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The protein encoded by the *rolB* plant oncogene hydrolyses indole glucosides

J.J.Estruch, J.Schell and A.Spena

MPI für Züchtungsforschung, 5000 Köln 30, FRG

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The *rolB* gene of *Agrobacterium rhizogenes*, whose expression stimulates the formation of roots by transformed plant tissues and other growth alterations in transgenic plants, codes for a β-glucosidase able to hydrolyse indole-β-glucosides. Indeed, we show that extracts of bacteria and/or plant tissue expressing the *rolB* protein hydrolyse indoxyl-β-glucoside (plant indican). Because of the structural similarity between indoxyl-β-glucoside and indole-3-acetyl-β-glucoside (IAA-β-glucoside), we propose that the physiological and developmental alterations in transgenic plants expressing the *rolB* gene could be the result of an increased intracellular auxin activity caused by the release of active auxins from inactive β-glucosides. Thus two of the oncogenes carried by the T-DNA of the plant pathogen *Agrobacterium rhizogenes* (*rolB* and *rolC*) perturb plant growth and development by coding for β-glucosidases with distinct specificities. Whereas the *rolC* β-glucosidase releases cytokinins from their glucoside conjugates, the *rolB* encoded protein hydrolyses indole-β-glucosides. The combined action of these two genes therefore is expected to modulate the intracellular concentration of two of the main growth factors active in plants.

Key words: Agrobacterium rhizogenes/β-glucosidase/indoxyl β-glucoside/plant oncogene/*rolB* gene

Introduction

The term auxin is used to describe a class of natural or synthetic plant growth substances such as indole-3-acetic acid (IAA), 4-chloro-indole-3-acetic acid (C1-IAA) and phenylacetic acid. IAA is regarded as the most important of natural auxins and is detectable in a variety of organs and in every plant species investigated (Bearder, 1980). IAA, as well as other phytohormones, is present in plants in free as well as in conjugated forms (Cohen and Bandurski, 1982). Most of the IAA extractable from plant tissue is in a conjugated form. These auxin conjugates consist of a variety of different molecules covalently attached to IAA. Thus IAA ester conjugates, IAA amide conjugates and IAA acyl anhydrides are known. The biological role of auxin conjugates is still largely unknown, although circumstantial evidence indicated that they might serve as storage forms of the hormone (Cohen and Bandursky, 1982).

The *rolB* oncogene from the T_L-DNA of the *Agrobacterium rhizogenes* Ri plasmid is known to be involved in the induction of hairy-root formation on *Kalanchoe daigremontiana* leaves (White *et al.*, 1985). When expressed under the control of a heterologous promoter (e.g. the 35S RNA promoter of cauliflower mosaic virus), the *rolB* gene has been shown to induce leaf necrosis in transgenic tobacco plants (Schmülling *et al.*, 1988). These developmental and physiological alterations are indicative of an increased auxin activity. Moreover, *rolB* gene expression increases auxin sensitivity in tobacco protoplasts (Shen *et al.*, 1988; Maurel *et al.*, 1990). Most of the IAA found in plants is present in a bound form (for review, see Cohen and Bandursky, 1982). Moreover, exogenously applied IAA can be converted to conjugated forms such as IAA-aspartate (Andrea and Good, 1955; Riov and Gottlieb, 1980) and IAA-β-glucoside (Zenk, 1961). No direct evidence for *in vivo* hydrolysis of auxin conjugates in plants has thus far been produced. However, the release of free auxin from its storage form is an attractive hypothesis to explain the increased auxin activity displayed by *rolB* transgenic plant tissues.

In this article, we show that the protein encoded by the *rolB* gene releases indoles from indoxyl-β-glucosides. Considering that IAA-glucosides are among the most abundant IAA conjugates found in plants (Cohen and Bandursky, 1982), it seems reasonable to propose that the release of IAA from its glucoside conjugates by the enzymatic activity of the *rolB*-encoded protein could be the first of a cascade of events leading to an alteration of plant physiological and developmental processes. In this respect, we have recently shown that the *rolC* gene, which is linked to the *rolB* gene on the T_L-DNA of Ri plasmids and positively interacts with *rolB* to induce root formation (Spena *et al.*, 1987; Schmülling *et al.*, 1988) codes for an enzyme able to release active cytokinins from their glucoside conjugates (Estruch *et al.*, 1991). Consequently, it appears that different *rol* genes are involved in a common strategy to perturb plant growth by modifying intracellularly the content of active auxins and cytokinins by releasing these growth factors from intracellular pools of inactive conjugates.

Results

Experimental strategy

On the assumption that the increased auxin activity and sensitivity displayed by transgenic plants or plant cells expressing the *rolB* gene is not due to *de novo* synthesis of indoleacetic acid (Schmülling *et al.*, 1988), we have interpreted the developmental and physiological alterations observed in *rolB* transgenic plants (Schmülling *et al.*, 1988; Shen *et al.*, 1988; Maurel *et al.*, 1990) as possibly resulting from an enzymatic activity able to release intracellularly free auxins from inactive conjugated forms. To test this hypothesis, the *rolB* protein was produced either in *Escherichia coli* or expressed specifically in the tapetum of transgenic tobacco plants and extracts were assayed *in vitro* for their capacity to hydrolyse specifically indole β-glucosides.

Expression of the rolB gene from A.rhizogenes in E.coli

The *rolB* gene codes for a putative peptide of 29 500 Daltons (Slightom *et al.*, 1986). A vector of the pDS family, pDS56/RBSII-1 (Stüber *et al.*, 1990), was used to express the *rolB* protein in *E.coli*. In this plasmid system (Figure 1), *rolB* expression is inducible by IPTG, and a protein, containing one extra amino acid at the amino-terminus, is produced (Figure 2).

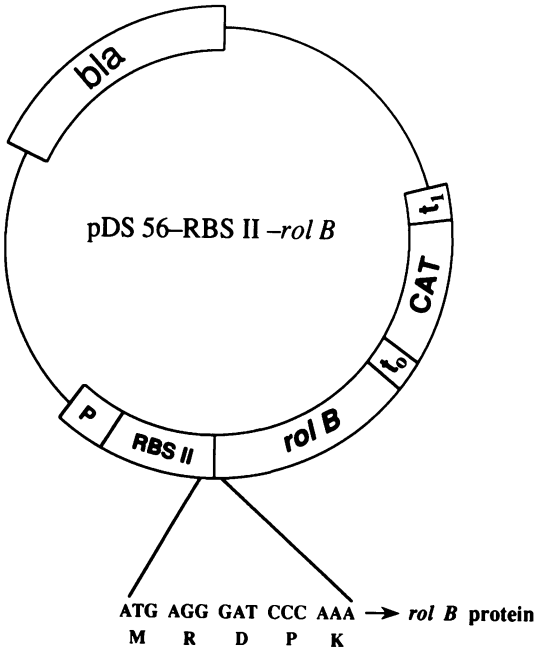

Fig. 1. Schematic representation of plasmid pDS56-RBSII-1-*rolB*. The promoter/operator element N250PSN250P29 is indicated by P, t_0 represents a terminator of phage lambda and t_1 is the terminator of the rrnB operon of *E.coli*. RBSII is a synthetic ribosomal binding site. For further details concerning the pDS vector family, see Stüber *et al.* (1990).

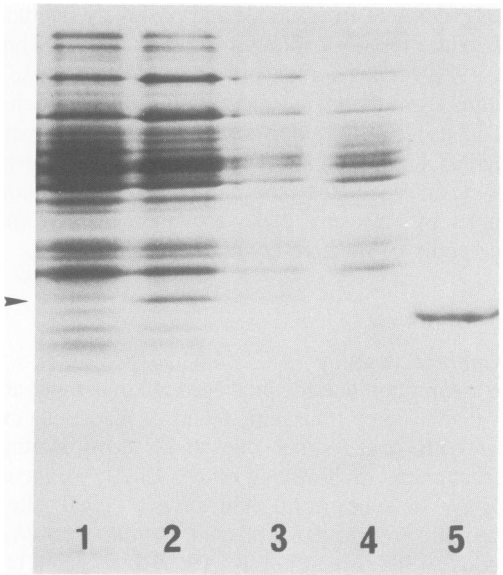

Fig. 2. Expression of the *rolB* protein in *E.coli*. Extracts of total proteins were prepared from *E.coli* harbouring the *rolB* expression vector after Coomassie staining. Lane 1, uninduced; lanes 2 and 3, IPTG-induced; lane 4, uninduced; lane 5, carbonic anhydrase (mol. wt ~29 kDa). Lanes 1 and 2 contain ~20 μg of total protein and lanes 3 and 4, half this quantity. The *rolB* protein is indicated by the arrowhead.

The rolB protein has β-glucosidase activity

To analyse whether the *rolB* protein has β-glucosidase activity, we used phenolic β-glucosides which release coloured products upon hydrolysis. Table I shows the results of β-glucosidase assays with extracts of sonicated bacteria harbouring a *rolB* expression vector. Extracts from IPTG-induced bacteria gave values up to 20 times higher than negative controls (i.e. extracts from uninduced bacteria).

The rolB protein hydrolyses indoxyl β-glucosides

Indoxyl-β-glucoside (plant indican) was used in an assay for hydrolysis of indole-glucosides. Figure 3 shows that *E.coli* extracts that contain the *rolB* protein are able specifically to metabolise indole-glucosides, provided that plant vascular sap (Estruch *et al.*, 1991) is added to the reaction mixture (compare lane 3 with lane 4 in Figure 3). In additional experiments vascular sap was substituted with UTP. Other substances (e.g. β-naphthyl β-D-glucoside, naphthol-AS-LC-β-D-galactoside, cytokinin-*N*-glucosides, IAA-aspartate, IAA-alanine, IAA-glycine and IAA-phenylalanine) were not hydrolysed by the *rolB* protein when tested under identical conditions (data not shown). The end products of the reaction comigrates, on TLC, with indoxyl-acetate and indoxyl-phosphate (not shown). LC-MS analysis of the assay has confirmed the presence of indole moieties.

Anthers transgenic for the expression of the rolB gene contain enzymatic activity able to hydrolyse indole glucosides

The *tap1* gene of *Antirrhinum majus* has been shown to be specifically expressed in the tapetum tissue of anthers (Nacken *et al.*, in press). The tissue specificity of expression of *tap1* is conserved in tobacco, indeed, tobacco plants transgenic for a chimeric gene composed of the *tap1* promoter and the *rolB* gene were shown to express the *rolB* gene during a narrow window of time in developing anthers (Spena *et al.*, in preparation). Anthers from plants transgenic for the *tap1*−*rolB* gene were assayed for the presence of enzymatic activity able to hydrolyse indoxyl-β-glucosides. Figure 4 shows a thin layer chromatogram of assays performed with extracts of anthers from two independent plants transgenic for the *tap1*−*rolB* gene (i.e. plant clones *tap-rolB* 3 and 7). While extracts of anthers from *tap1*−*rolB* plants hydrolysed the indoxyl-β-glucoside (Figure 4, lanes 2 and 3), no activity was present in extracts of anthers from wild type plants (or plants transgenic for the *tap1*−*rolB* gene but

Table I. β-glucosidase activity in sonicated extracts of *E.coli* harbouring the pDS56-RBSII-1-*rolB* expression vector

IPTG	pH		
	5.5	6.5	7.5
−	0.75	0.73	0.45
+	14.2	6.3	1.9

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 μmol/h/mg.

not expressing the *rolB* gene, data not shown). Addition of vascular sap or UTP was not required in the assays performed with extracts of anthers.

Discussion

Agrobacterium tumefaciens, the aetiological agent of the crown gall disease, alters plant cell physiology by introducing into the plant genome genes coding for enzymes able to catalyse the synthesis of auxin and cytokinin (for recent review, see Zambrisky *et al.*, 1990). Similarly, *Agrobacterium rhizogenes*, the aetiological agent of the hairy-root disease, alters plant cell physiology by natural genetic engineering (Chilton *et al.*, 1982). However, *Agrobacterium rhizogenes* T_L-DNA *rol* genes can modulate phytohormone activity in ways other than by *de novo* phytohormone synthesis (Cardarelli *et al.*, 1987; Jouanin *et al.*, 1987; Oono *et al.*, 1987; Sinkar *et al.*, 1987; Spena *et al.*, 1987; Vilaine and Casse-Delbart, 1987; Schmülling *et al.*, 1988).

One of the relevant Ri T_L-DNA genes, called *rolC*, was recently shown to code for an enzyme able to release free and active cytokinins from inactive glucoside conjugates (Estruch *et al.*, 1991). Here, we show that the *rolB* gene, similarly, codes for an enzyme possessing β-glucosidase activity and able to hydrolyse indole-β-glucoside conjugates. This finding allows us to propose that *in planta* the biochemical target of the *rolB* gene would be one or more auxin-glucosides structurally related to the indoxyl-β-glucoside (for example, see Figure 5).

Although no increase in IAA content has so far been reported in *rolB* transgenic plants, the biological effects of the *rolB* gene product (i.e. root initiation, leaf necrosis) are indeed reminiscent of auxin-mediated effects (Spena *et al.*, 1987; Schmülling *et al.*, 1988). More accurate (e.g. cell-specific) phytohormone analysis could perhaps clarify this aspect, provided that the active free hormone is not elusive due to chemical lability. We have now shown that two of the *rol* oncogenes responsible for the pathogenesis of the hairy-root disease (Ryker *et al.*, 1930) and of the hairy-root syndrome (Tepfer, 1984; Jouanin *et al.*, 1987; Spena *et al.*, 1987), code for enzymes able to release free forms of phytohormones from their conjugated forms (this article and Estruch *et al.*, 1991). These results, allow us to understand to a large extent the pathogenesis of the hairy-root disease and of the hairy-root syndrome and show that plant developmental and physiological processes can be influenced by enzymatic systems leading to conjugation and deconjugation of auxin and cytokinin phytohormones.

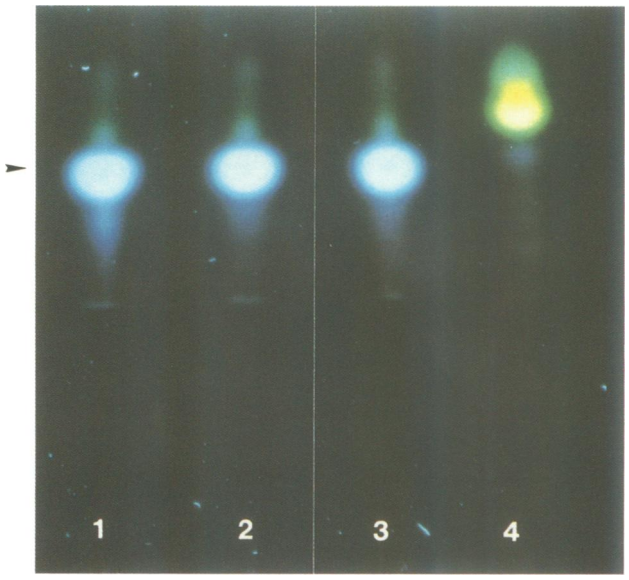

Fig. 3. Thin layer chromatogram of the products of the indole-β-glucosidase assays performed with bacterial extracts expressing the *rolB* protein. The substrate was indoxyl-β-glucoside (plant indican, Sigma). Lanes 1 and 2, uninduced extracts without and with vascular sap; lanes 3 and 4, IPTG-induced extracts (containing *rolB* protein) without and with vascular sap respectively. The indoxyl-β-glucoside (arrowhead) and the products of its hydrolysis (indoxyl-acetate, indoxyl-phosphate) were visualized under UV light (at a wavelength of 254 nm).

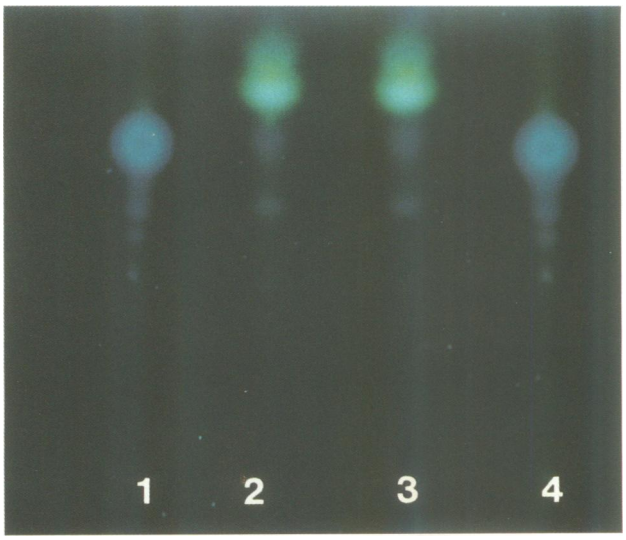

Fig. 4. Thin layer chromatogram of the products of the indole-β-glucosidase assays performed with extracts of anthers expressing the *rolB* gene. Lane 1, indoxyl-β-glucoside; lane 2, extracts of anthers from a plant transgenic for the *tap1*−*rolB* chimeric gene (plant clone −7, R0); lane 3, extract of anthers from a plant transgenic for the *tap1*−*rolB* gene (plant clone −3. F1 progeny); lane 4, negative control, extracts of anthers from a wild type SR1 plant.

Materials and methods

Plasmid construction

Plasmid pDS56-RBSII-1/*rolB* was constructed by subcloning the *rolB* gene as a *Bam*HI (partial digestion)−*Hin*dIII fragment spanning from base 11 278 to base 9814 of the T_L-DNA of Ri plasmid A4 (Slightom *et al.*, 1986). The *Bam*HI site at position 11 278 removes the ATG initiation codon, which is provided, together with an extra amino acid, by the vector pDS56-RBSII-1

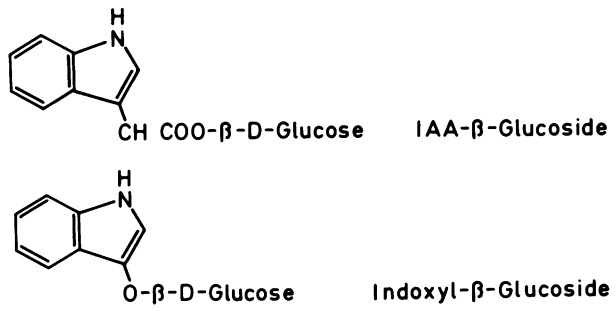

Fig. 5. Chemical structures of indoxyl-β-glucoside and indole-3-acetyl-β-glucoside. Chemical structures of two indole-β-glucosides: plant indican (indoxyl-β-glucoside) and IAA-glucoside (indol-3-acetyl-β-glucoside).

(Stüber *et al.*, 1990). *E.coli* strain M15 bearing repressor plasmid pDMI.1 was used to produce the *rolB* protein (for details, see Stüber *et al.*, 1990).

Preparation of sonicated extracts, estimation of protein concentration and protein analysis by polyacrylamide gel electrophoresis were done as previously described (Estruch *et al.*, 1991).

Enzymatic assays, preparation of vascular sap and chromatographic conditions

These were performed as previously described (Estruch *et al.*, 1991).

Plant material

Tobacco (SR1) plants transformed with the *tap1–rolB* chimeric gene will be described elsewhere (Spena *et al.*, in preparation).

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