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Single genes from *Agrobacterium rhizogenes* influence plant development

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The combined expression of the *rol A, B* and *C* loci of *Agrobacterium rhizogenes* Ri-plasmids establishes, in transgenic tobacco plants, a pathological state called hairy-root syndrome. However, when expressed separately they provoke distinct developmental abnormalities characteristic for each *rol* gene. Moreover, changes in their mode of expression obtained by replacing the promoters of the *rol B* and *C* genes with the cauliflower mosaic virus 35S promoter elicit new and distinct developmental patterns. These results indicate that the different *rol* gene products have either different targets, or have a qualitatively different effect on the same target. The target(s) must be involved in the control of plant development. Although each of the three *rol* genes are independently able to promote root formation in tobacco, efficient root initiation and growth is best achieved through the combined activities of more than a single *rol* gene. Models explaining the biological effects of *A. rhizogenes*-derived TL-DNA genes are discussed.

Key words: developmental genes/*Agrobacterium rhizogenes*/transgenic plants/male sterility

Introduction

An understanding of the mechanisms underlying plant pathological conditions has in many cases contributed to our knowledge of hormone-mediated physiological processes (for reviews see Dekhujzen, 1976; Pegg, 1976; Schröder, 1987). Indeed, the discovery of gibberellins stemmed from the investigation of the bakanae fungal disease of rice (Kurosawa, 1926). Similarly the study of fasciation disease by *Corynebacterium fascians* (Thimann and Sachs, 1966; Murai *et al.*, 1980), and of the genesis of galls on olives and oleanders by *Pseudomonas savastanoi* (Smidt and Kosuge, 1978; Comai and Kosuge, 1980) have contributed to our understanding of the effects of cytokinin and auxin. In each of these pathogenetic plant–bacterial interactions, hormone biosynthesis by the phytopathogen was shown to be a decisive factor.

A special case is represented by *Agrobacterium tumefaciens*, the aetiological agent of the crown gall disease, where hormone biosynthesis takes place in transformed plant cells. This phytopathogen relies on genetic transformation of plant cells to achieve hormone biosynthesis (for review see Schell, 1986). A fragment of DNA, called T-DNA, is delivered to the plant cells and, after nuclear integration and expression, alters the hormonal metabolism of the host cell which in turn induces uncontrolled tumorous growth. Genetic transformation of plant cells is also at the basis of pathogenesis of hairy-root disease (for reviews see Birot *et al.*, 1987; White and Sinkar, 1987). However, while crown galls are chimaeric tissues because the auxin and cytokinin synthesizing enzymes coded for by the T-DNA genes (Akiyoshi *et al.*, 1984; Barry *et al.*, 1984; Inze *et al.*, 1984; Schröder *et al.*, 1984; Thomashow *et al.*, 1984; Buchmann *et al.*, 1985; Yamada *et al.*, 1985) modify the growth of both transformed and associated untransformed cells, roots induced by infection with *A.rhizogenes* appear to be composed only of transformed cells (Chilton *et al.*, 1982; Bercetche *et al.*, 1987). Consequently the products of T-DNA linked genes of *A.rhizogenes* (so-called *rol* genes; White *et al.*, 1985) have to act mainly, if not only, in transformed cells, and consequently do not appear to be directly involved in the synthesis of growth factors which are transported.

The so-called 'hairy-root syndrome' is displayed by plants regenerated from Ri-transformed roots (Ackermann, 1977; Tepfer, 1984). The characteristic symptoms of this syndrome are adventitious root formation, high growth rate of roots in culture, reduced apical dominance both in stems and roots, altered leaf and flower morphology, plagiotropic root growth (i.e. with altered geotropism) and reduced pollen and seed production (Tepfer, 1984). Establishment of the full hairy-root syndrome correlates with expression of the *rol A, B* and *C* loci, whose products show synergistic activities involved both in rhizogenesis and in generating plant growth abnormalities (Cardarelli *et al.*, 1987b; Spena *et al.*, 1987; Vilaine *et al.*, 1987).

In this article we have further characterized the biological effects of the *rol A, B* and *C* genes in transgenic plants. This was achieved by cloning the *rol A, B* and *C* genes either singly or in combination into Ti plasmid-based gene vectors and by isolating and analysing transgenic tobacco plants carrying and expressing these genes. By positioning the coding regions of *rol B* or *rol C* genes under the transcriptional control of the cauliflower mosaic virus 35S promoter, deregulated expression of these genes in transgenic plants was achieved and its consequences studied.

Results

Distinct biological effects are caused by the separate expression of the rol A, B and C loci in transgenic tobacco plants

Transgenic tobacco plants expressing a combination of the *A.rhizogenes* Ri plasmid derived *rol A, B* and *C* loci display phenotypic alterations, typical of the hairy-root (hr) syndrome, whereas transgenic tobacco plants expressing single *rol* genes or their pair-wise combinations (Figure 1) show abnormalities of growth which are specific for each gene or for a given combination of genes. For example, Figure 2a shows a tobacco plant transgenic for the

combination of *rol A* and *B* genes compared to either a normal SR1 or a *rol A, B* and *C* transgenic plant. Although the morphology of the *rol A* plus *B* plant deviates from the normal one (i.e. SR1 plant), the full hr syndrome is displayed only in transgenic plants carrying all three loci. Similarly transgenic plants carrying the *rol B* plus *C* or the *rol A* plus *C* combinations do not show all the phenotypic traits of the hr syndrome, although they deviate from normality (Figure 2b and c respectively). An identical conclusion is reached through the analysis of the offspring from crosses between *rol A* plus *B* with *rol C* transgenic plants. Indeed 29 out of 100 plants resulting from the cross of a tobacco plant transgenic for a single *rol C* gene (e.g. C9a) with plants transgenic for a single *rol A* plus *B* gene combination (e.g. AB3−5) displayed the full hr syndrome.

Transgenic tobacco plants carrying only the *rol C* locus show growth alterations consisting of altered leaf morphology (Figure 2d), reduced flower size and pollen production (Figure 2e). Seed capsules are smaller (Figure 2f) and the plant is more branched. Transgenic tobacco plants expressing the *rol B* locus show other abnormalities of growth consisting mainly of different alterations of leaf morphology (Figure 2d), increased stigma and flower size, of heterostyly (Figure 2e) as well as of increased formation of adventitious roots on the stem. Pollen production is only slightly reduced and seed capsules are not impaired (Figure 2f). *Rol A, B, C* transgenic flowers are smaller than those of normal SR1 plants, whilst *rol B* flowers are larger and abnormal (Figure 2e). Small flowers were also shown by plants transgenic for the *rol C* gene in combination with the other *rol* genes (i.e. pPCV002-AC, -BC, -CaMVBT+C; data not shown).

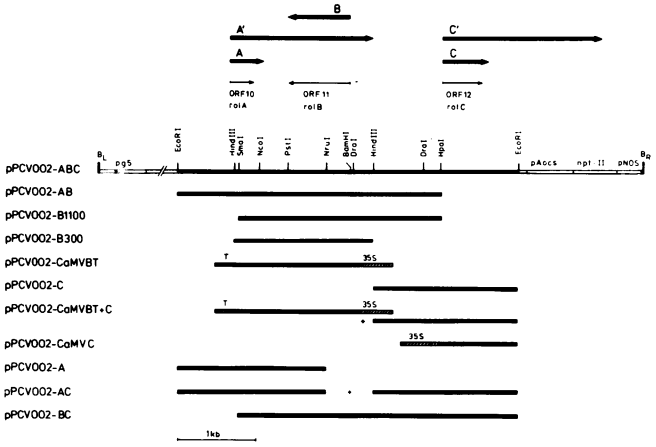


Fig. 1. Schematic drawing of the constructions. pPCV002-ABC contains the *Eco*RI fragment 15 of the Ri plasmid A4 in both orientations called pPCV002-ABC1 and pPCV002-ABC2. The location of the kanamycin-resistant gene and of the gene 5 promoter of the pPCV002 vector (Koncz and Schell, 1986) are also indicated. Above the map with relevant restriction sites are shown the positions of ORFs 10, 11 and 12 (Slightom *et al.*, 1986) corresponding to the *rol A*, *rol B* and *rol C* loci respectively. The approximate location and size of the *rol* transcripts as determined in Spena *et al.* (1987) are also indicated. Below the map are shown the different fragments and chimaeric constructions delivered to the plant genome. Abbreviations: 35S, CaMV 35S promoter; T, CaMV 35S terminator; kb, kilobase; B_L and B_R, left and right border sequences of vector T-DNAs; pg5, truncated promoter of T_L-DNA gene 5; pNOS, promoter of nopaline synthase gene; pAocs, polyadenylation sequence of octopine synathase gene; NPT-II, neomycin phosphotransferase gene of transposon Tn*5*. All the constructions were described previously (Spena *et al.*, 1987), with the exception of pPCV002-BC.

Tobacco plants transgenic for the *rol A* locus differ in their growth abnormalities from both *rol B* and *rol C* transgenic plants. Their most typical feature is the presence of wrinkled leaves (Figure 2d), and of condensed inflorescence with abnormal (i.e. increased) stigma size and larger flower morphology (Figure 2e).

These observations were obtained from at least 20 independent regenerants from at least four independent transformation events (i.e. either callus derived from *rol*-induced roots or transgenic plants raised via leaf disc transformation; see Materials and methods) for each construction (Figure 1). Transgenic plants displaying morphological alterations were analysed by Northern blot analysis of poly(A)$^+$ extracted from stems, to test whether the phenotypic alterations correlate with *rol* gene expression. In all cases abnormalities of growth correlate with the presence of *rol* transcripts (Figure 3), although in plants transgenic for *rol A* and *rol A* plus *C*, other *rol A* transcripts than those observed in plants transgenic for all three *rol* genes are detectable (Figure 3, lanes 2 and 9). These transcripts are probably readthrough products of the *rol A* gene due to inefficient termination of transcription. Progeny of transgenic plants showed abnormal morphologies similar to those described for parental plants. These morphological traits co-segregated with the *Npt*II marker present in the pPCV002 binary vector. Variability in the expressivity of the traits was observed not only among different regenerants, most likely due to position effect on the level of expression, but also in the progeny of transgenic plants. Similar variability in expressivity was described in hr tobacco plants regenerated from hairy roots and correlated with alteration of *rol* gene expression (Durand-Tardif *et al.*, 1985). Another indication that the reason for this phenomenon is most likely modulation of promoter activity is that progeny of plants transgenic for the CaMVC chimaeric gene, where the *rol C* promoter is replaced by the cauliflower mosaic virus 35S promoter, are phenotypically uniform.

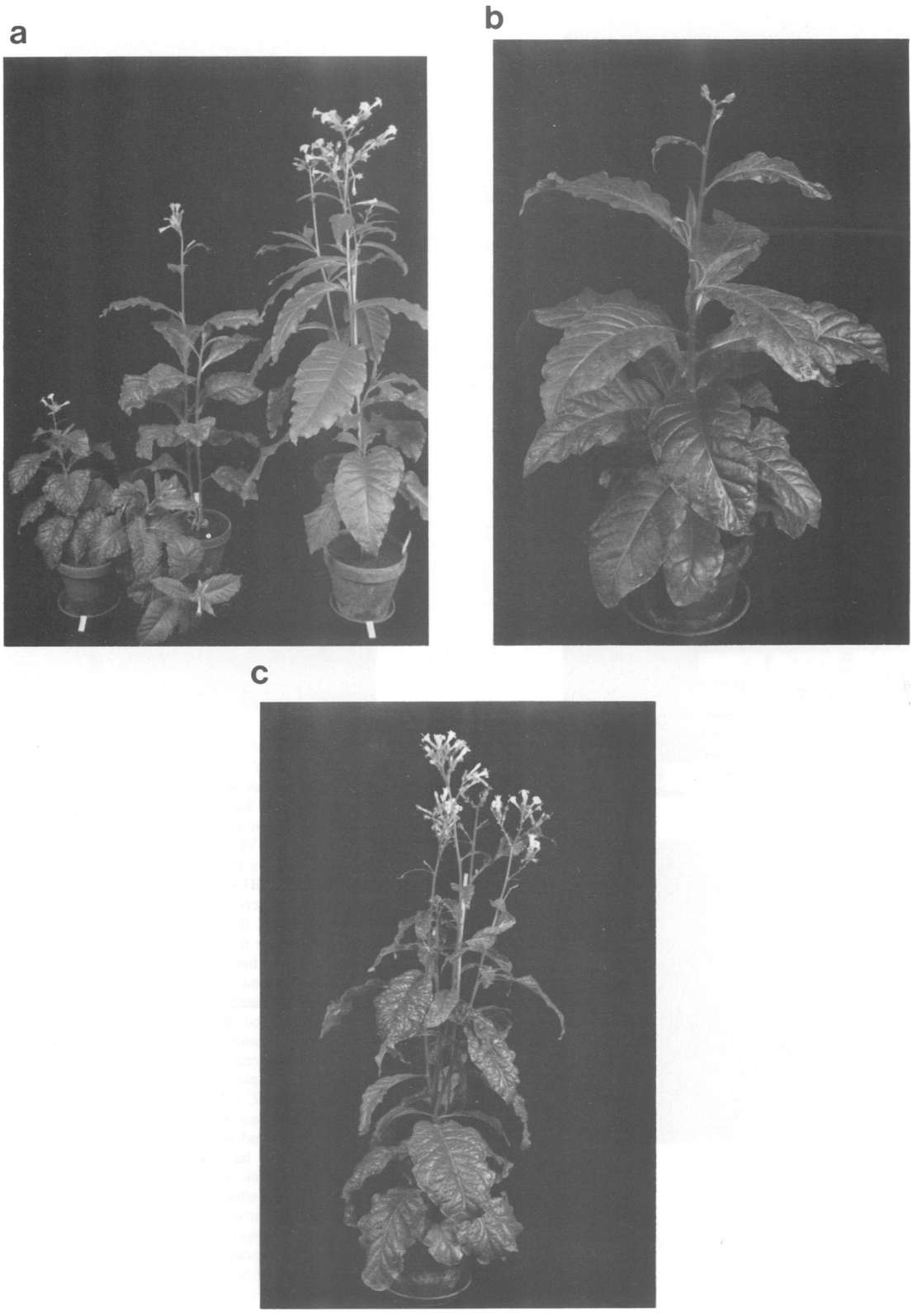
New abnormalities of growth are generated by altering the expression of rol B or C genes. Rol B and C gene products have different biological effects

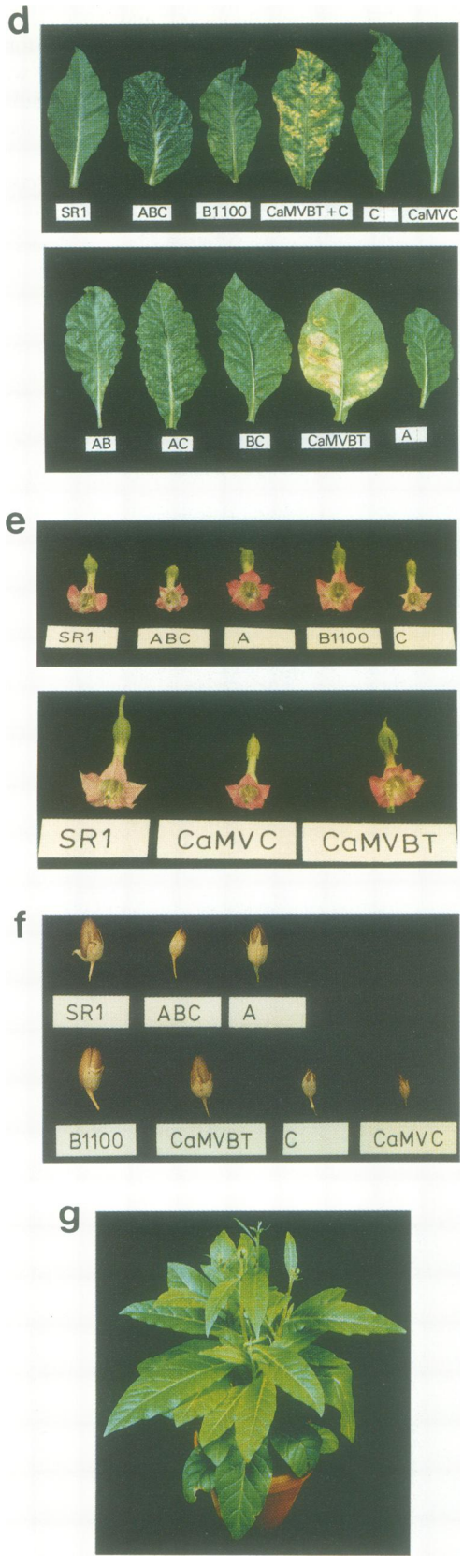
A different phenotype to that observed in *rol C* plants was displayed by tobacco plants transgenic for the CaMVC chimaeric gene, where the *rol C* coding region is under the control of the CaMV 35S promoter (Figure 1). These transgenic plants (Figure 2g) have a dwarf and bushy phenotype due to decreased internode length and an increased number of shoots and leaves. CaMVC flowers are tiny (Figure 2e) and the leaves are small and lanceolate (Figure 2d). Pollen production is severely impaired and consequently CaMVC plants are male sterile. Female fertility is reduced, though not abolished. These data were obtained from the analysis of 27 plants from 16 independent clones.

These abnormal phenotypes were dominant as was shown by analysing the offspring of crosses in which CaMVC transgenic plants were pollinated by a wild-type SR1 plant. For example, 73 out of 162 offspring plantlets of clone CaMVC1-VIII crossed with SR1 displayed the dwarf and male sterile phenotype. Similarly in the offspring of clone CaMVC1-I 23 out of 85 plantlets showed the altered phenotype. Northern blot analysis performed on the poly(A)$^+$ RNA extracted from transgenic plants expressing either the wild-type or the CaMV 35S driven *rol C* gene

(Figure 4, compare lanes 1–3 with lanes 5–7) shows that the *rol C* gene driven by the CaMV 35S promoter is strongly expressed in leaves, whereas the original *rol C* gene is expressed at a low level in leaves. This was also confirmed by the analysis of the expression in transgenic plants of a chimaeric gene consisting of the β-glucuronidase coding region (Jefferson *et al.*, 1987) positioned under the control of the *rol C* promoter (T.Schmülling *et al.*, in preparation). We conclude that altering the level and specificity of expression of the *rol C* coding region is responsible for the reduction in internode length and apical dominance which results from an increased number of shoots with small and lanceolate leaves, and gives a bushy appearance to tobacco plants.

The CaMVBT chimaeric gene, where the *rol B* coding region is under the control of the CaMV 35S promoter





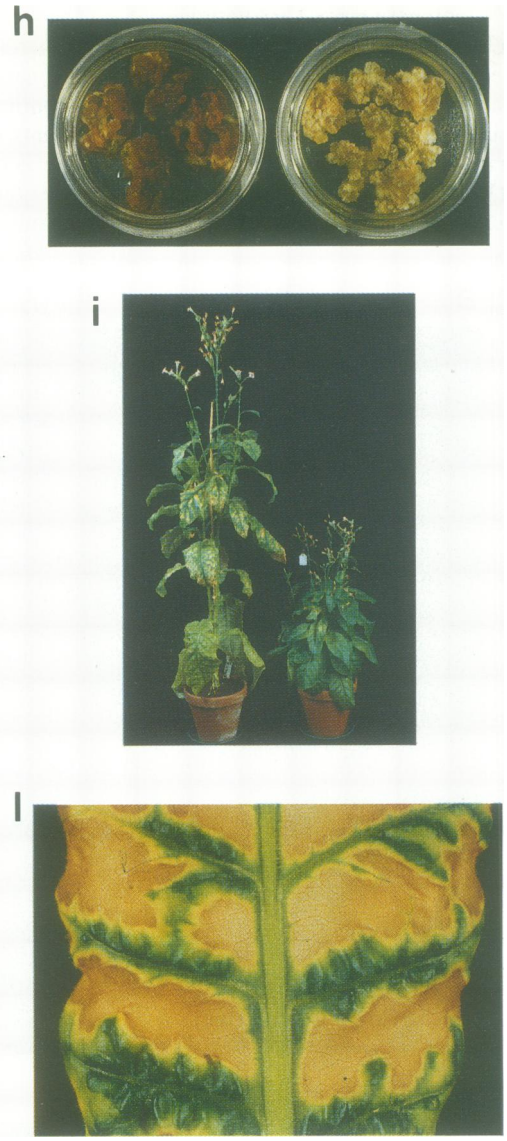


Fig. 2. (**a**) Comparison of a wild-type SR1 tobacco plant (right) with a *rol A, B* (middle) and *rol A, B, C* (left) transgenic plants. (**b**) Tobacco plant transgenic for the *rol B* and *C* genes. (**c**) *rol A, C* transgenic plant. (**d**) Comparison of leaves from wild-type SR1 tobacco plants and from plants transgenic for pPCV002-ABC, -B1100, -CaMVBT+C, -C, -CaMVC, -AB, -AC, -BC, -CaMVBT and -A. (**e**) Comparison of flowers from wild-type SR1 plant and from plants transgenic for ABC, A, B1100, C, CaMVC and CaMVBT. *Rol A* and *rol B* transgenics have increased flower size, while *rol C* plants have smaller flowers. CaMVC flowers are very small and male sterile. CaMVBT flowers, where the same promoter controls the expression of the *rol B* coding region, have protruding stigma and reduced length of the stamen. (**f**) Comparison of seed capsules from wild-type and transgenic plants as indicated. While the capsule size of *rol A* and *rol B* transgenics is not altered, the seed capsules of *rol A, B, C* and *rol C* transgenics are significantly reduced. CaMVC seed capsules are even smaller. All these seed capsules were obtained by cross-pollination of female recipient with SR1 pollen. (**g**) Tobacco plant transgenic for the CaMVC chimaeric gene. CaMVC transgenics have decreased apical dominance, resulting in a dwarf with increased numbers of side shoots and leaves which are lighter green and lanceolate. Inflorescences have small flowers which are male sterile. (**h**) Tobacco calli transgenic fo the CaMVBT chimaeric gene (left) are necrotic. At the right a callus transformed wtih the pPCV002 vector by itself is shown as control. (**i**) Tobacco plants transgenic for the CaMVBT+C gene combination. Two types of growth habit are shown: a tall (left) and stunted one (right) (see also Figure 5). (**l**) Transgenic leaf displaying signs of necrosis due to the expression of the *rol B* gene under the control of the CaMV 35S promoter.

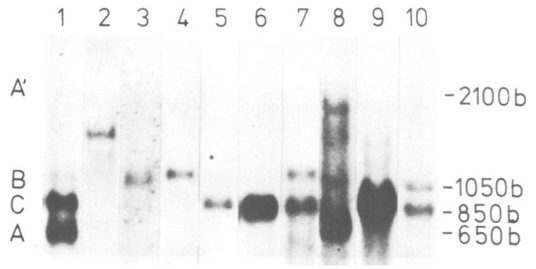


Fig. 3. Northern blot analysis of poly(A)$^+$ RNA extracted from stems of plants transgenic for the different constructions shown in Figure 1. **Lane 1**, pPCV002-ABC transgenic plant (clone ABC2-e2); **lane 2**, pPCV002-A transgenic plant (clone A5-I); **lane 3**, pPCV002-B1100 transgenic plant (clone B1100, 4-III); **lane 4**, pPCV002-CaMVBT transgenic plant (clone CaMVBT6-I); **lane 5**, pPCV002-C transgenic plant (clone C3-I); **lane 6**, pPCV002-CaMVC transgenic plant (clone CaMVC4-I); **lane 7**, pPCV002-CaMVBT+C transgenic plant (clone CaMVBT+C1-XI); **lane 8**, pPCV002-AB transgenic plant (clone AB3-V); **lane 9**, pPCV002-AC transgenic plant (clone AC2,2-I); **lane 10**, pPCV002-BC transgenic plant (clone BC10-III). the Northern blot was hybridized to a probe obtained by nick translation of the 4.3-kb *Eco*RI fragment spanning the *rol A, B* and *C* genes. **Lane 9** was rehybridized to a probe derived from the *Hin*dIII/*Pst*I fragment containing part of the *rol A* coding region to identify *rol A*-specific transcripts (data not shown). Each lane contains 3 μg of poly(A)$^+$ RNA, except for **lanes 3, 8** and **9** which contain 8 μg of poly(A)$^+$ RNA.

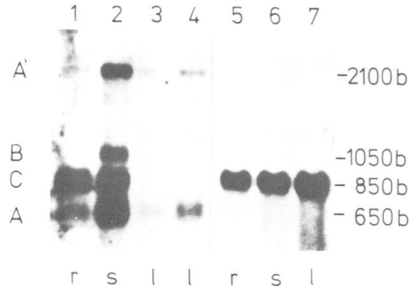


Fig. 4. Northern blot analysis of poly(A)$^+$ RNA extracted from roots (r), stems (s) and leaves (l) of a plant transgenic for the *rol A, B* and *C* genes (clone ABC2q-I; **lanes 1–4**) and of a plant transgenic for the CaMVC chimaeric gene (clone CaMVC2-II; **lanes 5–7**). Each lane contains ~3 μg of poly(A)$^+$, except for **lane 4** where 7 μg were loaded. The probe was derived by nick translation of the *Eco*RI fragment spanning the *rol A, B* and *C* genes. *Rol A, B, C* transgenics express the *rol C* gene in an organ-specific fashion, while in CaMVC plants its expression is constitutive.

(Figure 1), was shown to be less efficient in root induction than the wild-type *rol B* gene (Spena *et al.*, 1987). Calli derived from CaMVBT induced roots showed necrosis (Figure 2h). Indeed out of 26 kanamycin-resistant calli obtained from CaMVBT roots, 14 were so severely affected that they died. Moreover calli transgenic for the CaMVBT chimaeric gene (i.e. pPCV002-CaMVBT, -CaMVBT+C) were recalcitrant to the generation of shoots (see Materials and methods). The most remarkable feature of tobacco plants transgenic for the CaMVBT chimaeric gene is leaf necrosis (Figure 2d and l). The necrotic process is initiated in the leaf tissue between veins and then expands to the whole leaf. Other traits displayed by CaMVBT transgenics are heterostyly and round-edged leaves. These data were obtained on eight plants from two independent transformation events. Offspring obtained by pollinating a CaMVBT transgenic plant with pollen from a wild-type SR1 plant show the same

Fig. 5. Northern blot analysis of poly(A)$^+$ RNA extracted from roots (r), stems (s) and leaves (l) of independent CaMVBT+C transgenic plants. The two types of plants, tall and stunted, differ in the ratio between *rol C* and *B* transcripts. Compare **lanes 2, 4** and **5** with **lanes 6–9.**

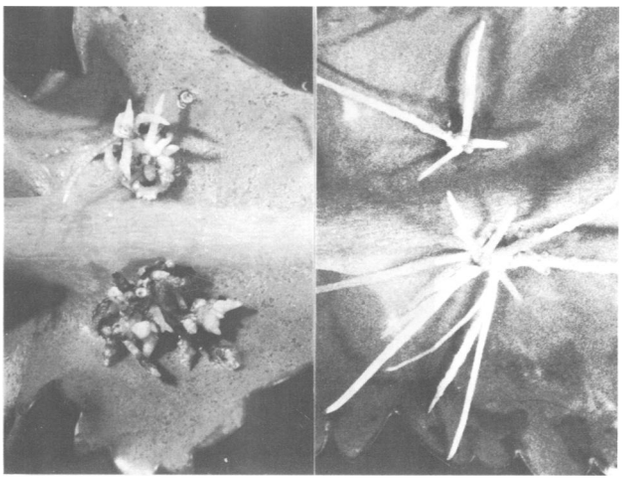


Fig. 6. Comparison of roots elicited on kalanchoe leaves by inoculation with *A.tumefaciens* strains containing the *rol A, B* and *C* (left) or the *rol B* (right) genes. Inoculations were performed as previously described (Spena *et al.*, 1987).

traits. Northern blot analysis performed on poly(A)$^+$ RNA extracted from transgenic plants shows that the CaMVBT chimaeric gene is expressed in leaves at a level higher than that observed in plants transgenic for the wild-type *rol B* gene (compare Figure 4, lanes 3 and 4 with Figure 5, lane 3). A new phenotype was thus obtained by altering the expression of the *rol B* gene.

Also plants transgenic for combinations of the CaMVBT chimaeric gene and the *rol C* gene (CaMVBT+C, Figure 1) show leaf necrosis (Figure 2d and i) typical of CaMVBT transgenic plants, whilst flower morphology is similar to that displayed by *rol C* transgenic plants (not shown). Some transgenic CaMVBT+C plants have an increased number of shoots and leaves and in these plants the necrotic process is less pronounced (Figure 2i). Northern blot analysis of poly(A)$^+$ RNA extracted from stems of the two types of CaMVBT+C transgenic plants indicates that plants which are stunted and have an increased number of shoots and less pronounced leaf necrosis express the CaMVBT gene at a level lower than *rol C* and, consequently, they have an increased C/B transcript ratio (Figure 5).

Rol-induced tobacco roots are all transgenic

Roots induced by inoculation of carrot discs with *A. rhizogenes* are usually transformed (Chilton *et al.*, 1982). However, in cucumber some transgenic plants, regenerated from roots induced by inoculation of inverted hypocotyl

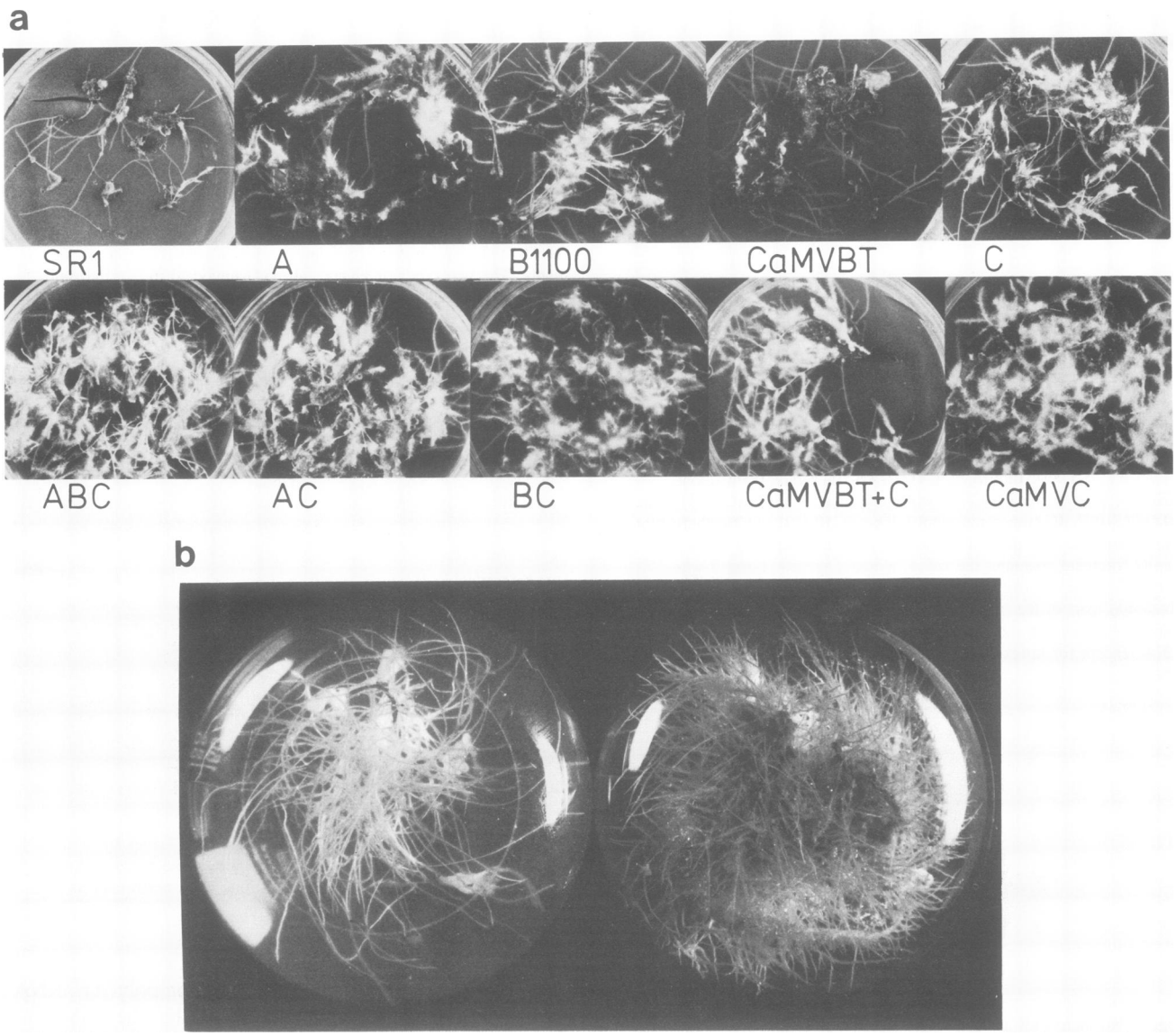


Fig. 7. (**a**) Comparison of *in vitro* root growth of roots transgenic for different *rol* genes and different combinations of the *rol* genes on solid MS medium. (**b**) *In vitro* root cultures. Comparison of growth of roots from a wild-type SR1 tobacco plant (left) with roots from a CaMVC transgenic plant cultivated in the dark in hormone-free liquid medium (MS) for 3 weeks.

sections with *A.rhizogenes*, did not contain DNA from the Ri plasmid (Trulson *et al.*, 1986). Therefore we have tested whether *rol*-induced roots (e.g. pPCV002-ABC, pPCV002-B1100, pPCV002-AC; Figure 1) were able to grow on MS medium supplemented with kanamycin (see Materials and methods for details) as a consequence of harbouring the neomycin phosphotransferase II gene linked on the same T-DNA vector as the *rol* genes. A total of 17 individual *rol*-induced roots (58 *rol ABC*, 55 *rol B* and 58 *rol AC*) were tested and 159 were clearly able to grow on medium supplemented with kanamycin sulphate. Twelve roots (seven *rol B* and five *rol AC*) gave a somewhat ambiguous result, so their transformed state was also checked by *in situ* assays for *Npt*II activity (data not shown), and/or for the presence of the genes in the plant genome by Southern blotting (data not shown). The results of these analyses indicate that these roots were also transformed.

Growth properties of roots transgenic for the rol genes

Kalanchoe leaves were transformed with our constructions in order to estimate their capacity to induce root formation (Spena *et al.*, 1987). The results indicated that only the *rol B* locus was able, by itself, to induce root formation of kalanchoe leaves, while both *rol A* and *C* were able to influence root formation only if delivered in combination with the *rol B* locus. However the growth properties of the different *rol*-induced roots are distinct. Figure 6 shows that *rol ABC*-induced kalanchoe roots are curling whilst *rol B*-induced roots grow straight away from the leaf surface. Similarly, *rol AB* kalanchoe roots are curling, whilst *rol BC* roots are straight (not shown).

In vitro cultures of tobacco roots transgenic for different *rol* loci or their combinations, on hormone-free MS medium, show that the different *rol* loci have distinct effects on root

Table I. Phenotypic traits of *rol* transgenic tobacco plants

Transgenic plant	Phenotypic traits
pPCV002-ABC	'hairy-root syndrome', i.e. reduced apical dominance, wrinkled and epinastic leaves, reduced internodal distance, reduced seed production, small flowers
pPCV002-A	wrinkled leaves, condensed inflorescences, bigger flower size, reduced internodal distance
pPCV002-B1100	altered leaf and flower morphology, heterostyly, increased formation of adventitious roots on stems
pPCV002-CaMVBT	round-edged and necrotic leaves, heterostyly
pPCV002-C	reduced apical dominance and internodal distance, altered leaf morphology, small flowers, reduced seed production
pPCV002-CaMVC	pale green and lanceolated leaves, drastically reduced apical dominance and internodal length, very small, male sterile flowers

growth (Figure 7a and b). Tobacco roots transgenic for single *rol* loci grew better than untransformed roots, and *rol C* transgenic roots branched more frequently than *rol A* or *B* roots. However, combinations of the *rol C* gene with *rol A* and/or *rol B* (i.e. pPCV002-ABC, pPCV002-BC and pPCV002-AC; Figure 1) grew more vigorously (Figure 7a), with *rol B* having a stronger effect than *rol A*. Combinations of *rol A* and *B* (pPCV002-AB; Figure 1) did not elicit much better root growth than single constructions. Overexpression of the *rol C* gene (CaMVC) led to increased root growth (Figure 7a and b), while roots transgenic for the CaMVBT chimaeric gene grew less vigorously than roots transgenic for the wild-type *rol B* gene (pPCV002-B1100, pPCV002-B300). However, when the CaMVBT chimaeric gene was combined with the *rol C* gene (pPCV002-CaMVBT+C), transgenic roots grew fast, highly branched and plagiotropic (Figure 7a). Growth of transgenic roots on solid medium was in all cases accompanied by slight formation of callus. Roots transgenic for CaMVBT had a more pronounced tendency to form callus on solid medium.

Discussion

Transgenic plants harbouring a set of genes derived from the transferable T-DNA of Ri plasmids of *A.rhizogenes* show a number of morphological abnormalities known as the hr syndrome (Tepfer, 1984; see Introduction). The full hr syndrome is established only in transgenic plants expressing the *rol A, B* and *C* loci of the Tl-DNA of Ri plasmid A4 (Jouanin *et al.*, 1987; Spena *et al.*, 1987), whereas plants transgenic for single *rol* genes do not show all the phenotypic traits of the hr syndrome, but each of them elicits distinct and specific growth abnormalities which correlate with expression of defined *rol* genes (Figures 2 and 3). The analysis of plants transgenic for different combinations of these three *rol* loci (Figure 1) has shown that they display phenotypic alterations typical for a given combination of *rol* genes. Consequently in the hr phenomenon, genes which by themselves are able to induce distinct developmental effects in transgenic plants must interact to establish the phenotypic alterations of the hr syndrome (see Table I).

When transcription of *rol B* or *rol C* is driven by the 35S CaMV promoter, the growth-regulating functions of these genes become more clearly visible, probably as a consequence of a systemic high level of expression (Odell *et al.*, 1985). It becomes apparent that the effects of these two genes are very different and that in fact they counteract or balance each other. Whereas systemic expression of *rol C* is responsible for a general 'juvenilization' of tobacco plants, high levels of expression of *rol B* apparently lead to cellular death (necrosis) both in callus and in leaves of young plants. That both effects balance one another can best be illustrated by the two plants shown in Figure 2i. Both plants contain the same set of genes, i.e. *rol B* driven by the 35S promoter plus *rol C* under its own promoter (CaMVBT+C). Probably due to position effects, the transcription of *rol B* is relatively low in the stunted, bushy plants and is high in the tall but very necrotic plants, whereas *rol C* expression is similar in both types of plants (Figure 5). Taken together our data show that *rol C* expression is responsible for stunting and for an increased number of side shoots as well as for reduction in flower size and male fertility; *rol A* expression, on the other hand, can be correlated with the formation of wrinkled leaves, condensed inflorescence and larger flower size. The product of *rol B* appears to stimulate adventitious root formation and to affect flower and leaf morphology and its overexpression in leaves leads to early necrosis.

The *rol* genes have distinct and synergistic activities involved both in root formation and growth (Cardarelli *et al.*, 1987a; Vilaine *et al.*, 1987; Spena *et al.*, 1987). Analysis of root induction on kalanchoe leaves by different combinations of *rol* genes (Spena *et al.*, 1987) has indicated that only the *rol B* locus was able to induce root formation by itself, whilst the *rol A* and *C* loci were not able by themselves to induce root formation. However, when combined with the *rol B* locus these genes increased root formation. Roots induced on kalanchoe leaves by *rol AB* or *rol ABC* constructions were curling, while *rol B* or *rol BC* roots grew straight from the leaf surface. A similar observation was reported by White and collaborators in their analysis of insertional mutants in the A4 Ri-plasmid (White *et al.*, 1985). These phenomena, indicative of distinct biological effects on root growth of the *rol A* and *C* loci, are confirmed and extended by the analysis of growth habits of transgenic tobacco root cultures. Indeed the *rol B* locus is the most efficient of the *rol A, B* and *C* loci in inducing root formation (Spena *et al.*, 1987); however, fast and branched root growth requires the presence of the *rol C* locus. Conversely the *rol C* gene product has a rather weak capacity to induce root formation; however, *rol C*, and even more pronounced CaMVC transgenic roots, are highly branched and show fast and plagiotropic growth (Figure 7a and b).

Taken together our results indicate that a combination of the *rol B* and *C* genes (or *rol A* and *C*), is necessary to achieve substantial improvement of root growth in *in vitro* root cultures. This is in contrast to a previous report (Vilaine *et al.*, 1987), where roots transgenic for *rol A* or *rol A, B, C* were said to 'display the same phenotype and growth capacity in axenic culture on hormone free medium'.

Arguments have been proposed to explain the hr disease as resulting from an increased sensitivity to auxin caused by a *rol*-determined modification of the reception–

transduction system of the auxin signal (Cardarelli *et al.*, 1987b; Shen *et al.*, 1988). On the other hand, it was suggested that 'one function of the *rol* genes involves alterations in cytokinin synthesis or a cytokinin-like effect' (White and Sinkar, 1987). The biological effects of the *rol B* gene product, as described in this paper, are indeed reminiscent of auxin-mediated effects (i.e. root initiation, leaf burning) whereas the action of the *rol C* gene is suggestive of a cytokinin activity, if one neglects the fact that root stimulation rather than root inhibition is observed. However, the activities of the *rol* genes cannot be explained by models invoking synthesis of transported phytohormones like indoleacetic acid or isopentenyladenosin because the action of these genes is, at least as far as root formation is concerned, limited to transformed cells (Chilton *et al.*, 1982; Bercetche *et al.*, 1987; this article). Moreover, plants transgenic for auxin-synthesizing genes (Klee *et al.*, 1987), or plants transgenic for the *ipt* gene (Barker *et al.*, 1983) either under the control of its own promoter or under the control of several heterologous promoters, do not resemble *rol* transgenic plants (our unpublished results). *Rol B*-mediated auxin-like effects can be explained by an alteration of the reception–transduction system of the auxin signal (Cardarelli *et al.*, 1987b; Shen *et al.*, 1988). This could result directly from a modification of the auxin signal transduction system, but other explanations are equally conceivable such as a *rol B*-mediated synthesis of a substance with auxin-like activity but which is not transported in plant tissues. For example, phenylacetic acid is an auxin which is not transported within the plant (Wightman and Lighty, 1982; Morris and Johnson, 1987). The result, in all cases, would be an increased auxin effect without an alteration of IAA concentration. The phenotypic alterations displayed by CaMVC tobacco plants are somewhat similar to the developmental phenotype of the auxin-resistant mutants (axr-1) of *Arabidopsis thaliana* (Estelle and Somerville, 1987). The phenotypic alterations of the axr-1 mutants result in the formation of a short bush suggesting a reduction in apical dominance. Moreover the flowers on the mutant are smaller than the wild-type and most of the mutants are self-sterile. Since the axr-1 mutants are recessive, they probably result in a loss of function, the CaMVC gene on the other hand is dominant and consequently its effects are likely to result from a gain of function such as auxin-modifying or auxin-inactivating activity, or synthesis of an auxin-antagonizing agent such as a cell autonomous cytokinin.

Materials and methods

Bacterial strains and cultures

Bacterial strains and cultures were previously described (Spena *et al.*, 1987).

Construction of plasmids

Standard techniques were used for the construction of recombinant DNA plasmids (Maniatis *et al.*, 1982). Constructions shown in Figure 1 were described previously (Spena *et al.*, 1987) with the exception of pPCV002-BC, which resulted from the subcloning of a 3581-bp *SmaI*/*EcoRI* fragment into the binary vector pPCV002 (Koncz and Schell, 1986).

All constructions were transferred to *Escherichia coli* strain SM10 and then mobilized to *A.tumefaciens* strain GV3101 as described (Koncz and Schell, 1986).

Plant tissue culture and transformation

Root induction on *Kalanchoe diagremontiana* leaves and on leaf discs of sterile shoot cultures of *Nicotiana tabacum* cv. Petit Havana SR1 (Nagy and Maliga, 1976) were done as described (Spena *et al.*, 1987).

To test whether *rol*-induced roots represented clones of transformed cells, leaf discs were incubated with the different constructions and then kept on MS medium (Murashige and Skoog, 1962) without kanamycin selection. After 4 weeks roots were harvested and split into two parts. Only one of the two explants was exposed to kanamycin selection (50 mg/l) during callus induction on MS medium supplemented with 0.6 mg/l of naphthyl acetic acid (NAA) and 0.2 mg/l of kinetin. Transgenic calli were derived from *rol*-induced roots placed on MS medium supplemented with 0.6 mg/l of NAA, 0.2 mg/l of kinetin and 50 mg/l of kanamycin sulphate, and so transgenic plants were usually raised from these root-derived transgenic calli on MS medium containing 0.5 mg/l benzylaminopurine (BAP) and 0.1 mg/l NAA. However, callus clones transgenic for the CaMVBT gene (i.e. pPCV002-CaMVBT, -CaMVBT+C) were very difficult to regenerate. Therefore in these cases shoot formation was obtained after 10–12 weeks of callus growth on MS medium supplemented with 7.5 mg/l isopentenyladenosine and 0.1 mg/l chlorophenoxyacetic acid (Firoozabady, 1986). Alternatively leaf discs were transformed as described (Horsch *et al.*, 1984). Shoots were rooted on hormone-free MS medium under kanamycin selection. Root cultures were established either on solid or in liquid MS medium.

DNA and RNA analysis

DNA and RNA were extracted from transgenic tissues as described (Taylor and Powell, 1983). Poly(A)$^+$ RNA was selected by chromatography on oligo(dT) cellulose according to the manufacturer (Boehringer Mannheim), and then separated on 1.5% agarose–formaldehyde gel, transferred to nylon membranes and hybridized to radioactive probes. Purified DNA fragments were labelled using the BRL nick-translation kit. Hybridization of Northern blots was carried out in 1 M NaCl, 1% SDS, 10% dextran sulphate, 100 μg/ml of herring sperm DNA at 65°C. Washes were done in 2 × SSPE, 1% SDS at room temperature and then in 0.2 × SSPE, 1% SDS at 65°C. DNA blots were performed according to established methods (Maniatis *et al.*, 1982).

Assays for activity of neomycin phosphotransferase II

In situ assays for the activity of the neomycin phosphotransferase II enzyme were done as previously described (Spena *et al.*, 1985).

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References

Ackermann,C. (1977) *Plant Sci. Lett.*, **8**, 23–30.
Akiyoshi,D.E., Klee,H., Amasino,R.M., Nester,E.W. and Gordon,M.P. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5994–5998.
Barker,R.F., Idler,K.B., Thompson,D.V. and Kemp,J.D. (1983) *Plant. Mol. Biol.*, **2**, 335–350.
Barry,G.F., Rogers,S.G., Fraley,R.T. and Brand,L. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 4776–4780.
Bercetche,J., Chriqui,D., Adam,S. and David,C. (1987) *Plant Sci.*, **52**, 195–210.
Birot,A.-M., Bouchez,D., Casse-Delbart,F., Durand-Tardif,M., Jouanin,L., Pautot,V., Robaglia,C., Tepfer,D., Tepfer,M., Tourneur,J. and Vilaine, F. (1987) *Plant. Physiol. Biochem.*, **25**, 323–335.
Buchmann,I., Marner,F.J., Schröder,G., Waffenschmidt,S. and Schröder,J. (1985) *EMBO J.*, **4**, 855–859.
Cardarelli,M., Spano,L., Mariotti,D., Mauro,M.L., Van Sluys,M.A. and Costantino,P. (1987a) *Mol. Gen. Genet.*, **208**, 457–463.
Cardarelli,M., Mariotti,D., Pomponi,M., Spano,L., Capone,I. and Costantino,P. (1987b) *Mol. Gen. Genet.*, **209**, 475–480.
Chilton,M.D., Tepfer,D.A., Petit,A., David,C., Casse-Delbart,F. and Tempe,J. (1982) *Nature*, **295**, 432–434.
Comai,L. and Kosuge,T. (1980) *J. Bacteriol.*, **143**, 950–957.
Dekhujzen,H.M. (1976) In Heitefuss,R. and Williams,P.H. (eds), *Encyclopedia of Plant Physiology*. Springer-Verlag, Berlin, Vol. 4, pp. 526–559.
Durand-Tardif,M., Broglie,R., Slightom,J. and Tepfer,D. (1985) *J. Mol. Biol.*, **186**, 557–564.
Estelle,M.A. and Somerville,C. (1987) *Mol. Gen. Genet.*, **206**, 200–206.
Firoozabady,E. (1986) *Plant. Sci.*, **46**, 127–131.
Horsch,R.B., Fraley,R.T., Rogers,S.G., Sanders,P.R., Lloyd,A. and Hoffman,N. (1984) *Science*, **223**, 496–498.

Inze,D., Follin,A., Van Lijsebettens,M., Simoens,C., Genetello,C., Van Montagu,M. and Schell,J. (1984) *Mol. Gen. Genet.*, **194**, 265–274.
Jefferson,R.A., Kavanagh,T.A. and Bevan,M.W. (1987) *EMBO J.*, **6**, 3901–3907.
Jouanin,L., Vilaine,F., Tourneur,J., Tourneur,C., Pautot,V., Muller,J.F. and Caboche,M. (1987) *Plant. Sci.*, **53**, 53–63.
Klee,H.J., Horsch,R.B., Hinchee,M.A., Hein,M.B. and Hoffmann,N.L. (1987) *Genes Dev.*, **1**, 86–96.
Koncz,C. and Schell,J. (1986) *Mol. Gen. Genet.*, **204**, 383–396.
Kurosawa,E. (1926) *Trans. Natl. Hist. Soc. Formosa*, **16**, 213–227.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Morris,D.A. and Johnson,C.F. (1987) *Planta*, **172**, 408–416.
Murai,N., Skoog,F., Doyle,M.E. and Hanson,R.S. (1980) *Proc. Natl. Acad. Sci. USA*, **77**, 619–623.
Murashige,T. and Skoog,F. (1962) *Physiol. Plant.*, **15**, 473–496.
Nagy,J.I. and Maliga,P. (1976) *Z. Pflanzenphysiol.*, **78**, 453–455.
Odell,J.T., Nagy,F. and Chua,N.-H. (1985) *Nature*, **313**, 810–812.
Pegg,G.F. (1976) In Heitefuss,R. and Williams,P.H. (eds), *Encyclopedia of Plant Physiology.* Springer-Verlag, Berlin, Vol. 4, pp. 582–616.
Schell,J. (1986) In Gustafson,P.J., Stebbins,G.L. and Ayala,F.J. (eds), *Genetics, Development and Evolution.* Plenum, Washington, DC, pp. 343–353.
Schröder,J. (1987) In Kosuge,T. and Nester,E.W. (eds), *Plant–Microbe Interactions.* Vol. III, pp. 40–63.
Schröder,G., Waffenschmidt,S., Weiler,E.W. and Schröder,J. (1984) *Eur. J. Biochem.*, **138**, 387–391.
Shen,W.H., Petit,A., Guern,J. and Tempe,J. (1988) *Proc. Natl. Acad. Sci. USA*, in press.
Slightom,J.L., Durand-Tardif,M., Joanin,L. and Tepfer,D. (1986) *J. Biol. Chem.*, **261**, 108–121.
Smidt,M. and Kosuge,T. (1978) *Physiol. Plant Pathol.*, **13**, 203–214.
Spena,A., Hain,R., Ziervogel,U., Saedler,H. and Schell,J. (1985) *EMBO J.*, **4**, 2739–2743.
Spena,A., Schmülling,T., Koncz,C. and Schell,J. (1987) *EMBO J.*, **6**, 3891–3899.
Taylor,B.H. and Powell,A. (1983) *BRL Focus*, **4**, 4–6.
Tepfer,D. (1984) *Cell*, **37**, 959–967.
Thimann,K.V. and Sachs,T. (1966) *Am. J. Bot.*, **53**, 731–739.
Thomashow,L., Reeves,S. and Thomashow,M. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 5071–5075.
Trulson,A.J., Simpson,R.B. and Shahin,E.A. (1986) *Theor. Appl. Genet.*, **73**, 11–15.
Vilaine,F., Charbonnier,C. and Casse-Delbart,F. (1987) *Mol. Gen. Genet.*, **210**, 111–115.
White,F.F. and Sinkar,V.P. (1987) In Hohn,T. and Schell,J. (eds), *Plant DNA Infectious Agents.* Springer-Verlag, Wien, pp. 149–178.
White,F.F., Taylor,B.H., Huffman,G.A., Gordon,M.P. and Nester,E.W. (1985) *J. Bacteriol.*, **164**, 33–44.
Wightman,F. and Lighty,D.L. (1982) *Physiol. Plant*, **55**, 17–24.
Yamada,T., Palm,C.J., Brooks,B. and Kosuge,T. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 6522–6526.

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