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Rol genes and root initiation and development

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Abstract

Due to their extensive growth potential, transgenic root systems arising from inoculation with *Agrobacterium rhizogenes* became popular in the last decade as model systems in domains as diverse as production of secondary metabolites, interactions with pathogens and symbionts, examination of gene importance in control of root development or in regulation of gene expression in roots. Wild-type bacterial strains have also been considered as useful tools to stimulate rooting on recalcitrant cuttings or microcuttings as they cause abundant root initiation at the site of inoculation.

Root initiation and the in vitro growth characteristics of transformed roots result from the transfer of genes located on the root-inducing plasmid (Ri) to plant cells and their expression therein. Two sets of pRi genes are involved in tlhe root induction process: the *rol* (root *loci*) genes located in the TL region and the *aux* genes of the TR region. Some of these genes being able to interact, the system appears also as a new tool to study the role of auxin in the process of root initiation. The distinctive phenotype of the transformed roots which are capable of hormone autonomous growth seems to be controlled mainly by the *rol* genes. These *rol* genes, i.e. the genetic *loci rol A, rol B, rol C* and *rol D* correspond to open reading frames ORFs 10, 11, 12 and 15. In vitro experiments determined the functions of the Rol B and Rol C proteins but the functions of Rol A and Rol D are still unknown. Altered metabolism of developmental regulators or modified sensitivity to auxin have been suspected to mediate root induction and morphological abnormalities of transformed roots and plants.

The target cells for transformation and the cells which are competent for root initiation will be characterized as well as the subsequent development of transgenic roots provided with various constructs from the whole T-DNA to single *rol* genes. Results dealing with auxin contents in relation with root growth kinetics, phenotype and structure, will also be presented and discussed with the potential use of the *rol* genes to control root biomass.

Introduction

Phylogenetically, roots are recent organs the appearance of which was correlated with landing, vascular tissue differentiation and new metabolic pathways leading to phenolics and lignins. Rhizogenesis is now a developmental process interesting for both traditional and biotechnological strategies of plant production in agronomy, horticulture aad forestry. However, for many species of commercial importance, this process lacks of efficient root-inducing factors and/or fundamental knowledge on what determines the rooting potential and the subsequent root morphogenesis. In other sectors such as biotechnological production of secondary metabolites, it appeared during the last past years that cell suspensions were frequently too variable to insure a stable production and could be usefully substituted by axenic cultures of isolated roots that are apparently characterized by a high biosynthetic capacity and a good genetic and biochemical stability (Nautila et al., 1994). Also rhizosphere studies about interactions of roots with pathogens and symbionts are looking for model systems including root clones provided with efficient growth potential (Mug-

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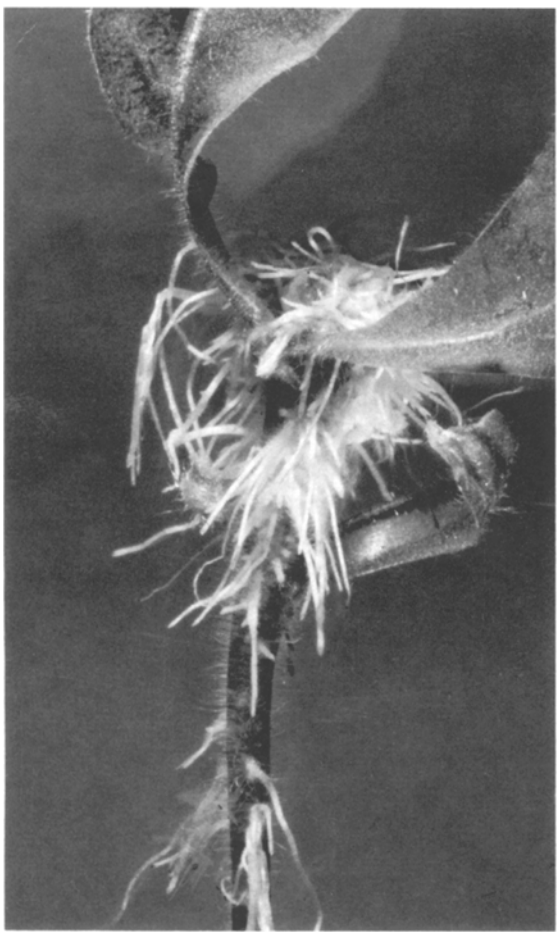

Figure 1. Hairy root disease induced on decapitated tobacco vitroplantlet by inoculating the wounded surface with *Agrobacterium rhizogenes*, strain A4. Abundant root production both at the decapitated surface (transformed roots) and along the stem (untransformed roots).

nier and Mosse, 1987). In this context, transgenic root systems arising from inoculation with *Agrobacterium rhizogenes* became popular in the last decade and the wild strains of this bacterium were also considered as useful to manipulate the rooting potential of recalcitrant cuttings or microcuttings as they cause abundant root initiation at the site of inoculation.

Agrobacterium rhizogenes, the Ri plasmid and the *rol* genes

Agrobacterium rhizogenes, a Gram-negative soil-born bacterium belonging to the Rhizobiacea family, is the etiological agent of the hairy root disease. It was studied mainly as a pathogen since its discovery (Riker et al., 1930) until a recent period. The disease is characterized by an abundant root formation at the infected sites (Figure 1) for a wide host range (De Cleene and De Ley, 1981; Tepfer, 1989) mainly restricted to dicotyledonous plants. Experimentally, hairy root induction was also reported for some Gymnosperms (McAfee et al., 1993; Magnussen et al., 1994). It is also possible that some species were reported as non susceptible for the only reason that inoculations were performed at inadequate developmental stages, during unfavourable seasons or on non-competent tissues (Chriqui et al., 1988, 1991). As for the crown gall induced by *A. tumefaciens*, the hairy root disease is neoplastic and results from a natural gene transfer from a plasmid, the root-inducing plasmid (pRi), to the host nuclear genome (Chilton et al., 1982), and the molecular basis of the phenomenon is very similar to that of crown gall. The pRi carries a transferable region, the T-DNA, that becomes integrated into the plant DNA during the course of infection (Tempé and Casse-Delbart, 1989). The integration and the expression of the transferred genes induce many changes in growth and developmental pathways in the transformed cells, in the subsequent transformed roots and in the transgenic plants that can be regenerated from these roots and their progeny (Tepfer, 1984).

The expression "hairy root phenotype" has been used to define the morphology of the clones established from transformed roots that are frequently able of extensive growth on hormone-free medium (Tepfer and Temp, 1981). Also the modified phenotype of transgenic plants harbouring the Ri T-DNA and provided with wrinkled leaves and shortened internodes was referred to as "hairy root phenotype". So this label covers quite different sitations and might be used circumspectly. For agropine-type strains in which the Ri T-DNA is present as two sub-fragments, it was demonstrated that most of the phenotype modifications were due to the expression of genes from the leftward subfragment (TL-DNA) (Cardarelli et al., 1987; Durand-Tardif et al., 1985; Ooms et al., 1986; Schmülling et al., 1988; Vilaine et al., 1987). It was also found that the various TL-DNA genes interplay to confer specific traits to the hairy roots (Capone et al., 1989; Schmülling et al., 1988) and that the TR-DNA auxin synthetic genes *aux1* and *aux2* (Huffmann et al., 1984) play a rather accessory role, being useful merely when endogenous plant auxin is insufficient to trigger differentiation of cells made competent to respond to the hormone by the expression of the TL-DNA genes (Car-

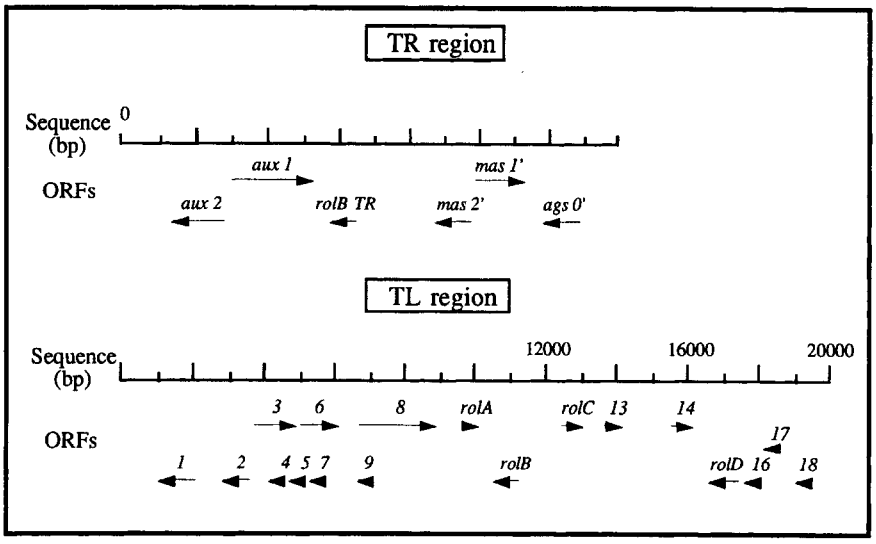

Figure 2. Functional organization of the T-DNA of the agropine-type strains of *Agrobacterium rhizogenes* (from Huffman et al., 1984; Jouanin, 1984; White et al., 1985; Slightom et al., 1986; Bouchez and Camilleri, 1990).

darelli et al., 1987). As most of the common traits of the phenomenon were observed when only the open-reading frames ORF10, 11 and 12 of the TL-DNA (Figure 2) were transferred and expressed together, these ORFs were qualified as root *loci*, respectively *rol A, B* and *C* (White et al., 1985) and special attention was directed to their key role. These genes are capable of triggering, to different extents, root differentiation and morphogenesis. They represent transferrable "root-forming" genes susceptible to have had an important impact during evolution (Barlow, 1994; Harper et al., 1991) although this is very speculative. They represent also morphogenetic genes that can be used as tools to study morphogenesis.

The reported effects of single *rol* genes on root induction and root development are summarized on Table 1. Their functions are still undetermined or discussed. The Rol A protein has a highly basic isoelectric point, so it was suggested that it could interact with positively charged macromolecules as a regulatory protein (Levesque et al., 1988). It was also found that the vegetative shoot apices of transgenic plants harbouring the *rol A* gene were characterized by reduced auxin contents (Prinsen et al., 1994) and a 40-60% reduction in immunoreactive gibberellin A1 was found in leaves of *rolA* and *35S-rol A* transgenic clones (Dehio et al., 1993). In addition, the rol A protein was found to be located in the plasma membrane of transgenic tobacco lines harbouring a *rol A-gus* gene fusion under the control of the *rolA* promoter (Rembur et al., submitted). The Rol B peptide is a cytosolic enzyme able to hydrolyse in vitro indoxyl-glucoside and other glucosides (cytokinin-O-glucosides, indoxyl-galactoside) (Estruch et al., 1991a; Spena et al., 1992). On the other hand, it was shown that *rol B* expression enhances the sensitivity to auxin of transformed cells (Filippini et al., 1994; Maurel et al., 1991). Recently, it was reported that the product of the *rolB* gene has a tyrosine phosphatase activity and that it is localized in the plasma membrane of transformed cells (Filippini et al., 1996). This suggested that a kinase/phosphatase cascade could be involved in the signal transduction of auxin. The Rol C peptide is a cytosolic enzyme able to hydrolyse in vitro cytokinin-O-glucosides (Estruch et al., 1991b). However, these functions failed to be confirmed in vivo. It is clear that each of these genes provokes specific effects and that these effects could vary according to the host genotypes and experimental conditions. In situ localization of *rol* gene expression in transgenic tobaccos indicate that the *rol A* promoter is constitutive (Guivarc'h et al., 1996a) while the *rol B* promoter is mainly expressed in the shoot and root apical meristems (Altamura et al., 1991; Maurel et al., 1990; Schmülling et al., 1989). The *rol C* promoter is expressed in the phloem-companion cells in all the organs and in the initials of root protophloem (Guivarc'h et al., 1996b).

Table 1. Effects of single *rol* genes on root induction and root develoomment

	rol A	*rolB*	*rol C*
Gene function	Not determined	Indoxyl-β-glucosidase (1,2)	Cytokinin-β-glucosidase (2,3)
Effects on root induction	- Carrot discs [4] + Tobacco Xanthi [5] ++Tobacco SR1 [6] - Kalanchoe [6]	- Carrot discs [4] - Tobacco Xanthi [5] +++Tobacco SR1 [6] - Kalanchoe [6]	- Carrot discs [4] ++ Apple rootstock [7] - Tobacco SR1 [6] - Kalanchoe [6]
Rooting potental of transgenic organs	++Tomato [8] ++Juvenile Tobacco - Mature Xanthi [5] ++Tobacco SR1 [6]	++ Tomato [8] +++Tobacco SR1 [9]	+++Tomato [9]
Development of roots - excised	- Light Tobacco + Dark Xanthi [5]	+ Tobacco SR1 [9] + Carrot discs [4]	++ Tobacco SR1 under darkness [9]
- attached to normal or transgenic plants	- Tobacco Xanthi [11]	No effect [12]	++ Apple rootstock [7] +++Tobacco SR1. [13]

Sources: [1] Estruch et al. (1991a); [2] Spena et al. (1992); [3] Estruch et al. (1991b); [4] Capone et al. (1989); [5] Vilaine et al. (1987); [6] Spena et al. (1987); [7] Lambert and Tepfer (1991); [8] Van Altvorst et al. (1992); [9] Schmülling et al (1988); [10] Grelon (1991); [11] Guivarc'h et al. (1996a); [12] Nilsson et al. (1993); [13] Guivarc'h (personal observation).

Early steps of transformed root initiation

It is now accepted that the plant response to *A. rhizogenes* depend upon various parameters including host genotype and bacterial strains, some strains acting with respect to polarity. On carrot root discs, mannopine-type strains induced transformed roots only at the apical surface while agropine-type strains may act indistinctly on both apical and basal sides (Ryder et al., 1985). This polarity suggested the influence of endogenous compounds that move basipetally. Kinetic studies of free endogenous IAA levels on both sides after wounding indicated a rapid IAA accumulation at the apical side resulting from both migration from the basal side and IAA neosynthesis at the wounded surface (Guivarc'h et al., 1993). The developmental stage is also a limiting factor to get transformed roots. For example, only juvenile seedlings of both *Eucalyptus gunnii* and *E. globulus* are able to respond to inoculations by a rooting response and mature plants never display any symptom (Chriqui et al., 1991) but whether the absence of root initiation results from a lack of transformation or from a lack of cells competent for rhizogenesis was not determined. Consequently, a disarmed *Agrobacterium* strain provided with the *gus* reporter gene modified by an intron in the coding region to prevent its expression in bacteria (Vancanneyt et al., 1990) and controlled by the 35S CaMV promoter was used to localize the target cells for transformation. On carrot root discs, only the cells of the intrafascicular cambium and the immature phloem strands displayed a β-glucuronidase activity, the first GUS positive cells being detected 48 h following inoculation. This suggested a relationship between transformation efficiency and the dividing capacity of the host cells. This was evidenced by pretreating the carrot discs by 25 μM acetosyringone; this treatment both advanced the reentry of potentially dividing cells into the S phase of the cell cycle and increased the number of transformed cells in the cambial ring (Guivarc'h et al., 1993).

Usually, the transformed roots are not of direct origin and arise from a callus previously differentiated at the inoculated region. Wounding and inoculation are rapidly followed by cell proliferation, then cambial-like layers redifferentiate inside the neoformed callus and root meristems organize from these layers (Bercetche et al., 1987; Grima-Pettenati et al., 1989). Using a co-integrated strain harbouring a wild pRi and the *gus* reporter gene controlled by a 35S CaMV promoter with a double enhancer (Robaglia et al., 1987)

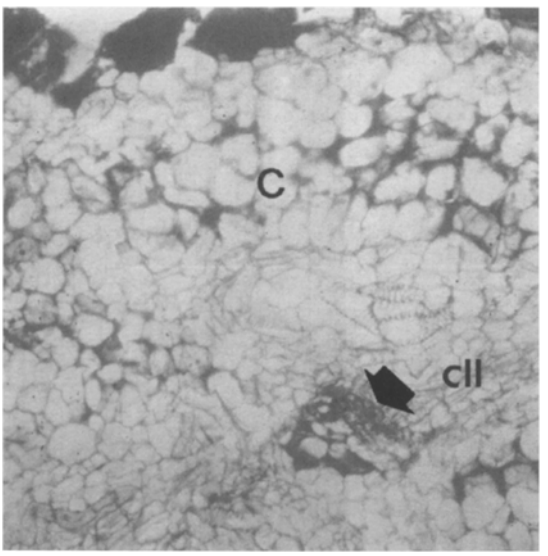

Figure 3. Early step of transformed root initiation from cambial-like layers in a callus resulting from inoculation with the A4 M70 GUS strain. The arrow indicates the GUS-positive cells. C: callus; cll: cambial-like layers. *Bar*: 50 μm.

inside the TL-DNA, *gus* expression was found only in a few number of callus cells, either isolated or clustered, but belonging to the cambial-like layers (Figure 3). In this way the clonal origin of hairy root lines predicted by Chilton et al. (1982) and David et al. (1984) after genetic analysis was visualized.

Development of hairy root lines

Although the hairy roots are usually reported as provided with the capacity of extensive and hormone-autonomous growth and lateral branching, it must be noted that not all the root lines described in the literature display such characteristics and that detailed mapping of the T-DNA was not included in most reports. Following inoculation with wild strains, it is clear now that spontaneous deletions of Ri T-DNA in hairy roots and their subsequent regenerants occur frequently (Amselem and Tepfer, 1992; Hänisch ten Cate et al., 1990).

Inoculation of carrot discs with the strain A4 M70 GUS harbouring the wild T-DNA supplemented with the *gus* gene inside the left subfragment leads to roots that display a diversity of phenotypes and behaviours when excised and grown in vitro on hormone-free MS medium (Guivarc'h et al., submitted). Apices of roots arising from inoculated discs were first subcultured on liquid or solid media containing antibiotics to define the optimal conditions for root development. 30 root clones were established on solid MS medium added with 500 mg L^{-1} cefotaxime and subcultured every two weeks. After 20 subcultures, only 8 clones were still able to develop while the others progressively lost their growth capacity and died. These clones varied in their phenotypes with more or less lateral branching and growth ability. They were characterized using the opine test to reveal the expression of the opine synthetic genes (*ops*) located on the TR-DNA and the GUS assay to check the expression of the *gus* gene as a marker of the TL-DNA. Also tests for response to naphthalene acetamide (NAM) were carried out in order to reveal the presence of an active *aux2* gene product. The *aux2* gene, located on the TR-DNA, is able to convert NAM into active auxin (Depicker et al., 1988), naphthalene acetic acid, leading to root growth inhibition. The study was completed by Southern blot analysis using a probe corresponding to the *gus* gene. In this way, the root clones were characterized for their TL and/or TR content. Only clones provided with TL or TL + TR survived and displayed a good potential for longitudinal growth with more or less ramifications, leading to the idea that genes of the TL-DNA are essential for long-term growth. In addition, some clones provided with many copies of the *gus* gene were GUS negative raising the question of a possible methylation of the gene (DeVries-Uijtwaal et al., 1989; Ottaviani et al., 1993). This was not observed for the native genes *ops* and *aux2* of the TR-DNA (Guivarc'h et al., submitted). These results revealed that large variations in morphology, molecular constitution and physiology characterized the carrot hairy root lines as it was also the case for potato (DeVries-Uijtwaal et al., 1988) but no strict relation between phenotypes and transformation events was found. This was not the case for pea and *Cucumis* root clones.

In pea root clones established following inoculation with strains harbouring either the wild T-DNA, or only the TL- or the TR-subfragment, the TL roots were long and thin, the TR roots were short and ramified and the TL + TR roots were intermediate. Interestingly, high endogenous IAA and IAM levels characterized roots bearing only the TL-DNA (Prinsen et al., 1992). This perhaps reflected the functionality of the earliest observed homology of the TL *orf8* with a tryptophane monoxygenase gene (Levesque et al., 1988).

Culture of transformed roots resulting from the inoculation of cucumber stem explants with a wild-

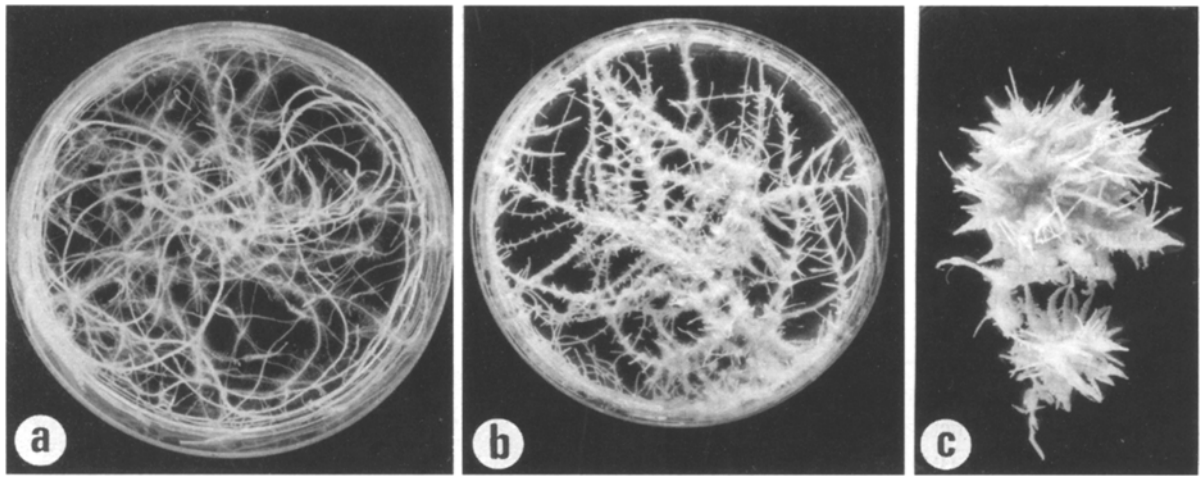

Figure 4. Phenotypes of cucumber root lines established following inoculation of stem fragments with the wild *A. rhizogenes* strain A4. **a**: roots provided only by the TL-DNA, **b**: roots containing both the TL- and the TR-DNA; **c**: roots harbouring the TR-DNA.

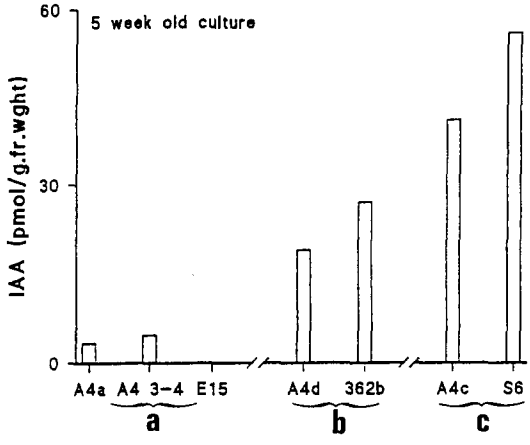

Figure 5. Endogenous auxin levels in 5 week-old culture root lines displaying the phenotypes a, b or c described on Figure 4. A4a: TL-DNA; $A4_{3-4}$: TL-DNA and TR-DNA partly deleted of *aux2*; E15: part of TL-DNA covering the *rolA+B+C* genes; A4D, 362B and A4C: TL- and TR-DNA; S6: part of TR-DNA covering the *aux1* and *aux2* genes.

type agropine *A. rhizogenes* strain A4 were established and several classes of phenotypes were described and characterized at the molecular level (Amselem and Tepfer, 1992). A physiological study of these root lines revealed that roots provided only by genes from the TL-DNA or by the set of *rol A + B + C* genes were long, poorly ramified and with a reduced growth potential in long-term cultures (Figure 4a); this was accompanied by low levels of endogenous auxin (Figure 5). Roots provided with both TL and TR were either intermediate (long and ramified) (Figure 4b) or short and very ramified (Figure 4c). The extent of reduced growth and lateral branching was closely related to the presence of the TR-subfragment with functional *aux* genes and to high auxin levels (Figure 5, Dewitte et al., submitted). Roots provided with high auxin levels displayed many structural modifications, including large central cylinder, triarchy instead of diarchy and exfoliation of peripheral tissues. It seemed that roots with the intermediate phenotypes appeared to be more adaptated for a good growth potential in long-term culture.

As it was reported that transformed roots induced by *A. rhizogenes* on whole plants or on various explants seems to be modified in their gravitropic behaviour (Spano et al., 1987; White et al.,1985), root gravitropism was studied on normal and transgenic rapeseed seedlings harbouring the whole T-DNA of pRi A4 and it was found that rapeseed hairy roots were less sensitive to gravity than normal roots (Legué et al., 1994). This was probably related with the difficulty of amyloplast sedimentation in the statocysts as shown at ultrastructural level in pea hairy roots (Bercetche, 1987).

Conclusions

Important progress has been made in the last few years concerning the effects of the *rol* genes from *A. rhizogenes* on plants. A number of factors involved in host susceptibility and transformed root initiation have been determined and it is clear now that not only the

host genotype but also the developmental stages and the cell types have to be considered when transformed roots are expected. Root formation appears secondarily following inoculations with *A. rhizogenes*, the first observed effects being transformation and cell proliferation. Later, transformed root primordia organized from cells redifferentiated inside the callus. These cells correspond to prerhizogenetic cells previously identified in normal aerial tissues (frequently cells from the vascular strands) submitted to rooting treatments.

The growth potential of hairy root lines is influenced by the host genotype as root clones from woody species frequently failed to display the high growth rates observed in many herbaceous species. In addition, transformation events (transferred sequences, copy number...) strongly influence the patterns of growth and development of the various hairy root lines that can be established following inoculation with wild strains and the long-term stability of gene expression following subcultures. It could not be excluded that conditions of culture could also interfere with the expression of transferred genes but little is known about the factors that controlled the expression of their promoters. The mechanism by which the *rol* genes control root initiation and differentiation is not yet fully understood. Much evidence at this time indicates that expression of each *rol* gene leads to a succession of events including modifications in hormone balances and sensitivity but the primary effects are still unknown. Open questions are also raised concerning the possible interrelations between *rol* gene expression and the normal metabolic pathways of the roots. Up to now, optimization of root biomass through genetic engineering was mainly achieved with wild-type strains of *A. rhizogenes*. Further investigations on the function and regulation of the single *rol* genes and other genes born on the Ri T-DNA would allow a rational use of hairy roots for both secondary metabolite production and recalcitrant cuttings.

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