A4-RolB in *E. coli* under IPTG-inducible promoter control and tested its tyrosine phosphatase activity. In order to avoid any negative influence of a tag noticed in our previous experiments

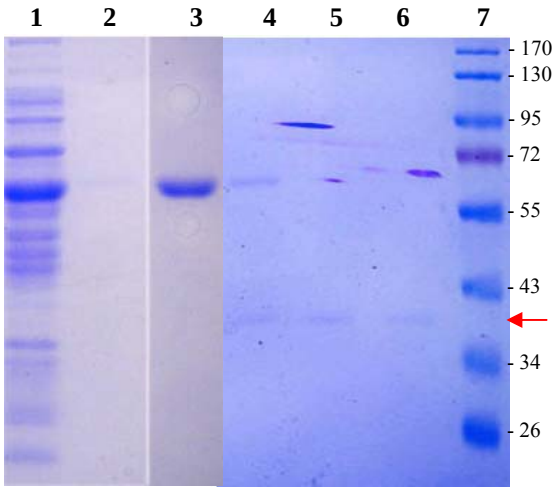


Figure 20. Expression and purification of AtPTP1 from an AtPTP1-GST fusion protein.
Lanes: 1. Non-bound protein. 2. Run-through after washing and before elution. 3. Eluted material, AtPTP1-GST band at expected position (26 kD GST + 38 kD AtPTP1: 64 kD). 4-6: Cleavage products at three different thrombin concentrations (0,1, 0,2 and 0,3 mg/ml), the 38 kD protein (represented by the arrow) corresponds to AtPTP1. 7. Protein molecular weight ladder.

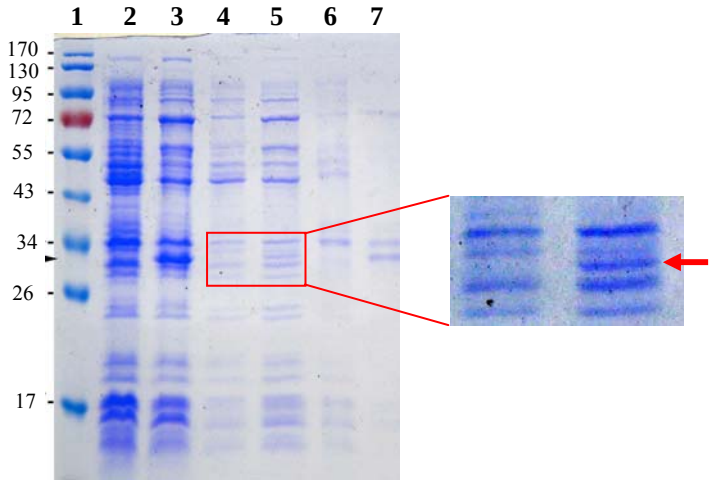


Figure 21. Expression of the RolB protein in *E. coli*. Lanes : 1. Protein molecular weight ladder. 2. Total protein of non-induced cells. 3. Total protein of induced cells. 4. Supernatant of non-induced cells. 5. Supernatant of induced cells. 6. Pellet (1/10 diluted) of non-induced cells. 7. Pellet (1/10 diluted) of induced cells. The arrow indicates a band that is only present in induced fractions and has the expected RolB size of 30 kD.

(see above), we expressed the native protein. As a positive control, an *Arabidopsis thaliana* AtPTP1-GST fusion protein (Xu *et al*., 1998), kindly provided by the Dr. Sheng Luan (University of Berkeley, California), was also expressed in *E. coli* and purified by GST cleavage. Figure 20 shows the induction of the AtPTP1-GST fusion protein and the purification of the native AtPTP protein after thrombin cleavage. A4-*rolB* was cloned in the pQE60 bacterial expression vector and expressed by induction with IPTG. Upon induction, a protein band of the expected size of 30 kD appeared in the crude extract. The crude extract was cleared by centrifugation, part of the A4-RolB protein was found in the supernatant, the remainder was found in the pellet (Figure 21). The purified AtPTP1 enzyme, and the A4-RolB pellets and supernatants were tested for tyrosine phosphatase activity with p-nitrophenylphosphate (pNPP) as a substrate. Similar amounts of protein were used (as estimated by Coomassie staining). Whereas AtPTP1 had a very high and reproducible phosphatase activity (4500 arbitrary units per hour), the activity of the A4-RolB protein, either in the pellet or in the supernatant was undetectable (less than 1). We therefore consider it unlikely that the A4-RolB protein has tyrosine phosphatase activity.

III. Conclusions

Our study about the A4-*rolB* gene led to the following conclusions:

- The A4-*rolB*-induced necrosis does not represent a classical HR reaction because *rolB* expression had no effect on the expression of the PR protein(s).

- *ipt* gene expression inhibits the necrosis induced by A4-*rolB* indicating that this necrosis could be related to a senescence reaction.

- We invalidated the earlier reported hypothesis for A4-RolB tyrosine phosphatase activity by the following findings: a) sequence comparison showed that the PTPase conserved motif (I/V)HCXAGXXR(S/T)G is not present in the various RolB proteins, b) A4-RolB expressed in *E. coli* did not show detectable phosphatase activity, whereas the control enzyme AtPTP1 had a very high and reproducible phosphatase activity under the same conditions, c) A4-RolB proteins with a mutation in the essential residues of the CX5R motif induced a necrotic phenotype exactly like the intact A4-RolB protein.

- Tetracycline-inducible A4-*rolB* tobacco seedlings did not show alterations in the growth rate of roots or root hairs. These results are in strong contrast to the effects of the A4-*rolC, trolC,* A4-*orf13* and T-*6b* genes which cause a dramatic decrease in root growth and root hairs density.

DISCUSSION AND PERSPECTIVES

I. *N. tabacum* cT-DNA structure

Among the plants belonging to the genus Nicotiana, *N. glauca* is the only one whose cT-DNA structure has been completely determined. To increase our knowledge about the structure of other cT-DNAs we have studied the structure of *N. tabacum* cT-DNA and found two non-adjacent fragments, one carrying the *torf8, trolA, trolB* and *trolC* genes and the other one the *torf14* and *tmis* genes flanked by a right border. At present we have no information about the structure of cT-DNA downstream of *torf8,* so it would be interesting to determine the sequence of this region up to the left end to complete the *N. tabacum* cT-DNA structure.

Aoki and Syono (1999a) reported that the *NgrolB* sequence of *N. glauca* is mutated and biologically inactive, but this activity can be restored by site directed mutagenesis. We have demonstrated that *N. tabacum* contains a *trolB* sequence with a 40 nt duplication in the coding region. A similar experiment as has been utilized for *NgrolB* can be envisaged to restore *trolB* (by deleting the insertion) in order to reactivate its biological function and compare it with the "classical" *rolB* genes both by generating transgenic plants and by transient expression of the gene. In addition to *trolB,* the reactivation of the mutated *torf8, trolA, torf13* and *tmis* genes will help to better understand their potentially original activities in the plant. The approach that we used sofar to study the biological function of *trolC* was over-expression in tobacco and Arabidopsis plants. A complementary approach that should be used is the extinction of the gene in tobacco. We have already transformed *N. tabacum* (cv. Samsun nn) plants with a hair-pin construct directed against *trolC* under control of a 35S promoter. The regenerants will be analyzed to identify the homozygous plants with a single copy of T-DNA and will then be characterized. Fründt and colleagues (1998) reported that in *N. tabacum* (cv. Havana 425), *torf13* is transcriptionally active and induces dense green callus on carrot discs. It would be interesting to determine the effect of *torf13* on plant development by a hair-pin strategy and therefore we have recently transformed *N. tabacum* (cv. Havana 425) plants with a hairpin construct directed against *torf13* under 35S promoter. For the *N. tabacum* cT-DNA *plast* gene, *torf14,* it will be important to determine whether it is transcribed and possibly has a biological function.

II. *rolC*

The *(t)rolC* functions have been discussed previously in Chapter I and to avoid repetition we will only disscuss them briefly here.

1. *(t)rolC* and flowering

rolC accelerates flowering time in tobacco and in other plant species (Faiss *et al.,* 1996; Casanova *et al.,* 2005), our studies showed that this is also the case for *(t)rolC* transgenic Arabidopsis plants. Most studies demonstrated that flowering is induced in at least some plant species by increase of the sucrose levels or its transport. In tomato, flowering is stimulated by high levels of sugar (Dielen *et al.,* 2001) and in Arabidopsis, the application of exogenous sucrose allows flowering in complete darkness (Roldán *et al.,* 1999). In addition, a correlation between export of carbohydrates from the leaves and increased flower induction has been identified in Arabidopsis (Corbesier *et al.,* 1998).

Therefore it would be interesting to investigate whether *(t)rolC*-induced flowering is related to sucrose accumulation and/or its transport. The use of sucrose transport blockers or the production of plants in which the genes involved in sucrose transport have been extinguished could help to elucidate, although partially, the mechanism by which the *(t)rolC*-induced flowering occurs. The comparison of gene expression in *(t)rolC* transgenic and wild-type plants, using microarray experiments, will also provide useful information to understand more in detail which genes are involved in *(t)rolC*-induced flowering.

2. (t)RolC interaction with the cellular protein TCP13

To discover the mechanism by which A4-*rolC* and *trolC* act in plants to cause the various growth alterations we have screened an Arabidopsis cDNA library using a yeast two-hybrid system and identified a protein which interacts with proteins RolC and tRolC. The (t)RolC-cellular partner corresponds to a member of the *TCP* class of genes, *TCP13*. These genes are only found in plants (Cubas *et al.,* 1999) and are represented by the first three identified members Teosinte branched1(***T****B1*) in maize (*Zea mays*), Cycloidea (***C****YC*) in *Antirrhinum majus*, and ***P****CF* (Proliferating Cell Factor) in rice (*Oryza sativa*). Members belonging to this class are nuclear-encoded transcription factors that control multiple developmental traits in diverse plant species, such as flower morphogenesis (Luo *et al.,* 1996), leaf morphology and senescence (Nath *et al.,* 2003; Schommer *et al.,* 2008), embryo growth (Tatematsu *et al.,* 2007) and plant architecture (Aguilar-Martínez *et al.,* 2007). It has been reported that TCP transcription factors regulate leaf growth negatively and leaf senescence positively (Schommer *et al.,* 2008).

Leaf senescence is characterized by a progressive yellowing of the leaves due to

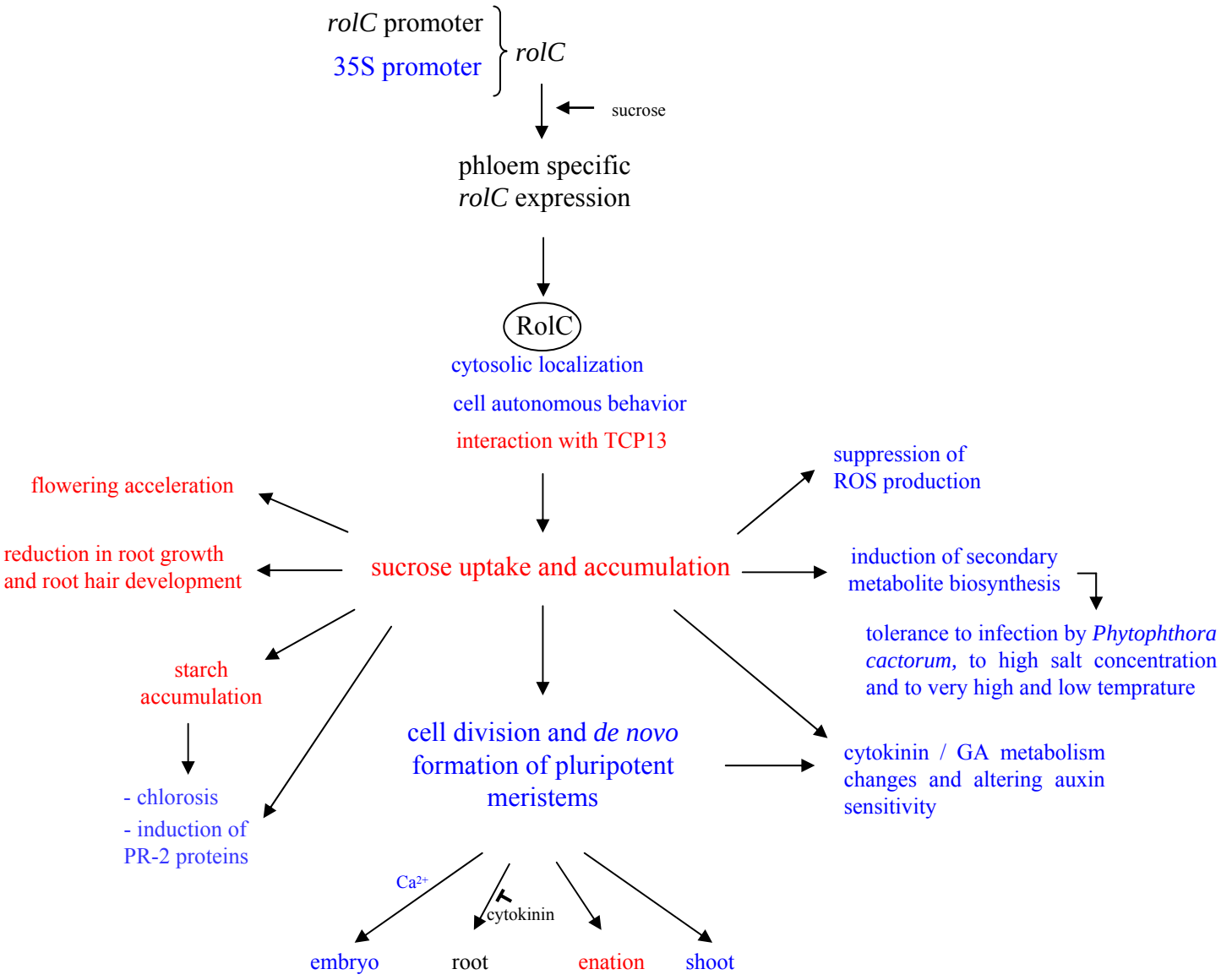


Figure 22. A model summarizing *rolC*-induced effects. The results that we obtained are presented in red (See Figure 13 for more information).

chlorophyll and photosynthetic protein degradation (Buchanan-Wollaston *et al.,* 2003). The plants expressing *(t)rolC* show a pale green phenotype. The nature and the agent of this chlorotic phenotype have not been identified so far. This phenotype could be an indication of a precocious senescence phenomenon which may be due to the interaction of TCP with (t)RolC.

A common feature for many members of the *TCP* genes family is a role in meristem growth and cell division: The *CYC* gene is expressed in floral meristems and controls primordium initiation (Luo *et al.,* 1996); *TB1* affects axillary meristem growth (Doebley *et al.,* 1997; Aguilar-Martínez *et al.,* 2007); the proteins encoded by *PCF1* and *PCF2* bind to the promoter of proliferating cell nuclear antigen gene (*PCNA*) involved in meristematic cell division (Kosugi and Ohashi, 1997) and the Arabidopsis *TCP2* and *TCP3* genes are expressed in rapidly growing floral primordia (Cubas *et al.,* 1999). RolC and tRolC are also capable to induce meristem formation. Therefore it could be hypothesized that meristem induction is the result of (t)RolC interaction with TCP.

Antirrhinum *cin* (a member of the *TCP* family, Nath *et al.,* 2003) and Arabidopsis *tcp2, 4* and *10* loss-of-function mutants (Schommer *et al.,* 2008) have crinkled leaves. Small crinkly leaves were also observed in *(t)rolC*-induced tobacco plantlets. A possible explanation for the presence of crinkly leaves in *(t)rolC* expressing plants is that (t)RolC interaction with TCP inactivates TCP and therefore results in a phenotype similar to *tcp* loss-of-function mutants. Studying TCP mutants will help to clarify whether the induction of meristems and chlorosis and the presence of crinkly leaves in *(t)rolC*-expressing plants are related to interaction of (t)RolC with TCP.

Although the analysis of the protein interaction networks in transformed plant cells is a valuable tool to understand the molecular function of a gene, these interactions should be confirmed by several methods (*in vitro* and *in vivo*) and therefore, the confirmation of the interaction between (t)RolC and TCP is indispensable to define correctly the mechanism of action of (t)RolC and the pathway by which they influence plant morphology and development.

Figure 22 presents a model summarizing the earlier reported *rolC* characteristics and the results that we obtained for this gene. In this model we propose that sucrose uptake and accumulation is an early and essential step that leads to many of the observed *rolC* effects.

III. *rolB*

1. RolB and necrosis

rolB gene activity can be easily and rapidly measured by transient expression in tobacco leaves, since 2x35S::A4-*rolB* leads to strong necrosis after 3-4 days. The necrotic effect remains completely limited to the patch. This simple test has allowed us to test various A4-RolB point mutants and tagged A4-RolB constructs, and the influence of *ipt* and *iaa* genes on A4-RolB-induced necrosis (see below). The same necrosis test might be used in the future to test *rolB* genes of different origin of tobacco (for example, the *trolB* gene (after restoration of its reading frame) or the $rolB^{TR}$ gene). It might also be used on Arabidopsis leaves and leaves of various Arabidopsis mutants, in order to characterize *rolB*-induced necrosis with respect to other potentially involved functions like senescence.

2. RolB, senescence and cytokinins

According to the results obtained from the inhibitory effect of cytokinins on A4-*rolB*-induced necrosis which imply that this could be related to a senescence phenomenon, the best way to continue these studies is by introduction of an inducible A4-*rolB* gene in Arabidopsis and testing the transcriptome by DNA microarray analysis. In Arabidopsis, the dex promoter leads to strong background induction of many endogenous genes, and our constructs can therefore not be used for this species, instead, new constructs are required that use the estradiol promoter. Utilization of other senescence markers such as metabolites (i.e. sugar, anion and organic nitrogen content (Diaz *et al.,* 2005) in addition to proteomic or genomic approaches could also provide new insights in the characteristics of A4-*rolB*-induced necrosis. It will be interesting to further explore the inhibitory effect of cytokinins on A4-*rolB*-induced necrosis, at different times before or after A4-*rolB* induction. At the same time, A4-*rolB* might influence some of the A4-*rolC* effects that we studied in Chapter I (see below for antagonistic *rolB*/*rolC* effects). In a later stage, it should be attempted to combine the individual activities of the three essential *rol* genes *rolA*, *rolB* and *rolC* (with their own specific promoter) and to explain the induction and growth of hairy roots.

3. *rolB* and antagonistic effect with *rolC*

Several studies reported the presence of an antagonistic effect between *rolB* and *rolC*.

Schmülling and co-workers (1988) observed that constitutive *rolB* expression suppressed growth of tobacco cells but that *rolC* gene was able to overcome this growth inhibition. Likewise, *rolC* diminished the intensity of *rolB*-induced rooting on carrot discs (Capone *et al.*, 1989) and also diminished *rolB*-induced sensitivity to auxin in transformed tobacco cells (Maurel *et al.,* 1991). *rolA* and *rolC* inhibited the stimulatory effect of *rolB* on the biosynthesis of secondary metabolites and also attenuated the *rolB*-induced inhibition of callus growth (Shkryl *et al.,* 2007; Bulgakov *et al.,* 2008). In this respect it would be interesting to study whether *rolC* is also capable of inhibiting *rolB*-induced necrosis. Two simple approaches for this would be a) infiltration of B5-7 plants with tetracycline and subsequently by an *Agrobacterium* strain containing the A4-*rolC* gene and b) infiltration of transgenic dex-*rolC* plants with dexamethasone and subsequently with an *Agrobacterium* strain containing A4-*rolB*. If the necrotic phenotype is reduced, further tests can be envisaged to analyze this antagonism.

4. RolB and Nt14-3-3 proteins

Although Moriuchi and co-workers (2004) reported a nuclear localization for RolB that depended on its interaction with Nt14-3-3 ωII, it should be pointed out that there is evidence that 14-3-3 proteins can modulate the localization of the proteins to different extents. In most cases, 14-3-3 binding sequesters the target protein in a particular subcellular compartment, and the release of the 14-3-3 protein allows the partner to relocate (Dougherty and Morrison 2004). The nuclear retention of the proteins due to their interaction with 14-3-3 proteins and their relocalization to other cellular compartment has been reported for the human telomerase reverse transcriptase (TERT) (Seimiya *et al.,* 2000), Txl-2 (Tang *et al.,* 1998) and Chk1 (Jiang *et al.,* 2003) proteins. Therefore, the possibility that RolB also localizes in other(s) cell compartment(s) such as the plasma membrane as reported by Filippini *et al*., (1996) cannot be excluded. In addition, Moriuchi *et al*., (2004) observed that the RolB protein partly moved from the nucleus to the cytoplasm during the treatment required to produce the protoplasts necessary for the microscopic observations. Apparently, the subcellular localization of RolB can be changed in response to some conditions. It is not known whether the necrosis phenotype also requires nuclear localization. It seems difficult to reconcile the membrane localization of the RolB protein with its nuclear localization, unless the protein binds to the nuclear membrane. Clearly, more data are required on the localization of the protein under different conditions and in different tissues if we want to understand its mechanism of action.

It should be noted that RolB and Nt14-3-3 ωII do not contain NLS motifs (Moriuchi *et al.,* 2004). It would be important to search for another protein containing an NLS motif which could interact with RolB or Nt14-3-3 ωII, or both, and transport the complex into the nucleus. In animals, it has been reported that the 14-3-3 proteins play a role in cellular death (apoptosis) (Dougherty and Morrison 2004). One question that arises is whether A4-*rolB*-induced necrosis requires interaction with Nt14-3-3 protein.

It has been demonstrated that 14-3-3 proteins activate the P-type H^+-ATPase proteins (Bunney *et al.,* 2002). On the other hand, Maurel *et al.,* (1991) showed that *rolB*-transformed protoplasts display a high polarization of the plasma membrane after auxin treatment. Thus the question whether the interaction between the RolB and 14-3-3 proteins plays a role in this hyperpolarization, also remains to be investigated.

The role of Nt14-3-3 proteins in *rolB*-induced effects might be further studied by RNA interference constructs that target Nt14-3-3 expression.

5. *rolB*, auxin and NtBBF1 protein

As described, the biological effects of the A4-*rolB* gene product are reminiscent of some auxin-mediated effects. It will be interesting to test if A4-*rolB* expression can partly mimic an auxin-like expression pattern and to investigate the influence of A4-*rolB* expression on auxin perception mutants. It has been shown that NtBBF1 protein can bind to the auxin-responsive ACTTTA sequence of the *rolB* promoter. However, the mechanism of *rolB* regulation by this protein is still unknown. Studying mutants of NtBBF1 (for example by using RNA interference) will help to clarify the way in which this protein regulates *rolB* expression.

6. *rolB*, seedlings growth and sugar uptake

Our results demonstrated that induction of A4-*rolB* B5-7 seedlings did not provoke any alterations in the growth rate of roots or root hairs. These results are in strong contrast to those obtained with the A4-*rolC, trolC* (Chapter I) and T-*6b* genes (Clément *et al.,* 2006; 2007) which cause a dramatic decrease in root hairs and an overall reduction in root growth. Recent experiments in our lab have shown a similar strong root inhibition in plants expressing an inducible A4-*orf13 plast* gene (L. Otten, non published). The absence of any root effects for A4-*rolB* seedlings is unexpected since this gene is the most important Agrobacterium gene in

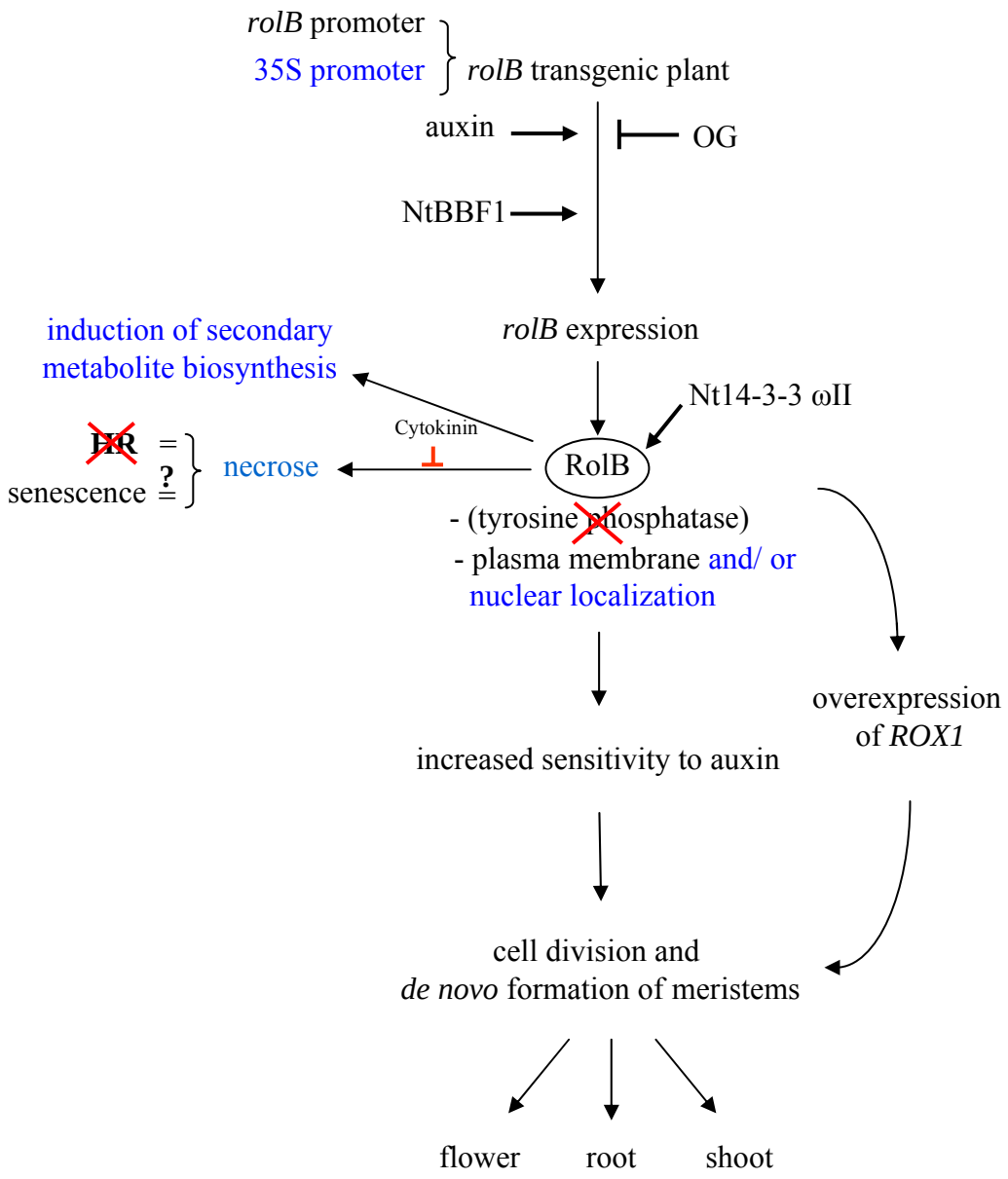


Figure 23. A model summarizing *rolB*-induced effects.
The results that we obtained are presented in red. *rolB*- induced necrosis does not have the characteristics of an HR and can be inhibited by co-infiltration with *ipt*-expressing agrobacteria in leaf patches expressing *rolB*, therefore it could be related to a senescence phenomenon. The RolB protein does not have tyrosine phosphatase activity (See Figure 11 for more information).

induction of the roots on various explants and also the "hairy root" phenotype in *rolB*-expressing plants. This prompts the question how efficiently the B5-7 A4-*rolB* gene was induced under these experimental conditions. This should be checked in future experiments using RT-qPCR.

When starting this work we speculated that *rolB*-induced cell division and meristem formation could be due to sucrose accumulation in *rolB*-expressing cells like in the case of other *plast* genes such as the *rolB*-like *Norf8* gene (Umber *et al*., 2002) and the T-*6b* gene, which cause increased sucrose uptake and accumulation in roots (Clément *et al*., 2007) and additional cell division in the root pericycle and vasculature (Grémillon *et al.,* 2004). However, results on the glucose, fructose and sucrose levels in A4-*rolB*-expressing leaf discs (not shown) were not reproducible so far, we hope to improve them with more standardized plant growth conditions. Another approach to find a possible influence of *rolB* on sucrose accumulation is to induce root fragments in a medium containing ^{14}C-sucrose with subsequent quantification of radioactivity in the medium as done for *rolC*.

Figure 23 presents a model summarizing the earlier reported *rolB* characteristics and the results that we obtained for this gene.

At the beginning of this work we started with the assumption that *plast* genes have a basic common mechanism. This expectation could only partially be fulfilled. The *rolC* and *trolC* do indeed share some important, unexpected characteristics with the distantly related *6b* gene, the most important one of which are sucrose uptake and accumulation, highly similar root growth effects and the induction of enations. If sucrose uptake is caused by an interaction between RolC/tRolC and TCP13, one would expect that the 6B protein also binds to TCP13. However, other partners have been reported for the 6B protein (NtSIP1, NtSIP2 and H3) (Kitakura *et al.,* 2002; 2008; Terakura *et al.,* 2007), and the latter have not been found to interact with RolC/tRolC (this work).

The RolB protein effects do not resemble the RolC/6B effects (except for chlorosis), and the RolB protein has been reported to interact with yet another cellular partner, the Nt14-3-3 ωII protein (Moriuchi *et al.,* 2004). However, it is striking that the RolB-like N-terminal part of Orf8 protein leads to hexose and starch accumulation (but not to necrosis, Umber *et al.,* 2002; 2005) as do 6B and RolC. Therefore, this protein (and possibly RolB^{TR}, equally without necrotic effects but sofar little studied) might be a "missing link" between RolB on the one hand and RolC/6B on the other hand.

MATERIALS AND METHODS

Biological Materials

I. Plant materials

1. *Nicotiana tabacum*: Three cultivars were used during this research: cv. Samsun nn, cv. Havana 425 (kindly provided by Dr. Fred Meins) and cv. Wisconsin 38. The transgenic Wisconsin 38 lines, W38tetR and B5-7, have been kindly provided by Dr. Thomas Schmülling (Röder *et al.,* 1993).

2. *Nicotiana benthamiana*: seeds of *N. benthamiana* were provided by the IBMP greenhouse.

3. *Arabidopsis thaliana:* Ecotype Colombia (Col).

II. Bacteria

1. *E. coli* MC1022 strain

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

2. *E. coli* Top 10 strain

Top 10 is a strain of *E. coli* that is used for cloning and amplification of plasmids. Its genotype is : *F–, mcrA, Δ(mrr-hsdRMS-mcrBC), φ80lacZΔM15, ΔlacX74, deoR, recA1, araD139, Δ(ara, leu)7697, galU, galK, rpsL(strr), endA1, nupG.*

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

This strain is 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 the 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 LBA4404 (Hoekema *et al.*, 1983)

This strain was used for infiltration of leaves of *N. tabacum* and *N. benthamiana* in the Patch Test and also for transformation of tobacco plants. It contains rifampicin and streptomycine resistance genes on its chromosome. It also contains a disarmed pAL4404 Ti-plasmid with *vir* genes allowing the T-DNA integration into the plant genome. The T-DNA is supplied by a binary pBIN vector.

5. *Agrobacterium tumefaciens* strain GV3101 (Koncz and Schell, 1986)

This strain was used for transformation of Arabidopsis plants. It contains a rifampicin resistance gene on its chromosome and a disarmed pMP90 Ti-plasmid with *vir* genes. The T-DNA is supplied by a binary pBIN vector.

The bacterium strains are stored at -80ºC in 50% glycerol.

III. Yeast

1. *Saccharomyces cerevisiae* strain AH109 (CLONTECH)

This strain is designed for detecting protein interactions in a two-hybrid screen. AH109 contains distinct *ADE2, HIS3, lacZ*, and *MEL1* reporter genes that are only expressed in the presence of GAL4-based protein interactions. 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 or binding domain. Then they are double transformed into the yeast cells. Positive interaction between two proteins leads to the transcriptional activation of a selective marker gene containing a binding site for GAL4 and allowing the yeast colony to grow in the absence of the corresponding amino acid. The *LacZ* reporter gene allows quantification of the interaction level (Keegan *et al*., 1986). The yeast strains are stored at -80ºC in 25% glycerol.

IV. Vectors

1. Cloning vectors:

1.1. pGEM-T promega (Promega)

This plasmid is used for high efficiency cloning of PCR products. The presence of a 3´ terminal thymidine at both ends of the cut vector facilitates the insertion of PCR products

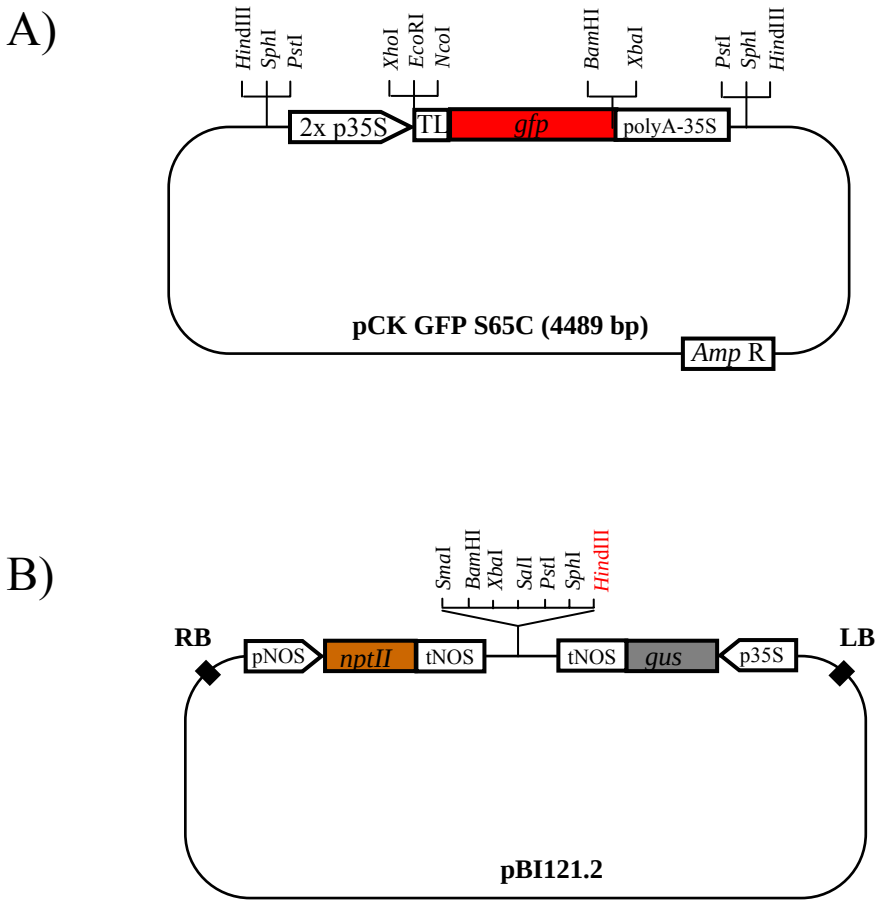


Figure 24. Maps of the plant expression vectors. A) pCK GFP S65C and B) pBI121.2.
Some restriction enzyme sites are represented. *AmpR*: ampicillin resistance gene; p35S: CaMV-35S promoter; TL: translational enhancer from tobacco etch virus (TEV); *gfp*: green fluorescent protein gene; polyA-35S: polyadenylation signal of the CaMV 35S gene; pNOS: the nopaline synthase gene promoter; *nptII:* the neomycin phosphotransferase II gene*;* tNOS: the NOS terminator; *gus*: the ß-glucuronidase gene; RB: right border; LB: left border.

carrying a single deoxyadenosine at their 3´-ends that is provided by the activity of the Taq polymerase. This plasmid contains T7 and SP6 RNA Polymerase promoters flanking a multiple cloning site (MCS) within the α-peptide coding region of the enzyme ß-galactosidase. Insertional inactivation of the α-peptide allows recombinant clones to be directly identified by color screening on indicator plates.

1.2. pCK GFP S65C (Carrington *et al.*, 1991)

This plasmid is derived from pUC18 (Yanisch-Perron *et al.,* 1985) and allows the expression of genes under control of the 2x35S promoter. It contains the ampicillin resistance gene (β-lactamase) (Figure 24A). In different experiments PCR fragments were cloned in this vector by replacing the *gfp* gene, and checked by DNA sequence analysis. The *Hin*dIII fragments containing the gene of interest were removed from this vector and inserted into the binary vector pBI121.2 (see below).

2. Expression vectors:

2.1. pBI121.2 (Clontech)

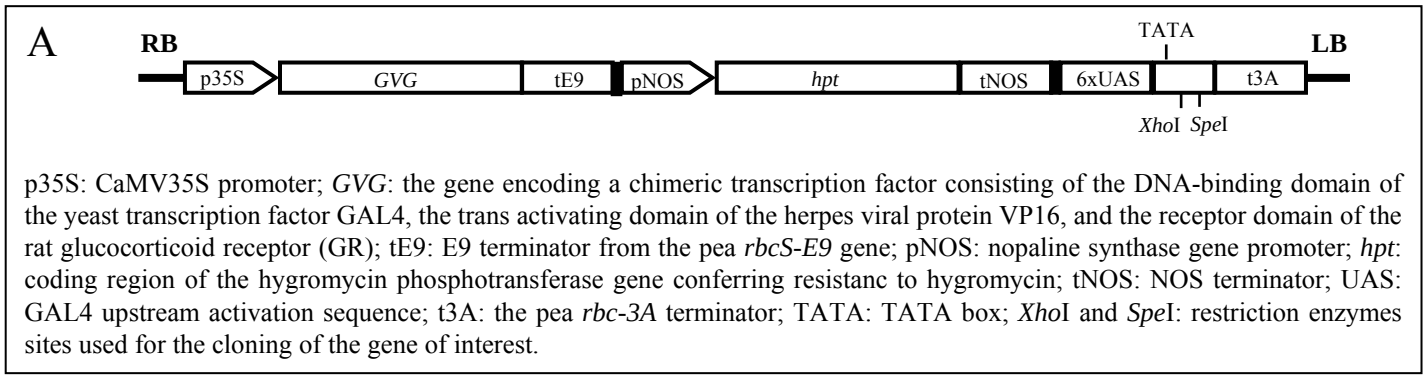
This binary plasmid allows the transfer of genes to plant cells and was used for several *plast* genes placed under control of 2x35S promoter. It contains a T-DNA with a kanamycine resistance gene (*nptII*) and a ß-glucuronidase (*gus*) gene (Figure 24B).

2.2. pTA7002 (Aoyama and Chua, 1997)

This binary plasmid allows the expression of genes under control of a dexamethasone inducible promoter in transformed plant cells (in leaf patches and transgenic plants). It contains a T-DNA with a gene encoding a chimeric transcription factor, GVG, as well as a hygromycin phosphotransferase (*hpt*) gene conferring resistance to hygromycin in plants (Figure 25A). It also contains a *nptII* gene for bacterial selection.

2.3. pFGC5941 (Arabidopsis Biological Resource Center, ABRC)

This is a RNAi binary plasmid, a derivative from pCAMBIA1300, that was used for producing hair-pin constructs of *trolC* and *torf13*. It contains the following key features: a kanamycin resistance gene for bacterial selection, a Basta resistance gene for plant selection, a CaMV 35S promoter to drive the expression of the inverted repeat target sequence, and a 1352 bp *CHS*A intron (from the petunia chalcone synthase A gene) to stabilize the inverted repeat of the target gene fragment (Figure 26).



p35S: CaMV35S promoter; *GVG*: the gene encoding a chimeric transcription factor consisting of the DNA-binding domain of the yeast transcription factor GAL4, the trans activating domain of the herpes viral protein VP16, and the receptor domain of the rat glucocorticoid receptor (GR); tE9: E9 terminator from the pea *rbcS-E9* gene; pNOS: nopaline synthase gene promoter; *hpt*: coding region of the hygromycin phosphotransferase gene conferring resistanc to hygromycin; tNOS: NOS terminator; UAS: GAL4 upstream activation sequence; t3A: the pea *rbc-3A* terminator; TATA: TATA box; *Xho*I and *Spe*I: restriction enzymes sites used for the cloning of the gene of interest.

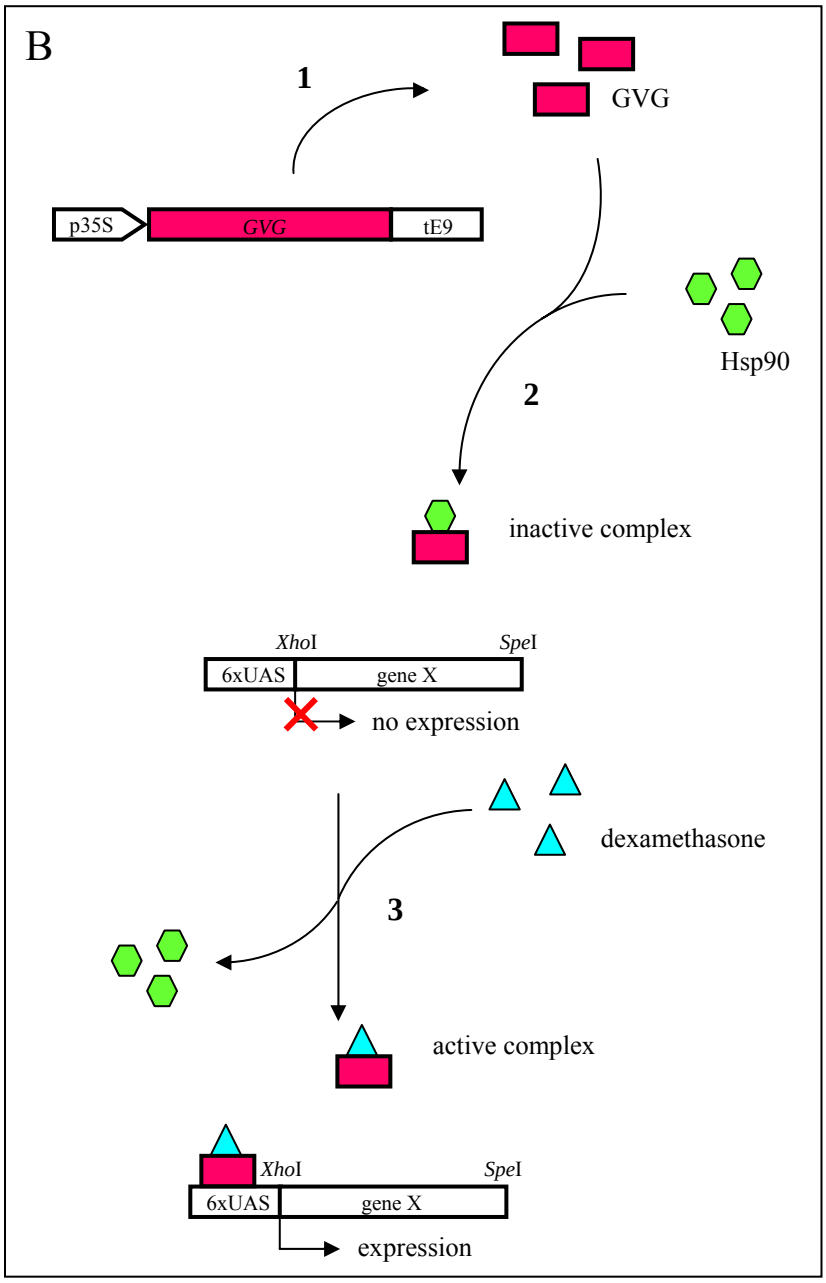


Figure25. A) Schematic representation of T-DNA in binary plasmid pTA7002 and B) the principle of gene expression induction by dexamethasone in transformed plants. 1. The GVG transcription factor is constitutively produced in transformed plant. 2. In the absence of inducer, dexamethasone , GVG interacts with the Hsp90 chaperon protein and forms an inactive complexe which is incapable to associate with the UAS elements of the promoter. 3. Addition of dexamethasone leads to the dissociation of the Hsp90 and the formation of an active dexamethasone/GVG complex which binds to the promoter and initiates the expression of gene X.

2.4. pGEX2TK (Kaelin *et al.*, 1992)

This vector was used to express GST fusion proteins. It contains the *lac*I^q gene allowing its amplification in any *E. coli* host, a *tac* promoter for chemically inducible, high level expression by IPTG and a 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 (MCS). Expressed protein can be directly labeled using protein kinase and γ^{32}P-ATP and readily detected by autoradiography. pGEX2TK contains anampicillin resistance gene.

2.5. Yeast two-hybrid vectors: pGBKT7 and pGADT10 (CLONTECH)

These vectors are used in the yeast two hybrid system for testing protein-protein interaction or for screens of a cDNA library of Arabidopsis. The bacterial resistance and yeast selection genes in pGBKT7 are kanamycin resistance and tryptophan synthesis genes (*TRP*) and in pGADT10 are ampicillin resistance and leucine (*LEU2*) synthesis genes, respectively (Figure 27A and B).

Methods

I. Plant techniques

1. Leaf patch test

A. tumefaciens strain LBA4404 was transformed with pBI121.2 or pTA7002 recombinant plasmids by electroporation and then cultured on solid YEB medium with antibiotics at 28ºC for 48 h. One colony of Agrobacterium was cultured in 3 ml liquid YEB medium containing appropriate antibiotics at 28ºC for 48h with shaking. After two days the bacterial culture was centrifuged 15 min at 5000x g at RT. The liquid medium was discarded and the pellet was washed by $MgSO_4$ (10 mM) and then resuspended in $MgSO_4$ (10 mM) and its density adjusted to an OD600 of 0.5. Infiltration of plant leaves was done using a 1 ml needleless syringe.

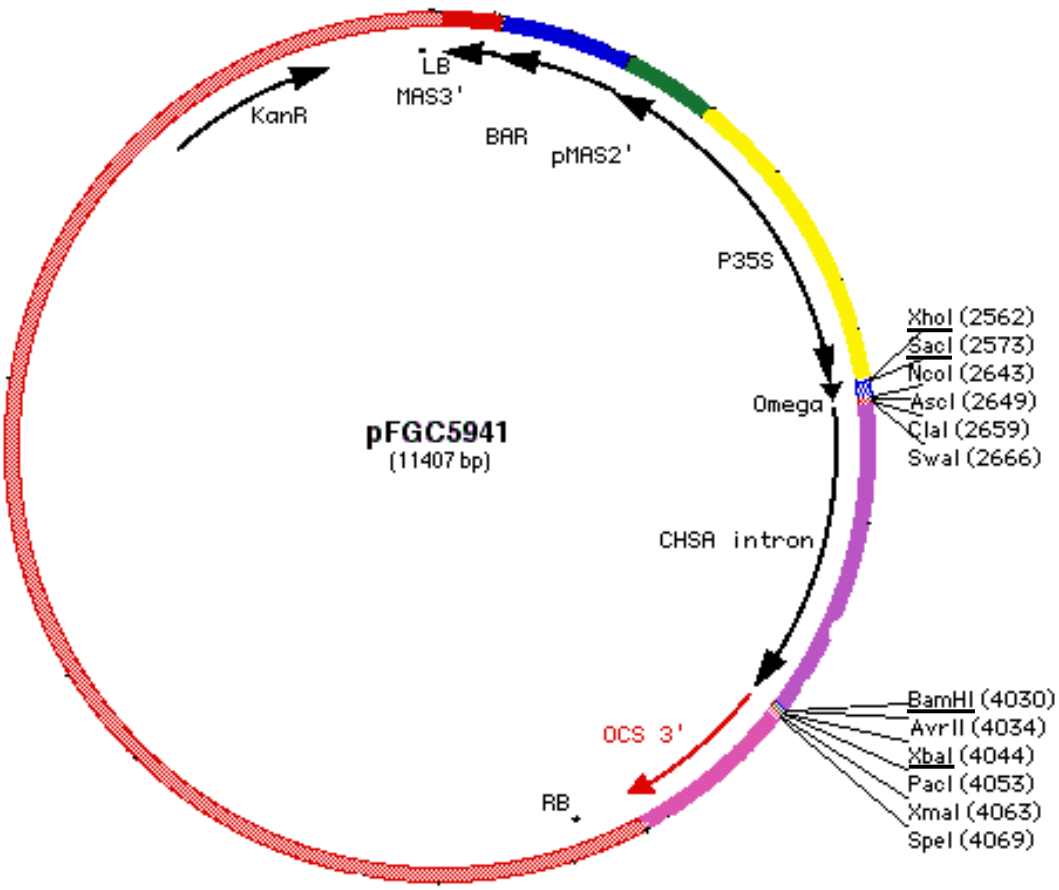


Figure 26 . Map of binary vector pFGC5941.
The orientations of genes and promoter are indicated with arrows. The underlined enzymes represent the cloning sites for the *trolC* and *torf13* fragments. kanR: kanamycin resistance gene for bacterial selection; MAS3': polyadenylation signal of the mannopine synthase gene; BAR: basta resistance gene for plant selection; pMAS2': promoter of the mannopine synthase gene; p35S: a CaMV35S promoter to drive the expression of the inverted repeat target sequence, Omega: TMV omega leader sequence; CHSA intron: a 1352 bp *CHS*A intron from the petunia *Chalcone synthase A* gene to stabilize the inverted repeat of the target gene fragment; OCS3': polyadenylation signal of the octopine synthase gene.

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

2. Tobacco transformation

2.1. Preparation of bacterial culture

After two days of preculture in 2 ml of selective YEB medium, the agrobacteria were recultured in 50-100 ml fresh medium for 24 h and then centrifuged 15 min at 5000x g at RT. The pellet was washed by $MgSO_4$ (10 mM) and resuspended in this solution and its density adjusted to an OD600 of 1.0.

2.2. Infection of tobacco leaf fragments

Young leaves of greenhouse-grown tobacco plants (*N. tabacum* cv. Samsun nn) were sterilized by a brief passage in 70% ethanol and then 20 min in a 5% sodium hypochlorite solution to which a few drops of Tween 20 had been added. Sodium hypochlorite was removed by three successive washing with sterile water (each one 5 min). After drying the leaves on sterile paper, squares of about 2 cm were taken and placed in bacterial suspension. They were then placed on a modified MS (Murashige and Skoog, 1962) medium (MS238, this medium was supplemented with 30 g/l sucrose, 825 mg/l NH_4NO_3 and the MS vitamins (Duchefa M0409)) and containing hormones (0.05 mg/L NAA, 2 mg/l BA). After 2 days, the leaf discs were washed in liquid MS238 medium containing 350 mg/l cefotaxime and transferred onto Petri dishes containing solid MS medium with hormones and appropriate antibiotics. The plates were sealed with urgopore and were placed in the culture room (12 h days, 24 ° C; 12 h night, 20 °C).

After 3-4 weeks, the shoots appeared on the leaf fragments. The medium was changed with the same medium containing the antibiotics without hormones. After 2-3 weeks seedlings grew from the calli. When the seedlings had sufficiently developed, they were individualized and placed on a complete MS rooting medium containing antibiotics (Duchefa M0222 supplemented with 10 g/l sucrose). After rooting, they were transferred to soil and placed in a mini-greenhouse (about 89% humidity, day/night cycle: 12h/12h) during one

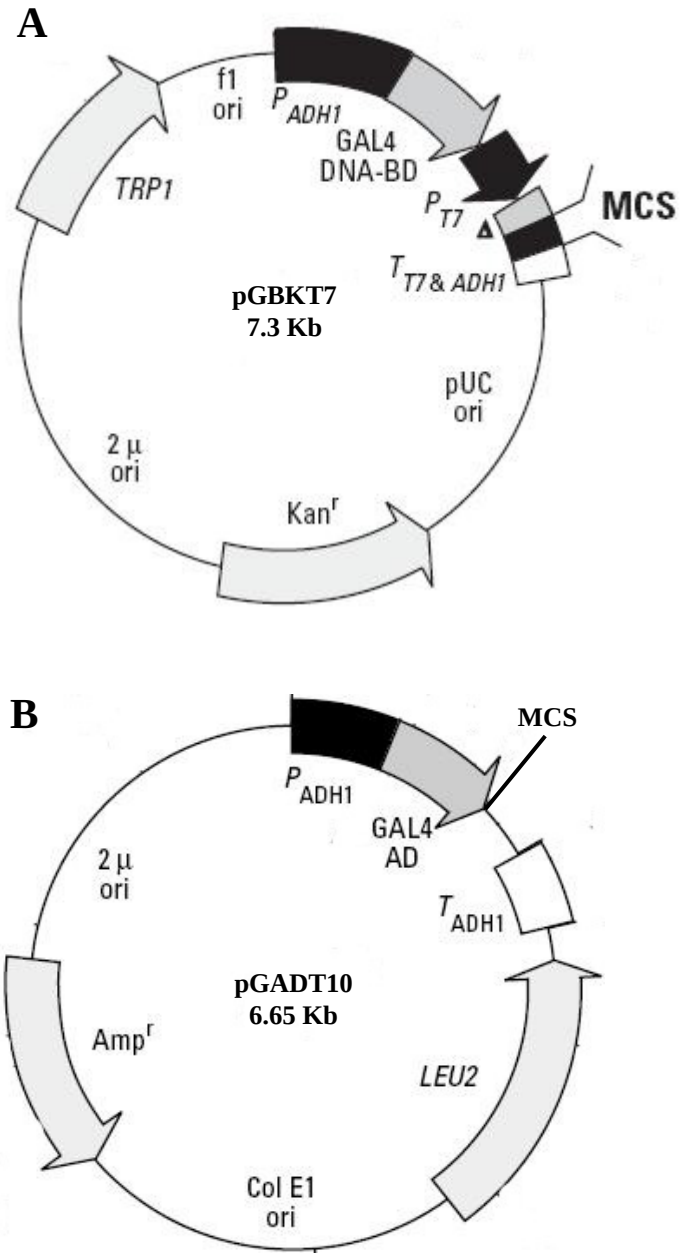


Figure 27. Yeast expression vectors: A) pGBKT7. B) pGADT7.
TRP1: TRP1 coding sequence (for yeast selection); f1 ori: f1 bacteriophage origin of replication; P_{ADH1}: ADH1 promoter; GAL4 DNA-BD: GAL4 DNA binding domain; P_{T7}: T7 polymerase promoter; Δ: c-myc epitope tag; MCS: multiple cloning site; T_{T7}: T7 polymerase terminator; T_{ADH1}: ADH1 terminator, pUC ori: pUC plasmid replication origin; Kan^r: kanamycin resistance gene (for bacterial selection); 2 μ ori: yeast 2 μ replication origin. GAL4AD: GAL4 DNA activation domain; LEU2: LEU2 coding sequence (for yeast selection), , ColE1ori: colE1 plasmid replication origin ; Amp^r: ampicillin resistance gene (for bacterial selection).

week, then transferred to the greenhouse for seed production.

3. Arabidopsis transformation by floral dip

A. thaliana 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 5000x g 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 high humidity during 48h in the dark.

For *in vitro* culture, Arabidopsis seeds were sterilized with 75% ethanol for 2 min, then with 10% bleach +Tween20 (one drop/tube) for 15 min. The seeds were rinsed three times with sterile H_2O and spread on culture medium (Duchefa M0255, supplemented with 20 g/L sucrose) and placed in a growth chamber (16h day/ 8h night at 20-22ºC).

4. Protein analysis

4.1. Protein extraction from plant tissue

Leaf discs (1 cm in diameter) were ground in 100 µl protein denaturing buffer and then heated at 95˚C for 5 min. After 5 min centrifugation at 5000x g, the supernatant was transferred to a new tube and used for gel analysis.

protein denaturing buffer	
Tris-HCl pH 6.8	62.5 mM
urea	4 M
SDS	3 %
β-mercaptoethanol	3 %
glycerol	10 %
bromophenol blue	0.1 %

4.2. Gel for protein analysis

Stacking and resolution denaturing gels were prepared as follows:

	Resolution gel: 12.5%	**Stacking gel:**
37.5 % Acryl-bisacryl	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 a solution of 10% acetic acid, 25% ethanol and 0.5 g/l Coomassie blue. Destaining was carried out in 10% acetic acid.

4.3. Western blot

For Western blots, an electroblot apparatus (BioRad) was used to transfer the proteins from the gel onto a nitrocellulose membrane or a Immobilon^{TM}-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, pH 8.3) at 500 mA and 80 V for 2h at 4ºC. The membrane was blocked with 2.5% milk powder in Tris buffer saline (TBS, Tris-HCl 20 mM pH7,4, NaCl 150 mM et Triton X-100 0,1 %) 30 min at RT. The membrane was then incubated with the primary antibody for 3h at RT. washed 3 times for 5 min in TBS-Tween. It was then incubated with the secondary antibody conjugated with goat anti mouse peroxidase (GAM) for 2h at RT and washed again as described. Proteins of interest were revealed by chemiluminescence with Lumi-Light^{PLUS} Western Blotting kit (Roche) by exposure for different times using 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 destained as described for gels or with TBS for Red Ponceau.

5. Nucleic acid analysis

5.1. Rapid DNA extraction from plants

100 mg of young leaf tissue was removed and inserted into an eppendorf tube containing glass beads (diameter 1 mm). The fragments were frozen in liquid nitrogen and ground using the Silamat® S5 apparatus (Ivoclar, Vivadent). 1 ml of extraction buffer (100 mM Tris-HCl, pH 8.8; 50 mM EDTA, pH 8; 500 mM NaCl; 0.07 % ß-mercaptoethanol) was added to the resulting powder and the mixture was homogenized by vortexing. 130 µl of SDS (10%) was added to the mixture which was incubated at 65°C for 15 min, in order to promote cell lysis. After this, the mixture was neutralized by 300 µl of 5 M potassium acetate and the tube was placed in ice for 30 min to allow precipitation of proteins. After centrifugation at 5000x g for 5 min, the supernatant was transferred to a new tube and isopropanol (v/v) was added in order to precipitate the DNA. Further centrifugation at 11000x g for 15 min yielded a DNA pellet. It was dried and taken up in 50 µl double-distilled sterile water. DNA concentration was evaluated by measuring the absorbance at 260 nm (1 unit of OD at 260 nm corresponds to 50 µg/ml of DNA).

5.2. PCR

This technique allows the exponential amplification of a DNA fragment using a thermo-stable DNA polymerase isolated from *Thermus aquaticus* (*Taq*). It requires the utilization of two primers that are specific to the sequence that will be amplified. The adding of restriction enzyme sites in the extremity of primers allows the cloning of the PCR product in the desired vector.

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
TaqDNA Polymerase	0.5 U
H_2O	Up to 20 µl

HiFi PCR reaction:

DNA	50-100 ng
Primers	0.5 µM
HiFiTaq DNA Polymerase mix (the mixture of dNTP, buffer and enzyme)	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	Repeated 30 cycles
Annealing at 50˚C for 30 sec	
Elongation at 72˚C for 45 sec	
Final elongation at 72˚C for 15 min	
Hold at 10˚C	

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

5.3. RNA extraction

200-300 mg of young leaf tissue was inserted into an eppendorf tube containing glass beads (diameter 1 mm). They were frozen in liquid nitrogen and ground using the Silamat® S5 apparatus (Ivoclar, Vivadent). Total RNA was extracted using "TRIzol®Reagent" kit (Invitrogen) following the manufacturer's indications. This reagent, a monophasic solution of phenol and a chaotropic agent (guanidine isothiocyanate), maintains the integrity of RNA and allows its extraction by denaturation of cellular components. The addition of chloroform followed by a centrifugation step separated the solution into an aqueous phase and organic phase. RNA is found exclusively in the aqueous phase. After precipitation using 1 volume of isopropanol, the pellet was washed with 70% ethanol and then dissolved in 30 µl of water. RNA concentration was evaluated by measuring the absorbance at 260 nm (1 unit of OD at 260 nm corresponds to 40 µg/ml of RNA).

5.4. RT-PCR

Total RNA extracted from leaf tissues was treated with RNase-free DNase during 90 min (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_2O 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), 1 µl of 0.1 M 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 1 h. Control reactions were performed without reverse transcriptase.

The synthesized cDNA was used as the template for PCR. Control reactions to normalize

RT-PCR amplification were run with the EF-1α specific primers.

EF-1α-forward	5′-tcgccttgtggaagtttgagac-3′
EF-1α-reverse	5′-caccaacagcaacagtttgacg-3′
PR-1-forward	5’-gatgcccataacacagctcg-3’
PR-1-reverse	5′- tttacagatccagttcttcagagg-3′
rolB-forward	5′-gatttgaaggaaaactctccaccg-3′
rolB-reverse	5′-ccaagcaaggttgtgaacacg-3′

5.5. RT-quantitative PCR

This method was used to quantify *(t)rolC* mRNA levels in transgenic tobacco plants. It was performed in 384-well optical plates on a BioRad i-cycler apparatus using PCR master 2× mix (Roche) containing 480 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. *EF2* (GenBank: AJ299248) was used as an internal standard for equalization of RNA levels. Quantifications were then normalized to the RNA level of non-induced plants. 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.

EF2-Forward:	5’-ctgaaccagaagcgtggaca-3'
EF2-Reverse:	5’-ccagatgtagcagccctcaag-3'
trolC-Forward	5’-ctgaaccagaagcgtggaca-3'
trolC-Reverse	5’- ccctgacattcagtctttatgcac-3'
rolC-Forward	5’-gggcagtcgacgtagagg -3'
rolC-Reverse	5’-cgatggatattgacgaagaagg -3'

5.6. 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 (50 µg) were prepared in loading buffer and denatured at 65°C for 5 min and chilled on ice for 5 min. 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 rinsed with 2× SSC and RNA was UV cross-linked to the membrane twice in a Stratalinker apparatus (1200 Joules X100) photographed under UV light to check for efficient RNA transfer.

RNA loading buffer:	
HEPES buffer 10×	500 µl
formaldehyde	800 µl
formamide (deionized)	2.5 ml
glycerol 50%	250 µl
ethidium bromide	1 µg/ml
bromophenol blue	a little bit

5.6.1. Probe preparation

RNA blots were hybridized with DNA probes. *rolC* and *trolC* sequences were amplified by PCR and the products were denatured at 95–100°C for 2 minutes and rapidly chilled in ice. The labeling was done using α^{32}P-dCTP as shown in the following table according to the Promega, prime-a-gene Labeling Protocol.

Component	Add	Final concentration
Nuclease free water	to achieve final volume of 50 µl	-
Labeling 5x buffer	10 µl	1x
Mixture of unlabeled dNTPs	2 µl	20 µM each
Denaturated DNA template	50 ng	500 ng/ml
Nuclease-Free BSA	2 µl	400 µg/ml
[α^{32}P]dNTP, 50 µCi, 3,000 Ci/mmol	5 µl	333 nM
DNA Polymerase I Large (Klenow) Fragment	5 units	100 u/ml

The mixture was incubated for 1h at RT and then EDTA (0.5 M, 9 µl) was added. Subsequently, the mixture was heated at 95–100°C for 2 minutes followed by chilling in ice. Purification of the labeled probes was done by commercial Riboprobe spin columns and 3 successive centrifugation steps (each: 6 min at 10000x g) in the presence of TE buffer. The purified probes attached to the filter of the column were detached by adding 100 µl TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) with pipeting.

5.6.2. Hybridization of RNA probes

The membrane was prehybridized for at least 1 h, hybridized with the appropriate probe overnight and washed.

Hybridization steps (using Perfect Hyb Plus buffer, SIGMA)	
Pre-hybridization (1 h)	42ºC
Hybridization (overnight)	42ºC
First washing step (15 min, two times)	50ºC with SSC 2×, SDS 2%
Second washing step (15 min)	55ºC with SSC 1×, SDS 1%

Small RNA blots were rehybridized with a probe complementary to U6 to provide loading controls. RNAs were detected by autoradiography.

6. Quantitative analysis of sugars by enzymatic tests

Leaf discs with a diameter of 1.8 cm were taken from plants and the soluble sugars were extracted in two times with 1 ml 80% ethanol at 65°C for 30 min. The alcohol was evaporated under vacuum in a Speed-Vac apparatus and the precipitate was dissolved in 500 µL water. This solution was used for the determination of glucose, fructose and sucrose using Sigma enzymatic kits (glucose (HK) assay kit G-3161, fructose assay kit F-2668, sucrose assay kit S-1299). The enzymes operate at room temperature. The appearance of NADH was measured by its absorbance at 340 nm, in a 96-well microplate, using the EL_X808 spectrophotometer (BIO TEK).

II. Bacterial techniques

1. Bacterial competent cell preparation

A bacterial strain was inoculated in 5 ml of LB (*E. coli*) or YEB (Agrobacterium) medium and cultured 16 h at 37°C (*E. coli*) or 32 h at 28°C (Agrobacterium). This pre-culture was transferred into 500 ml of fresh medium, and shaken strongly until A_{600nm}= 0.8-1. This corresponds to cells in late exponential growth phase. The following steps were performed with cold sterile equipment.

The resulting culture was centrifuged for 15 min at 5000x g, washed with 500 ml H_2O and after a second step of centrifugation and washing the pellet (bacterial cells) was resuspended in 20 ml of 15% glycerol. This bacterial solution was aliquoted in individual tubes (40 µl / tube), frozen in liquid nitrogen and stored at -80°C. One aliquot was used for each transformation.

2. Transformation of bacteria by electroporation

In this method, 1-2 µl of plasmid (DNA solution) was added to 40 µl of electro-competent bacteria cells. The mixture was transferred to the cuvette and loaded into a cell-electroporator apparatus (BioRad) for electroporation with 125 µF capacitance, 200 Ω resistance and 2.5 V voltage. After electroporation, cells were mixed with 1 ml of LB (Luria-Bertoni) medium and incubated at 37˚C (*E. coli*) or at 28˚C (*A. tumefaciens*) 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*).

3. Culture of bacteria

One colony of transformed bacteria was cultured in 1.5 ml medium containing the appropriate antibiotic with incubation overnight at 37°C (*E. coli*) or 48h at 28°C (*A. tumefaciens*) with shaking.

4. Extraction of plasmid DNA

The amplified plasmid from a 1.5 ml bacterial culture was extracted by an alkaline lysis method. Cells were sedimented by centrifugation at 10000x g for 2 min and the medium was

discarded. 100 µl of solution I was added and the cells were resuspended by vortexing. 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 mixed by inverting the tube. Centrifugation was performed at 15000x g for 5 min and the supernatant was transferred to a new tube. The solution was extracted with one volume of phenol/chloroform and plasmids were precipitated with 2 volumes of ethanol and resuspended in 50 µl H_2O.

Recombinant colonies were analyzed by enzymatic restriction analysis or PCR. The selected plasmid preparation were treated by RNase (10 ng/µl) in TE buffer (10 mM Tris-HCl and 1 mM EDTA) in a 50 µl volume at 37˚C for 30 min. DNA was then extracted by phenol/chloroform and precipitated in ethanol in the presence of 2 M ammonium acetate. Finally, the plasmids were taken up in 50 µl H_2O and 50 ng was used for sequencing.

DNA isolation buffers		
Solution I	**Solution II**	**Solution III**
25 mM Tris-HCl pH 8	200 mM NaOH	58.8 g/200 ml potassium acetate
10 mM EDTA	1% SDS	23 ml acetic acid
50 mM glucose		

The sequence of genes in plasmids was verified by sequencing.

5. Overexpression of the recombinant AtPTP1 protein

The coding region of the *AtPTP1* cDNA, subcloned in pGEX-4T-3 (Pharmacia, Piscataway, NJ) and transformed into *E. coli* BL21 (DE3) (Novagen, Madison, WI) was used for overexpression and purification of the GST-AtPTP1 fusion protein. Overexpression was performed as described (Luan *et al*., 1994, 1996), with some modifications. After inducing expression with 0.25 mM isopropyl ß-D-thiogalactopyranoside (IPTG), bacterial cultures were pelleted and resuspended in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 2 mM phenylmethylsulfonylfluoride, 1 mM benzamidine, and 2 mM EDTA before lysis by sonication. The cell lysate was pelleted at 15000x *g* to collect the supernatant containing the fusion protein that was subsequently purified by glutathione Sepharose 4B (Pharmacia). Cleavage was performed using 0,1, 0,2 and 0,3 mg/ml thrombin (Sigma).

III. Yeast yechniques

1. Yeast transformation

Several methods can be 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. Yeast (*S. cerevisiae* strain AH109) competent cells were 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 a 10 ml culture was used to inoculate 50 ml YPD and incubated for 4h at 28ºC with shaking until A_{600}= 0.6. The yeast cells were sedimented by centrifugation at 4000x g for 3 min and resuspended in 2 ml sterile H_2O. Centrifugation was performed at 6500x g 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×: 1 M lithium 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 for 5 min and chilling 3 min on ice) was mixed with 1 µl of each plasmid solution (pGBKT7 and pGADT7 derivatives) 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 solution 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 6500x g 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. Growth of colonies in the absence of **H**istidine implies a weak interaction between candidate fusion proteins fused to AD and BD and 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 (t)RolC in an Arabidopsis cDNA library. To this end, the pGBKT7-(t)RolC vector (encoding BD-(t)RolC

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 x g 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 x g 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 x g 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 up in 20 ml SD selective medium (–AHWL or –HWL) containing 10 µg/ml tetracycline and kept at 4ºC overnight. The day after, cells were sedimented by centrifugation at 2000 x g 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.

3. DNA extraction from yeast

5 ml of yeast culture was centrifuged at 4000 x g 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 x g for 5 min, 200 µl of supernatant was transferred to a new tube. DNA was precipitated with 3 volumes ethanol in the presence of 1/10 volume 4 M ammonium acetate. Finally, after 15 min centrifugation at 14000 x g and washing with 70% ethanol, DNA was taken up in 40 µl H_2O.

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Résumé de la thèse en français

Etude de deux gènes plast d'Agrobacterium rhizogenes, *rolB* et *rolC* et leurs homologues chez le tabac

INTRODUCTION

Agrobacterium rhizogenes est connue pour sa capacité à induire des chevelus racinaires sur un grand nombre de plantes. Cette bactérie contient un plasmide, appelé pRi (Root-inducing plasmide) qui porte un fragment d'ADN-T qui est transféré dans la cellule végétale. La séquence d'ADN-T est différente d'une souche à l'autre mais la partie centrale qui comporte les gènes *rolA, rolB* et *rolC* est bien conservée. Selon l'homologie de séquences protéiques, les gènes *rolB* et *rolC* appartiennent à une famille de gènes appelée plast (pour plasticité phénotypique). L'homologie entre les différentes protéines de cette famille est très faible mais significative, et jusqu'à maintenant aucune séquence homologue n'a été décrite en dehors d'un contexte d'ADN-T. Les phénotypes induits par les gènes *plast* sont extrêmement divers et les mécanismes d'action de ces gènes restent controversés. Les séquences d'ADN-T sont également présentes dans certaines espèces de Nicotiana, notamment *N. glauca* et *N. tabacum* où elles sont appelées ADN-T cellulaires ou cT-DNAs). Ils sont probablement le résultat d'un événement de transfert très ancien d'une agrobactérie vers une plante. Le cT-DNA de *N. glauca* est totalement séquencé et il s'agit d'une séquence répétée et inversée. Les gènes *NgrolC* et *Ngorf13* sont transcrits et ils ont une activité morphogénique. *NgrolB* est muté, mais peut être restauré à un gène fonctionnel et *rolA* est absent.

Dans le cas de *N. tabacum*, la structure de cT-DNA est très peu connue et seulement quelques fragments de cT-DNA de différents cultivars ont été décrits : un *trolC*, une version complète et une version partielle de *torf13* et un *torf14*. Une séquence similaire au gène *mis* (mikimopine synthase) a été aussi identifiée. Parmi ces gènes seulement le gène *torf13* du cultivar Havana-425 a montré une activité biologique, il induit des cals sur des rondelles de carottes. Nous avons étudié dans un premier temps la structure de cT-DNA de *N. tabacum* et dans un deuxième temps la fonction du gène *trolC* du cT-DNA afin de le comparer avec le gène *rolC* de la bactérie.

RESULTATS

1. La structure de cT-DNA du tabac (*N. tabacum* cv. Samsun nn)

Depuis les différentes publications indiquant la présence des gènes *trolC*, *torf13* et *torf14*, un grand nombre de ‘’expressed sequence tags (EST)’’ et ‘’genomic survey sequences (GSS)’’ pour tabac ont été rapportés. Grâce à ces informations et aussi la séquence de cT-DNA de *N. glauca*, nous avons dessiné les oligos pour PCR. Nos resultats ont montré que la séquence cT-DNA de *N. tabacum* cv. Samsun nn, diffère de celle du cT-DNA de *N. glauca.* Deux fragments non-adjacents de cT-DNA existent dans le tabac: Le premier fragment porte *torf8, trolA, trolB, trolC* et *torf13*, le deuxième *torf14* et *tmis*. La région *torf8-torf13* est le plus similaire aux séquences correspondantes chez la souche A4 d’*A. rhizogenes* et *N. glauca* (70 et 80% d'homologie respectivement), *torf14-tmis* à 96% homologie avec la séquence de cT-DNA de *N. glauca* jusqu'à la frontière droite, mais pas d'homologie au-delà du cT-DNA, indiquant que *torf14-tmis* n'est pas inséré dans le même endroit que *Ngorf14-Ngmis* de *N. glauca*. Nous avons observé que contrairement à *N. glauca*, *N. tabacum* porte un gène *rolA* (*trolA*), mais *trolA* a un codon stop en position 112 (ORF normale: 270 nt). *trolB* porte à la position 24, une répétition directe de 40 nt avec un ‘’mismatch’’ (TCAACGCTT-(T/A)- CACCCAAGAAATCTCACTCGAGCATTGAGC) provoquant un décalage du cadre de lecture (ORF normale: 783 nt), *trolC* est intact, *torf13* est tronqué au-delà de 400 nucléotides, *torf8* a un codon stop à la position 1873 (ORF normale: 2286 nt), *torf14* est intact, *tmis* a également un codon stop précoce en position 199 (ORF normale: 987 nt). Ainsi, *trolC* est le seul gène potentiellement capable de modifier la croissance du tabac cv. Samsun nn (Figure 1). Il y a des évidences qui indiquent que *trolC* est transcrit, mais son activité biologique n'a pas encore été testé. Il a été montre que le gène *rolC* de la souche A4 *d’A. rhizogenes* (*A4-rolC*) provoque un nanisme et des feuilles vertes pâles lancéolées dans le tabac. Nous avons donc décidé de tester l'activité de *trolC* et la comparer avec celle de *A4-rolC* par expression dans des plantes de tabac et d’Arabidopsis.

2. Les tabacs transgéniques *A4-rolC* et *trolC* et les phénotypes associés

Nous avons transformé des plantes de tabac (cv. Samsun nn) par les constructions dex-*A4-rolC*-HAHIS et dex-*trolC*-HAHIS dans lesquelles les gènes *A4-rolC* ou *trolC* étaient fusionnés au tag HAHIS et sous le contrôle de promoteur inductible par dexaméthasone (dex). Plusieurs lignées indépendantes ont été obtenues montrant des niveaux élevés de protéines A4-RolC-HAHIS ou tRolC-HAHIS, 24 h après induction. Une induction de plantules de 14 jours par dex ont dans la condition *in vitro* aboutit à un ralentissement de croissance des racines avec une diminution de taille et de densité des poils racinaires. En plus les cotylédones et les feuilles deviennent vertes pâles et plus petites. Aux stades plus avancés (environ 10 jours après induction), les feuilles et les racines latérales ultérieures deviennent progressivement normales pour les plantules dex-*trolC*-HAHIS-7, alors que les plantules dex-*A4-rolC*HAHIS gardent toujours l'aspect réduit de leur taille et les feuilles chlorotiques (Figure 2).

Afin de tester les effets de ces gènes dans la condition *in vivo*, les graines ont été semées en terre et pulvérisées avec une solution de dex au stade deux cotylédones. Les deux lignées transgéniques ont montrés une réduction de croissance considérable par rapport aux plantes sauvages. Aux stades ultérieurs, les pousses latérales remplacent les pousses principales qui semblaient être bloquées. Étonnamment, plusieurs plantes ont montré des ''énations'' le long de la nervure centrale de la feuille. Des énations ont été observées dans certaines maladies virales des plantes et chez des plants de tabac exprimant les gènes *plast AB4-6b* et *T-6b*. Lorsque des plantes matures *A4-rolC* ou *trolC* ont été pulvérisées par dex, les feuilles sont devenues étroites avec un aspect chlorotique irrégulier, démontrant que *A4-rolC* et *trolC* interfèrent non seulement avec le développent de la plante dans un stade précoce, mais sont aussi capables de modifier le développement de plantes matures. Les zônes chlorotiques correspondent à des zones riches en amidon, indiquant la rétention locale du saccharose (Figure 3). Une accumulation d'amidon a également été décrite pour les plantes *AB4-6b*.

L'inhibition de la croissance observée dans la partie aérienne de la plante pourrait être la conséquence indirecte de *(t)rolC* sur les racines (ou l'inverse), donc afin de simplifier l'analyse des gènes *(t)rolC*, nous avons testé leurs effets sur les plantules et les racines isolées.

Lorsque les plantules dépourvues des racines ont été transférées sur un milieu inductible avec 1% de saccharose ils ont ralenti leur croissance et sont devenues chlorotiques indiquant que ces modifications ne dépendent pas de racines. Etant donné que les gènes *(t)rolC* conduisent à l'accumulation d'amidon dans les feuilles, nous avons supposé que ce phénotype était dû à l'accumulation anormale du saccharose. Cela a été testé en utilisant la méthode d'expansion de disques foliaires dans un milieu liquide. Il a été montré que l'expansion des disques foliaires de tabac est stimulée par la présence de saccharose. Dans le cas de disques induits de type *A4-rolC* et *trolC,* nous avons observé une expansion plus importante qui dépendait de la présence de saccharose (Figure 4). Il est intéressant de savoir que la stimulation de l'expansion des disques a été également rapportée pour le gène *T-6b,* ce qui confirme la relation entre *rolC* et *6b* suggérée par l'induction des énations.

Pour savoir si les racines sont influencées par la partie aérienne, nous les avons coupées et placées sur milieu inductible avec 1% de saccharose. Les racines des plantes de type sauvage ne poussent pas sur ce milieu alors que les racines des plantules *A4-rolC* et *trolC* continuaient à pousser (Figure 5a). Cela signifie que *A4-rolC* et *trolC* peuvent directement influencer la croissance des racines. Nous avons supposé que la croissance des racines sous ces conditions était due à l'absorption ou l'utilisation améliorée de saccharose. Le placement des racines sur un milieu inductible avec des concentrations croissantes de saccharose (de 0 à 2 %) a montré une absence de croissance sur milieu sans saccharose alors que la croissance des racines était déjà visible à 0,1% de saccharose montrant une forte capacité d'absorption ou d'utilisation du saccharose par les racines transgéniques (Figure 5a). Les racines *A4-rolC* et *trolC* ont montré une dépendance très similaire par rapport à la concentration du saccharose. Il est important de noter que les racines *trolC* ont cessé leur croissance au delà du 5^{eme} jour, alors que les racines *A4-rolC* continuaient à croître (Figure 5b). Une croissance de racines isolées sur un milieu avec des concentrations faibles en saccharose avait déjà été notée pour des racines *T-6b* et il a été démontré que ceci est dû à une absorption accrue du saccharose. Nous avons donc mesuré l'absorption de saccharose radioactif par des fragments subapicaux des racines *A4-rolC* et *trolC*. Les résultats obtenus ont montré une absorption de saccharose deux fois plus élevée pour les racines *A4-rolC*, *trolC* et *T-6b* (comme témoin positif) par rapport aux racines de type sauvage (Figure 5c).

Au cours de nos études, nous avons remarqué que certains changements induits par *trolC* ont été rapidement perdus malgré la présence continue de l'inducteur (dex), par exemple les

nouvelles feuilles et les racines latérales se sont normalisées et les racines isolées ont arrêté leur croissance, pourtant les changements dus à *A4-rolC* semblaient être stables. La normalisation des plantes *trolC* pourrait être le résultat d'une adaptation physiologique, en augmentant la dégradation des protéines ou en diminuant l'expression du transgène. Les analyses des protéines et les transcrits de ces gènes après induction (mesurés par western blot et RT-qPCR) ont montré que le niveau de protéines et transcrits de *A4-rolC* reste stable, alors que celui de *trolC* montre une forte diminution au cours du temps et en parallèle les plantes *trolC* accumulent des quantités croissantes de siARN de 21 nt confirmant l'implication d'un PTGS (post transcriptional gene silencing) pour les lignées *trolC* mais pas pour les plantes *A4-rolC* (Figure 6).
Dans les comparaisons entre les différentes constructions géniques il est important de tenir compte de ce phénomène inattendu.

3. Les plantes Arabidopsis *A4-rolC* et *trolC* et les phénotypes associés

La surexpression de *trolC* ou *A4-rolC* dans le tabac peut interférer avec la fonction du *trolC* endogène. *A. thaliana* est dépourvu de cT-DNA et permet ainsi des études de *A4-rolC* et *trolC* en absence d'une activité endogène. Les plantes *A. thaliana* transformées avec *A4-rolC* ou *trolC*, avec ou sans tag HAHIS, sous le promoteur 35S de CaMV ont montré un phénotype caractéristique similaire ; ralentissement de la croissance racinaire, floraison précoce et chlorose légère (Figure 7), et rappelle ce que nous avons observé pour les plantes transgéniques du tabac. Ainsi, *A4-rolC* et *trolC* induisent des effets biologiques similaires en absence d'une activité endogène *trolC* indiquant d'une part que l'activité biologique de *trolC* est conservée pendant l'évolution et d'autre part que ces gènes influencent directement le développement des plantes.

4. Les protéines Plast en dehors des contextes T-DNA et cT-DNA

Les protéines Plast retrouvées chez les agrobactéries et certaines espèces de Nicotiana, constituent une classe unique de protéines sans similarité avec d'autres protéines. Les recherches dans les bases de données en utilisant la séquence de *A4-rolC* ou *trolC*, nous ont permis de trouver 5 protéines de *Laccaria bicolor* (un Basidiomycète ectomycorrhizien) avec une faible homologie (environ 20%) par rapport à ces protéines (Figure 8). Nous

proposons d'appeler ces protéines protéines L-Plast (Laccaria-Plast protéines). La présence de ces protéines en dehors d'un contexte d'ADN-T, peut fournir de nouveaux moyens d'élucider la fonction des gènes *plast*.

PERSPECTIVES

Nos résultats indiquent que les modifications induites par les gènes *(t)rolC* et *6b* sont causées par un mécanisme de base: l'absorption et rétention renforcée du saccharose par les cellules transgeniques. Cependant les caractéristiques de ce transport et sa relation avec le transport normal de saccharose restent encore à élucider.

Les gènes *(t)rolC* accélèrent la floraison chez le tabac et l'Arabidopsis. Il a été montré que la floraison est aussi influencée par le transport du saccharose. Les plantes inductibles *(t)rolC* pourraient constituer de nouveaux modèles pour étudier la relation entre le transport du saccharose et la floraison.

Il reste aussi à élucider si les autres gènes *plast* partagent les mêmes fonctions que *(t)rolC* et *6b*.

Il sera intéressant d'étudier la distribution des gènes L-PLAST dans d'autres champignons, d'étudier leur rôle dans Laccaria et de les exprimer dans les plantes. Si les protéines L-Plast stimulent l'absorption du saccharose comme *RolC* et *6b*, ils pourraient jouer un rôle important dans le transport du saccharose à l'interface plante-champignon, un processus essentiel pour la symbiose ectomycorhizienne.

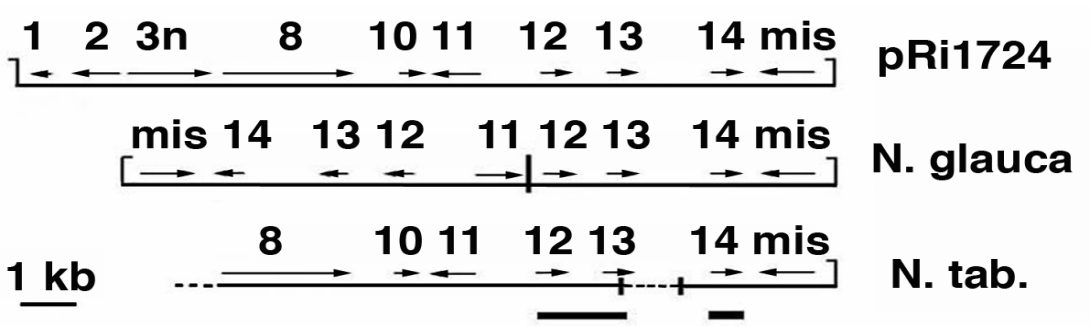


Figure 1. Les cartes d'ADN-T et d'AND-T cellulaire (cT-DNA).
pRi1724: AND-T d'*A. rhizogenes* souche 1724 (NC_002575.1). cT-DNA de *N. glauca* (Suzuki *et al.*, 2002). cT-DNA de *N. tabacum*. Les fleches: les ORF (open reading frame). 10, 11 et 12: *rolA*, *rolB* et *rolC*. mis: le gène de mikimopine synthase. Les lignes épaisses horizontale: les séquences publiées de *N. tabacum.*

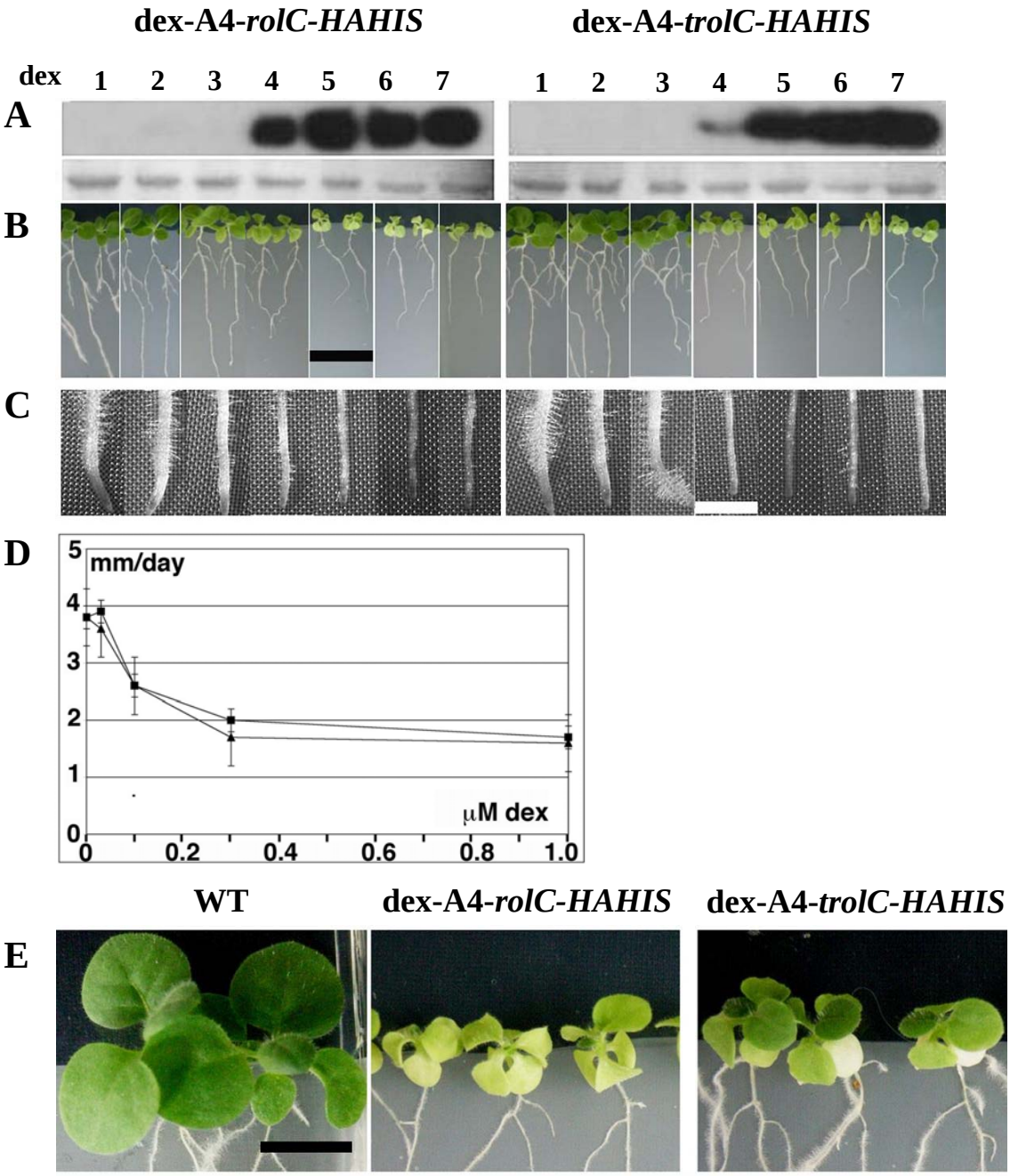


Figure 2. Contrôle de l'expression de dex-A4-*rolC-HAHIS* et dex-*trolC-HAHIS* par different concentration de dex et les phénotypes associés.

Les plantules dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7* ont été intuits sur le milieu avec 0, 0.01, 0.03, 0.1, 0.3, 1 and 3 µM de dex (les numéros 1 à 7 respectivement). A. Analyse par Western des protéines A4-RolC-HAHIS et tRolC-HAHIS qui ont été détectés par anticorps anti-HA 24 heures après induction. haut: les protéines A4-RolC-HAHIS et tRolC-HAHIS, bas: le côntrole de la charge. B. Les plantules 8 jours après induction. Bar: 2 cm. C. Les racines 8 jours après induction. Bar: 0.25 cm. D. La croissance racinaire (mm/jour) diminue avec la concentration accrue de dex. carré: dex-*trolC-HAHIS-7*, triangle: dex-A4-*rolC-HAHIS*. E. Les plantules Wild-type, dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7*, 18 jours après induction. Les plantules dex-A4-*rolC-HAHIS-7* restent toujours anormal mais les plantules dex-*trolC-HAHIS-7* se normalizent: les nouvelles feuilles sont normales avec la couleur vert foncé et les racines latérales contient des poiles racinaire. Bar: 1 cm.

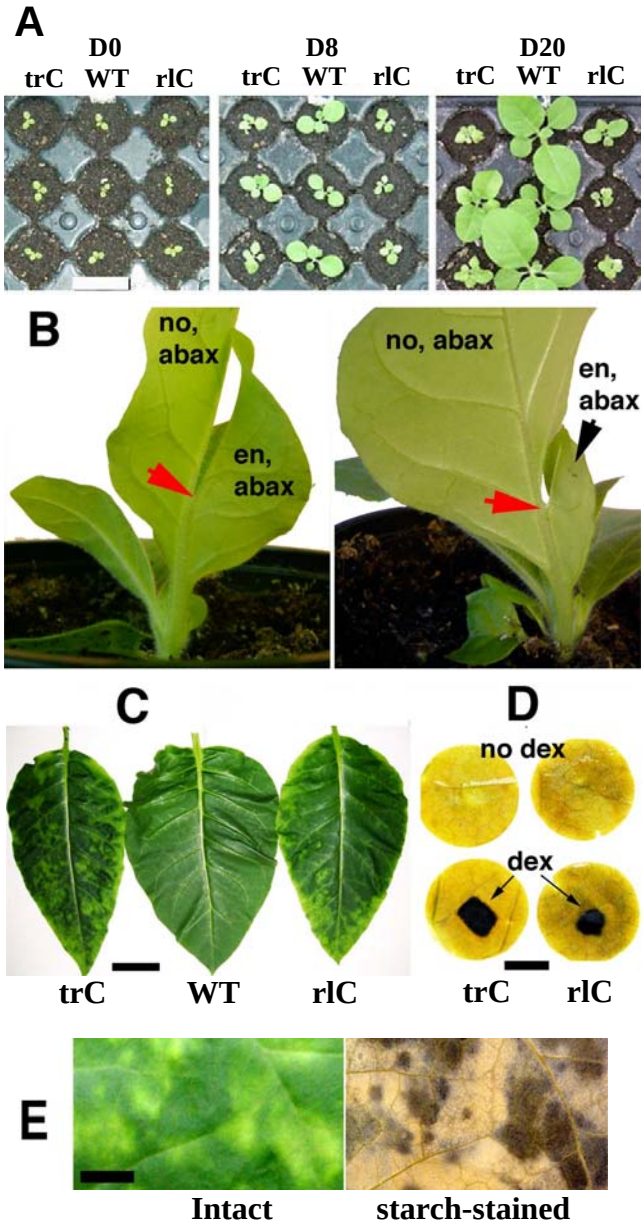


Figure 3. *trolC* et A4-*rolC* modifient la croissance des plantes de tabacs dans le sol.
Les plantes wild-type (WT), dex-*trolC-HAHIS-7* (trC) et dex-A4-*rolC-HAHIS-7* (rC) ont été pulvérisées par 3 µM dex. A. Les plantules en stade de 2-cotyledon ont été induites par dex. Les plantes wild type se développent normalement, les plantes dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7* montrent une réduction de la croissance. Bar: 3 cm. B. Enations sur les plantes dex-*trolC-HAHIS-7* (trC) et dex-A4-*rolC-HAHIS-7* (rC) àpres 40 jours d'induction. Bar: 2 cm. C. Induction des plantes matures. Après 7 jours d'induction les feuilles des plantes dex-*trolC-HAHIS-7* (trC) and dex-A4-*rolC-HAHIS-7* (rC) montrent la chlorose irrégulière. Bar: 2 cm. D. Coloration à l'Iode révèle la présence de l'amidon (zones noires) dans les feuilles dex-trolC-HAHIS-7 (trC) et dex-A4-rolC-HAHIS-7 (rC), 7 jours après l'induction locale avec une sphère de lanoline contenant dex. Haut: les disques traités avec des sphères de la lanoline, sans induction, bas: les disques traités avec des sphères de la lanoline avec 10 dex uM. L'amidon s'accumule dans une petite zone autour de la sphère de lanoline contenant dex (zone noire) . Bar: 1 cm. E. Accumulation d'amidon dans une des feuilles de la plante dex-A4-*rolC-HAHIS-7* pulvérisée par dex. Coloration à l'Iode montre que les zones chlorosés correspondent aux zones d'accumulation d'amidon.

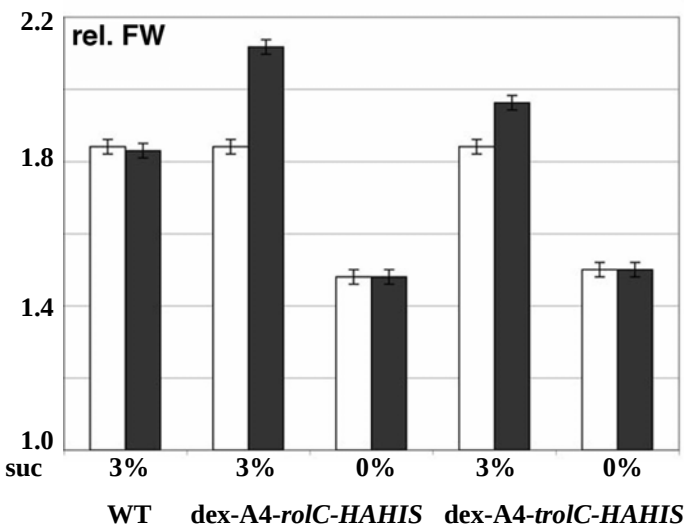


Figure 4. Augmentation de l'expansion des disques foliaire par *trolC* et A4-*rolC* et la dépendance en saccharose.
Les disques des plantes wild-type, dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7* ont été placées sur milieu liquide et l'expansion a été mesurée en poids freshe des disques en jour 5 par rapport au jour 1. bars blanches: non induit, bars noires: induit par 3 µM de dex.

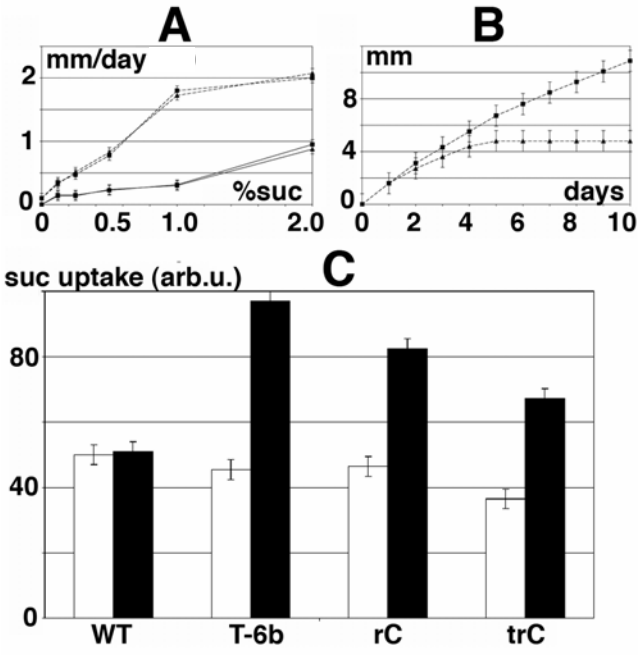


Figure 5. Influence de *rolC* et *trolC* sur la croissance de racines et sur l'absorption de saccharose.
A. Les racines dex-A4-*rolC-HAHIS-7* (triangle) et dex-*trolC-HAHIS-7* (carré) ont été cultivées sur different concentration de saccharose. Croissance en mm/jour sur une période de 5 jours. Les lignes continues: non-induit, les lignes discontinues: induit. B. Augmentation de la longueur (en mm) de racines dex-A4-rolC-HAHIS-7 (triangles) et dex-trolC-HAHIS-7 (carrés) sur un milieu avec 1% de saccharose et 3 dex uM, au cours d'une période d'induction de 10 jours. Les racines dex-A4-*rolC-HAHIS-7* montrent une croissance régulière alors que les racines dex-*trolC-HAHIS-7* arrêtent leur croissance des 5 jours. C. Absorption du saccharose par les fragments de racine sauvage (WT), dex-T-*6b* (T-*6b*), dex-A4-*rolC-HAHIS-7* (rC) et dex-*trolC-HAHIS-7* (trC), sans induction (barres blanches) ou après induction avec 3 dex uM (barres noires). Les lignées dex-T-*6b*, dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7* montrent une augmentation de l'absorption du saccharose, le contrôle WT ne montre pas. Les données sont exprimées en pourcentage de saccharose repris après 24 heures.

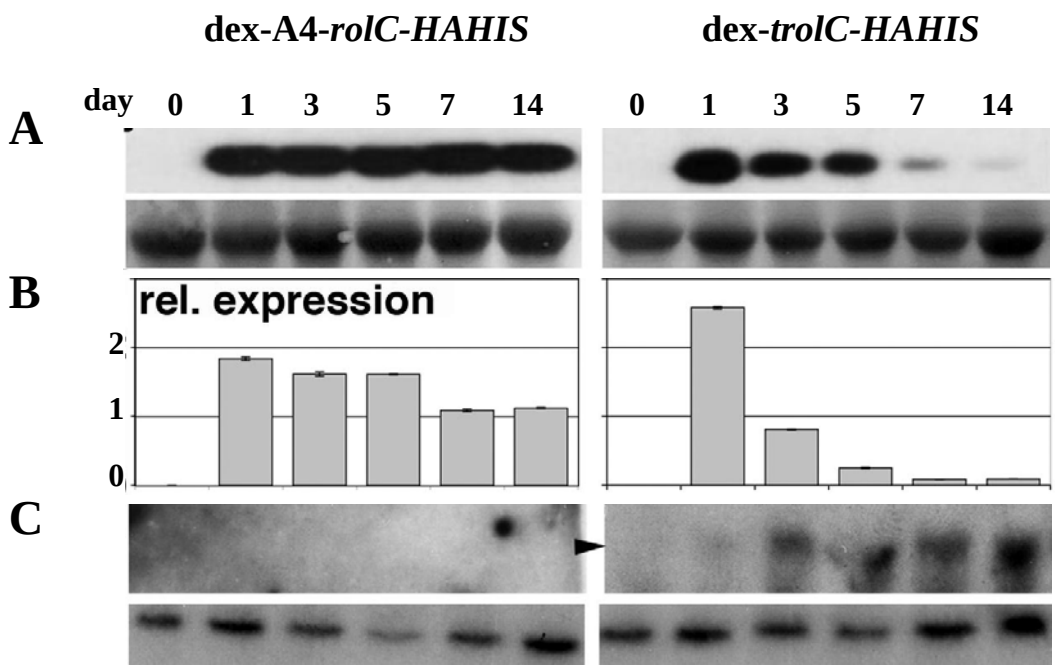


Figure 6. Extinction progressive de dex-A4-*rolC-HAHIS-7* et dex-*trolC-HAHIS-7* après induction. Les plantules ont été placés sur un milieu avec 3 µM de dex et analysé 0, 1, 3, 5, 7 et 14 jours après induction. A. Analyse des protéines: haut: A4-RolC-HAHIS et tRolC-HAHIS, bas: contrôle de la charge. La quantité des protéines A4-RolC-HAHIS restent stables alors que la niveaux des protéines tRolC-HAHIS diminuent rapidement. B. Analyse des mRNA par RT-qPCR. Unités: expression relative x 0,0001 (calculé par rapport aux valeurs au jour 0, fixé à 1). La niveaux de mRNA de la lignée dex-A4-*rolC-HAHIS* diminue lentement, tandis que les mRNA de la lignée dex-*trolC-HAHIS* diminuent rapidement. C. Analyse des siRNA. Haut: les échantillons dex-A4-*rolC-HAHIS* ne montrent pas de signal, alors que les échantillons dex-*trolC-HAHIS* montrent des quantités croissantes de siRNA. Bas: contrôle de la charge de RNA qui est obtenus en hybridant la sonde de RNA U6 avec la filtre (Gy *et al*, 2007).

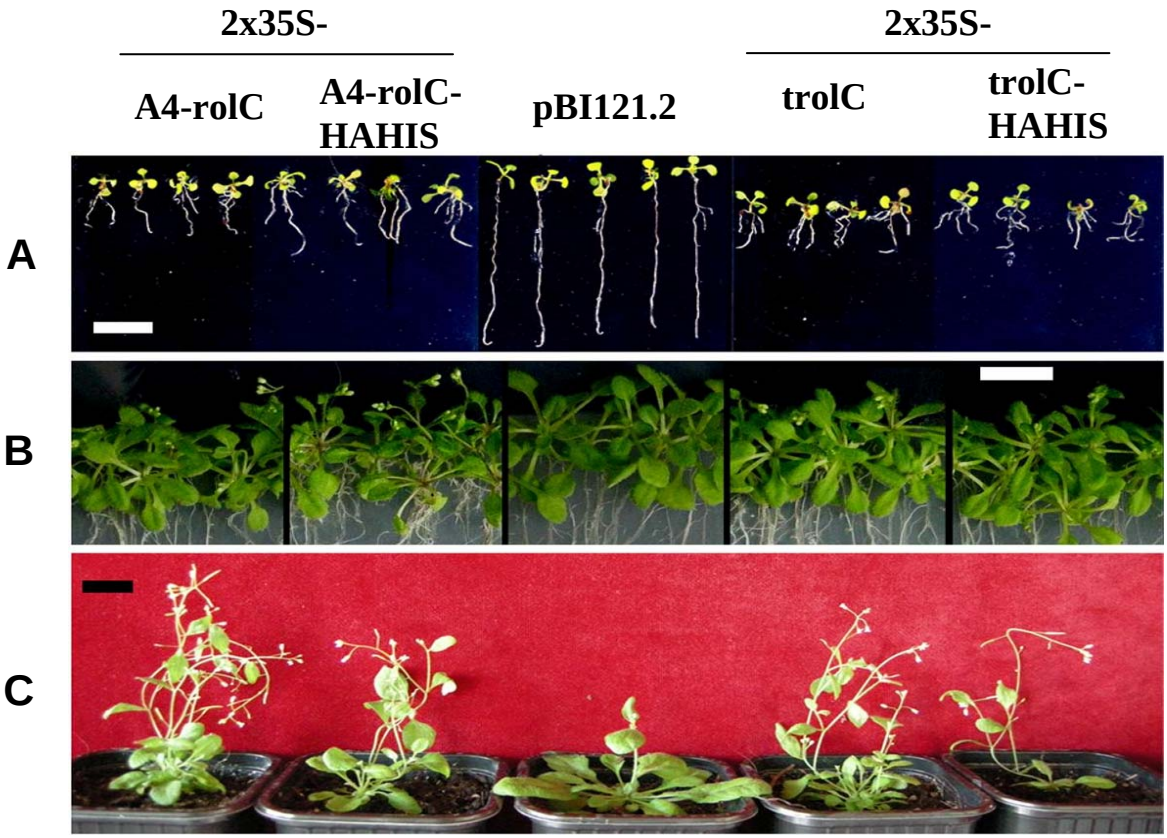
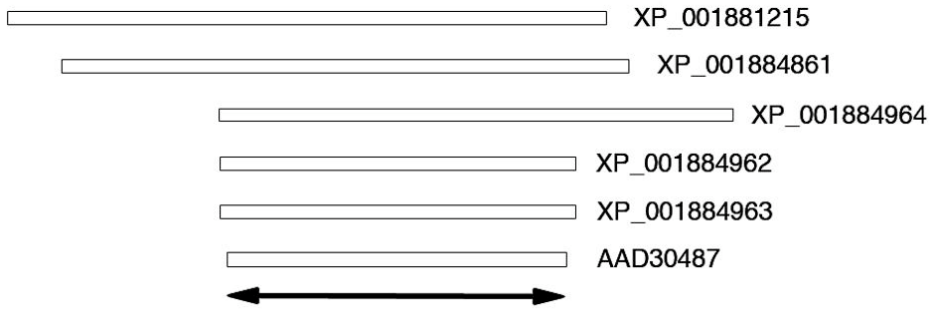


Figure 7. Expression de A4-*rolC* et *trolC* chez *Arabidopsis thaliana*.
La version taggée (ou non) à la sequence HAHIS de A4-*rolC* et *trolC*, sous la côntrole du promoteur 2x35S et la construction du vecteur vide pBI121.2 ont été utilisé pour la transformation des plantes *Arabidopsis thaliana* ecotype Col-0. A. 10 jour après induction (*in vitro)*. B. 21 jour après induction (*in vitro*). C. 32 jour après avoir poussé dans le sol. Bars (A et B): 1 cm, (C): 2 cm.

A



B

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6    YLLPQAFTPSDCSGITNPTELEAMLKYALSRYKLHI-ERTYKSQVAWAALLLPPFLNEPA    64
     Y     F   D S I +  EL+ +L++    Y+    E    +Q +W   ++    + P
2    YHSRPIFNIIDSSNIQDRRELKLVLRHTEIAYRSFAQEDLIPAQRSWMNSIINT--DVPI    59

65   DAPTLTMAQRIIEKRELMPHYQPFGEKHYEPGQPQDDIHEIRDTILIDAQPLFTYSCADR   124
     D P +     ++++         F E    PG        I   I+++    + Y
60   D-PAI---DEVVKR---------FCEVACLPGP-----AGIPLNIILNDSLTYVYCSFQA   101

125  VEQQVTHHGLDISGGAECIYQHQQSSFPVGAYGITIPPYNQDISQAAMRSFMQTPIQT--   182
     + +             +  Y         G    T+PPY + I++  MRS+     Q
102  MRKY----------AHKRFYD---GVSDEGVVISTVPPYAEGITKETMRSWHNNVCQNTS   148

183  ------NHWIMFIPTTGFSLKSQLIFSHPNSGFKEVFNHYGLATPHLSPGTYLPY--DIV   234
           + +I F+PT+         FSH   G              L+P    P+  +I+
149  NETHDLDAYIAFLPTS----LQNPSFSHMKIG----------CDSFLAPSRVDPFCVEII   194

235  AIGDTL  240
     A+G  L
195  AVGKAL  200
```

Figure 8. Les protéins homologues de protéines Plast chez *Laccaria bicolor*.
A. Alignement schématique de 5 protéines Plast (Plast-like) chez *Laccaria bicolor* avec une de protéines Plast d'*Agrobacterium* AAD30487 (protéine 5 d' *A. tumefaciens*, souche C58). La flèche à deux têtes: la partie commune de protéines Plast. B. Alignment de protéine XP_001884962.1 de *Laccaria bicolor* (haut) avec la protéine 5 (bas). En gras: résidus conservés dans la famille Plast d'Agrobacterium.