# The TR-DNA Region Carrying the Auxin Synthesis Genes of the Agrobacterium rhizogenes Agropine-Type Plasmid pRiA4: **Nucleotide Sequence Analysis and Introduction into Tobacco Plants**

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We have determined the nucleotide sequence of a 6-kilobase fragment of the Agrobacterium rhizogenes plasmid pRiA4 TRregion that carries genes (aux1 and aux2) responsible for auxin biosynthesis in transformed plant cells. Sequence analysis revealed two open reading frames corresponding to proteins of 749 amino acids for the aux1 gene and 466 amino acids for the aux2 gene. We observed significant similarity between the amino acid sequences deduced from the pRiA4 aux genes and those of the auxin biosynthesis genes of A. tumefaciens octopine-type Ti plasmids, the iaaM and iaaH genes of Pseudomonas savastanoi, and

different genes of the pRiA4 TL-region; however, the 5'-flanking regions of the pRi and pTi auxin biosynthesis genes were found to be completely different. Transgenic tobacco plants containing this entire 6-kilobase fragment of the pRiA4 TR-region have been obtained. Regenerated plants are phenotypically normal. The aux1 gene is not or is very weakly expressed in these plants, but expression of the aux2 gene leads to a modified root phenotype when plants are grown on medium containing an auxin precursor (naphthalene acetamide).

genes (White et al. 1985; Offringa et al. 1986). The right

Crown gall and hairy root diseases induced by Agrobacterium tumefaciens (Smith and Townsend) Conn or A. rhizogenes (Riker et al.) Conn on dicotyledonous plants are the result of the transformation of plant cells by a specific DNA fragment, the T-DNA. This T-DNA, bordered by direct imperfect repeats of 24 base pairs (bp) required for its transfer, is part of a large bacterial plasmid, the tumorinducing (Ti) or the root-inducing (Ri) plasmid (for reviews, see Melchers and Hooykaas 1987; Binns and Thomashow 1988). Crown gall and hairy root cells are able to proliferate in the absence of phytohormones, due to the expression of T-DNA genes, especially of genes involved in the synthesis of growth factors. In crown gall cells, the tmr gene is associated with cytokinin synthesis, and two genes, tms1 and tms2, are involved in auxin production: the tms1 gene product catalyzes the conversion of tryptophan to indole-3-acetamide (IAM), which is then converted to indole-3acetic acid (IAA) by the product of the tms2 gene. This biosynthetic pathway is not used in normal plant metabolism but occurs in the bacterium Pseudomonas savastanoi E. F. Smith, which harbors genes (iaaM and iaaH) similar to the A. tumefaciens auxin synthesis genes (Yamada et al. 1985). In agropine-type Ri plasmids, such as pRiA4 which we have studied, the T-region is split in two parts, TL and TR, and it has been shown by crosshybridizations that the TR exhibits homology with pTi T-DNA only in loci responsible for agropine synthesis and in the tms locus (Willmitzer et al. 1982; Jouanin 1984; Huffman et al. 1984). The occurrence of two auxin synthesis genes has been shown by complementation of the pTi tms

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part of the pRiA4 TR-region harbors agropine synthesis genes, and the left part harbors the auxin synthesis genes. Here we present the 5,995 nucleotide sequence of the left part of the TR-region that carries the two auxin synthesis genes. The exact location of these genes has been determined, their coding sequences were compared to those of the homologous genes of A. tumefaciens and P. savastanoi, and their nontranslated regions were analyzed. (To avoid confusion, we chose here to systematically call the auxin synthesis genes tms when they are from A. tumefaciens and aux when they are from A. rhizogenes; the corresponding P. savastanoi genes will be named iaaM and iaaH.) The sequenced fragment was then introduced into tobacco and its effects in transgenic plants were studied.

# MATERIALS AND METHODS

Plasmids, bacterial strains, and culture conditions. The cosmid pLJ85 containing the pRiA4 TR-region (Jouanin 1984) served as a source for the 6-kilobase (kb) SalI fragment encompassing the left part of the TR-DNA. This 6-kb SalI fragment was cloned in both orientations in pEMBL19 (Dente et al. 1985), giving plasmids named p19S7 and p19S25. To introduce the auxin synthesis genes in plants, the 6-kb SalI fragment was cloned in the XbaI site of the binary vector pMRK62 (Vilaine et al. 1987), which confers kanamycin resistance to transformed plant cells; the resulting plasmid was designated pMRKS6 (Vilaine et al. 1987).

Strain HB101 of Escherichia coli (Boyer and Roulland-Dussoix 1969) was used for recombinant DNA techniques, and strain NM522 (Gough and Murray 1983) was used for the production of single-stranded DNA. They were grown at 37° C in Luria-Bertani medium. The filamentous phage M13K07 (Vieira and Messing 1987) was used for the production of single-stranded DNA from pEMBL vectors for sequencing.

A. tumefaciens GV3101 (pMP90) (Koncz and Schell 1986), a rifampicin- and gentamycin-resistant derivative of nopaline A. tumefaciens C58, was grown at 28° C in Luria-Bertani medium supplemented with the appropriate antibiotics.

Antibiotic concentrations added in the bacterial media were 200 mg/L ampicillin, 10 mg/L tetracycline, and 50 mg/L kanamycin for E. coli, and 100 mg/L neomycin, 50 mg/L rifampicin, and 25 mg/L gentamycin for A. tumefaciens.

Recombinant DNA techniques for clone preparation. Most DNA techniques were performed essentially as described by Maniatis et al. (1982). Subclones presenting nested deletions of the cloned fragments were obtained from the plasmids p19S7 and p19S25 by random DNase I treatment according to Lin et al. (1985). Resulting subclones were sized on agarose gels before sequencing.

DNA sequencing and sequence analysis. DNA sequencing was conducted by the chain termination method of Sanger et al. (1981) on single-stranded DNA obtained from such subclones by using the phage M13K07 (Vieira and Messing 1987). When necessary, gaps were sequenced with synthetic oligonucleotide primers. Sequences were analyzed using the programs supplied by the computer services of CITI2 (BISANCE, French Ministry of Research and Technology, Paris).

Transformation of tobacco plants. A. tumefaciens GV3101 (pMP90) (Koncz and Schell 1986) was transformed by pMRKS6 according to Holsters et al. (1978). The resulting strain was used for *Nicotiana tabacum* L. (cv. Xanthi) leaf disk transformation as described by Budar et al. (1986). Kanamycin-resistant shoots were selected on Murashige and Skoog (1962) (MS) agar medium containing 100 mg/L of kanamycin and 500 mg/L of cefotaxime, then grown on MS medium without cefotaxime. Transformed plants were regenerated and analyzed by Southern and northern hybridizations using the 6-kb SalI fragment as a probe as described by Jouanin et al. (1987).

# **RESULTS**

We determined the complete nucleotide sequence of the pRiA4 6-kb SalI fragment that covers the left part of the TR-region. A map of the sequenced region is shown in Figure 1, and the sequence is displayed in Figure 2. Jouanin et al. (1989) have established that the pRiA4 TR-region is flanked by 24-bp functional borders showing strong homology with the border consensus sequence of Ti plasmid T-DNA. We determined the left border sequence to be located at position 1,021 to 1,045 (Fig. 2). Eight open reading frames (ORFs) larger than 225 bp were found in the sequenced region (Fig. 1). Characteristics of these ORFs are given in Table 1. We have identified the two larger ORFs (6 and 2') as the coding sequences of the pRiA4 auxin synthesis genes (aux1 and aux2, respectively) by comparison with the auxin synthesis region of pTiAch5 (Gielen et al. 1984). As in the pTi genes, intervening sequences appear to be absent in both the aux1 and aux2 genes (Fig. 2).

Analysis of the aux1 and aux2 coding regions. The coding regions of the aux genes present significant sequence similarity with those of the pTiAch5 tms1 and tms2 genes (Gielen et al. 1984) and with the iaaM and iaaH genes of P. savastanoi (Yamada et al. 1985). The aux1 gene was also compared with members of a pRiA4 TL-DNA gene family that present homologies with tms1: ORFs 8, 11 (rolB), 12 (rolC), 13, and 14 (Levesque et al. 1988). The coding region of the pRiA4 aux1 gene (Fig. 3A) has an organization similar to ORF 8 (28% amino acid identity) and tms1 (60% identity): in its N-terminal domain, the aux1 gene product shows weak similarity (about 20%) with parts of the pRi ORFs 11, 12, 13, and 14, and the C-terminal region presents 52% identity with iaaM of P. savastanoi. The FAD-binding site of tms1 (Klee et al. 1984), strongly conserved in iaaM (Yamada et al. 1985), is also present in ORF 8 and in the aux1 gene product (Fig. 3A). The larger size of ORF 8 and the aux1 and tms1 genes compared to iaaM is due to the additional N-terminal polypeptide that is weakly homologous to the pRi TL-DNA ORFs 11, 12, 13, and 14, and whose function is unknown.

The aux2 and tms2 gene products can be aligned over the entire sequence with 71% homology, and there is 30% identity between aux2 and iaaH (Fig. 3B) (27% for tms2 and iaaH, Yamada et al. 1985).

Analysis of nontranslated regions of the aux1 and aux2 genes. The sequences of the regions comprised between the two aux genes and between the two tms genes (393) bp for pRiA4 and 345 bp for pTiAch5 [Gielen et al. 1984]), which contain possible promoter elements, were found to be largely different. The only homology is with a 12-bp sequence, located 76-87 bp upstream from the aux2 ATG (position 2,912-2,923, Fig. 2) and 35-46 bp upstream from the tms2 ATG.

The majority of the genes expressed in plants contain a TATA box (Joshi 1987; see Bruce and Gurley 1987; De Pater et al. 1987a; Bandyopadhyay et al. 1989 for T-DNA genes). TATA elements close to Joshi's consensus are found in the 5' regions of the aux1 gene (84 bp upstream from the ATG, position 3,134-3,146) and the aux2 gene (79 bp from the ATG, position 2,927–2,915) (Fig. 2). For the aux2 gene, the TATA motif is located in the 12-bp sequence

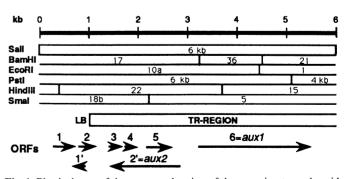


Fig. 1. Physical map of the sequenced region of the agropine-type plasmid pRiA4. Restriction fragment numbering or lengths are from Huffman et al. (1984) for HindIII, Jouanin (1984) for EcoRI and SmaI, Jouanin et al. (1986) for BamHI, and L. Jouanin (unpublished) for PstI and SalI. The extent of the TR-region, delimited by the left border sequence (LB), is indicated by an open box. Arrows indicate position and polarity of the open reading frames (ORFs, see Table 1).

homologous to a sequence of the pTiAch5 and pTiA6NC tms2 promoter, which contains a TATA element (Klee et al. 1984).

Sequences homologous to the polyadenylation signal AATAAA were found in the 3' regions of the aux1 and aux2 genes (Fig. 2).

Many T-DNA genes contain upstream elements that modulate the transcriptional level (e.g. Ellis *et al.* 1987; Bouchez *et al.* 1989; Bruce *et al.* 1988; Leisner and Gelvin 1988, 1989; Mitra and An 1989). Sequences related to the 9-bp motif (TTTCAAGGA) observed by Lichtenstein

GTCGACAGTCGCAACAGCAATCGAGGGGTGTTGATCAACCTTGGCCAGTTGCCCTTCGTCCCTATCAGTAAC GACAACCTTGTRATCGCCGGTTTCCGCRAGCRTCRGAGCRATGGCTCCGCCRATCTTACCCGCGCCGATAAC CACTATTTCTTTCATACAAATCCCCTGTGTTCTGTATATAGGATATTTATAAAAATAACCGGGGATTGGTCG 216 AATCATAGCGGCAAGGTGGGCATTATGTTATATTTTTTGTGCAAAAAGACGACTGCACTTGGTAATATGAA 288 GGGGTTCCGCATGCAATTGACCGAAAGGGATCGGGAAGCTCCTGTCGTTACTCGGCGAAAATGCCAGAACGCC 360 TGTGGCGACGCTCGCGAAAAAGCTTTCTCTATCCAGGACCACAGTGCAGGCTCGCTTGGAGCGCCTTGAGAG 432 AGRGGGRGTTATTGTCGGCTATGGTGTARGGCTATCGRATGRATATTCCTCGAGCTTGGTTCGAGCCCATAT 501 TCTGATCACCATTACGAAGGCGCTTTCACAGGTGACGGCCTCCCTTGGGAAGGTCACGGCCGTTATCGCTCT 576 TCATTCTGTGAGTGGCACTTTTGATTTGATTGCGATTATCGAAGCCCCTTCGATTTCAGAGCTCGATCAGTT 648 CATCGATCAAATCGGCATAATTGAAGGTGTCGAGCGGACGCTCTCTTCGATTATTCTTTCAACACGCATTTC 720 ACCCTG MANG AGCARGAGGCCACAGGCCGTGCGCCTGCTGCCACCTTTCCGACGATGACCATGCAGAACTTAC GGCCTCTATGTCGGTGTATTCCACCCACCGACGCGCCATTTCCGTGTGCATTAAGATAATGCTTTCCGCCCC ATCGGATTGCTGTTCTGACATGGTATATCATTACGACAGCCAATGTTTGGCGGCTCGTACGAATCATCAAGC 936 TIGITGGCTGTTTGGCAGGATATATGCCAACGTAAAAAATGAGGGCAATCGATTGTACTGAATCGGATTTTCA 1000 AGGGTCTGGCCAAAACTATTCCGTGGGCACCTGGCACACGCCCTGGAGTCCGGCCCGTTTCCAGTTGAGGGT 1152 TGTCTACGCTTAGATGAGAAGGAAAGTTGTCCAAGACGAATCCCAGTGTCCTATTACCAATAGCCGGCGCTA 1224 GTIIGAIITCAGAATAAAGAGAAATTCGTCACACCAAATATTAGAAGCAATGTIIGAIIGACCATCATACIT 1296 ARGRIGARCATTCCARACACRGITATARTACGCallaliatigacacartatarartitatartgitgatati 1368 CCTTGAAATATAATATTACAATAGATAAAGTAGAGGAAATTATGTCAGAAATTTTGGTTTGGCTCTGCTTA S L T P R H R F D I A E E I A L G I A L L R E D CGACAGAGTCGGACGATGCCTAACAGGTCTATTGCTTCTTCTTCTCTGCTAGTCCAATTGCTAACAGACGTTCATC 1510 S S M S G D I E M G I P L G M S S L S U P L S L CGAGCTTGCAGAGCCATCGATTCCATGCCATAGGCAGACCTTGGAACTAAGAAAAACCGGAAGACCTTAG 1582 G P L G A M S S P D U M R U F I K F T D T M S G GCCCGGCAGGCCCGCATTACTGCTGGGATCTACATTCGGCAGGATTTTAAAGGTATCGGTCATTGAGCC 1654 M H I U S L D H G I P K A T L P A T P F L I A D attgtgaatcaccgataggtcatggccaattggctgctgcgaggactgttgggaaagattgcatc 1726 L Q H A K F Y S H Y A A Q L R P R F F R R A L C Tagctgatgegecttgaagtaactgtggtaggecgcttgagtctcggtctgaaaraacgtcggccagaca 1798 Y E S K S I L M D S L Q A M L I S A U D P S R I atactegetittggaratargattateegagattgtgeattgagaatacttgeaacateeggactgegaat 1870 A R U U E S F S U G E U F M Q I Y H E L S L P F CGCTCTGACAACCTCGAGAAACCACCCTCTACGAAGTTCTGAATATAATGTTCAAGGGACAACGGAAA 1942 E Y I A T P F S U G E H H H A L D P I D A E U F ttcgtagatggcagtcggaragctgacccttcattgtgatgcgctaratcaggartatctgcttcarcrap 2014 T U D K R R L U R I I T E A A L A U D P E L D M Agtarcatctigggtgccagarctctgataatcgtctgggtgctaagggcgatcgggtccaggtcgt 2086 Y F Y A T P L G ! R L G K L R U T Q M U P P R G GTARARGTARGCGGTTGGCARGCCCTTCAGGCGGTCCCCTTCAGGCGGTCTCCC 2158 C I I G D L L I U D P U M Q A I U G P T D R T P GCARATGATACCGTCAGAAGAATCACGTCCGGATCATTCTGTGCGATAACGCCAGGGGTGTCCCGGGTGGG 2230 S U P U I G D T P Y R G U T P R F G U U G C L A GCTTACCGGACTATTCCGTCGCTTGGATATCGCCCCCACGGTAGGACGARACCCCACCACGCCGCACAAGGC 2302 A U G G S S G G P I L S P M U P M R U A G T A F GGCCACACCCCCACTIGATCCCCCTGGGATGAGGACTAGGGTTCCACGGGTTTCGTACGGCGCCTGTGGGGAA 2446 Q D I K S A T W R L H A W D T E L F A M L S R A TIGATEGATITIGETGEGEGETECACCGTAGGTGEGECCCAGTCGGTTTCAGGAGGTTTAAGGATCTTGC 2734 T E T I S S L T U M ← aux2
CGTCTCGGTGATCGAGGAGAGGGTCACCATTITCGTTGTGCTGAGGAACTGAGGTAGATCTCGCCAGAGAA 2878 ACCTICARTGATITITGCTTGGAGTGAAAAAGGCAAATAATTATTAGAGGAAGTCAGAAATGCTGCGCA 2950 GTAGGGCCACTTGTATAAGTGCCGGTCGAACACTGCTGGTGGAAAGTCAAAAGCGTGAAGTATTAGTTGAAC 3022 TCTGTTACTAAATTGAGATAAATGGGATATTTTATTCGAAAGTACTGTTTGAGATCTAGCGACAATAATAAT 3094 GTCATCTTATGAGATTGCATGGCAATATGGATCTAATAT<u>ITGGCATAAATAG</u>ATGGTGGTTTTGTCTCCACT 3166 TTTAAACCTTCACAGCGTTACCCTAACACCTCTTAATTGCGTACACTCCTTTCAACCGCATCAATGGCTGGA 3238 TCCTCCTTCACATTGCCATCAACTGGCTCAGCGCCCCTTGATATGATGCTTATCGATGATTCAGATCTGCTG 3310

SFTLPSTGSAPLDMMLIDDSDL

et al. (1984) in a number of pTi T-DNA genes and by Klee et al. (1984) (TgTCAAcGA) 85 bp upstream from the pTiA6NC tmsl translation start were found in the pRiA4 sequence: gTTCAAtGA at 349 bp upstream from the auxl ATG (position 2,881) and TTgaAAGGA at 385 bp from the aux2 initiation codon (position 3,221). A sequence (CCAtATTGCCATG) 288 bp upstream from the pRiA4 aux2 ATG (position 3,124) is related to a consensus sequence [CCACAN(T/A)NNNN(T/A)G] established by Memelink et al. (1987) from a transcriptional activator element found in the 5'-untranslated region of the pTi

CARTTGGGTCTCCAGCAGGTATTCTCGAAGCGGTACACAGAGACACCGCAGTCACCACTACAAACCGGCAGCCAGG 3302 Q L G L Q Q W F S K R V T E T P Q S R V K L T R AGGECTTCTCCAGACGTCTCATCTGGCGAAGGCAATGTGCATGCCCTTGCGTTCATATATGTCAACGCTGAG 3454 BUSSGEGNUNALAFIYUNAE ACGTTGCAGATGATCAARAACGCTCGATCGCTAACCGAAGCGAACGGCGTCAAAGATCTTGTCGCCATCGAC 3526 GTTCCGCCATTTCGAAACGACTTCTCAAGAGCGCTACTCCTTCAAGTGATCAACTTGTTGGGAAACAACCGA 3598 U P P F R M D F S R A L L L Q U I M L L G N N R ANTGCCGNTGNCGNTCTTAGTCNCTTCCTNGCNGTTCCCCNNACNGCGCCCGCTCTNNGNTCCTNACC 3670 H A D D D L S H F I A U A L P H S A R S K I L T CTTTATGACTATGGCAAGTTCTTCGAGAGTTGCGCGGATGGACGTTATCGGTTATTTTCCTGAAGGCGTT 3006 Lybyg ffeg AGTGCTCCAAATGTGATAGCCGAGATGGGGCCATGCGTTTTCCGCGAAGTGAATCATGCTTGTTCTTCTAT 4102 Saphula en gantagagaga CGCGCATTTTTGCAAGATGGCTATCTCCATGATGGAGTCATGTTGGCGTCACCGTTAGCAATTGTTGACGCC

R A F L Q D G Y L H D G U H L A S P L A I U D A TTGARTTTAGGGCATCTACAGCAGGCGCATGGCTTCTGGCATCTTTGGCTCACATATTTTGAGCGAGGCTCT
LMLGHLQQAHGFNQSNLTYFERES TTCTCTTCTGGCATCGARAAATGTTCTTGGGCATCATCCTCCGGGGGGTGAACAATGGAATTCCCTAGAT 4162 F S S G I E K M F L G M W P P G G E Q W N S L D GACTIGGATCTITICAAAGCGCTGGGTATTGGATCCGGCGGATTCGGCCCTGTATTTGAAAGTGGGTTTATC 4534 D L D L F K A L G I G S G G F G P U F E S G F I CCTCATAGGATCGCCTCACAGGTAATTAACGGCAGGTCTATTCGCGAGGCGTACAATTCACGTTCAAGTCGAG 4678
PHRIASQUINGRSIRERTIHUQUE CAGATTGATAGAGAGGAGGATAAAATAAATATCAAGATCAAAGGAGGAAAGGTTGAGGTCTATGATCGAGTA 4750 OIDREEDKINIKIKGGKU CTGGTTACATCCGGGTTTGCGAACATCGAAATGCGCCATCTCCTGACATCAAGCAACGCATTCTTCCATGCA 1822 L u t s g f a m i e m r m l l t s s m a f f h a GATGTAAGCCATGCAATAGGGAACAGTCATATGACTGGTGGGTCAAAACTGTTCTTGCTGACCTAACGAAAAA 1894 D U S H A I G H S H H T G A S K L F L L T H E K TTCTGGCTACAACATCATTTGCCATCGTGCATACTCACCACCGGCGTTGCAAAGGCAGTTTATTGCTTAGAC 4966 H L Q H H L P S C I L T T G U A K A U TATGATCCGCGAGATCCAAGCGCGCAAAGGACTGGTGTTGATAAGCTATATCTTGGGAGGATGACTCACATAAG 5038 Y D P R D P S G K G L U L I S Y T W E D D S H K CTCCTAGCCGTCCCCGACAAAAGGAAAGGTTCGCATCGCTGCAGCGCGATATTGGGAGGGCATTCCCAGAT 5110 L L A U P D K R E R F A S L Q R D I G R A F P D AKHLTPADGNYDDHI CCCTTTGACGTAATGCATCCCGGGGGGGGATAAGGGACTTTACTTGGCCGGTTGTAGCTGTTCCTTCACCGGA 5326
PFDUNNPADDKGLYLAGCCSCSFTG GGGTGGGTTCATGGTGCCATTCAGACCGCATGCAACGCTACGTGTGCGATCATTTATGGTTCCGGACACCTG 5398 NU H G A I Q T A C H A T C A I I CARGAGCTRATCCRCTGGGGGCACCTCARAGAAGGTAATCCACTGGGGCACGCTTGGAAGGGGTATAGGTAT 5470 0 E L I H W R H L K E G M P L A H A W K A Y A Y CARGCGTGATAATGCAACAGTTAGAAIAAITAGTTTGCCCTAGCCGGTATTCCTTGGTGTTCCAATAGGGTT 5542 CCGARGCCAATAGGCGAAAAAGCTGACTTTTCAGTCCCTTTTATTATTCAATTCGCTTCGGTCCAAGCATAA 5614 GTCAAGCTAAAACATGTAATACGTA<u>aatata</u>tggaaactttatgtctgaaaagacacattattattgatc 5758 GTRATACACTGAACTGGTCATAACAGGGAAGGCTAACTGCAACATATCCTATAAATACTCAGTGAAAATGGC 5830 CGCTCCCCAATGTTAAGCCATTTTTGCGGTCGGGCTAAGCGCTCGTCCGTGTCTCCCCTGGCCCGAGTGTCG 5902 GCTCTCCATCAGCGGCCTCATCATCTGTCGCTGACACCGGTGGCCCCAATTTCAAATCGAGGAAAGACGATG 5974 CCCTCGCCGGCAAACGTCGAC

Fig. 2. Nucleotide sequence of the 6-kilobase Sall fragment of the pRiA4 TR-region. The 24-base pair left border sequence (position 1,021 to 1,045) is boxed. The amino acid translation of the open reading frames corresponding to the auxin synthesis genes, aux1 and aux2 (see Fig. 1 for location), is presented. In the intergenic region, sequences homologous to a sequence upstream of the pTiAch5 tms2 ATG (position 2,912 to 2,923) and to a sequence from the 3'-flanking region of pTiAch5 gene 6b (position 3,039 to 3,059) are indicated by dotted lines. Potential transcriptional signals are underlined: potential TATA boxes are in the promoter region (position 3,134 to 3,146 for aux1 and position 2,927 to 2,915 for aux2), and possible polyadenylation signals are in the 3'-flanking regions of the genes.

tmr gene (De Pater et al. 1987b). A 21-bp motif (position 3,039-3,059, Fig. 2) is homologous (18 identical bases) to a sequence of the 3'-flanking region of pTiAch5 gene 6b (position 10,139–10,159; Gielen et al. 1984). Gene 6b is an oncogene (Hooykaas et al. 1988) that interacts with the auxin synthesis genes (Tinland et al. 1989). A search for a consensus sequence (GATAAATGNNATATTTNATTC) derived from these two 21-bp elements in the bank of nucleic acid sequences of GenBank has not allowed us to find this exact motif in other sequences.

Effects of the aux genes in transgenic tobacco plants. The entire 6-kb SalI fragment carrying the two pRiA4 auxin synthesis genes was introduced into tobacco plants. We have obtained eight plants transformed by the two A. rhizogenes aux genes. Southern hybridization analysis of two of these plants, Sal6.1 and Sal6.5, which correspond to different transformation events, is presented in Figure 4. All the regenerated plants transformed by the two A. rhizogenes aux genes are fertile and show a normal phenotype. We were not able to detect transcripts corresponding to the aux1 or aux2 genes by northern hybridization of mRNA extracted either from the leaves or from the roots of transformed plants (data not shown).

To investigate the expression of the aux genes at the seedling stage, seeds of homozygous transformed plants Sal6.1 and Sal6.5 were germinated on MS medium supplemented with or without 1 mg/L of naphthalene acetamide (NAM, a more stable IAM analogue). Nontransformed seedlings develop normally on both media. On the medium without NAM, seeds of the Sal6.1 plant germinate normally, but seedlings of the Sal6.5 plant show longer and thicker roots with abundant root hairs (Fig. 5A). This modified root phenotype is the same as observed when normal seeds are germinated on medium containing low levels (0.1 mg/L) of naphthalene acetic acid (NAA). On the MS medium containing NAM, all the transformed seeds form modified roots and callus (Fig. 5B) as found when normal seeds are germinated on medium containing 1 mg/ L of NAA. It can be seen that the response to NAM of Sal6.5 seedlings is more accentuated than that of Sal6.1 seedlings.

Cuttings of the Sal6.5 plant also showed modified roots on MS medium without NAM, whereas cuttings of untransformed and Sal6.1 plants root normally. Cuttings of Sal6.1 and Sal6.5 plants produce an abnormal root system on MS medium with 1 mg/L of NAM, while the untransformed plants develop a normal root system, and roots of Sal6.5 show a more marked phenotype than Sal6.1 (results not shown).

#### DISCUSSION

In this TR-DNA region, only two transcripts of 1.6 and 2.5 kb were observed by Taylor et al. (1985) in N. glauca Graham tumor lines transformed by pRiA4. The respective locations and sizes determined for the aux2 (1,398 bp) and aux1 (2,247 bp) ORFs are in agreement with these results. A single transcript of 1.6 kb was also observed in the region carrying the aux2 ORF in transformed cucumber roots (J. Amselem, unpublished results). ORFs 3, 4, and 5 are located on the direct strand of the sequence in the region

carrying on the complementary strand the aux2 gene (Fig. 1). No transcript corresponding to these ORFs has been observed in this region, so there is no evidence that they correspond to functional genes in plants.

None of the ORFs located outside of the TR-region (1, 2, and 1') showed a procaryotic ribosome binding site, and we do not know if they are actually transcribed in Agrobacterium. ORF 1 (147 amino acids) shows similarity with a 153-amino acid protein of the E. coli replication origin oriC (Buhk and Messer 1983) whose function is not known.

In plants, translation generally begins at the first AUG codon present on the mRNA, which is often flanked by the consensus sequence TAAACAATGGCT (Joshi 1987). For the aux2 gene, a second ATG in frame is located at a distance of 354 bp downstream of the first one. Considering that the sequence flanking the first ATG (AAAATGG) matches Joshi's consensus, we suggest that it is the functional initiator of translation in plants. For the aux1 gene, after the first ATG, we found two ATG codons in frame at the beginning of the ORF, 51 and 54 bp farther. The sequences flanking these three ATG codons are, respectively, GCATCAATGGCT, CTTGATATGATG, and GATATGATGCTT. The first ATG environment corresponds well to Joshi's consensus, and there is no evidence that this ATG is not functional, but the exact determination of the initiation codon would need amino acid sequence determination of the translation product.

The coding regions of the pTiAch5 tms genes and the pRiA4 aux genes present the same organization and comparable sizes: 749 amino acids for aux1 and 755 for A. tumefaciens tms1, 466 amino acids for aux2 and 467 for tms2. The protein sequences deduced from the A. rhizogenes auxin synthesis genes are very similar to those of the corresponding genes of A. tumefaciens. This is consistent with the similarity of function of these proteins and an evolutionary relationship among these genes. The similarities observed between aux1 and ORF 8 or aux1 and iaaM are comparable to those reported for tms1 and ORF 8 (31%, Levesque et al. 1988) and tms1 and iaaM (50%, Yamada et al. 1985). Considering there is 38% homology between ORF 8 and iaaM (Levesque et al. 1988), tms1 and ORF 8 are the most divergent, with iaaM closer to tms1 than to ORF 8. The results obtained for the aux1

Table 1. Coordinates of open reading frames (ORFs) of more than 75 amino acids

ORF	First base	Last base	Number of amino acids	Calculated mol. wt.
Orientatio	on a <sup>a</sup>			
1	284	724	147	16,072
2	800	1,162	121	13,055
3	1,410	1,658	83	8,662
4	1,733	1,987	85	9,038
5	2,163	2,663	167	18,055
6 b	3,230	5,476	749	83,084
Orientatio	on b <sup>c</sup>	·		,
1'	885	658	76	8,789
2' <sup>d</sup>	2,836	1,439	466	49,477

Orientation as read from left to right.

<sup>&</sup>lt;sup>b</sup>ORF 6 corresponds to the aux1 gene.

<sup>&</sup>lt;sup>c</sup> Orientation as read from right to left.

dORF 2' corresponds to the aux2 gene.

gene seem to indicate identical ways of evolution for aux1 and tms1. ORF 8 is only weakly related to the aux1 gene; an explanation could be that these sequences are derived from a common ancestor and that ORF 8 has evolved

more rapidly than the others, perhaps due to the presence of the homologous auxI gene on the same plasmid. Whereas aux2 and tms2 have diverged from the P.  $savastanoi\ iaaH$  gene more than auxI and tmsI from iaaM, they have only

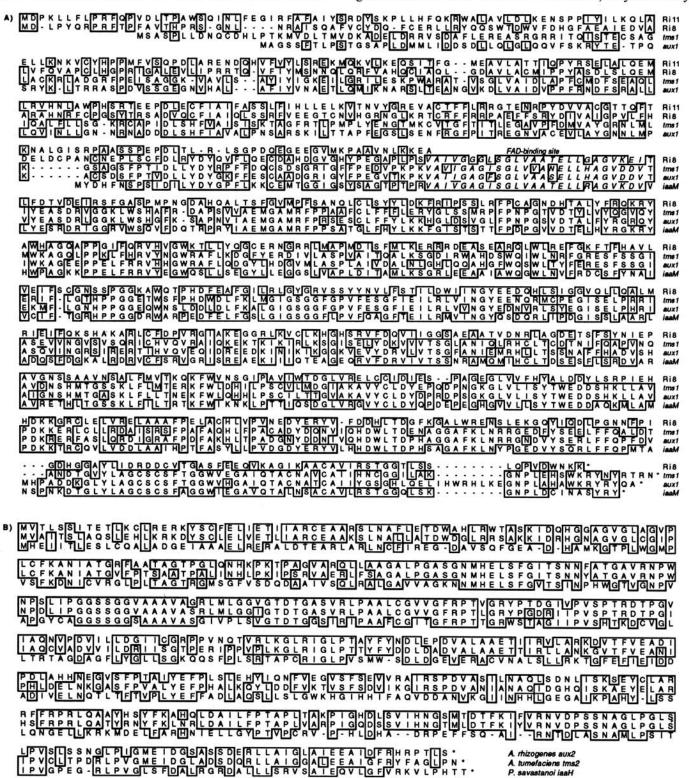


Fig. 3. Alignment of the protein sequences derived from the pRiA4 aux genes with those of related genes from pTiAch5 (Gielen et al. 1984), pRiA4 TL-DNA (Slightom et al. 1986), and Pseudomonas savastanoi (Yamada et al. 1985). Identical amino acids are boxed. A, Comparison of the pRiA4 aux1 sequence with pTiAch5 tms1, pRiA4 TL-DNA ORFs 8 (Ri8) and 11 (Ri11), and P. savastanoi iaaM. The conserved FAD-binding site is in italics. B, Comparison of the pRiA4 aux2 sequence with pTiAch5 tms2 and P. savastanoi iaaH sequences.

slightly diverged from one another.

In contrast, the promoter regions of the auxin synthesis genes were found to be substantially different in Ti and Ri plasmids, which could be related to different forms of regulation. In fact, several loci in the pRi T-DNA are implicated in the control of root proliferation, which results from the influence of several different mechanisms (Cardarelli et al. 1985; Vilaine and Casse-Delbart 1987; Cardarelli et al. 1987). Auxin biosynthesis is only one of these mechanisms; other mechanisms are under the control of TL-DNA genes. It is possible to imagine in A. rhizogenes a particular set of mechanisms resulting in the determination of the hormonal balance in which the promoter activity level of the auxin synthesis genes could be regulated by other T-DNA gene products. Functional analysis of the promoter region will determine if the potential regulatory sequences found in this region are actually implicated in the transcriptional regulation of the pRiA4 auxin synthesis genes. The expression of the aux1 and aux2 genes with deleted promoters is being studied.

There is no report in the literature of plants containing both the A. tumefaciens tms1 and tms2 genes. Tobacco plants have been transformed by the A. tumefaciens tms1 gene with its own promoter, giving phenotypically normal plants (Follin et al. 1985), whereas the tms1 gene under the control of the cauliflower mosaic virus 19S promoter gave petunia and tobacco plants an extremely abnormal morphology due to high levels of IAM and IAA (Klee et al. 1987). High level of expression of the tms1 gene alone is thus sufficient to lead to auxin levels resulting in highly modified plants. The plants transformed with the two pRiA4 auxin synthesis genes are phenotypically normal, indicating that the auxl gene is not sufficiently expressed to lead to auxin overproduction resulting in morphological alterations. The aux1 gene appears therefore

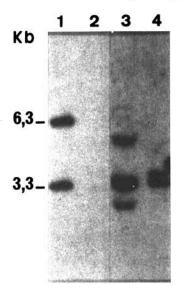


Fig. 4. Southern hybridization of HindIII-digested DNA with the 6kilobase (kb) SalI fragment. Lane 1 contains DNA from the plasmid pEMBL19 containing the 6-kb Sall fragment. The 3.3-kb fragment is an internal fragment corresponding to HindIII fragment 22 in Figure 1. Lane 2 contains genomic DNA from a normal tobacco plant. Lanes 3 and 4 contain genomic DNA from tobacco plants containing the 6-kb SalI fragment (lane 3, Sal6.1; lane 4, Sal6.5).

to be weakly active in seedlings and cuttings of certain Sal6 plants (i.e. Sal6.5), producing enough IAM and IAA to give slightly modified roots but not enough to deeply change the plant morphology. This modified root phenotype is soon outgrown to give normal plants, which may be related to the regulation of the genes in entire plants. Seeds and cuttings of plants transformed with the aux2 gene are able to convert NAM into NAA, producing high levels of auxin that inhibit rooting, as has been found in transgenic plants expressing the A. tumefaciens tms2 gene (Budar et al. 1986). Different levels of expression of the aux genes, leading to a more or less accentuated phenotype, have been observed. Preliminary results indicate that the level of DNA methylation of the Sal6.1 and Sal6.5 plants is not responsible for the differential levels of expression of the aux genes in these plants. The aux2 gene under the control of its own promoter can be used by now in

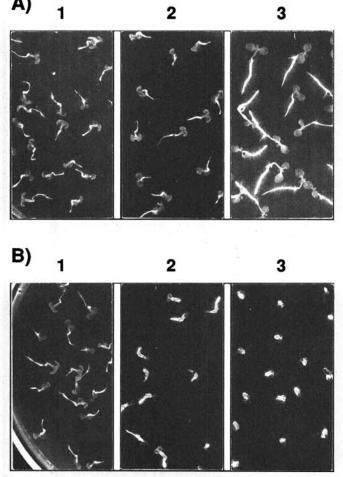


Fig. 5. Germination of seeds of wild-type and of homozygous transformed tobacco plants. A, Germination on Murashige and Skoog (1962) (MS) medium without naphthalene acetamide (NAM) (section 1, wild type; section 2, Sal6.1; and section 3, Sal6.5). Untransformed and Sal6.1 seeds germinate normally, but Sal6.5 seedlings show modified roots that are likely due to auxin overproduction. B, Germination on MS medium containing 1 mg/L of NAM (section 1, wild type; section 2, Sal6.1; and section 3, Sal6.5). Whereas the untransformed seeds germinate normally, seedlings expressing the aux2 gene are able to convert NAM into active auxin and present a modified phenotype (shorter and thicker roots in the Sal6.1 seedlings) amplified in Sal6.5 (callus instead of roots).

negative selection.

Knowledge of the auxin biosynthesis genes can be a very useful tool in studying the processes of plant differentiation. The possibility to overproduce hormones in plants constitutes an important step in studying the expression of genes regulated by auxin level and determining the role of phytohormones and the way in which they act.

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