The T-DNA Oncogene A4-*orf8* from *Agrobacterium rhizogenes* A4 Induces Abnormal Growth in Tobacco

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The related *orf8* and *iaaM* T-DNA genes from *Agrobacterium* are each composed of two distinct parts. The 5' parts (called *Norf8* or *NiaaM*) encode a 200-amino-acid (aa) sequence with homology to various T-DNA oncoproteins such as RolB, RolC, and 6b. The 3' parts (*Corf8* or *CiaaM*) encode a 550-aa sequence with homology to IaaM proteins from *Pseudomonas* and *Pantoea* spp. Whereas *iaaM* genes encode flavin adenine dinucleotide (FAD)-dependent tryptophan 2-monooxygenases that catalyze the synthesis of indole-3-acetamide (IAM), A4-*orf8* from *Agrobacterium rhizogenes* A4 does not. Plants expressing a 2x35S-A4-*Norf8* construct accumulate soluble sugars and starch. We now have regenerated plants that express the full-size 2x35S-A4-*orf8* and the truncated 2x35S-A4-*Corf8* gene. 2x35S-A4-*Corf8* plants accumulate starch and show reduced growth like 2x35S-A4-*Norf8* plants but, in addition, display a novel set of characteristic growth modifications. These consist of leaf hypertrophy and hyperplasia (blisters); thick, dark-green leaves; thick stems; and swollen midveins. Mutations in the putative FAD-binding site of A4-*Orf8* did not affect the blister syndrome. Plants expressing 2x35S-A4-*Corf8* had a normal phenotype but contained less starch and soluble sugars than did wild-type plants. When 2x35S-A4-*Corf8* plants were crossed to starch-accumulating 2x35S-A4-*Norf8* plants with reduced growth, A4-*Corf8* partially restored growth and reduced starch accumulation. A4-*Corf8*xA4-*Norf8* crosses did not lead to the blister syndrome, suggesting that this requires physical linkage of the A4-*Norf8* and A4-*Corf8* sequences.

Agrobacterium rhizogenes induces hairy roots on a large number of plant species (Meyer et al. 2000). The closely related *orf8* genes of the T-DNAs from *A. rhizogenes* strains A4, HRI, 8196, 1724, and 2659 are homologs of the well-known *Agrobacterium* tryptophan 2-monooxygenase (*iaaM*) T-DNA genes (Levesque et al. 1988) and the *iaaM* genes from *Pseudomonas savastanoi* (Yamada et al. 1985) and other bacteria. *iaaM* genes encode enzymes that carry out the oxidative decarboxylation of tryptophan to indole-3-acetamide (IAM) (Kosuge et al. 1966; Thomashow et al. 1986; van Onckelen et al. 1986) and are closely physically linked to *iaaH* genes whose products catalyze the final conversion of IAM to indole-3-acetic acid (IAA). Unlike *iaaM* genes, *orf8* genes are not associated with

iaaH genes and the A4-*orf8* gene from *A. rhizogenes* A4 does not encode an enzyme with detectable tryptophan 2-monooxygenase activity (Otten and Helfer 2001), in the case of the closely related strain HRI, only very low levels were reported (Lemcke et al. 2000). All *iaaM* and *orf8* genes from *Agrobacterium* carry a 700-bp 5' extension absent from the *iaaM* genes of *P. savastanoi*, *Pantoea agglomerans* (*Erwinia herbicola*), and other bacteria. This 5' sequence is related to a large group of strongly divergent T-DNA genes called *plast* or *rolB*-like genes and probably became fused to an ancestral *iaaM* gene during T-DNA evolution (Helfer et al. 2002; Levesque et al. 1988; Otten and Schmidt 1998). The 5' moieties of the *Agrobacterium iaaM/orf8* genes have been called *NiaaM/Norf8* and their 3' moieties *CiaaM/Corf8*. In transient expression assays, A-*CiaaM* (from *A. tumefaciens* Ach5) induced the same levels of IAM synthesis as did the full-size A-*iaaM* gene (Otten and Helfer 2001), showing that the A-*NiaaM* moiety plays no role in IAM synthesis. The *plast* family includes *NiaaM/Norf8*, *rolB*, *rolBTR*, *rolC*, *orf13*, *orf14*, *iso*, 5, 3', 6a, and 6b. Most of these genes induce specific and apparently unrelated growth effects (Helfer et al. 2002; Levesque et al. 1988; Meyer et al. 2000; Nilsson and Olsson 1997). In spite of numerous studies, it is not clear how these genes induce their various morphogenetic effects and whether they act through a common mechanism. Recently, it has been shown that the AKE10-6b and 1724-RolB proteins enter the plant nucleus with the help of host plant proteins: NtSIP1 for AKE10-6b (Kitakura et al. 2002) and Nt14-3-3 ω II for 1724-RolB (Moriuchi et al. 2004). The authors proposed that the nucleus-localized 6b and RolB proteins induce changes in gene expression and thereby lead to growth modifications. Because the Orf8 protein carries a RolB-like extension, it can be expected that it, too, leads to growth changes. Lemcke and Schmülling (1998) obtained transgenic tobacco plants that expressed the *orf8* gene from *A. rhizogenes* HRI under control of a modified 35S promoter. These plants had a normal phenotype and synthesized low levels of IAM (Lemcke et al. 2000). Germinating seedlings were tolerant to normally inhibitory concentrations of the synthetic auxin naphthalene acetic acid (NAA). Similar results were reported recently for tobacco plants expressing the *orf8* gene from *A. rhizogenes* 2659 under 35S promoter control (Ouarts et al. 2004). The 2659-*orf8* plants had lanceolate cotyledons but mature plants were normal. In view of the HRI-*orf8* results, it came as a surprise that the 5' terminal sequence of A4-*orf8* (A4-*Norf8*, from *A. rhizogenes* A4, identical to the HRI sequence) induced significant changes in transgenic plants. Some A4-*Norf8* regenerants contained very high levels of soluble sugars and starch, and showed intercostal leaf chlorosis and strong growth reduction (Otten and Helfer 2001). Starch

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accumulation in A4-*Norf8* plants resulted from the inhibition of sucrose export from source leaves to sink tissues (Umber et al. 2002). The lack of phenotype of HRI-*orf8* plants might be reconciled with the strong phenotype of A4-*Norf8* plants if the *Corf8* moiety would somehow reverse the *Norf8*-induced effects. In order to gain more insight into the activities of *orf8* genes and their *Norf8* and *Corf8* sequences, we regenerated and analyzed tobacco plants that express the full-size A4-*orf8* gene and the 3' terminal A4-*Corf8* moiety. In addition, we combined the two parts in the same plant by crosses.

RESULTS

Regeneration and properties of 2x35S-A4-*orf8* and 2x35S-A4-*Corf8* tobacco plants.

The 2x35S-A4-*orf8* and 2x35S-A4-*Corf8* genes lack IAM synthesis in transient leaf patch expression assays, contrary to

2x35S-A-*iaaM* and 2x35S-A-*CiaaM* constructs (Otten and Helfer 2001). The 2x35S-A4-*orf8* and 2x35S-A4-*Corf8* constructs were used to obtain stably transformed *Nicotiana tabacum* Samsun nn plants. In addition, transformed plants carried a kanamycin resistance marker and a 35S-*gusA* gene. For each construct, a series of 30 T0 regenerants were obtained. Single-locus plants were identified by growth of T1 seed on kanamycin, and homozygous single-locus plants were identified by T2 analysis. The T0 lines are indicated by 2x35S-A4-*orf8*-X and 2x35S-A4-*Corf8*-Y, and hemizygous and homozygous single-locus lines by the suffixes HE and HO, respectively. All 30 2x35S-A4-*Corf8* T0 lines were phenotypically normal (not shown), whereas 21 of 30 2x35S-A4-*orf8* T0 lines had an abnormal phenotype. Compared with a 2x35S-*gusA*-7HO control line (Toquin et al. 2003) (Fig. 1A), several 2x35S-A4-*orf8* plants were stunted (Fig. 1B through D) and had thick fleshy midribs (Fig. 1E and F) and a rough, mottled leaf surface (Fig.

Fig. 1. Phenotypes of 2x35S-A4-*orf8* plants and Northern analysis. **A**, 2x35S-*gusA*-7HO control plant: normal growth. **B–D**, Lines 2x35S-A4-*orf8*-14HO, 2x35S-A4-*orf8*-5HO, and 2x35S-A4-*orf8*-20HO show various levels of growth inhibition. **E**, Leaf of 2x35S-*gusA*-7HO control line. **F**, Leaf detail of line 2x35S-A4-*orf8*-19 with dark-green leaf surface and swollen midvein. **G**, Leaf detail of line 2x35S-A4-*orf8*-8 with rough surface. **H**, Older leaf of line 2x35S-A4-*orf8*-8 with mosaic leaf chlorosis at the leaf edge and dark-green surface at the center of the leaf. **I**, Leaf of a 2x35S-A4-*Norf8*-32HE plant with mosaic leaf chlorosis but no dark-green leaf center. **J**, Appearance of blisters on an early leaf of line 2x35S-A4-*orf8*-14HO. **K**, Normal tobacco flower (left) and an enlarged flower of line 2x35S-A4-*orf8*-5HO (right). Bars: 10 cm (A–D), 1 cm (E–K). **L**, Northern analysis of total RNA for three selected homozygous 2x35S-A4-*orf8* lines. The *orf8* coding region was used as a probe. Lane 1, untransformed tobacco; lane 2, 2x35S-A4-*orf8*-14HO; lane 3, 2x35S-A4-*orf8*-5HO; lane 4, 2x35S-A4-*orf8*-20HO; 25S = 25S RNA from the ethidium bromide-stained gel used as an RNA loading control.

1G). Changes appeared progressively. At later stages, leaves became dark green (Fig. 1F) and intercostal leaf chlorosis appeared (Fig. 1H). Chlorosis also was found on 2x35S-A4-*Norf8* leaves (Otten and Helfer 2001), but the latter did not have a rough surface and did not become dark green (Fig. 1I). Leaf modifications in 2x35S-A4-*orf8* plants sometimes appeared locally, in an apparently random fashion (Fig. 1J). Flowers generally were larger (Fig. 1K). RNA analysis of F2 progeny of three selected homozygous single-locus lines, 2x35S-A4-*orf8*-14HO, -5HO, and -20HO, showed increasing levels of expression related to the severity of the phenotypes (Fig. 1L).

The rough, mottled leaves were investigated in more detail using the 2x35S-A4-*orf8*-5HO line (Fig. 2). On early leaves, three distinct and spatially separated types of abnormal growth appeared: dark green spots (Fig. 2A), adaxial blisters (Fig. 2C), and abaxial blisters (Fig. 2E). The different zones later merged, forming the rough, mottled pattern shown in Fig. 1G. Anatomical analysis showed that the dark-green spots were due to hypertrophic growth of palisade parenchyma cells (Fig. 2B), whereas adaxial blisters resulted from hypertrophy of adaxial epidermal cells, hyperplasia of adaxial cells, and an increase of intercellular spaces (Fig. 2D). Abaxial blisters were due to hypertrophy of abaxial epidermal cells (Fig. 2F).

The late-appearing chlorosis of 2x35S-A4-*orf8* leaves (Fig. 1H) might be due to accumulation of soluble sugars and starch, as in 2x35S-A4-*Norf8* plants. Therefore, 2x35S-A4-*orf8*-5HO, 2x35S-A4-*orf8*-14HO, and 2x35S-A4-*orf8*-20HO F2 plants were compared with 2x35S-*gusA*-7HO and 2x35S-A4-*Norf8*-32HO (Otten and Helfer 2001) control plants. Results obtained 10 weeks after germination, are shown in Figure 3. Leaves of 2x35S-A4-*orf8*-5HO had levels close to those of control plants. Leaves of 2x35S-A4-*orf8*-14HO had lower val-

ues for glucose, fructose, and starch. Finally, glucose, fructose, and starch values for 2x35S-A4-*orf8*-20HO leaves, although variable, were always higher than for normal leaves. In addition, 2x35S-A4-*Norf8*-32HO had high levels of all compounds, as found previously (Umber et al. 2003). Both 2x35S-A4-*orf8*-20HO and 2x35S-A4-*Norf8*-32HO showed chlorosis. At later growth stages, 2x35S-A4-*orf8*-5HO and 2x35S-A4-*orf8*-14HO plants also developed chlorosis and accumulated starch contrary to *gusA* control plants (not shown).

NAA sensitivity of A4-*orf8* and A4-*Corf8* seedlings.

A4-*orf8* (Lemcke and Schmülling 1998) and 2659-*orf8* (Quarts et al. 2004) tobacco seedlings grow on a normally inhibitory NAA concentration of 5 μ M. In contrast, 2x35S-A4-*Norf8* seedlings are slightly more NAA sensitive than wild-type seedlings (Umber et al. 2002), suggesting that NAA insensitivity requires the *Corf8* part. The A4-*orf8*-induced blister syndrome could be due to a modification of auxin sensitivity. Therefore, we tested seedlings of the 2x35S-A4-*orf8*-14HO, 2x35S-A4-*orf8*-20HO, 2x35S-A4-*Corf8*-14HO, and 2x35S-A4-*Corf8*-26HO lines (each with strong transgene expression as tested by Northern analysis; not shown), on 0 and 5 μ M NAA. On medium without NAA, all grew normally; whereas, on 5 μ M NAA, all showed a similar degree of growth inhibition (results not shown). Thus, A4-*orf8* does not provide NAA resistance to seedlings and the abnormal blister phenotype does not seem to be correlated with it.

A4-*orf8* and A4-*Norf8* plants

do not transmit their phenotype across a graft junction.

Rootstocks of tobacco plants expressing A4-*orf13* (Hansen et al. 1993), AB-6b (Helfer et al. 2003), or T-6b (Grémillon et al. 2004) have the remarkable capacity to transmit their pheno-

Fig. 2. Anatomical analysis of growth modifications in line 2x35S-A4-*orf8*-5HO. **A**, Dark-green spot. **B**, Transverse section through dark-green spot: hypertrophic palisade parenchyma cells. **C**, Adaxial blister. **D**, Transverse section through adaxial blister: hypertrophic adaxial epidermis cells, hyperplasia of subepidermal adaxial cells, large intercellular spaces. **E**, Abaxial blister. **F**, Transverse section through abaxial blister: hypertrophic abaxial epidermis cells. Thin lines in A, C, and E indicate investigated areas. Double arrows in B, D, and F indicate modified regions. Thick bars in A, C, and E = 2 mm and in B, D, and F = 50 μ m.

types to wild-type scions across graft junctions. We tested whether this also was true for A4-*Norf8* or A4-*orf8* plants. The 2x35S-A4-*Norf8*-32HO and 2x35S-A4-*orf8*-20HO plants (both with a strong phenotype) were used as rootstocks and wild-type Samsun nn tobacco as scions. No graft transmission was observed (not shown). Thus, phenotype graft transmission is not a general characteristic for genes of the *plast* family.

Mutation of the putative A4-Orf8 and A-IaaM flavin adenine dinucleotide (FAD)-binding sites.

Tryptophan 2-monooxygenases (IaaM or TMO enzymes) belong to a large group of flavoenzymes that catalyze the oxidation of a variety of substrates (Kubo et al. 1997) and the even larger group of L-amino acid oxidases (LAAO) (Sobrado and Fitzpatrick 2002). In the *Pseudomonas savastanoi* IaaM enzyme, the R98 residue is essential for tryptophan binding. R98 is conserved in nearly all members of the LAAO family. Orf8 proteins constitute a remarkable exception: they carry a proline instead of arginine. In the *P. savastanoi* IaaM enzyme, several other highly conserved residues have been reported: G75, G76, H338, and C511, the latter two being involved in maintaining protein structure (Sobrado and Fitzpatrick 2002). Orf8 proteins carry RS instead of GG and have N (I in 1724-Orf8) and R (S in 1724-Orf8) at the H338 and C511 positions. The above-mentioned changes (Fig. 4A) strongly suggest that the Orf8 proteins do not have a classical TMO activity.

In spite of these modifications, Orf8 proteins seem to possess a bona fide FAD-binding domain with a canonical GxGxxG motif, as in IaaM and other flavin-binding enzymes (Levesque et al. 1988) (Fig. 4A and B). The first two G residues of the GxGxxG motif are extremely conserved in FAD-dependent enzymes, the third G somewhat less (Stehr et al. 1998). A4-Orf8, therefore, could have an FAD-dependent enzyme activity that would be likely to play a role in the induction of the blister

syndrome. Kubo and associates (1997) studied the FAD-binding domain of rat liver FMO1 (GAGVSG) in detail. GAAVSG and GAGVSV mutations abolished enzyme activity. We introduced the same mutations, separately and combined in the A4-Orf8 and A4-Corf8 GGGLSG sites by site-directed mutagenesis, obtaining GGALSG, GGGLSV, and GGALSV mutants. The constructs were cloned in the 2x35S promoter cassette of the pCK vector (Carrington et al. 1991) and stably introduced in tobacco (Materials and Methods). Transgenic plants carrying the A4-Orf8 FAD site mutations had blister phenotypes similar to those of 2x35S-A4-*orf8* plants; those carrying the A4-Corf8 FAD site mutations had a normal phenotype, as expected (not shown). Rat FMO1 is only distantly related to IaaM enzymes; therefore, the GxGxxG residues in Orf8 might not have the same important roles as in FMO1. A-IaaM from the *A. tumefaciens* octopine strain Ach5 is a close homologue of A4-Orf8 and leads to high IAM levels in a transient tobacco leaf expression system (Otten and Helfer 2001). Therefore, we mutated the putative A-IaaM FAD-binding site GAGISG to GAAISG and GAGISV. The GAGISV mutant showed reduced levels of IAM synthesis, whereas the GAAISG mutant had completely lost its capacity to synthesize IAM (Fig. 4C). Thus, as in the case of rat FMO1, A-IaaM has an absolute requirement for a G at position 3 of its FAD-binding site and has a much lower activity when the G at position 6 is replaced by alanine. Because the same mutation that eliminates IAM synthesis in A-IaaM does not affect the capacity of the closely related A4-Orf8 to induce the blister syndrome, the blister syndrome does not require a monooxygenase activity.

Combining A4-Norf8 and A4-Corf8 by crosses.

Neither 2x35S-A4-*Norf8* (Otten and Helfer 2001) nor 2x35S-A4-*Corf8* plants (this study) show the A4-*orf8* phenotype. Therefore, we postulated that only the simultaneous pres-

Fig. 3. Soluble sugars and starch accumulation in 2x35S-A4-*orf8* plants. Levels of soluble sugars and starch were determined for 2x35S-*gusA*-7HO control plants, 2x35S-A4-*orf8*-14HO, 2x35S-A4-*orf8*-5HO, and 2x35S-A4-*orf8*-20HO plants, and 2x35S-A4-*Norf8*-32HO control plants (for the latter, only four plants were analyzed). Because of high variability between plants of line 20HO, values for individual plants are shown. The 2x35S-A4-*orf8*-20HO and 2x35S-A4-*Norf8*-32HO plants have higher and 2x35S-A4-*orf8*-14HO plants lower hexose values than the 2x35S-*gusA*-7HO control plants.

ence of both genes induces the blister syndrome. In order to test this, a 2x35S-A4-*Corf8*-26HE plant (this study) was crossed with a 2x35S-A4-*Norf8*-5HE plant (Otten and Helfer 2001). Care was taken to select two lines with similar transgene expression levels, as tested by Northern analysis. Plants of the F1 population were analyzed by Northern analysis. As expected, four categories were found: plants expressing A4-*Norf8* (N plants), A4-*Corf8* (C), A4-*Norf8* and A4-*Corf8* (N+C), or none of the transgenes (WT) (Fig. 5A and B). N+C plants showed leaf chlorosis like A4-*Norf8* plants but no blistering. Thus, combining the A4-*Norf8* and A4-*Corf8* constructs in one plant was not sufficient to induce the blister syndrome; a physical linkage between the two parts apparently is essential. However, the combination of the two genes led to another, unexpected phenomenon. Growth rates of N+C plants clearly were intermediate between those of the parents, the slow-growing N plants and normal-growing C plants (Fig. 5C and D). Because growth reduction in A4-*Norf8* plants is due to soluble sugar and starch accumulation, this suggested that A4-*Corf8* partially reversed this effect. Therefore, we measured soluble sugars and starch in N, C, N+C, and WT plants (Fig. 5E). As expected, N plants had high levels of all compounds. C plants had lower glucose, sucrose, and starch levels than WT plants, whereas N+C plants had sucrose and starch levels that were significantly lower than those of N plants. A cross between a 2x35S-A4-*Norf8*-5HE plant and a plant from another A4-*Corf8* line, 2x35S-A4-*Corf8*-33HE, yielded essentially the

same results (not shown). Remarkably, A4-*Corf8* can reduce sucrose and starch accumulation in a wild-type background and, even more clearly, in an A4-*Norf8* background that normally leads to high levels of these compounds.

DISCUSSION

Lemcke and Schmölling (1998) reported that an HRI-*orf8* construct under control of a modified 35S promoter did not alter the normal tobacco phenotype but allowed germination at normally inhibitory NAA levels. These results have been confirmed by Ouarts and associates (2004) for 2659-*orf8*. In this article, we describe several independent 2x35S-A4-*orf8* tobacco lines with strong, highly specific growth changes. None showed an increase in NAA tolerance. The discrepancies between our results and those of the two other groups might be due to differences between the *orf8* sequences and

Fig. 4. Conserved sites in *Pseudomonas savastanoi* IaaM and related proteins and IaaM mutant analysis. **A**, Schematic structure of *P. savastanoi* IaaM sequence with flavin adenine dinucleotide (FAD)-binding site and conserved GG, arginine, histidine, and cysteine residues and corresponding residues in A4-Orf8. **B**, Putative FAD-binding sites of *Calloselasma rhodostoma* L-amino acid oxidases, *Rattus norvegicus* FMO1, *P. savastanoi* TMO, *A. tumefaciens* A-IaaM, *A. rhizogenes* A4-Orf8, and GxGxxG consensus sequence. **C**, Indole-3-acetamide (IAM) synthesis in leaf patches infiltrated with *Agrobacterium* strains carrying different 2x35S-A-*iaaM* gene constructs, four patches per construct. 1 = Empty vector control. 2 = 2x35S-A-*iaaM* with the nonmutated FAD-binding site sequence GAGISG: high levels of IAM synthesis. 3 = GAAISG mutant: no IAM synthesis. 4 = GAGISV mutant: low levels of IAM synthesis.

Fig. 5. Cross between a 2x35S-A4-*Norf8*-5HE plant and a 2x35S-A4-*Corf8*-26HE plant. Properties of F1 plants. **A**, Northern analysis of F1 plants; A4-*Norf8* and A4-*Corf8* messenger positions are indicated by N and C, and S indicates start of migration. Lanes 1–3 and 5, N+C plants; lanes 4 and 10, C plants; lanes 6–9 and 11–14, N plants. **B**, Same Northern analysis as in A, ribosomal RNA loading controls. **C**, C, N, and N+C plants after 12 weeks of growth. N+C plants are intermediate in size between N and C plants. **D**, Heights of wild-type (●), N (▲), C (○), and N+C (×) plants in centimeters (average values with standard errors of the mean). **E**, Soluble sugars and starch in wild-type, N, C, and N+C plants (starch levels × 0.1) 11 weeks after germination. Shown are average values with standard errors of the mean.

their promoters or to the use of another tobacco cultivar (Samsun nn instead of Petit Havana SR1). The striking 2x35S-A4-*orf8* tobacco phenotype has not been reported before. Its main characteristics are hypertrophy (cell size increase) and hyperplasia (increased cell division). The AvrBs3 protein of *Xanthomonas campestris* induces mesophyll hypertrophy in tobacco after transfer from the bacterium to the plant nucleus (Marois et al. 2002); the protein is unrelated to RolB-like proteins. The AvrBs3 effects were obtained by patch infiltration and, so far, no transgenic AvrBs3 plants have been reported, so that no effects on stem or flower growth are known. The related *pthA* gene from *X. citri* induces cell enlargement, division, and death in *Citrus sinensis* cells but not in tobacco or in other species (Duan et al. 1999). Recently, virus-induced gene silencing of the *NbDEK* gene, which encodes a calcium-dependent cysteine protease (phycalpain), has been shown to induce generalized hyperplasia and ectopic cell division in *N. benthamiana* (Ahn et al. 2004), but the mechanism involved remains obscure. Interestingly, the *T-6b* oncogene (distantly related to *Norf8*) induces hypertrophy in cotyledons and roots of transgenic tobacco plants (Grémillon et al. 2004), but no blisters.

A detailed comparison between the C-terminal Orf8 part and related sequences revealed that several functionally important conserved residues are modified in Orf8 proteins, but the FAD-binding site seemed intact. A point mutation in the FAD-binding site of *A. tumefaciens* A-*IaaM* abolished IAM synthesis, but the same mutation in A4-Orf8 did not affect the blister phenotype. Therefore, we conclude that A4-Orf8 does not require an FAD-dependent enzyme activity to induce the growth modifications. It is likely that the induction of the blister syndrome by A4-Orf8 involves the A4-NOrf8 moiety because of its homology to other RolB-like growth-modifying oncoproteins. Co-expression of the A4-*Corf8* and A4-*Norf8* constructs, obtained by crosses between A4-*Corf8* and A4-*Corf8* plants, did not induce blisters; therefore, we conclude that blister formation requires a physical link between the A4-NOrf8 and A4-*Corf8* moieties. Possibly, this link provides a special localization to the A4-NOrf8 part required for full activity. Further studies should be aimed at the cellular localization of the A4-Orf8 protein and its constituent parts.

Although A4-*Corf8* did not confer a visible phenotype, it reduced the levels of soluble sugars and starch. This unexpected phenomenon was confirmed in A4-*Corf8* × A4-*Norf8* crosses: the introduction of the A4-*Corf8* gene into A4-*Norf8* plants reversed the slow growth and high soluble sugar and starch levels typical of A4-*Norf8* plants. The fact that A4-*Corf8* also causes soluble sugar and starch reduction in the absence of A4-NOrf8 makes it unlikely that its effects require direct interaction with A4-NOrf8. Whether the A4-*Corf8* protein reverts the A4-*Norf8*-induced sucrose export inhibition by favoring sucrose export or acts more indirectly awaits further analysis. As part of the intact A4-Orf8 protein, the A4-NOrf8 and A4-*Corf8* moieties might have the same opposing effects. Therefore, the physiological and phenotypic outcome of A4-*orf8* expression might depend on the relative activities of A4-*Corf8*, A4-NOrf8, and A4-Orf8. The complex interplay of these activities possibly might explain the stochastic appearance of blisters on leaves and the paradoxically low levels of soluble sugars and starch observed at early stages of development in some A4-*orf8* regenerants.

MATERIALS AND METHODS

Mutagenesis of putative FAD-binding sites.

Putative FAD-binding sites were mutated by polymerase chain reaction (PCR) amplification of the complete A4-*orf8*,

A4-*Corf8*, and A-*iaaM* pCK derivatives constructed earlier (Otten and Helfer 2001). The complementary PCR primers carried one or both of the point mutations G260A (GGG→GCG) and G263V (GGC→GTC). The same method was used for A-*iaaM* mutagenesis with primers carrying the point mutations G245A (GGG→GCG) and G248V (GGC→GTC). After *DpnI* digestion of the plasmid templates, PCR products were introduced into *Escherichia coli* TG2 and sequenced, and *HindIII* fragments were introduced into pBI121.2 (CLONTECH, Palo Alto, CA, U.S.A.) and introduced into *Agrobacterium* LBA4404 (Hoekema et al. 1983) by electroporation.

Transient expression, stable transformation, genetic analysis, and Northern analysis.

Transient expression assays were carried out by agroinfiltration of mature *N. rustica* leaves (patch assay) (Helfer et al. 2002). IAM synthesis in patches was obtained by infiltration of patches with 10 mM tryptophan in water 5 days after agroinfection. After 7 h of incubation, patches were extracted and analyzed for IAM by thin-layer chromatography as described (Otten and Helfer 2001). Transgenic *N. tabacum* cv. Samsun nn plants were obtained with the method described in Otten and Helfer (2001). Seed of transformants were sterilized with 10% commercial bleach, washed with sterile water, and germinated on MS222 medium (Duchefa, Haarlem, The Netherlands). The number of T-DNA loci for each line was determined by growing 300 T1 seed on plates with kanamycin at 300 mg/liter. Hemizygous (HE) and homozygous (HO) lines with a single T-DNA locus were identified by growth of T2 seed on kanamycin. Transgene expression was analyzed by Northern analysis. RNA was isolated according to Verwoerd and associates (1989), and 15 µg of total RNA was loaded on a denaturing gel. For hybridization, ³²P-labeled probes prepared by nick translation of the relevant coding sequences were used.

Soluble sugars and starch measurements.

Glucose, fructose, sucrose, and starch were measured with enzymatic assays as described (Umber et al. 2002), using early source leaves from plants of the same age (11 weeks after germination) and grown in the same batch. The standard deviation due to experimental errors was less than 0.1 mmole/m².

Microscopy.

One-micron leaf sections were prepared as described (Helfer et al. 2002) and viewed and photographed with a Nikon Eclipse E800 microscope, equipped with a Sony DXC950 camera. Images were analyzed with image analysis software (VisioLab2000, Biocom, Paris).

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LITERATURE CITED

- Ahn, J.-W., Kim, M., Lim, J. H., Kim, G.-T., and Pai, H.-S. 2004. Phycalpain controls the proliferation and differentiation fates of cells in plant organ development. *Plant J.* 38:969-981.
- Carrington, J. C., Freed, D. D., and Leinicke, A. J. 1991. Bipartite signal sequence mediates nuclear translocation of the plant potyviral NIa protein. *Plant Cell* 3:953-962.
- Duan, Y. P., Castaneda, A., Zhao, G., Erdos, G., and Gabriel, D. W. 1999. Expression of a single, host-specific, bacterial pathogenicity gene in plant cells elicits division, enlargement and cell death. *Mol. Plant-Microbe Interact.* 12:556-560.
- Grémillon, L., Helfer, A., Clément, B., and Otten, L. 2004. New plant growth-modifying properties of the *Agrobacterium* T-6b oncogene re-

- vealed by the use of a dexamethasone-inducible promoter. *Plant J.* 37:218-228.
- Hansen, G., Vaubert, D., Héron, J. N., Clérot, D., Tempé, J., and Brevet, J. 1993. Phenotypic effects of overexpression of *Agrobacterium rhizogenes* T-DNA ORF13 in transgenic tobacco plants are mediated by diffusible factor(s). *Plant J.* 4:581-585.
- Helfer, A., Pien, S., and Otten, L. 2002. Functional diversity and mutational analysis of *Agrobacterium* 6B oncoproteins. *Mol. Gen. Genomics* 267:577-586.
- Helfer, A., Clément, B., Michler, P., and Otten, L. 2003. The *Agrobacterium* oncogene AB-6b causes a graft-transmissible enation syndrome in tobacco. *Plant Mol. Biol.* 52:483-493.
- Hoekema, A., Hirsch, P. R., Hooykaas, P. J. J., and Schilperoort, R. A. 1983. A binary plant vector strategy based on separation of vir- and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179-180.
- Kitakura, S., Fujita, T., Ueno, Y., Terakura, S., Wabiko, H., and Machida, Y. 2002. The protein encoded by oncogene 6b from *Agrobacterium tumefaciens* interacts with a nuclear protein of tobacco. *Plant Cell* 14:451-463.
- Kosuge, T., Heskett, M. G., and Wilson, E. E. 1966. Microbial synthesis and degradation of indole-3-acetic acid. I. The conversion of L-tryptophan to indole-3-acetamide by an enzyme system from *Pseudomonas savastanoi*. *J. Biol. Chem.* 241:3738-3744.
- Kubo, A., Itoh, S., Itoh, K., and Kamataki, T. 1997. Determination of FAD-binding domain in flavin-containing monooxygenase 1 (FMO1). *Arch. Biochem. Biophys.* 345:271-277.
- Lemcke, K., and Schmülling, T. 1998. Gain of function assays identify non-rol genes from *Agrobacterium rhizogenes* TL-DNA that alter plant morphogenesis or hormone sensitivity. *Plant J.* 15:423-433.
- Lemcke, K., Prinsen, E., van Onckelen, H., and Schmülling, T. 2000. The ORF8 gene product of *Agrobacterium rhizogenes* TL-DNA has tryptophan 2-monooxygenase activity. *Mol. Plant-Microbe Interact.* 13:787-790.
- Levesque, H., Delépelaire, P., Rouzé, P., Slightom, J., and Tepfer, D. 1988. Common evolutionary origin of the central portions of the Ri TL-DNA of *Agrobacterium rhizogenes* and the Ti T-DNAs of *Agrobacterium tumefaciens*. *Plant Mol. Biol.* 11:731-744.
- Marois, E., Van den Ackerveken, G., and Bonas, U. 2002. The *Xanthomonas* type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. *Mol. Plant-Microbe Interact.* 15:637-646.
- Meyer, A., Tempé, J., and Costantino, P. 2000. Hairy root: A molecular overview. Functional analysis of *Agrobacterium rhizogenes* T-DNA genes. Pages 93-139 in: *Plant Microbe Interactions*. G. Stacey and N. T. Keen, eds. American Phytopathological Society Press, St. Paul, MN, U.S.A.
- Moriuchi, H., Okamoto, C., Nishihama, R., Yamashita, I., Machida, Y., and Tanaka, N. 2004. Nuclear localization and interaction of RolB with plant 14-3-3 proteins correlates with induction of adventitious roots by the oncogene *rolB*. *Plant J.* 38:260-275.
- Nilsson, O., and Olsson, O. 1997. Getting to the root: The role of the *Agrobacterium rhizogenes* *rol* genes in the formation of hairy roots. *Physiol. Plant.* 100:463-473.
- Otten, L., and Helfer, A. 2001. Biological activity of the *rolB*-like 5' end of the A4-*orf8* gene from the *Agrobacterium rhizogenes* TL-DNA. *Mol. Plant-Microbe Interact.* 14:405-411.
- Otten, L., and Schmidt, J. 1998. A T-DNA from the *Agrobacterium tumefaciens* limited-host-range strain AB2/73 contains a single oncogene. *Mol. Plant-Microbe Interact.* 11:335-342.
- Ouartsi, A., Clérot, D., Meyer, A., Dessaux, Y., Brevet, J., and Bonfill, M. 2004. The T-DNA ORF8 of the cucumopine-type *Agrobacterium rhizogenes* Ri plasmid is involved in auxin response in transgenic tobacco. *Plant Sci.* 166:557-567.
- Sobrado, P., and Fitzpatrick, P. F. 2002. Analysis of the roles of amino acid residues in the flavoprotein tryptophan 2-monooxygenase modified by 2-oxo-3-pentynoate: Characterization of His338, Cys339 and Cys511 mutant enzymes. *Arch. Biochem. Biophys.* 402:24-30.
- Stehr, M., Diekmann, H., Smau, L., Seth, O., Ghisla, S., Singh, M., and Macheroux, P. 1998. A hydrophobic sequence motif common to N-hydroxylating enzymes. *Trends Biochem. Sci.* 23:56-57.
- Thomashow, M. F., Hugley, W. G., Buchholz, L. S., and Thomashow, L. S. 1986. Molecular basis for the auxin-independent phenotype of crown gall tumor tissues. *Science* 231:616-618.
- Toquin, V., Grausem, B., Geoffroy, P., and Legrand, M. 2003. Structure of the tobacco caffeic acid O-methyltransferase (COMT) II gene: Identification of promoter sequences involved in gene inducibility by various stimuli. *Plant Mol. Biol.* 52:495-509.
- Umber, M., Voll, L., Weber, A., Michler, P., and Otten, L. 2002. The *rolB*-like part of the *Agrobacterium rhizogenes* *orf8* gene inhibits sucrose export in tobacco. *Mol. Plant-Microbe Interact.* 15:956-962.
- van Onckelen, H., Prinsen, E., Inzé, D., Rüdelsheim, P., van Lijsebettens, M., Follin, A., Schell, J., van Montagu, M., and de Greef, J. 1986. *Agrobacterium* T-DNA gene 1 codes for tryptophan 2-monooxygenase activity in tobacco crown gall cells. *FEBS (Fed. Eur. Biol. Soc.) Lett.* 198:357-360.
- Verwoerd, T. C., Dekker, B. M. M., and Hoekema, A. 1989. A small-scale procedure for the rapid isolation of plant RNAs. *Nucleic Acids Res.* 17:2362.
- Yamada, T., Palm, C. J., Brooks, B., and Kosuge, T. 1985. Nucleotide sequences of the *Pseudomonas savastanoi* indoleacetic acid genes show homology with *Agrobacterium tumefaciens* T-DNA. *Proc. Natl. Acad. Sci. U.S.A.* 82:6522-6526.