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Molecular basis for novel root phenotypes induced by *Agrobacterium rhizogenes* A4 on cucumber

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Abstract

We have used the wild-type *Agrobacterium rhizogenes* strain A4 to induce roots on cucumber stem explants. Cultures of transformed roots obtained that were capable of hormone-autonomous growth could be grouped in three phenotypic classes. Of particular interest were extremely thick roots of a type not previously described. Characterization of the transferred DNA and of the expression of the corresponding genes allowed us to determine that the genes *rolABC* of the TL region of the Ri plasmid are sufficient to induce thin roots similar to those observed in other species, while the *aux* genes of the TR region are sufficient to induce thick roots. Among clones bearing the *aux* genes, there was a correlation between level of expression of *aux2* and root phenotype.

Introduction

Agrobacterium rhizogenes induces the differentiation of genetically transformed roots on many dicotyledonous plants. Root initiation and the *in vitro* growth characteristics of transformed roots are due to transfer of Ri plasmid genes to plant cells and their expression therein [7, 22, 29; for review see 1]. Two primary groups of pRi genes are involved in the root induction process, the *rol* genes located in the TL region and the *aux* genes of the TR region [30]. Interactions among genes may also be important as it has been shown, for instance, that interactions between auxin biosynthesis due to the *aux* genes and expression of the *rolB* gene play a particularly critical role in root induction on carrot root discs [6]. However, the distinctive phenotype of Ri-transformed roots grown *in vitro*, which are capable of long-term hormone-autonomous growth, seems to be essentially attributable to the *rol* genes of the TL-DNA [28]. In tobacco, for instance, the *rolA* gene is sufficient to induce transformed roots capable of vigorous hormone-autonomous growth *in vitro* [28].

We have induced genetically transformed roots on cucumber stem explants. *A. rhizogenes* strain A4 induced roots, some of which were essentially as described for transformed roots of other species (rapid elongation, moderate branching, abundant root hairs), whereas others were extremely thick, highly branched and without root hairs *in vitro*. Through detailed mapping of the T-DNA of transformed roots of different phenotypes, as well

as of the T-DNA transcripts, we have shown that the *aux* genes from the TR region play a key role in determining the atypical root phenotype observed. The results described here provide a further example of the role played by the host plant in determining the response to expression of Ri T-DNA genes.

Methods

Induction and culture in vitro *of transformed cucumber roots*

Genetically transformed roots were induced essentially as described previously for other species [26]. Internode segments of greenhouse-grown cucumber plants (cv. Noval) were surface-sterilized and implanted in Murashige and Skoog (MS) medium, basal end upwards. The basal end was inoculated with a fresh culture of *Agrobacterium*. Roots were removed from the explants 10–15 days later, and cultured on solid MS medium in which the nitrogen content had been reduced 80% (MS N/5), containing 250 mg/l cefotaxime. After two transfers, roots were routinely cultured at 25 °C on MS N/5 medium without antibiotics. Tests for response to naphthalene acetamide were also carried out on solid MS N/5 medium.

DNA gel blot analysis

Total DNA was extracted as described by Dellaporta *et al.* [9], followed by further purification on an isopycnic CsCl gradient. Ten μg DNA were digested with appropriate enzymes as suggested by the suppliers. After electrophoresis on 0.8% agarose gels, DNA was transferred to nylon membranes and hybridized with a probe which had been ^{32}P-labelled by random priming [13].

RNA gel blot analysis

Total RNA was isolated [8], purified [14] and analysed by electrophoresis in denaturing 1.2 M formaldehyde, 1.5% agarose gels [18]. After transfer to nylon membranes, blots were hybridized with random-primed ^{32}P-labelled probe [13].

Strand-specific RNA gel blots

The technique used was essentially as described by Boyer [4]. Denaturing RNA gels were prepared as above with 30 μg total RNA/sample. Unlabelled single-stranded pBluescript KS + and pBluescript KS −, into which the *Eco* RI-*Nru* I fragment covering ORF 10 had been cloned, was hybridized to the blot. Then ^{32}P-labelled double-stranded phagemid was hybridized to the transfer.

Results

Characteristics of transformed cucumber root cultures

The agropine-type *Agrobacterium rhizogenes* strain A4 was inoculated on cucumber stem segments *in vitro* essentially as described for other species [26]. The induced roots were decontaminated and maintained as root cultures on solid MS-N/5 medium [26]. Figure 1 shows representative root cultures, which could be grouped in three classes. Class I roots have the typical phenotype of Ri-transformed roots; they are similar to non-transformed roots, except that they have more numerous lateral roots and are capable of hormone-autonomous growth *in vitro* [25]. Classs III roots are of a type that has not been previously described; they are much thicker (several mm in diameter), elongate very slowly, are more highly branched than class I roots, and have no root hairs. Class II roots are intermediate between the other two classes. In several experiments, approximately equal proportions of root clones presenting each of the three phenotypes were obtained. The phenotype of the root cultures has been perfectly stable over several years of maintenance *in vitro*.

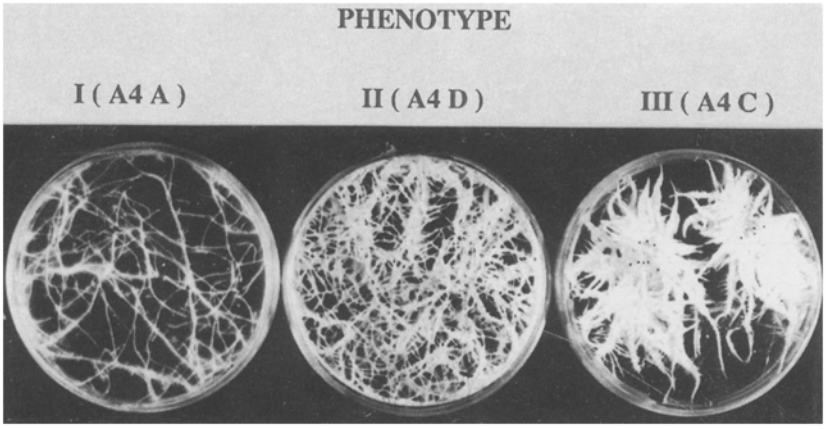

Fig. 1. Cultures of cucumber roots transformed by *Agrobacterium rhizogenes*. The root cultures obtained were grouped in three phenotype classes: (I) class I roots were similar to those obtained with other plant species; (II) class II roots were of intermediate phenotype; (III) class III roots were short, thick, highly branched and without root hairs.

DNA gel blot analysis

Molecular studies were carried out on a total of nine root clones representing the three phenotypes in order to determine which genes are responsible for the different phenotypes observed. Genomic DNA extracted from the various root clones, digested with either *Bam* HI, *Kpn* I, *Hin*d III, *Nru* I or *Nde* I, was probed with ^{32}P-labelled pLJ1 DNA, which encompasses the entire TL-region [16], in order to determine the extent of the TL-DNA. Similarly, DNA was digested with either *Cla* I or *Bal* I and probed with labelled DNA of either pLJ85 [16] or restriction fragments *Sal* I 6 or *Sal* I 8 in order to determine the extent of the TR-DNA. Figures 2 and 3 show the resulting maps of the TL-DNA and TR-DNA. From relative hybridization intensities and numbers of border fragments, it was estimated that 1–5 copies of TL-DNA and/or TR-DNA are present in the different clones. No correlation between copy number and phenotype was observed. In contrast, a fairly consistent correlation between phenotype and presence or absence of TR-DNA was observed. All root clones of phenotype II or III had TR-DNA, while TR-DNA was absent in the phenotype I roots, except for clone A4 3–4, which had full-length TR-DNA and was of phenotype I.

Transformed roots obtained with pRiA4 T-region subclones

Cucumber stem explants were inoculated with disarmed *A. tumefaciens* strains bearing a binary transformation system based on the helper plasmid pLBA4404 and pMRK62 into which fragments of the pRiA4 T-regions had been subcloned [28]. A single clone of transformed roots of phenotype I was obtained with fragment *Eco* RI 15, which bears genes *rolA*, *rolB* and *rolC*, and three clones of roots of phenotypes II and III were obtained with fragment *Sal* I 6, which bears genes *aux1* and *aux2*. A single phenotype II clone containing both sub-fragments was obtained by co-inoculation with the two corresponding strains. In all cases 2–4 copies of the transferred fragment were detected, and no correlation between copy number and phenotype was observed. These results confirm the importance of these two groups of genes in inducing transformed roots, and also show that the *aux* genes are sufficient to induce roots of the novel phenotypes obtained with the wild-type Ri plasmid.

Transcript mapping

We carried out transcript mapping of both TL- and TR-DNA genes in several transformed root

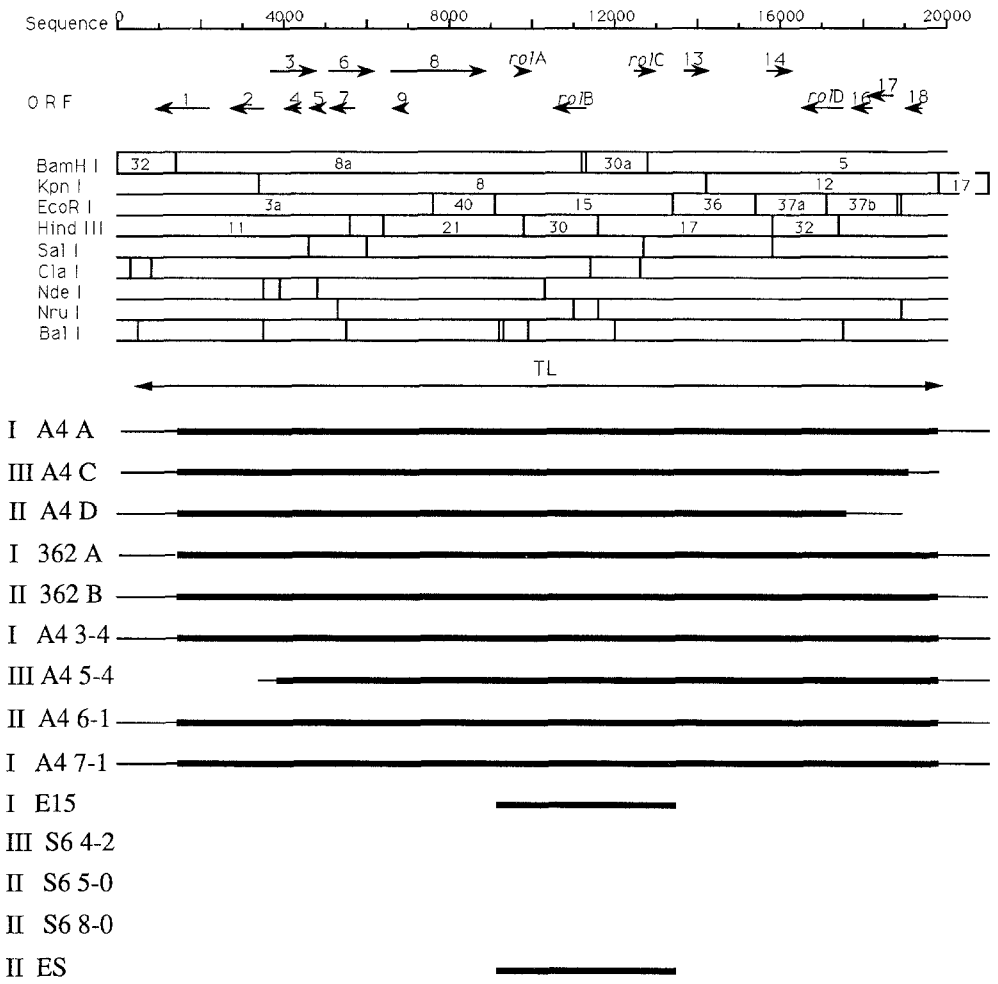

Fig. 2. Structure of TL-DNA in representative transformed root cultures. From top to bottom are shown successively: sequence coordinates, deduced open reading frames (ORFs), restriction map, usual extent of transferred DNA, extent of T-DNA determined by DNA blot hybridizations, in which internal fragments are indicated by thick lines and border fragments by thin lines. Phenotype of transformed root clones (I, II, III) is also indicated.

cultures of each of the three phenotypes. No correlation between T-DNA copy number and transcript levels was observed (not shown). If the phenotypic differences were due to differences in the level of expression of T-DNA genes, we predicted that, in particular among clones with both TL- and TR-DNA, we would find a correlation between level of expression and phenotype.

Figure 4 shows representative RNA gel blots using probes covering essentially all of the TL-DNA. In most cases, transcripts of sizes corresponding to those described in transformed tissues of other species were observed [12, 20, 24]. Three transcripts were detected in the RNA extracted from all transformants with probe E-3a. Those of 1.5 and 1.1 kb may correspond to ORFs 6 and 3 respectively, as transcripts of these sizes were observed in transformant A4 5–4, whose T-DNA does not include ORFs 1 and 2. The transcript of 2.4 kb probably corresponds to ORF 8, as it was also detected with probe H-21. A transcript of 1.5 kb also detected with this probe may correspond to ORF 6, as the 3′ noncoding region of this gene should extend into this restriction fragment. A transcript of 0.6 kb was detected with probe E-36, which should corre-

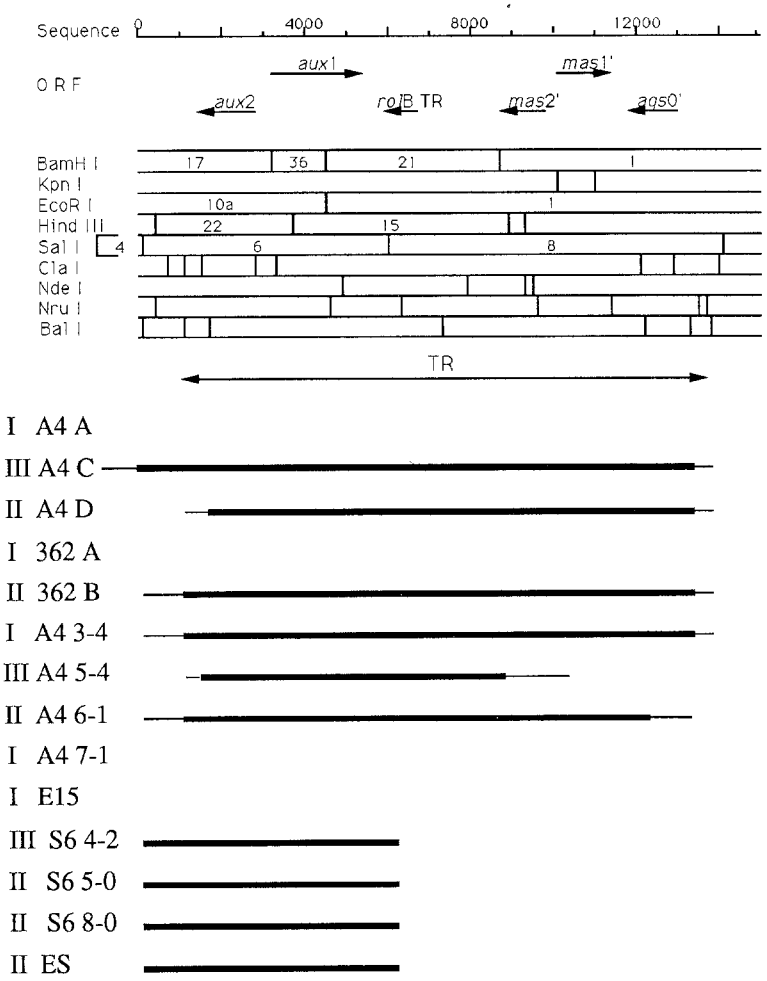

Fig. 3. Structure of TR-DNA in representative transformed root cultures. From top to bottom are shown successively: sequence coordinates, deduced open reading frames (ORFs), restriction map, usual extent of transferred DNA, extent of T-DNA determined by DNA blot hybridizations, in which internal fragments are indicated by thick lines and border fragments by thin lines. Phenotype of transformed root clones (I, II, III) is also indicated.

spond to ORF 13. The transcript of 1.3 kb detected with probe E-37ab probably corresponds to ORF 15, as the other ORFs in this region would be predicted to encode significantly shorter transcripts [21].

The central region of the TL-DNA, including ORFs 10–12, which correspond to loci *rolA*, *rolB* and *rolC*, was mapped in detail, as these genes have been shown to be particularly important in root induction [23, 28, 30]. Probe H-E hybridized with a transcript of 0.85 kb, which corresponds to the size expected from ORF 12 (*rolC*). No signal was observed with probe Nd-Nr, which should hybridize with transcripts of ORF 11 (*rolB*). Three transcripts were observed with probe E-Nd, which covers the ORF 10 (*rolA*) region. In other species, the major ORF 10 transcripts is of 0.6 kb, and a less abundant one of 2.0 kb is also often observed [20, 23]. In all clones of cucumber roots, we observed a much smaller major transcript of 0.35 kb in this region, but the 0.6 and 2.0 kb transcripts were also detected. All three transcripts were also observed with a smaller probe (Bg-Nc), which is specific for ORF 10. We also determined which strand encodes the major 0.35 kb transcript. The *Eco* RI-

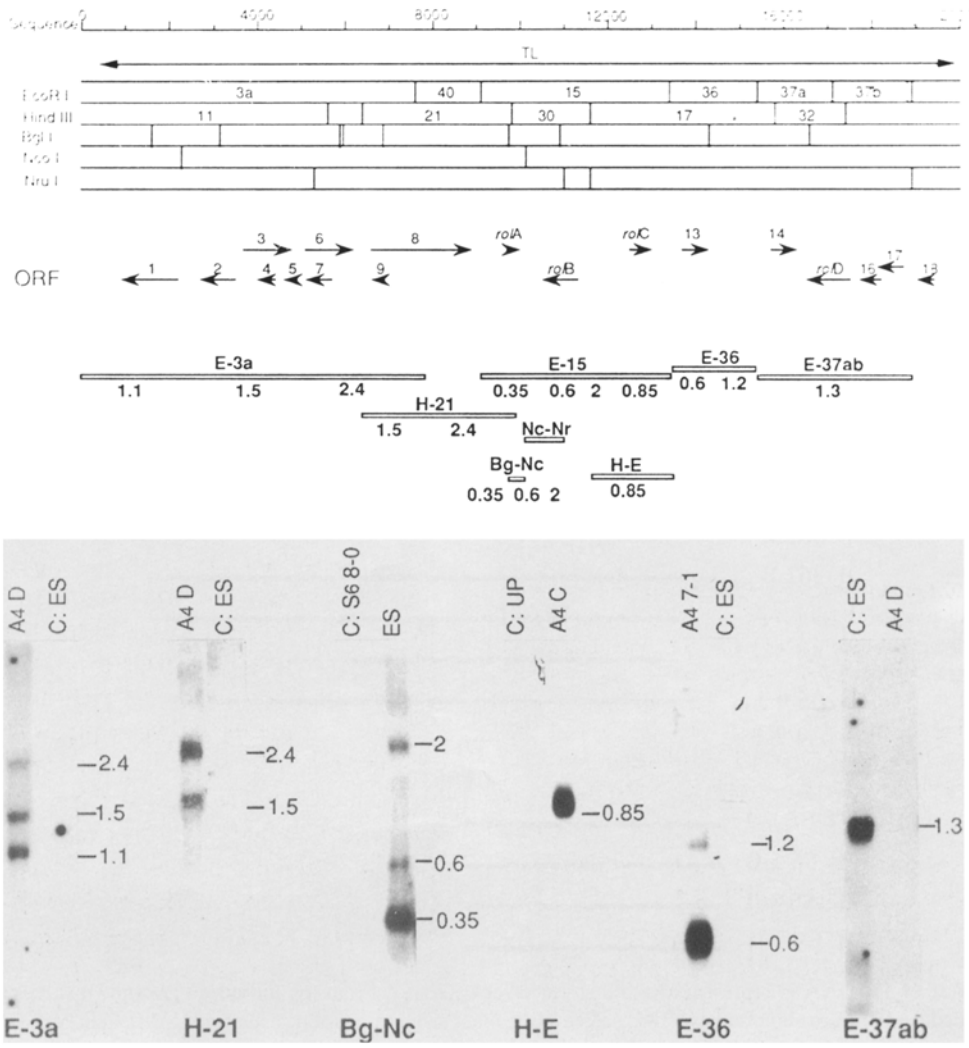

Fig. 4. Localization of TL-DNA transcripts. RNA gel blot experiments were carried out with probes covering the TL-DNA. Shown successively from top to bottom are: sequence coordinates, usual extent of TL-DNA, deduced ORFs. Probes are indicated by open boxes with probe name above and the size of transcripts observed below. Representative blots are shown for each probe, with one clone of transformed roots and one of several different controls: either RNA from leaves of non-transformed plants (C:UP) or from root clones lacking the T-DNA region probed (C:ES, C:S6 8–0).

Nco I fragment corresponding to ORF 10 was cloned in the phagemids pBluescript KS + and pBluescript KS − . Unlabelled single-stranded DNA was purified and hybridized to RNA gel transfers. Then ^{32}P-labelled phagemid was hybridized with the blots in order to detect transcripts which had bound single-stranded probe. Figure 5 shows the results obtained with the two strand-specific probes. Transcripts of 0.35 and 0.6 kb hybridized with the probe complementary to ORF 10, but did not hybridize with the probe for the other strand. The band at 1.6 kb is non-specific, as it is present on both blots, and also in control samples lacking the DNA region under study. The 2.0 kb transcript was not detected. No consistent differences were observed in TL-DNA transcripts extracted from roots of the three phenotypes.

Functions have been attributed to three groups of genes of the pRiA4 TR-DNA. Three genes

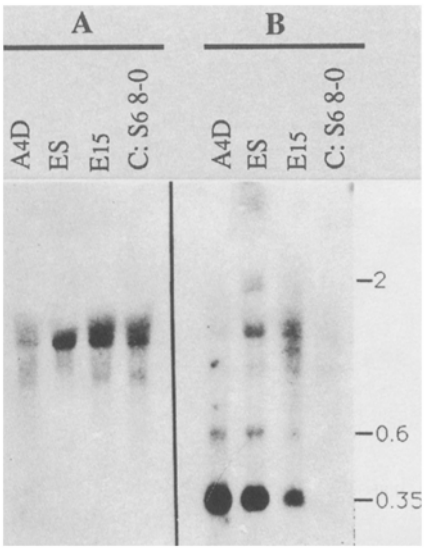

Fig. 5. Hybridization of strand-specific probes to transcripts of the ORF 10 region. RNA gel blots were first hybridized with non-labelled single-stranded DNA either corresponding to (A) or complementary to (B) ORF 10 (pBluescript KS + or pBluescript KS −, respectively, into which a fragment corresponding to ORF 10 had been cloned). Double-stranded ^{32}P-labelled vector was then hybridized to the blot in order to detect transcripts that had bound single-stranded probe.

involved in mannityl opine biosynthesis have been localized at the right of the TR-DNA [3, 15, 16], in the central region a gene homologous to the *rolB* gene, designated *rolB*-TR, has been described [3], and the genes *aux1* and *aux2*, found at the left of the TR-DNA, have been shown to be highly homologous to the pTi auxin biosynthesis genes [5]. Figure 6 shows the TR-DNA transcription map. The three genes involved in mannityl opine biosynthesis are of approximately the same size, so the 1.5 kb transcript observed with probe S-8 could be transcribed from any or all of the three genes concerned. As there is no evidence that these genes could be involved in the control of root development or growth, more detailed mapping of transcripts of the opine genes was not carried out. Probe S-8 also hybridized with a 0.6 kb transcript, which is significantly shorter than one would predict from *rolB*-TR. Indeed, a probe specific for this gene (Sl-Sc) did not hybridize with this transcript, whereas two probes corresponding to an adjacent region (Sl-Dr and Dr-Dr) did. With probe S-6, covering the *aux* genes, only a 1.6 kb transcript was observed, which presumably corresponds to *aux2*. We were unable to detect *aux1* transcripts, even using smaller probes, or with poly(A)$^+$ RNA (not shown). Among the TR-RNA genes, a correlation between higher expression of *aux2* and phenotype III was observed; expression of other genes was not related to phenotype.

Correlation between level of expression of gene expression and root phenotype

Our hypothesis at this point was that TR-DNA *aux* genes are responsible for the unique phenotypes (II and III) observed. Comparative studies of a more extensive collection of transformants bearing the *aux* genes were carried out with RNA blots probed with fragment *Sal* I 6. Figure 7 shows that among these transformants, there was a clear correlation between phenotype and level of *aux2* expression. Transcripts corresponding to *aux2* were not detected in RNA extracted from the only phenotype I clone bearing the *aux* genes (A4 3–4), but were present at moderate levels in phenotype II roots (clones A4 D, ES, A4 6–1, S6 5) and at high levels in phenotype III roots (clones A4 C, A4 5–4, S6 4–2). These results are consistent with the *aux* genes playing a critical role in determining phenotypes II and III.

Tests for gene functionality

Where possible, tests were carried out to determine if genes were not only transcribed, but also gave rise to a functional product. Agropine was detected in extracts of transformants A4C, A4D, 362B, A4 3–4 and A4 6–1 by high-voltage paper electrophoresis (not shown), confirming integrity and activity of the three mannityl opine biosynthesis genes. In the presence of an active *aux2* gene product, naphthalene acetamide (NAM) is transformed into the active auxin, naphthalene acetic acid, inducing callus formation (10). Figure 8 shows that all transformants bearing the

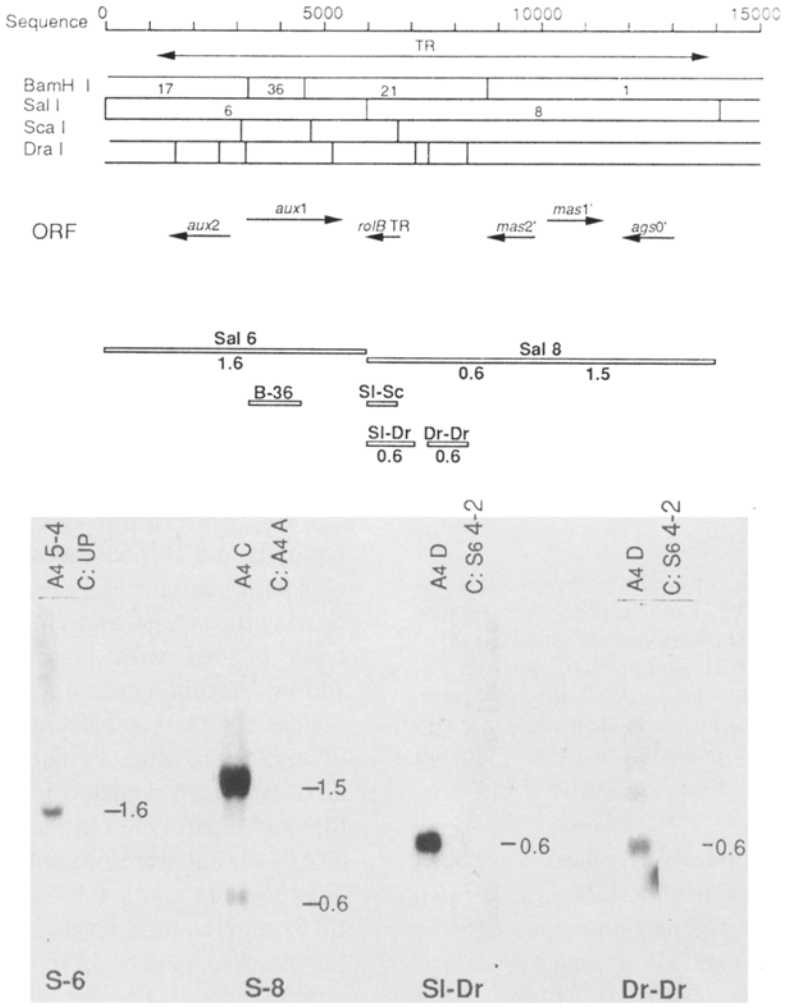

Fig. 6. Localization of TR-DNA transcripts. RNA gel blot experiments were carried out with probes covering the TR-DNA. Shown successively from top to bottom are: sequence coordinates, usual extent of TR-DNA, deduced ORFs. Probes are indicated by open boxes with probe name above and the size of transcripts observed below. Representative blots are shown, with one clone of transformed roots and one of several different controls: either RNA from leaves of non transformed plants (C:UP) or from root clones lacking the T-DNA region probed (C:A4A, C:S6 4–2).

aux2 gene are sensitive to NAM. This includes all roots of phenotypes II and III, as well as the single clone of phenotype I bearing the *aux* genes, A4 3–4. As expected, all other transformants were insensitive to NAM.

Discussion

Using *A. rhizogenes* strain A4 on cucumber explants, root cultures of three phenotypic classes were obtained. In addition to transformed roots with a phenotype similar to that described for other plant species [25], which we have designated phenotype I, we have obtained extremely thick roots lacking root hairs which we class in phenotype III, and roots of intermediate phenotype (II), all of which grow on hormone-free medium. The majority of the transformed cucumber root clones, including all those of phenotypes II and III, contained both TL- and TR-DNA. In transformation experiments with T-region sub-

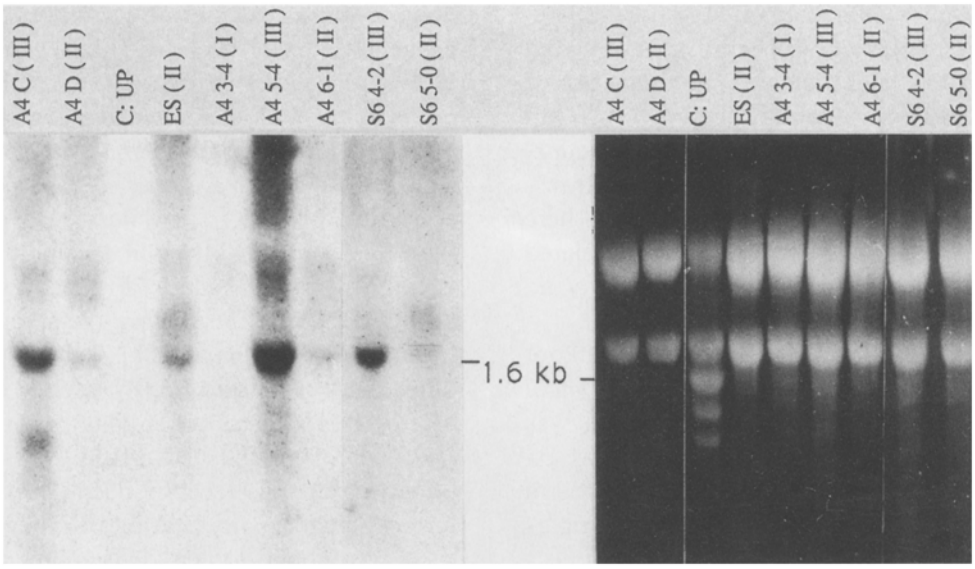

Fig. 7. Expression of *aux2* in transformed roots. A denaturing agarose gel with 30 μl total RNA extracted from root clones of the three phenotypes (right) was blotted to nylon membrane and probed with fragment *Sal*I 6 (left). Phenotype of the root clones (I, II, III) is also indicated.

fragments, fragment *Eco* RI 15, bearing genes *rolABC* was sufficient to induce roots of phenotype I, and *Sal* I fragment 6, bearing genes *aux1* and *aux2*, was sufficient to induce roots of phenotypes II and III. The results obtained with *Sal* I fragment 6 also show that the *rolB*-TR gene is not necessary for induction of class II and III roots. Furthermore, the level of transcript correspond-

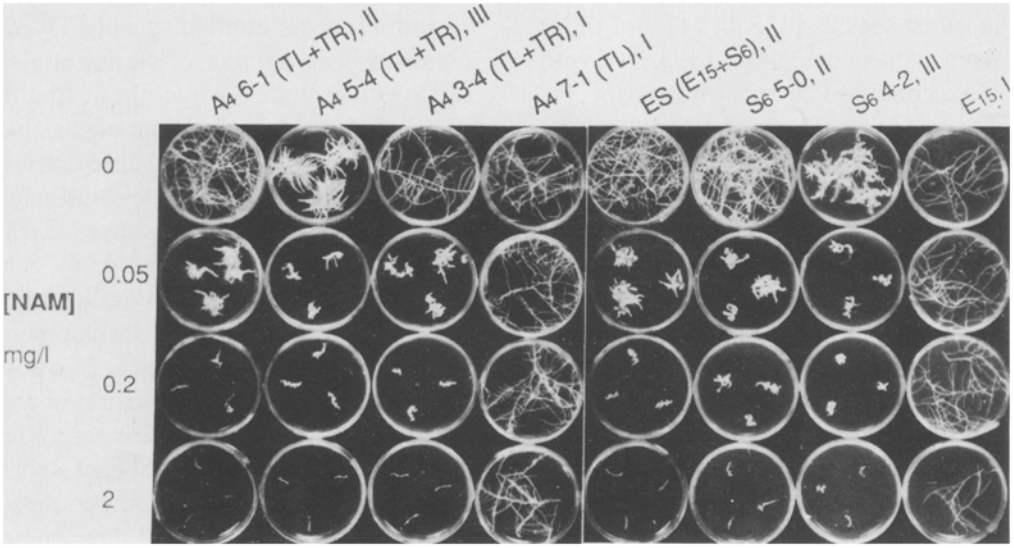

Fig. 8. Effects of naphthalene acetamide (NAM) on root growth. Root fragments were transferred to medium containing a range of concentrations of NAM, and photographed after 12 days. The T-DNA structure (presence/absence of TL and TR DNA) and phenotype are indicated for each root clone tested.

ing to the *aux2* gene is correlated with root phenotype, with highest levels of expression observed in class III roots, and the lowest level in a class I root clone. We conclude that phenotypes II and III are due to transfer and expression of the *aux* genes. Free auxin concentration in the transformed cucumber root clones characterized here has recently been shown to be closely correlated with the level of *aux2* transcripts (Dewitte *et al.*, in preparation).

It is interesting that even a phenotype I root clone bearing the *aux* genes, but in which *aux2* transcript could not be detected (A4 3–4), was sensitive to NAM, indicating that *aux2* was expressed. In the presence of NAM, conditions in which substrate for *aux2* is not limiting, apparently even such low levels of *aux2* gene product allow synthesis of sufficient active auxin to inhibit root elongation. This suggests that the limiting step in auxin biosynthesis via the *aux* genes, which determines root phenotype, may in fact be *aux1* expression. Since we have observed in RNA gel blot experiments a correlation between the level of *aux2* transcripts and root phenotype, this would suggest that expression of the two *aux* genes is co-regulated.

Most transcripts which we detected in RNA gel blot experiments were of sizes similar to those observed in other species [12, 20, 24] and those predicted from sequence analyses [3, 5, 21], with two notable exceptions. The major transcript corresponding to *rolA* in transformed cucumber roots (0.35 kb) was significantly smaller than that previously described in other species. The potential biological role of this transcript remains to be determined. Results concerning the TR-DNA transcript of 0.6 kb are also surprising. It is encoded by a small region between the *rolB*-TR and *mas2'* genes in which no ORF of appropriate size is known [3]. More careful mapping of this anomalous transcript should be of interest.

Different plant species have various sensitivities to different complements of T-DNA genes. For instance, TR-DNA genes induce roots more efficiently on tomato explants, while tobacco responds more strongly to TL-DNA genes [27]. Root induction on carrot root discs, on the other hand, requires not only *rolABC*, but also either exogenous auxin, the TR-DNA *aux* genes or TL-DNA ORFs 13 + 14 [6]. There are also differences in which T-DNA genes are integrated into the genome of transformed roots; for instance, transfer of TR-DNA genes is generally not observed in tobacco [1], while we have observed both TL- and TR-DNA in the majority of our cucumber transformants. Frequent transfer of both T-DNAs is also often observed in transformed *Brassica napus* [17], but no phenotypic differences correlated with presence or absence of TR-DNA *aux* genes has been observed (M. Tepfer, unpublished results). A subtle phenotypic effect has recently been described in tobacco transformants bearing pRi-TR *aux* genes; roots were found to be thicker than controls, but only at the earliest stages after germination [5].

Both TL-DNA (genes *rolA* + *rolB* + *rolC*) and TR-DNA genes (*aux1* + *aux2*) were able to induce roots capable of hormone-autonomous growth when transferred separately, perhaps via different mechanisms. Roots could be induced by auxin biosynthesis due to *aux* gene expression, but the functions of *rolABC* have not yet been clearly enough established to hypothesize on how they mediate root initiation and growth. When both T-regions were present in the inciting bacterium, no roots containing only TR-DNA were obtained. The number of clones analysed [9] is too low to exclude the hypothesis that this is simply a random effect, but one might also propose that the TR-DNA has an inherently lower frequency of transfer. Other possibilities include that roots develop only from a subset of transformed cells, ones in which *aux* and/or *rol* gene expression is sufficient for root induction, but low enough to allow continued development of organized roots. If this is the case, it is possible that the permissible latitude for expression of the two types of genes is different. Clearer understanding of the functions of individual Ri T-DNA genes is needed, before we can hypothesize on how they influence root initiation and development, either singly, or even more so in groups.

Genetically transformed roots of phenotypes II and III had not previously been described; the

only potential exception being the pRi-transformed potato roots described by de Vries-Uitewaal *et al.* [11], who obtained either transformed callus or root cultures of phenotype similar to I and II. It would be interesting to know if in this case as well, root phenotype is correlated with *aux* gene expression. While this article was in preparation, McInnes *et al.* [19] reported that the pRi TR-DNA can induce transformed roots on cucumber explants, though apparently they only observed phenotype I roots. This is particularly surprising, since we have obtained approximately equal proportions of the three root phenotypes in several other cucumber varieties, Straight Eight, Burpless and Cornichon de Meaux (not shown). Even so, this may be due to varietal differences, as de Vries-Uitewaal *et al.* [11] have found that only certain potato varieties produce the equivalent to phenotype II roots. It would be interesting to determine whether these differences in root induction are due to differences in responsiveness to a particular gene product (e.g. auxin due to *aux* gene expression) or to species-specific differences in expression of Ri plasmid T-DNA genes. In this regard, it should be noted that in the only other study of pRi-TR gene expression [24], certain *Nicotiana glauca* tumor lines expressed *aux1* at a high level, while we have been unable to detect *aux1* transcripts in transformed cucumber roots.

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