Theor Appl Genet (1992) 84: 520–527

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Anther-specific expression of the *rolB* gene of *Agrobacterium rhizogenes* increases IAA content in anthers and alters anther development and whole flower growth

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Received October 3, 1991; accepted January 10, 1992
Communicated by F. Salamini

Summary. The promoter activity of a 2.2-kb DNA fragment from the 5′ flanking sequence of the *tap1* gene of snapdragon has been characterised in tobacco with the *β-glucuronidase* reporter gene. The *tap1* promoter conferred to the reporter gene an expression that was limited to the early stages of anther development. Expression of the *rolB* gene of *Agrobacterium rhizogenes* under the control of the *tap1* promoter impaired the development of tobacco flowers. Tobacco plants transgenic for the *tap1-rolB* chimeric gene showed altered anthers and a reduction in whole flower growth. Such growth alterations were correlated with an increase in free IAA content and a decrease in gibberellin activity present in anthers. Since the *rolB* gene codes for an indole β-glucosidase (Estruch et al. 1991), we interpret the phenotypic alterations to be a consequence of the increased content and activity of auxin in anthers. The perturbation of anther development would in turn affect gibberellin production and content, which is reflected in a reduced elongation of flowers.

Key words: Anther-specific expression – *rolB* gene – Increased IAA content – Decreased gibberellin activity – Altered flower growth

Introduction

In early stages of flower development the removal of stamens negatively affects the growth of other floral organs, resulting in smaller flowers (Zanoni 1941; Marrè 1946). This growth dependence on the presence of stamens is limited to early developmental stages and ceases when anther development is complete and the tapetum starts to disappear (Marré 1946). Moreover, in several species it has been shown that stamens control corolla growth through gibberellin production (Plack 1957, 1958; Murakami 1973; Bala et al. 1985; Weiss and Halevy 1989).

We have used transgenic plants to test whether the expression of a morphogenetic gene under the control of a tapetum-specific promoter could cause developmental alterations similar to those obtained by stamen removal. For this purpose we first characterised the specificity of expression of the *tap1* promoter from snapdragon in tobacco (Nacken et al. 1991) and then built a chimeric gene consisting of the forementioned promoter of snapdragon and the *rolB* gene of *Agrobacterium rhizogenes*.

The *rolB* gene has been identified for its capacity to induce root formation on kalanchoe leaves (White et al. 1985). Expression of the *rolB* gene in transgenic plants causes biological effects reminiscent of those caused by increased auxin activity (Schmülling et al. 1988). Moreover, tobacco protoplasts transgenic for the *rolB* gene have a higher auxin sensitivity than wild-type protoplasts (Shen et al. 1988; Maurel et al. 1990).

We have recently shown that the *rolB* gene codes for an enzyme able to release free indoles from indoxyl β-glucosides (Estruch et al. 1991). In the present article we show that expression of the *rolB* gene in anthers causes morphological alterations of the anthers and a reduction in whole flower growth. Moreover, we show that extracts of anthers transgenic for the *tap1-rolB* gene have higher IAA content and lower gibberellin activity than those from control plants. Therefore, tobacco plants expressing the *rolB* gene in anthers have an altered hormone metabolism, which affects the development of the anther and, consequently, its production of plant growth substances (e.g. gibberellins). The resulting decrease in gib-

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berellin activity will then impair the elongation of flower cells causing a reduction in flower growth.

Materials and methods

Plant material and transformation

Leaf discs of *Nicotiana tabacum* cv 'Havana SR1' were transformed with *Agrobacterium tumefaciens* strains harbouring the two constructs shown in Fig. 1 following the method of Horsch et al. (1985). Kanamycin resistant shoots were transferred to soil and grown under greenhouse conditions.

Chimeric gene construction

The isolation and analysis of the genomic clone *tap1* of snapdragon (*Anthirrhinum majus*) has been described by Nacken et al. (1991). The 2.2-kb *Nco*I/*Hin*dIII DNA fragment containing 5′ flanking DNA of the *tap1* gene, including 56 bp of 5′ untranslated leader, was blunt ended and subcloned into pUC18 (Nacken et al. 1991). *Eco*RI/*Bam*HI and *Eco*RI/*Kpn*I fragments were used to construct the *tap1-gus*, *β-glucuronidase*) and *tap1-rolB* chimaeric genes, respectively. Both transcriptional fusions were subcloned into the pPCV002 binary vector (Koncz and Schell 1986) and then transferred to *A. tumefaciens* strain GV3101 (MP90RK) (Koncz and Schell 1986) as previously described (Spena et al. 1989).

RNA isolation and Northern blot analysis

RNA isolation, poly A^+RNA purification, Northern blotting and hybridization analysis were performed as previously described (Spena et al. 1987).

GUS assays

Fluorimetric and histochemical assays were carried out as described by Jefferson (1987).

Protein concentration in plant extracts was determined using the Bradford reagent (BioRad Laboratories) according to the manufacturer's instructions.

Indole β-glucosidase assays

Enzymatic assays were performed as previously described (Estruch et al. 1991).

Hormonal assays

Gibberellin activity present in ethanol extracts of anthers was estimated by the rice (Tan gingozu) dwarf test according to Murakami (1968). Ethanol solutions of gibberellic acid from 15–1500 ng were used as controls in a parallel set of experiments.

Hormonal analysis

Anthers were harvested from flower buds 0.7–1 cm long, homogenised in liquid nitrogen with a mortar and pestle and extracted twice with 2 ml methanol. [^{3}H]IAA (400 bq) was added to the extraction medium. After centrifugation (24,000 *g*, 20 min, 4 °C), solid phase extraction and a preparative IS-HPLC run (Rosil C18 RP column; methanol/water/acetic acid, 50/49.5/0.5; flow 0.5 ml/min), the amount of free IAA was analysed by analytical IP-HPLC (same column; methanol/0.01 *M* tetrabutylammonium hydroxide in 0.001 *M* phosphate, pH 6.6, 40/60; flow 0.5 ml/min) with on-line fluorimetric detection (Shimadzu RF-530, excitation at 285 nm, emission at 360 nm) (Van Onckelen et al. 1988).

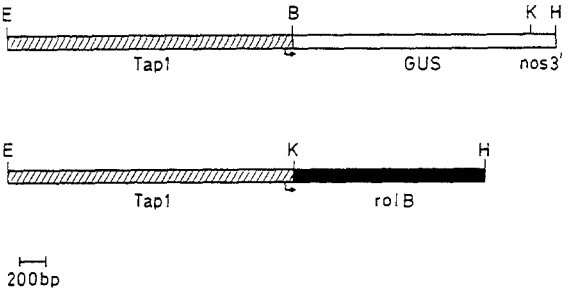

Fig. 1. Schematic drawing of the *tap1-gus* and *tap1-rolB* chimaeric genes. The 2.2-kb-long *tap1* promoter from *A. majus* was ligated either to the *β-glucuronidase* (GUS) or to the *rolB* gene. The two constructs, Tap1-GUS and Tap1-rolB, were subcloned into the binary vector pPCV002 (Koncz and Schell 1986). *E* *Eco*RI, *H* *Hin*dIII, *B* *Bam*HI, *K* *Kpn*I

Results

The tap1 promoter of snapdragon is able to drive anther-specific expression in tobacco

The isolation and tapetum-specific expression of the *tap1* gene of snapdragon has been described (Nacken et al. 1991). *Tap1* gene expression takes place in a narrow time period during organogenesis. *Tap1* mRNA starts to accumulate early during anther development, in 5-mm-long inflorescences, and it is undetectable in buds 10 mm long (Nacken et al. 1991). By means of *in situ* hybridization, its expression has been localised to the tapetum cells. To determine whether 5′ flanking sequences of the *tap1* gene are sufficient to direct anther-specific expression in a heterologous plant species, a transcriptional gene fusion was constructed utilising approximately 2.2 kb of *tap1* 5′ flanking DNA sequences and the *β-glucuronidase* reporter gene (Jefferson 1987). The *tap1-gus* chimeric gene (Fig. 1) was subcloned into the binary vector pPCV002 (Koncz and Schell 1986), and transgenic tobacco plants were raised by *Agrobacterium tumefaciens*-mediated transformation as described (Horsch et al. 1985). Tobacco plants transgenic for the *tap1-gus* chimeric gene were analysed by fluorimetric and histochemical assays. The results of GUS assays performed on four independent transgenic clones are presented in Fig. 2. High levels of GUS activity were detected only in anthers, with essentially no GUS activity in other vegetative or floral organs. Independent transformants showed approximately a 10-fold variation in the level of GUS activity, presumably due to a position effect frequently observed in transgenic plants. The temporal regulation of the *tap1* promoter was examined during the development of tobacco anthers by measuring GUS activity in extracts of anthers taken from tobacco flowers at different developmental stages. Figure 3 shows that maximum GUS activity is detected in anthers from flower buds less than 10 mm long, a finding consistent with the early expres-

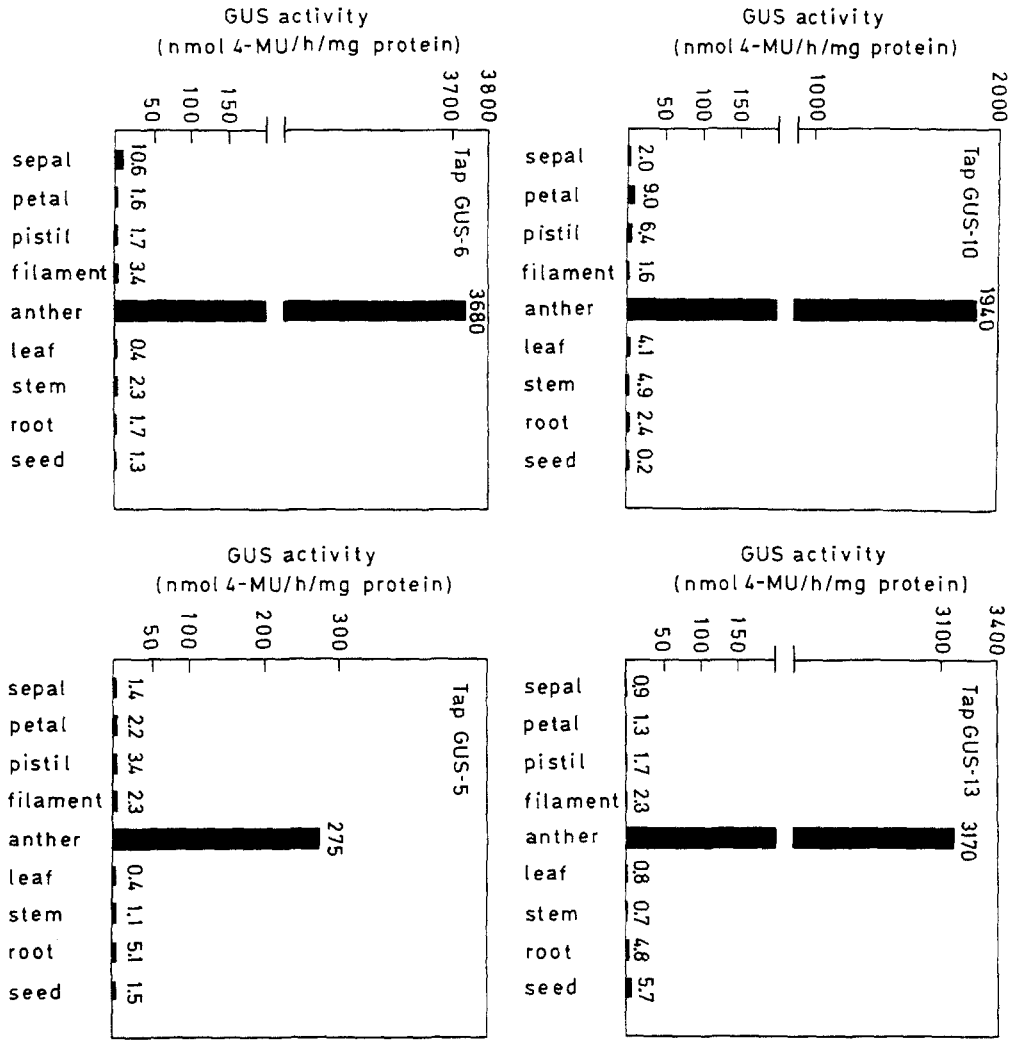

Fig. 2. GUS activity in vegetative and reproductive organs of four independent transgenic plant clones. Assays were performed on extracts of mature organs of R_0 plants. The results present the mean activity from at least three independent assays of the same tissue (SD between experiments ranged from 20% to 38%). Background GUS activity in extracts from untransformed plants was subtracted from GUS activity in transgenic plants

sion pattern observed in snapdragon flower development (Nacken et al. 1991). The localisation of GUS activity was performed by histochemical analysis. Figure 4a shows indigo staining in tapetum and sporogenous cells (Fig. 4c). In summary, a DNA fragment approximately 2.2 kb from the 5′ flanking sequences of the *tap1* gene of snapdragon is sufficient to ensure temporal and organ-specific expression in tobacco.

Tap1-rolB transgenic anthers have indole β-glucosidase activity

The *rolB* gene codes for an indole β-glucosidase able to hydrolyse indoxyl-β-glucoside (Estruch et al. 1991). Consequently, extracts of anthers less than 1 cm long and transgenic for the *rolB* gene were assayed for indole β-glucosidase activity. Figure 4d shows that anther extracts from independent plant clones (namely, -3, -6, -7, -12 and -17; lanes 2–6) have indole β-glucosidase activity detectable in assays with indoxyl β-glucoside (plant indican) as substrate. Therefore, anthers transgenic for the *tap1-rolB* gene possess the capacity to release free indoles from their β-glucoside conjugates.

Tap1-rolB transgenic anthers have an increased auxin content

The content of IAA in anthers transgenic for the *tap1-rolB* gene was compared to that of control plants (Table 1). When expressed as picomoles per gram fresh weight, all of the transgenic anthers contained a significantly higher concentration of free IAA than those of the con-

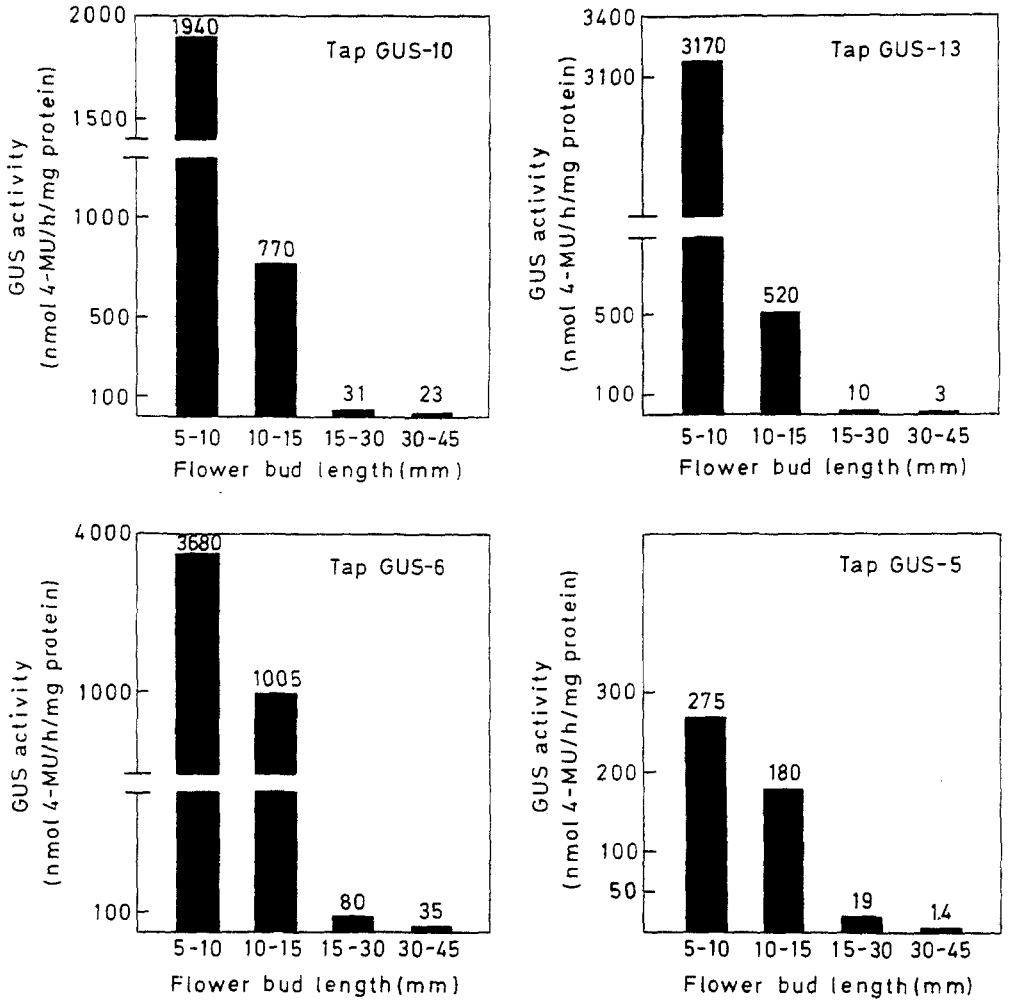

Fig. 3. GUS activity during anther development in four independent transgenic clones. Whole anthers were harvested from flowers where the bud length (from the top of the pedicle to the tip of the petals) was 5–10, 10–15, 15–30 or 30–45 mm. The results represent mean activity from at least three independent assays

Table 1. Endogenous levels of free IAA (expressed as pmol/g f.w. and as pmol/anther) in anthers of control and *tap1-rolB* transgenic tobacco plants

Sample	IAA free	
	pmol/g f.w.	pmol/anther
Control	790 ± 40	1.59 ± 0.08
TapB3	1522 ± 80	1.72 ± 0.09
TapB4	2292 ± 130	3.39 ± 0.19
TapB6	3060 ± 160	2.91 ± 0.15
TapB7	3273 ± 180	2.91 ± 0.16
TapB8	2591 ± 70	4.02 ± 0.11
TapB9	1214 ± 70	1.25 ± 0.07
TapB10	2655 ± 170	4.35 ± 0.27
TapB11	2080 ± 120	3.81 ± 0.22
TapB17	1071 ± 60	1.42 ± 0.08

± = uncertainty. It was calculated by means of standard deviation of the measurements

trol plants. As expression of the *rolB* gene alters the growth and development of the anthers, the results were also expressed as picomole per anther. In this case too, the anthers of seven out of nine independent *tap1-rolB* plant clones showed enhanced free IAA levels.

Levels of bound IAA did not decrease in anthers transgenic for the *tap1-rolB* gene (data not shown). Since bound IAA represents an heterogenous population of IAA-conjugates, its analysis needs to be carried out by mass spectroscopy. In this respect, the conjugation of IAA to aspartate and other compounds is induced by auxin treatment (Südi 1964, 1966; Venis 1964, 1972), and consequently a change in the composition of IAA conjugates in the *tap1-rolB* transgenic anthers might be expected.

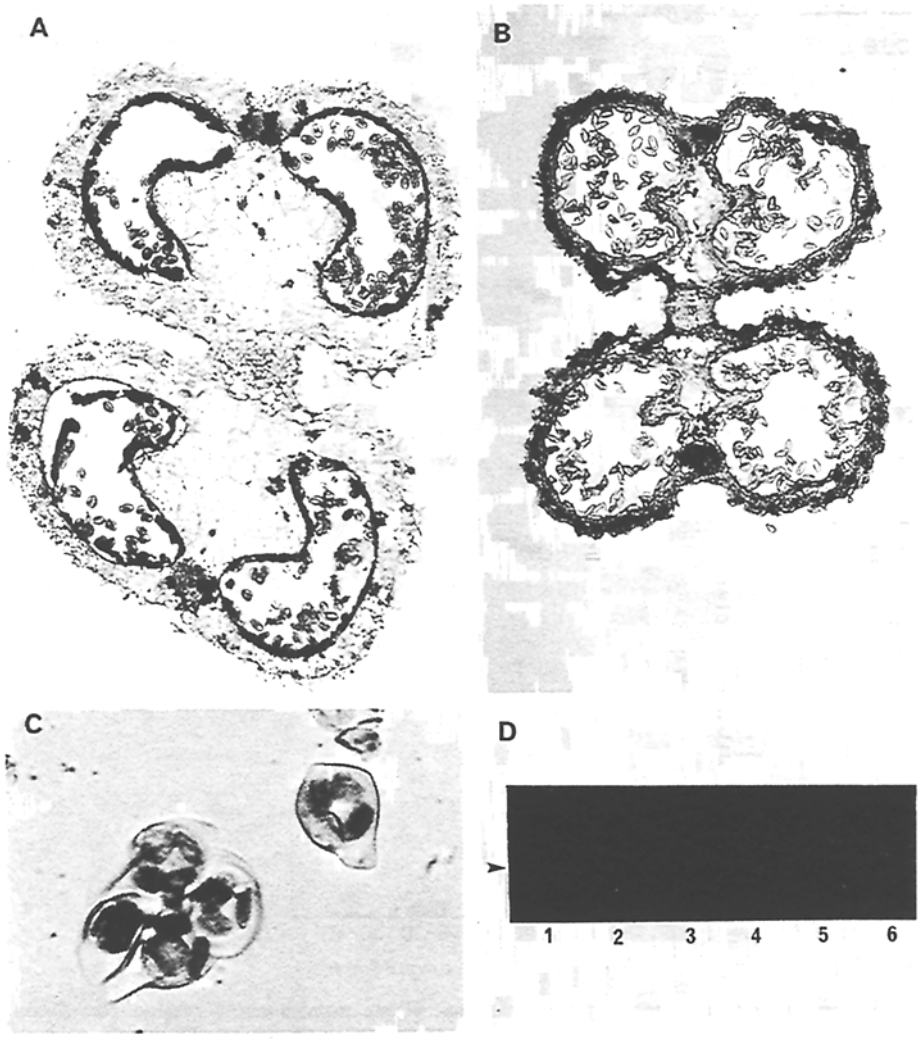

Fig. 4 A–D. GUS histochemical staining of anthers transgenic for the Tap1-GUS chimaeric gene and enzymatic assays for indole β-glucosidase activity in extracts from anthers transgenic for the *tap1-rolB* chimeric gene. **A** Section of a Tap-Gus transgenic anther from plant clone Tap-Gus-10 showing GUS-specific staining in tapetum and sporogenous cells. **B** Anther from negative control. **C** GUS activity is visualised in tetrads enclosed in pollen mother cells. **D** Thin-layer chromatogram of the products of the enzymatic assays performed with extracts from anthers of SR_1 untransformed plants (*lane 1*), clone Tap1rolB-3 (*lane 2*), clone Tap1rolB-6 (*lane* 3), clone Tap1rolB-7 (*lane 4*), clone Tap1rolB-12 (*lane 5*) and clone Tap1rolB-17 (*lane 6*). Enzymatic activity able to hydrolyse indoxyl β-glucoside is present in all transgenic anthers, but not in untransformed ones. The *arrow* indicates the indoxyl-β-glucoside

Tap1-rolB transgenic anthers have reduced gibberellin activity

Table 2 shows gibberellin biological activity in anthers transgenic for the *tap1-rolB* gene and control plants. Transgenic plants, both R_0 regenerants and F_1 progenies, had lower gibberellin activity than control plants when tested by a rice Tan gingozu dwarf test (Murakami 1968). Moreover, gibberellin activity inversely correlated with the expressivity of the traits being most reduced in plant clone TapB-7, which shows the most severe phenotype with anthers possessing the highest level of indole β-glucosidase activity (Fig. 4d) and the highest content of IAA (Table 1).

Expression of the rolB gene of Agrobacterium rhizogenes under the control of the tap1 promoter causes growth alterations in anthers and modifies whole flower development

Tobacco plants (R_0) transgenic for the *tap1-rolB* chimeric gene (Fig. 1) showed alterations in flower morphology (Fig. 5a). These alterations, absent in tobacco plants transgenic for the *tap1-gus* chimeric gene and other negative controls, consisted of shrivelled anthers (Fig. 5b) and reduced elongation (12.5%) of the calyx, corolla and peduncles (Fig. 5a and 5c). Seed capsules were also reduced, containing fewer seeds than those from control plants (data not shown). Inflorescences

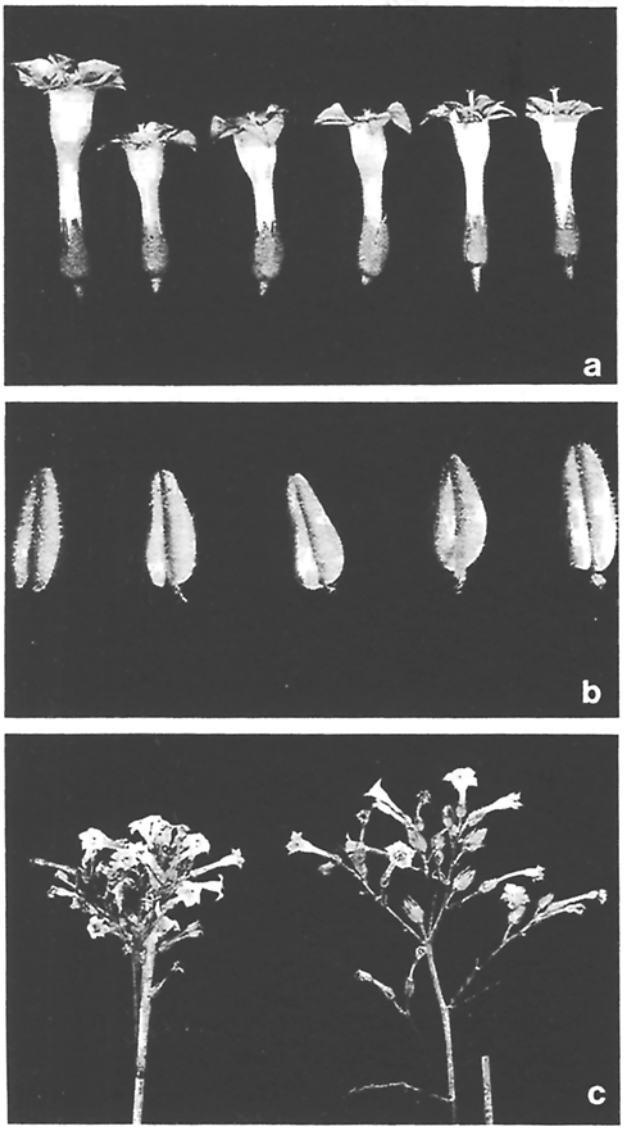

Fig. 5a–c. Phenotype of tobacco plants transgenic for the *tap1-rolB* gene. **a** Reduction in growth of flowers from tobacco plants transgenic for the *tap-1-rolB* gene. *Left*, normal flower from control plants. **b** Anthers from tobacco plants that are independent clones transgenic for the *tap1-rolB* chimeric gene have shrivelled anthers. *Right*, normal anther from control plant. **c** Reduction in the elongation of the peduncle of inflorescences in tobacco plants transgenic for the *tap1-rolB* gene. *Left*, tobacco plant transgenic for the *tap1-rolB* gene; *right*, control plant

were less elongated and, consequently, had a more compact appearance (Fig. 5c). The expressivity of the traits varied somewhat among individual transgenic clones.

At the whole plant level plants transgenic for the *tap1-rolB* gene presented no morphological alterations when compared to wild-type tobacco plants, but after flowering they were characterised by a growth of the axillary buds of the top leaves (data not shown). Northern blot analysis of mRNA extracted from flower buds

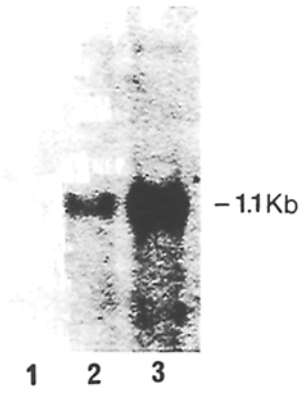

Fig. 6. Northern blot analysis of poly A^+RNA from *tap1-rolB* transgenic plants. *Lane 1* negative control, *lane 2* plant clone TapB-7, *lane 3* plant clone TapB-12. Approximately 10 μg of poly A^+RNA extracted from anthers was loaded in each lane. Anthers were from R_0 plants

Table 2. Gibberellin activity present in ethanol-extracts from anthers transgenic for the *tap1-rolB* chimeric gene

Anthers	GA_3-equivalents	Gibberellin activity
Control		
0.7 cm	1.5 ± 0.2 ng/anther	++++
1.0 cm	1.1 ± 0.1 ng/anther	+++
TapB1		
0.7 cm	0.8 ± 0.2 ng/anther	++
1.0 cm	0.7 ± 0.2 ng/anther	++
TapB3		
0.7 cm	0.7 ± 0.2 ng/anther	++
1.0 cm	0.6 ± 0.3 ng/anther	++
TapB7		
0.7 cm	< 0.5 ng/anther	+
1.0 cm	ND	±
TapB9		
0.7 cm	1.0 ± 0.1 ng/anther	+++
1.0 cm	1.1 ± 0.05 ng/anther	+++

ND, Not detectable; ± = SD
The data represent three independent experiments, each one consisting of 12 assays performed with anthers from primary transformants

Table 3. Progeny analysis of plants transgenic for the *tap1-rolB* gene

Crosses	Phenotypes		χ^2 Ratio	
	km^r	km^s	Tested	Value
TapB-1 × SR_1	62	77	1:1	1.62[a]
TapB-3 × SR_1	112	32	3:1	0.59[a]
TapB-5 × SR_1	106	46	3:1	2.25[a]
TapB-7 × SR_1	93	38	3:1	1.12[a]
TapB-9 × SR_1	112	48	3:1	2.13[a]
TapB-12 × SR_1	110	33	3:1	0.28[a]

Resistance to kanamycin sulfate (50 mg/l) was tested in vitro on MS medium (Murashige and Skoog 1962). The seeds analysed for each cross represent the progeny obtained from a single capsule [a] Not significant ($P = 0.05$)

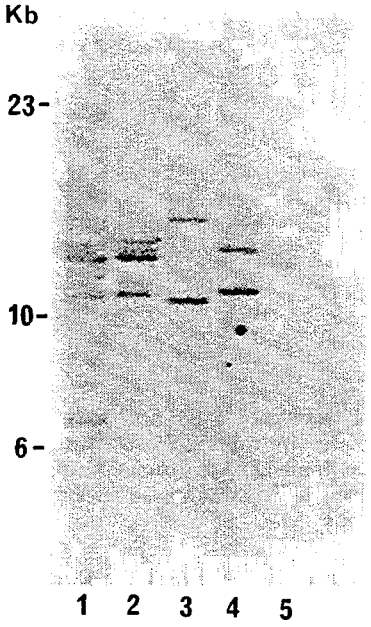

Fig. 7. Southern blot analysis of DNA from tobacco plants transgenic for the *tap1-rolB* chimaeric gene. *Hin*dII digests of DNA from plant clone TaprolB-12 (*lane 1*), -7 (*lane 2*), -3 (*lane 3*), -1 (*lane 4*) and normal SR_1 untransformed plant (*lane 5*). The DNA gel blot analysis detected three gene copies in clones -12 and -7, and two copies in clones -3 and -1

less than 7 mm long showed *rolB*-specific transcripts in plant clones TapB -7 and -12 (Fig. 6). Similar results were obtained in plant clones TapB-1, -3 and -9 (not shown). DNA blot analysis showed that all of the plant clones analysed have the *tap1-rolB* chimeric gene integrated in their genome (Fig. 7 and data not shown), albeit with different copy number. Progeny analysis for kanamycin resistance (Table 3) and DNA blot analysis together show that plant clones TapB-7 and -9 have two inserts on two unlinked chromosomal loci; clones TapB-5 and -12, three copies on two loci; clone TapB-1, five inserts at one locus; and clone TapB-3, three copies on two chromosomal loci.

Discussion

Flower growth is the result of a series of closely correlated influences. In particular, it has been reported that emasculation at the bud stage retards the elongation of petals and gynophores (Zanoni 1941; Marrè 1946; Weiss and Halevy 1989). Stamens control petal growth by acting, in early stages, as a source of gibberellins, which are produced in the anthers following the completion of meiosis (Plack 1958; Murakami 1973). Consequently, precisely timed perturbations of anther physiology early in flower development should affect not only anther growth but also the growth of other flower organs.

The analysis of GUS activity in tobacco plants transgenic for a chimeric gene composed of the *β-glucuronidase* coding region (Jefferson 1987) and the *tap1* promoter of snapdragon (Nacken et al. 1991) shows that this promoter confers an expression that is limited temporally to young flower buds and spatially localised to anthers. GUS-staining was also detected in sporogenous cells (Fig. 4c). This staining could be caused by the incorporation and subsequent precipitation as indigo inside the sporogenous cells of a product released by β-glucuronidase activity present in tapetum cells (D. Chriqui, personal communication). However, considering the ontogenetic proximity of tapetum and sporogenous cells, both derived from hypodermal initials (Gifford and Foster 1988), the *tap1* promoter might be also active at some stage of microspore differentiation. Whatever the case, the *tap1* promoter of snapdragon is able to confer anther-specific expression in tobacco.

Therefore, we have constructed plants transgenic for a chimeric gene placing the *rolB* coding region of *Agrobacterium rhizogenes* under the control of the *tap1* promoter. The *rolB* gene is involved in the pathogenesis of hairy-root disease (White et al. 1985), and is also the gene primarily responsible for root induction (Cardarelli et al. 1987; Spena et al. 1987). Moreover, the *rolB* gene product confers an increase in auxin sensitivity to tobacco protoplasts (Maurel et al. 1990), and when expressed under the control of the 35S promoter of cauliflower mosaic virus, it causes leaf necrosis in transgenic tobacco plants (Schmülling et al. 1988). The *rolB* protein has been recently characterised as an indole-β-glucosidase able to release free indoles from their conjugates (Estruch et al. 1991).

The use of the *tap1* promoter to control the expression of the *rolB* gene has allowed us to manipulate genetically the growth of tobacco flowers. Indeed, plants harbouring such a construction presented shrivelled anthers in which pollen production, although not completely abolished, was severely altered (not shown). The resulting flowers also had a reduction in corolla and calyx length (see Fig. 6b). We interpret the observed phenotypical alterations to be indications that the expression of the *rolB* gene under the control of the *tap1* promoter from snapdragon interferes with anther physiology and growth by altering the auxin metabolism. Consequently, the synthesis and accumulation of gibberellin(s) produced by the anthers in early developmental stages and required for the normal growth of the corolla, gynaecium as well as for the elongation of the inflorescence are also affected. This interpretation is confirmed by the finding of higher levels of IAA as well as lower gibberellin activity in anthers of plants transgenic for the *tap1-rolB* chimeric gene.

Recently, in the *Gib-1* mutant of tomato, gibberellin deficiency has been associated with an arrest in anther

and flower development (Jacobsen and Olszewsky 1991). This finding is again consistent with the existence of correlative influences of the anther on whole flower growth.

Considering that the *rolB* gene product alters auxin metabolism, more severe alterations of anther development leading to male sterility might be established by expressing under the control of the *tap1* promoter, either alone or in combination with the *rolB* gene, genes such as the *iaaM* and *iaaH* genes of *Pseudomonas savastanoi* and/or *Agrobacterium tumefaciens* (Yamada et al. 1985), which are able to increase, by virtue of synthesis, IAA content and auxin activity in plant tissues. The information presented here, the free availability of the *tap1* promoter and of genes able to affect plant cell viability might be useful to plant biologists interested in manipulating and/or studying male gametogenesis.

Acknowledgements. We thank S.C. Schulze and J. Dupont for skillful technical assistance. This work was supported by an E.E.C. BRIDGE grant to A. Spena and H. Van Onckelen, and an E.E.C. BRIDGE fellowship to J.J. Estruch.

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