The plant oncogene *rolC* is responsible for the release of cytokinins from glucoside conjugates

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The *rolC* gene of *Agrobacterium rhizogenes*, which drastically affects growth and development of transgenic plants, codes for a cytokinin- β -glucosidase. Indeed, *rolC* protein expressed in *Escherichia coli* as a fusion protein hydrolyses cytokinin glucosides, thus liberating free cytokinins. Furthermore, β -glucosidase activity present in *E.coli* extracts expressing the *rolC* protein was inhibited by affinity-purified antibodies specific for the *rolC* protein. Finally, *rolC* proteins expressed in transgenic plants were shown to be responsible for cytokinin- β -glucosidase activity. Morphological and phytohormonal analysis, performed on transgenic plants that are somatic mosaics for the expression of the *rolC* gene, extend and confirm our interpretation that the developmental, physiological and morphological alterations caused by *rolC* expression in transgenic plants are primarily due to a modification of the cytokinin balance. These observations shed new light on the control of growth and differentiation in plants by growth factors. Key words: *Agrobacterium rhizogenes*/cytokinin-*N*-glucosides/cytokinin- β -glucosidase/plant oncogenes/*rolC* gene

Introduction

Plants are fascinating systems for the study of developmental processes in eukaryotes. Nevertheless, much of our knowledge on plant development is descriptive rather than causal. Thus, although it is known that relatively simple molecules, such as auxins or cytokinins, play a crucial role in the elicitation of 'totipotency' in several types of plant cell and in the regulation of plant physiological processes, the molecular mechanisms underlying these cellular processes are still largely unknown.

The study of the structure and function of genes that control plant growth and differentiation is therefore of great interest. Plant pathogenic microbes are a remarkably rich source of such genes (for reviews see Agrios, 1988; Morris, 1988). Thus, genes involved in auxin synthesis have been found in *Agrobacterium tumefaciens* (Inze *et al.*, 1984; Schroeder *et al.*, 1984; Tomashow *et al.*, 1984) and in *Pseudomonas savastanoi* (Yamada *et al.*, 1985). Similarly, genes coding for enzymes involved in cytokinin synthesis have been isolated from *A.tumefaciens* and *P.savastanoi* (for review see Morris, 1988). Whilst the aforementioned genes affect morphology and development through the synthesis of phytohormones, another pathogen-derived gene, the *iaaL*

gene from *P.savastanoi* (Glass and Kosuge, 1986; Roberto *et al.*, 1990) alters physiological and morphological processes in plants (Spena and Schulze, 1990; Romano *et al.*, 1991; Spena *et al.*, 1991) through its capacity to conjugate the auxin IAA with amino acids such as lysine and ornithine.

Confident that the study of other genes derived from plant pathogens could reveal yet other ways by which phytohormone activity can be modulated, we have studied the *rol* genes of *Agrobacterium rhizogenes*. *A.rhizogenes* is the etiological agent of the hairy-root disease (Riker *et al.*, 1930). The first event in the pathogenesis of the hairy-root disease is the transfer of a DNA fragment (T-DNA) from an extrachromosomal plasmid (Ri) to the plant genome (for review see Ream, 1989). Expression of oncogenes located on the T-DNA of Ri plasmids is responsible for the formation of roots by transformed tissues (Chilton *et al.*, 1982). Several loci on the T-DNA of Ri plasmids were shown to be essential for root induction (so-called *rol* genes for root loci) on *Kalanchoe daigraemontiana* (White *et al.*, 1985). However, the expression of individual *rol* genes (i.e. the *rolA*, *B* and *C* genes) in transgenic plants not only affects rhizogenesis (Cardarelli *et al.*, 1987; Spena *et al.*, 1987; Vilaine and Casse-Delbart, 1987), but also causes major alterations in morphology, development and physiology of the entire plant (Sinkar *et al.*, 1987; Oono *et al.*, 1987; Schmülling *et al.*, 1988). The changes displayed by tobacco plants regenerated from hairy-roots (Ackermann, 1977; Tepfer, 1984) can be reproduced by the combined expression of only three of the *rol* genes (*A*, *B* and *C*) (Jouanin *et al.*, 1987; Spena *et al.*, 1987). Individually, the expression of the *rolC* gene, either under the control of its own promoter (Oono *et al.*, 1987; Schmülling *et al.*, 1988) or under the control of the 35S promoter from cauliflower mosaic virus (Schmülling *et al.*, 1988; Fladung, 1990) causes dwarfing in transgenic tobacco and potato plants. Transgenic plants expressing *rolC* under the control of the 35S promoter of cauliflower mosaic virus also exhibit the following abnormal phenotypes: (i) thin and lanceolate leaves, (ii) reduced leaf chlorophyll content, (iii) male sterility and (iv) increased root growth *in vitro* (Schmülling *et al.*, 1988). We have previously shown that the influence of the *rolC* gene action is cell autonomous (Spena *et al.*, 1989) and that its product is located in the cytosol (Estruch *et al.*, 1991). The cell-autonomous activity of the *rolC* gene seemed to indicate that it was unlikely to be involved in the synthesis of phytohormones, and would more likely play a role in phytohormone signal transduction. A cell specific and reversible release of active forms of plant growth regulators from inactive conjugates (Letham and Palni, 1983; Reinecke and Bandurski, 1988), which would not lead to significant transport of the growth factors away from their site of activation, was a relatively simple and therefore attractive hypothesis. Here we argue that this idea is supported by the fact that the *rolC* gene product can hydrolyse cytokinin-glucosides, releasing active forms of cytokinin.

Results

Experimental strategy

Measurements of hormone levels in crown gall tissue have been notoriously inconclusive for the identification of functions of T-DNA encoded oncogenes (for a brief and critical discussion on this topic see Serrano *et al.*, 1990). On the assumption that the developmental and physiological alterations observed in *rolC* transgenic plants (Oono *et al.*, 1987; Schmülling *et al.*, 1988) might be due to cell-autonomous alterations in phytohormone activity (Spena, 1990), we decided to study the possible biological function of *rolC* *in vitro* using proteins produced in *Escherichia coli*. Transgenic plants, mosaic for the somatic expression of the *rolC* gene (Spena *et al.*, 1989), were used to provide further evidence *in vivo*.

Expression of the *rolC* gene from *A.rhizogenes* in *E.coli*

The *rolC* gene codes for a putative peptide of 20 100 Daltons (Slightom *et al.*, 1985). A vector of the pDS family, pDS56/RBSII-2 (Stüber *et al.*, 1990), was used to achieve *rolC* expression in *E.coli*. In this system, *rolC* production is inducible and a fusion protein is produced (Figure 1). The production of the *rolC* protein in *E.coli*, induced by IPTG, is shown in Figure 2A. The protein was specifically visualized (Figure 2B) by anti-*rolC* affinity-purified antibodies (Estruch *et al.*, 1991).

The *rolC* protein is a cytokinin- β -glucosidase able to release free cytokinins from their *N*-glucosides

Plant cells contain several types of cytokinin conjugates (Letham and Palni, 1983). While O-glycosylation of cytokinins is known to result from a reversible reaction, N-glycosylation is not freely reversible in plants (Letham and Palni, 1983). Therefore, an enzyme that hydrolyses *N*-glucosyl linkages to release free cytokinins should have a profound effect on the pool of active cytokinins.

Two cytokinin-*N*-glucosides, 6-benzylaminopurine-9-(β -D-glucoside) (BAP-9-glucoside) (synthetic) and zeatin-9-glucoside (natural), were used in an assay for hydrolysis of cytokinin-*N*-glucosides. Figure 3 shows that *E.coli* extracts

that contain the *rolC* fusion protein are able to release free cytokinins from their *N*-glucosides. BAP-9-glucoside (lanes 5 and 7) and zeatin-9-glucoside (lanes 6 and 8) were converted to BAP and zeatin respectively. Indeed the compounds released by the action of the *rolC* protein on BAP- and zeatin-glucosides comigrate, under two different conditions, with BAP and zeatin respectively. Addition of vascular sap to the reaction assays resulted in the liberation of higher amounts of free cytokinins from their *N*-glucosides (Figure 3, compare lane 5 with lane 7, and lane 6 with lane 8). These results indicate that the *rolC* dependent reaction is limited, in our assay, by a still unidentified factor present in the vascular sap of tobacco plants. Considering the many possible acceptors of the glucose residue (see Walsh, 1979) that are known to be present in plant vascular sap, it is not surprising that their presence might enhance *rolC* enzymatic activity. No free cytokinin was liberated in negative controls (sonicated extracts from bacteria not induced with IPTG; lanes 1–4).

Biological assays, measuring cytokinin activity by the induction of fruit set in pea (Garcia-Martinez and Carbonell, 1980), have confirmed that samples used for lanes 5 and 6 of Figure 3 contained a cytokinin activity that was not detected in samples used for lanes 1 and 2, thus confirming our conclusion that the *rolC* protein releases active cytokinins from inactive cytokinin conjugates.

The glucosidase activity of the *rolC* fusion protein is inhibited by anti-*rolC* affinity-purified antibody

The covalent bond between the glucose residue and the cytokinin in the 7- and 9-cytokinin glucosides is β -type. Phenolic β -glucosides, which release coloured products in the presence of β -glucosidase activity, were used as substrate to confirm the enzymatic activity of the *rolC* fusion protein (Table I). Furthermore, immunoprecipitation of the *rolC* protein, present in sonicated extracts of *E.coli*, resulted in a parallel decrease of the β -glucosidase activity of those extracts (Figure 4A and B).

cytokinin- β -glucosidase activity of *rolC* protein produced in transgenic tobacco leaves

To prove that expression of the *rolC* gene from Ri plasmid A4 confers cytokinin- β -glucosidase activity on transgenic plants, we have immunopurified the *rolC* protein expressed in 35S-*rolC* transgenic plants (Schmülling *et al.*, 1988). Protein extracts of young leaves from SR1 wild-type and 35S-*rolC* transgenic plants were treated with anti-*rolC* affinity-purified antibody (Estruch *et al.*, 1991). *RolC* protein, eluted from antibodies covalently linked to protein A-Sepharose, released cytokinin aglycones from their glucosides (Figure 5). As previously shown for the *E.coli* extract, addition of vascular sap provided a factor that facilitated the reaction.

In transgenic plants, the *rolC* gene acts cell autonomously and increases IPA content

Transgenic plants carrying a *rolC* gene functionally inactivated by the presence of the transposable element *Ac* are mosaic for somatic expression of *rolC* (see Baker *et al.*, 1987 for this type of assay). *Ac* was inserted between the 35S promoter and the *rolC* coding sequence (Spena *et al.*, 1989). Transgenic tobacco plants harbouring such an *Ac*-interrupted *rolC* gene have a variegated phenotype, seen, in leaves, as pale green sectors intermixed with dark green

Fig. 1. Schematic representation of plasmid pDS56-RBSII-2-*rolC*. The promoter/operator element N25OPS/N25OP29 is indicated by P, *t*₀ represents a terminator of phage lambda and *t*₁ is the terminator of the *rrnB* operon of *E.coli*. RBSII is a synthetic ribosomal binding site. The ATG initiation codon of *rolC* is underlined. For further details concerning the pDS vector family, see Stüber *et al.* (1990).

tissue (Spena *et al.*, 1989). The variegation is caused by clonal activation of *rolC* gene expression as a result of *Ac* excision and consequent reconstruction of a functional 35S-*rolC* chimeric gene (Spena *et al.*, 1989). We have used leaves from such somatic mosaics (see Spena *et al.*, 1989 and Figure 6) because, *rolC* active and inactive sectors, apart from *rolC* expression, are genetically, physiologically, environmentally and developmentally identical (Spena, 1990).

Comparisons from five individual plants indicated that pale green (expressing the *rolC* gene) differed from neighbouring dark green areas by a reduced total thickness (Figure 7).

Fig. 2. Expression of the *rolC* fusion protein in *E. coli*. (A) 15% PAGE of total proteins extracted from *E. coli* harbouring the *rolC* expression vector after Coomassie staining. Lane 1, uninduced; lane 2, IPTG-induced. (B) Western blot analysis of *E. coli* extracts obtained from IPTG-induced (lane 2) and uninduced (lane 3). Lane 1 contains mol. wt markers. The arrows point to the *rolC* fusion protein.

Fig. 3. Thin layer chromatogram of the products of the cytokinin-N-glucosidase assays. Lanes 1 and 2, extracts from uninduced *E. coli* incubated with BAP-9-glucoside and zeatin-9-glucoside, respectively. The vascular sap was diluted 1:100; lanes 3 and 4, uninduced extracts incubated with BAP-9-glucoside and zeatin-9-glucoside, respectively, in the presence of a 1:10 dilution of vascular sap from wild-type tobacco plants; lanes 5 and 6, *rolC*-containing extracts (IPTG induced) incubated with BAP- and zeatin-9-glucosides, respectively, in the presence of a 1:100 dilution of vascular sap; lanes 7 and 8, *rolC*-containing bacterial extracts incubated with BAP- and zeatin-9-glucoside, respectively, in the presence of a 1:100 dilution of vascular sap. Lane 9, BAP-9-glucoside; lane 10, zeatin-9-glucoside; lane 11, BAP; lane 12, zeatin. The compounds were visualized under UV light.

The sizes and shapes of the mesophyll cells were less regular in pale green sectors. Although not always statistically significant, the differences in cell height and width of the various cell types indicated that the cells in pale green sectors were smaller and flatter, especially in the epidermis. These *rolC* sectors also displayed a higher cell density and larger intercellular spaces in the spongy mesophyll. Reduction in the height of palisade cells led to a reduction of dorsiventral differences in morphology across the leaf blade (Figure 8a and b).

These observations indicated that several developmental parameters have been altered specifically in cells expressing the *rolC* gene. Interestingly, several of the alterations observed in *rolC* transgenic plants (e.g. stimulation of cell division, inhibition of cell elongation and plant dwarfing, alterations of chloroplast development and of chlorophyll content) (Schmülling *et al.*, 1988; this article) could result from higher cytokinin activity (Matthysse and Scott, 1984; Smigocki, 1991; for inhibition of chloroplast development by cytokinin treatment see Stabel *et al.*, 1991).

Consequently, we have measured the levels of immunoreactive isopentenyladenosine (IPA), *trans*-zeatin riboside and dihydrozeatin riboside type cytokinins and abscisic acid (ABA) in pale green (*rolC* expressing) and dark green (not expressing) sectors of leaves from plants mosaic for the

Table I. β -Glucosidase activity in sonicated extracts of *E. coli* harbouring either the pDS56-RBSII-2 vector alone or containing the *rolC* gene

pH	5.5		6.5		7.5	
IPTG	-	+	-	+	-	+
Vector	0.23	0.11	0.06	0.1	0.02	0.03
Vector- <i>rolC</i>	0.18	3.67	0.10	1.2	0.02	0.46

Sonicated extracts were obtained from bacteria either induced (+) or not induced (-) with IPTG. The data represent the mean of three independent experiments with three measurements each. Activity is expressed as $\mu\text{mol/h/mg}$.

Fig. 4. β -Glucosidase activity and Western blot analysis of residual *rolC* fusion peptides in sonicated bacterial extracts after immunoprecipitation with affinity-purified anti-*rolC* antibodies. (A) β -Glucosidase activity remaining in supernatants after immunoprecipitation with either preimmune serum (filled circle) or with affinity-purified anti-*rolC* antibodies (open circle). (B) Lane 1, purified *rolC* fusion peptide (purification was done by electroelution of the protein after PAGE); lanes 2-5, supernatant of *E. coli* extracts incubated with 5, 25, 50 and 250 μg of affinity-purified anti-*rolC* antibody respectively. Lane 6 contains the same mol. wt markers as in Figure 2B.

Fig. 5. Immunopurification of the *rolC* protein expressed in *35S-rolC* transgenic plants and demonstration of cytokinin-glucosidase activity. (A) The immunopurified *rolC* protein (arrow) is visualized, after Coomassie staining, as a band of an apparent mol. wt of 20 kDa on a 12% polyacrylamide gel. The mol. wt markers are the same as in Figure 2. (B) Thin layer chromatogram showing that the *rolC* protein expressed in transgenic plants releases free cytokinins from their *N*-glucosides. Immunopurified *rolC* protein (~140 ng) was incubated: with BAP-9-glucoside, without (lane 1), or with (lane 2) vascular sap; with zeatin-9-glucoside, without (lane 3) or with vascular sap (lane 4); lanes 5 and 6, BAP- and zeatin-9-glucosides, respectively; lane 7, BAP; lane 8, zeatin.

Fig. 6. Pale green sector in a leaf transgenic for the *35S-Ac-rolC* construct. Leaf tissue expressing the *rolC* gene forms pale green sectors in plants which are somatic mosaics for *rolC* expression. [For further details see Spena *et al.* (1989) and Estruch *et al.* (1991).] The variegated leaf is from the progeny of plant clone *35S-Ac-rolC-1* described in Spena *et al.* (1989).

somatic expression of the *rolC* gene. Table II shows the data obtained with progeny from two independent transgenic tobacco plants (*35S-Ac-rolC-1* and -7) (Spena *et al.*, 1989). While ABA and dihydrozeatin riboside measurements were identical in *rolC* expressing and non-expressing tissues, IPA content increased in tissue expressing *rolC*. In pale green areas from *35S-Ac-rolC-7* and -1 progeny plants, we found respectively a 5-fold and a 2-fold increase in IPA. The higher IPA content in *rolC* expressing tissue is consistent with the biochemical role of the *rolC* gene product: the release of free cytokinin from the inactive cytokinin-glucoside by the hydrolytic activity of a cytokinin- β -glucosidase.

Fig. 7. Differences in thickness and cell size ($\pm$ SE) of pale and dark green sectors of the five youngest leaves. Leaves from fully developed plants before flowering were analysed. The data were obtained by analysing 150 cells from cross-sections of five individual pale or dark green sectors. Altogether, the most striking observation is the general reduction of cell sizes, the non-palisadic appearance of the palisade parenchyma and the irregularity of cell sizes and shapes in the two epidermis (see also Figure 8).

Discussion

Phytohormones are involved in the regulation of growth and development in plants (Thimann, 1977). Their activity can, in principle, be regulated at several levels. One of them is the control of the relative concentrations of different phytohormones. Experimentally, phytohormone content can be influenced through *de novo* synthesis, as is the case for plants carrying the *ipt* or *iaaH* and *iaaM* genes of *A.tumefaciens* (for review see Morris, 1988), or through conjugation, as in the case of the *iaaL* gene from *P.savastanoi* (Kosuge *et al.*, 1983; Romano *et al.*, 1991; Spena *et al.*, 1991). In this article we have shown that the *rolC* gene of *A.rhizogenes* codes for a β -glucosidase able to release free forms of cytokinin from their glucosides.

Cytokinin-*N*-glucosides possess a unique chemical structure because their sugar moiety is linked to the cytokinin through a nitrogen atom of the purine ring (Letham and Palni, 1983). *N*-Glucosides of cytokinins are only weakly, if at all, active as phytohormones (Letham *et al.*, 1975), and exhibit a remarkable metabolic stability in tobacco cell cultures (Gawer *et al.*, 1977) and other experimental tissues (Fox *et al.*, 1973; Parker and Letham, 1973). While *O*-glucosides are readily hydrolysed in several tissues, *N*-glucosides are resistant to emulsin (β -glucosidase from almond) (Letham *et al.*, 1975). Consequently it has been proposed (Letham and Palni, 1983) that *N*-glucosides of cytokinins are only weakly active because they are metabolically stable and not converted into the highly active aglycone (Letham and Palni, 1983).

Expression of the *rolC* gene in plants strongly affects some developmental, physiological and morphological processes (Oono *et al.*, 1987; Schmülling *et al.*, 1988) in a way which

Fig. 8. Semi-thin cross-sections of (a) pale and (b) dark green sectors ($\times 190$). S, spongy mesophyll; P, palisade mesophyll; ue, adaxial epidermis; le, abaxial epidermis. Note the reduction in the height of palisade cells and other leaf cells in (a) compared with (b).

Table II. Endogenous levels of immunoreactive IPA, DHZR and ABA (pmol equiv./g fresh wt) in leaf blade tissues, expressing and not expressing the *rolC* gene, obtained from variegated leaves transgenic for the *35S-Ac-rolC* construct (Spena *et al.*, 1989)

	ABA	IPA	DHZR
Clone <i>35S-Ac-rolC</i> -1, <i>rolC</i> ⁺	111	956	6.9
Clone <i>35S-Ac-rolC</i> -1, <i>rolC</i> ⁻	124	412	7.1
Clone <i>35S-Ac-rolC</i> -7, <i>rolC</i> ⁺	220	659	2.2
Clone <i>35S-Ac-rolC</i> -7, <i>rolC</i> ⁻	242	135	2.3

ABA, 2-*cis*(+)-abscisic acid; IPA, isopentenyladenosine; DHZR, dihydrozeatin riboside. *Trans*-zeatin riboside (ZR) contents were below the detection limit.

is indicative of a phytohormonal imbalance. The cytological and cytokinin analysis performed in somatic mosaics supports the interpretation (Spena *et al.*, 1987, 1989; Schmülling *et al.*, 1988; Spena, 1990; Estruch *et al.*, 1991) that the morphogenetic consequences of the action of the *rolC* gene are mediated by changing the activity of the cytokinin pool in these tissues. Here, we have shown that the *rolC* oncogene from the T-DNA of *A. rhizogenes* Ri plasmid codes for a β -glucosidase, which is able to hydrolyse at least two different cytokinin *N*-glucosides. These compounds are normally metabolically stable in plants and are resistant to emulsin, a widespread plant β -glucosidase (Letham and Palni, 1983). Thus, the plant pathogen *A. rhizogenes* has either developed a unique strategy to influence plant growth and development by targeting cytokinin-glucosides as a

source of active cytokinins, or this mechanism of phyto-hormone regulation exists in plants and was somehow mimicked by *A. rhizogenes*.

Whatever its evolution, the *rolC* gene has allowed the experimental demonstration that the conversion of an inactive and metabolically stable cytokinin conjugate to its free and active form can play a major role in a cell-autonomous control of plant growth and differentiation. Similar, thus far unknown biochemical routes influencing plant growth and development through the hydrolysis of inactive hormone conjugates, may well exist in plants.

As the name phytohormone implies, it is generally considered that plant growth factors, such as auxins and cytokinins, are synthesized in some tissues and transported to their target sites. While this process undoubtedly plays an important role in plant developmental and physiological processes, our data suggest an additional mechanism controlling the activity of some phytohormones, i.e. the interconversion from inactive conjugates to free forms. Considering that most plant cells could synthesize hormones, regulated enzymatic systems leading to activation – inactivation could regulate the active pool of phytohormones. If this hypothesis turns out to be correct, the role(s) and cellular localization(s) of putative phytohormone receptors would have to be reconsidered. Moreover, this and other work performed with plants which are somatic mosaics for a cytokinin-synthesizing gene (J.J. Estruch, J. Schell and A. Spena, in preparation) indicate that cytokinins can act in a cell-autonomous way in tobacco leaves.

It is evident that variations in the cytokinin-*N*-glucoside content of a plant tissue should result in variations in the expressivity of the *rolC* traits. Thus, differences in the severity of *rolC* induced phenotypes observed in different plant species (Fladung, 1990), as well as the variations observed in the phenotypes of isogenic plants grown under different greenhouse conditions (our unpublished observations) could be due to differences in cytokinin-glucoside content. These possibilities will be tested in future experiments.

Several *rolC*-induced phenotypes, such as stimulation of cell division, inhibition of cell elongation and plant dwarfing have been described as typical cytokinin controlled properties (Matthysse and Scott, 1984; Smigocki, 1991; our unpublished results) and can be readily explained by the *rolC* mediated release of active cytokinins from their inactive glucosides. However, a number of paradoxes come to mind. It has been clearly established that cytokinins can severely inhibit root initiation and growth. However, root growth in *rolC* transgenic tobacco roots is stimulated (Schmülling *et al.*, 1988) and the *rolC* gene has been shown to play an essential role in the formation of hairy roots (White *et al.*, 1985; Spena *et al.*, 1987). Obviously, we shall have to refine our knowledge and some of the general concepts relating to the mechanism of action of cytokinins. Incidentally, other biochemical and physiological parameters found to be altered in *rolC* transformed plants, such as increased auxin sensitivity, alteration in polyamine content (Shen *et al.*, 1988; Barbier-Brygoo *et al.*, 1989; Maurel *et al.*, 1990; Michael *et al.*, 1991) must be secondary effects resulting from the intracellular release of active cytokinins. Last but not least, considering that the action of the *A. rhizogenes rolB* gene results in an increased auxin sensitivity (Maurel *et al.*, 1990) and that the morphogenetic properties of *rolB* transgenic

plants suggest an increased auxin activity (Cardarelli *et al.*, 1987; Schmülling *et al.*, 1988), we would not be too surprised if the *rolB* gene turns out to code for an enzyme able to liberate free auxin from its inactive glucoside conjugates.

Materials and methods

Plasmid construction

Construction of pDS56-RBSII-2/*rolC* has been described previously (Estruch *et al.*, 1991). The *rolC* gene, contained in the *HpaI*–*EcoRI* fragment spanning from base 12 459 to base 13 445 of the T_L-DNA of Ri plasmid A4 (Slightom *et al.*, 1986), was first subcloned into pUC19 and then into pDS56-RBSII-2 (Stüber *et al.*, 1990). Consequently, the *rolC* peptide is produced as a peptide fusion, in which the *rolC* ATG initiation codon is preceded by 10 amino acids. Three of them are provided by the pDS56-RBSII-2 vector, six by polylinker sequences and one by the three bases preceding the ATG initiation codon. The production of anti-*rolC* antibodies has been described previously (Estruch *et al.*, 1991). *E. coli* strain M15 bearing repressor plasmid pDM1,1 was used to produce the *rolC* peptide fusions (for details see Stüber *et al.*, 1990).

Preparation of sonicated extracts

E. coli cells containing the repressor plasmid and the plasmid bearing the *rolC* construct were induced at 28°C with 1 mM isopropyl- β -D-thiogalactoside (IPTG) (for details see Stüber *et al.*, 1990). After 4 h, cells were harvested, resuspended in 10 mM Tris, pH 7.8, 50 mM NaCl, 1 mM EDTA, 1 mM DTT and 30 μ g/ml of lysozyme. After 1 h on ice, the suspension was sonicated until it was clear. After spinning at 16 000 g for 30 min the supernatant was aliquoted and frozen. Protein concentration was determined using a Pierce kit.

Protein and immunological techniques

Polyacrylamide gel electrophoresis, Western blots and immunodetection of *rolC* fusion protein were done by standard techniques (Harlow and Lane, 1988). Anti-*rolC* antibodies were covalently linked to protein A–Sepharose as described by Schneider *et al.* (1982). After immunoprecipitation and washing, *rolC* fusion protein was eluted as described by Schneider *et al.* (1982).

Enzymatic assays

β -Glucosidase activity was measured in 50 mM citrate buffer (pH 5.5), 50 mM MES–OH buffer (pH 6.5) and 50 mM Tris–HCl buffer, pH 7.5. The total reaction volume was 150 μ l. The reaction was initiated by the addition of the sonicated extracts using as substrate *p*-nitrophenyl- β -D-glucopyranoside (2 mM final concentration) (as substrate) and hydrolysis proceeded for 1 h at 25°C. The reaction was stopped by adding 350 μ l of 2 M sodium carbonate. The *p*-nitrophenol liberated was measured spectrophotometrically at 405 nm using an extinction coefficient of 18.6×10^6 cm²/mol (Walker and Axelrod, 1978).

Cytokinin-glucosidase activity was tested at pH 6.5 using as substrates BAP-9-glucoside or zeatin-9-glucoside (Sigma). Sap from the vascular system was described as described (Estruch, 1990). Tobacco midveins were frozen in liquid nitrogen. Before thawing, the vein sections were packed vertically into 2 ml syringes resting on porous polyethylene discs placed inside Eppendorf tubes. These vials were put in 15 ml glass centrifuge tubes and centrifuged at 4°C for 3 min at 200 g.

TLC was done on HPTLC pre-coated plates (Silica Gel 60 F254; Merck) which were run either in butanol:acetic acid:water (12:3:5) (Figures 3 and 5) or in ethanol:ammonia:water (20:1:4) (not shown).

Extraction and measurements of phytohormones

Extraction from tobacco leaf tissue was performed substantially as described (Grossman *et al.*, 1987). Further purification and separation of ABA and cytokinins in aqueous extract were done by HPLC, modifying the method according to Häuser *et al.* (1990). 0.8 ml of the cleared sample was injected onto a reversed phase Nucleosyl 120-5 μ m C₁₈ column (Mackerey-Nagel). The column had been equilibrated with 100 mM acetic acid. The mobile phase consisted of a linear gradient from 100 mM acetic acid to 100% methanol (Neill and Horgan, 1987). Gradient sweep time was at 30 min at a flow rate of 3 ml/min. The fractions containing ABA (26.4 min), IPA (25.8 min), DHZR (19.6 min) and ZR (19.4 min) were collected, evaporated to dryness and dissolved in 50 mM TBS, pH 7.8, 10% methanol for enzyme immunoassay using monoclonal antibodies for ABA (Mertens *et al.*, 1983), IPA (Weiler and Spanier, 1981), ZR and DHZR (Eberle *et al.*, 1986). The

detection limit was between 0.1 and 1 pmol as estimated from standard curves. All samples were assayed at least in triplicate and the concentrations were expressed as the equivalents of phytohormones in pmol/g fresh weight. In immunohistograms of the plant extracts no immunoreactive material was detected other than that eluting with the standards. No indication of interference was detected at various levels of added standard hormone in dilution analysis of the final purified extract fractions.

Recovery, as checked with internal radiolabelled standards in the same extract used for immunoassay, was >70% for all phytohormones. The standard deviation of results measured after parallel extraction of plant material and immunoassay was <10%.

Plant material

Tobacco (SR1) plants transformed with the 35S-*Ac-RolC* construct were crossed with untransformed tobacco (Spena *et al.*, 1989). The first generation progeny showing a mosaic expression (Spena *et al.*, 1989) of *rolC* were used for biochemical and anatomical analysis.

Light microscopy

3 mm pieces of pale and dark green sectors were excised from the fifth leaf from the apex, fixed with 4% glutaraldehyde in 0.2 M sodium cacodylate buffer at pH 7.2 for 4 h, washed in cacodylate buffer and post-fixed with 2% osmium tetroxide in the same buffer for 1 h. Samples were dehydrated in an alcohol series to absolute ethanol. After this, samples treated by three passages through propylene oxide, embedded in epoxy resin (Durcupan, Fluka) and cross-sectioned with a diamond knife in semi-thin (1 μ m) sections. The semi-thin sections were stained with toluidine blue before observation by light microscopy (Axioscop, Zeiss).

Chlorophyll determination

Chlorophylls *a* and *b* were extracted and measured according to Smith and Benitez (1955).

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Note added in proof

The rolC protein also hydrolyses BAP-0-glucoside, BAP-3-glucoside, BAP-7-glucoside, zeatin-7-glucoside, DHZ-7-glucoside, DHZ-9-glucoside, kinetin-9-glucoside, isopentenyl adenine-9-glucoside, zeatin-0-glucoside and DHZ-0-glucoside, although with different kinetics.