Research Note

The ORF8 Gene Product of *Agrobacterium rhizogenes* TL-DNA Has Tryptophan 2-Monooxygenase Activity

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The open reading frame 8 (*ORF8*) is located on the TL-DNA of the phytopathogenic soil bacterium *Agrobacterium rhizogenes* strain A4. The predicted *ORF8* protein has a particular structure and is possibly a natural fusion protein. The N-terminal domain shows homology to the *A. rhizogenes* *rolB* protein and may modulate the auxin responsiveness of host cells. The C terminus has up to 38% homology to tryptophan 2-monooxygenases (*t2m*). We show that *ORF8* overexpressing plants contain a fivefold higher concentration of indole-3-acetamide (IAM) than untransformed plants. Protein extracts from seedlings and *Escherichia coli* overexpressing *ORF8* show significantly higher turnover rates of tryptophan to IAM than negative controls. We conclude that the *ORF8* gene product has tryptophan 2-monooxygenase activity.

Additional keywords: *aux1* gene, auxin sensitivity, T-DNA.

Agrobacterium rhizogenes infection of most dicotyledonous plant cells causes the formation of roots at the site of infection (hairy root disease). The infectious agent, the T-DNA, is transferred from the bacterial Ri-plasmid to the plant cell nucleus where it is integrated and T-DNA-located genes that carry morphogenetic information are transcribed (for a review see Nilsson and Olsson 1997).

A. rhizogenes agropine type strains carry two T-DNAs, the TL- and the TR-DNA (Jouanin 1984). At least two pathogenic pathways, both leading to root formation by the host, are realized by genes of the TL- and TR-DNA. The TR-DNA harbors two auxin synthesizing genes, *aux1* and *aux2* (Camilleri and Jouanin 1991). The auxin biosynthetic pathway catalyzed by the *aux1* and *aux2* gene products is similar to that in *A. tumefaciens* and *Pseudomonas savastanoi* and comprises two steps. First, oxidative decarboxylation of tryptophan leading to indole-3-acetamide (IAM) is catalyzed by tryptophan 2-monooxygenase (*t2m*), encoded by the *tms1*, *aux1*, and *iaaM* genes of *A. tumefaciens*, *A. rhizogenes*, and *Pseudomonas savastanoi*, respectively (Camilleri and Jouanin 1991; Comai

and Kosuge 1982; Van Onckelen et al. 1986). The second step, the conversion of IAM to indole-3-acetic acid (IAA), is catalyzed by IAM hydrolase, the product of the *tms2*, *aux2*, and *iaaH* genes in the above bacteria (Jouanin 1984; Schröder et al. 1984; Thomashow et al. 1984).

On the TL-DNA of agropine type *A. rhizogenes*, the *rolB* gene is primarily responsible for root induction (White et al. 1985), possibly by enhancing the auxin sensitivity of cells (Maurel et al. 1991; Spano et al. 1988). In some instances, *rolB* and additional auxin act in concert to induce root formation; the factors alone are not effective individually (Spena et al. 1987; White et al. 1985). TR-DNA genes could provide this additional auxin if both T-DNAs are transferred. However, data indicate that TL-DNA-located genes in some hosts increase the auxin content, independent of the presence of the TR-DNA. For example, it was shown that TL-DNA transformed pea roots contain increased IAM and IAA concentrations (Prinsen et al. 1992). Interestingly, *iaaM/tms1* gene-expressing, transgenic asparagus and petunia accumulate both IAM and IAA while *tms1*-transformed tobacco accumulate only IAM (Klee et al. 1987; Prinsen et al. 1990; Van Onckelen et al. 1985). This indicates that conversion of IAM to IAA in cells that express only *t2m* depends on a host-specific factor such as an endogenous hydrolase (Klee et al. 1987; Prinsen et al. 1990).

A candidate gene responsible for the increased IAM content in TL-DNA transgenic tissue is the open reading frame 8 (*ORF8*). Transcripts possibly corresponding to *ORF8* have been reported for TL-DNA-transformed tissues (Durand-Tardif et al. 1985; Ooms et al. 1986), indicating that the gene is transcribed in infected cells. The predicted *ORF8* protein has in its C terminus 37.7% homology to the *iaaM* gene product of *Pseudomonas syringae* pv. *savastanoi* (Levesque et al. 1988). We have tested whether or not the *ORF8* protein has *t2m* activity and show that the gene product catalyzes the formation of IAM from tryptophan.

We compared the predicted protein sequences of the *ORF8* gene product and six other proteins with homology to *t2m* proteins, using the Clustal W program (Higgins et al. 1994). These proteins included the *t2m* homologs of the *A. tumefaciens* strains Ach5 (Gielen et al. 1984) and Tm4 (Bonnard et al. 1991), of the TR-DNA of the *A. rhizogenes* strain A4 (Camilleri and Jouanin 1991), of *A. vitis* (Canaday et al.

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1992), of *Erwinia herbicola* (GenBank accession Q47861), and the *iaaM* protein of *Pseudomonas savastanoi* (Yamada et al. 1985). The results of the comparison are summarized in Table 1. The T-DNA encoded proteins are 723 to 755 amino acids long and consist of at least two characteristic domains. The N terminus of 213 amino acids shows significant homology to the *rolB* protein of the *A. rhizogenes* strain A4 T-DNA (Slightom et al. 1986). Interestingly, the ORF8 N terminus has the highest sequence homology to *rolB* of all known t2m, namely, 26% compared with 12 to 15% for all others (Table 1). The t2m proteins of *P. savastanoi* and *E. herbicola* are missing this N-terminal extension (Table 1).

The C-terminal domain of approximately 506 to 524 amino acids of the *Agrobacterium* proteins shows homology to t2m proteins. The homology ranges from 26.6 to 36.1% for the ORF8 t2m domain aligned to all other t2m domains. For the other t2m domains the mutual similarity is between 45.2 and 91.9% (Table 1). Thus, the protein encoded by ORF8 is most similar to *rolB* and is less similar to t2m proteins than to any other member of the t2m family. On a phylogenetic tree for all known t2m proteins, ORF8 is located on its own branch, while the TR-DNA-derived *aux1* protein is closely linked to the *A. tumefaciens* proteins (data not shown). The 23-amino-acid-long FAD binding site, as identified by Levesque et al. (1988), a characteristic functional motif of t2m proteins, and neighboring amino acids are highly conserved in all predicted proteins (data not shown).

The product of t2m activity, IAM, accumulates in tobacco plants that overexpress the *tms1* gene of *Agrobacterium tumefaciens* (Van Onckelen et al. 1986). Therefore, we compared the IAM content of ORF8 overexpressing SR1 tobacco plants (clone 8-11; Lemcke and Schmölling 1998) with SR1 wild-type plants and 35S-*iaaM* transgenic SR1 tobacco (Sitbon et al. 1992). Table 2 shows that both transgenic lines contained 5.5-fold and 24-fold more IAM, respectively, than wild-type plants. The IAA content of both transgenic lines was similar to that of wild type (Table 2).

Next, we tested whether the turnover of tryptophan increased in protein extracts of ORF8-overexpressing plants. Table 3 shows that twice as much IAM was formed by protein extracts of 5-week-old seedlings of line 8-11 than by extracts

of wild-type seedlings. Similarly, protein extracts of the positive control line 35S-*iaaM* converted three times more tryptophan than extracts from wild type (Table 3).

To further test the enzymatic activity of the ORF8 protein, we expressed ORF8 in *E. coli*. To this end, ORF8 was subcloned from pUC19-ORF8 (Lemcke and Schmölling 1998) after restriction with *Bam*HI and *Sal*I into the corresponding sites of expression vector pQE30, which adds a His-tag to the N terminus of the protein (Qiagen, Hilden, Germany). The *aux1* gene of the *A. rhizogenes* strain HRI TR-DNA encoding a functional t2m protein (Camilleri and Jouanin 1991; Gaudin and Jouanin 1995) served as a positive control. It was amplified from plasmid pFC43 (Jouanin 1984) by polymerase chain reaction (PCR; 5' end primer: GGGGTACCATGGCTGGAA TCCTCCTTCACATT; 3' end primer: GGGGCGTCGACTC ACGCTTGATACCTATACCGCT; cloning procedures are described in Sambrook et al. [1989]) and inserted after restric-

Table 2. Indole-3-acetamide (IAM) and indole-3-acetic acid (IAA) content of tobacco plants

Plant ^a	IAM ^b	IAA ^b
	[pmol × g fw ⁻¹ ± SE]	[pmol × g fw ⁻¹ ± SE]
SR1	156 ± 60	30 ± 21
35S-ORF8	853 ± 469	39 ± 21
35S- <i>iaaM</i>	3833 ± 301	31 ± 14

^a Four-week-old tobacco seedlings of 35S-ORF8 transgenic clone 8-11 (Lemcke and Schmölling 1998), 35S-*iaaM* transgenic tobacco plants (Sitbon et al. 1992), and untransformed tobacco SR1 were sown in vitro on MS (Murashige and Skoog medium) agar plates and harvested after 4 weeks of growth under standard conditions (Lemcke and Schmölling 1998).

^b IAM and IAA were extracted and purified as described earlier (Prinsen et al. 1995). The stable isotope (phenyl-¹³C₆)-indole-3-acetamide and (phenyl-¹³C₆)-indole-3-acetic acid (50 ng; Cambridge Isotope Laboratory, Cambridge, MA) were added as internal tracer for recovery and analytical purposes. After methylation, IAA-Me and IAM were analyzed by micro liquid chromatography with column switch configuration coupled to positive ion electrospray tandem mass spectrometry with multiple reactant monitoring (Prinsen et al. 1997, 1998). Quantification was performed by single reactant monitoring (SRM) of the [MH]⁺ ion and the appropriate product ion (190→130 for IAA-Me, 196→136 for ¹³C₆-IAA-Me, 175→130 for IAM, and 181→136 for ¹³C₆-IAM). Data are the average of three replicates.

Table 1. Domain structure of the open reading frame 8 (ORF8) protein and sequence comparison to tryptophan 2-monoxygenase homologous proteins

Bacterial strain ^a	Protein name	N-terminal <i>rolB</i> domain	C-terminal t2m domain
<i>Agrobacterium rhizogenes</i> A4, TL-DNA	ORF8	25.9 ^b	26.6 to 36.1 ^c
<i>A. rhizogenes</i> A4, TR-DNA	<i>aux1</i>	13.8 ^b	47.6 to 64.9 ^d
<i>A. vitis</i>	—	11.8 ^b	45.2 to 56.7 ^d
<i>A. tumefaciens</i> Ach5	<i>tms1</i>	15.0 ^b	49.0 to 91.9 ^d
<i>A. tumefaciens</i> Tm4	<i>tms1</i>	14.2 ^b	47.9 to 91.9 ^d
<i>E. herbicola</i>	—	—	45.2 to 68.8 ^d
<i>P. syringae</i> pv. <i>savastanoi</i>	<i>iaaM</i>	—	46.8 to 68.8 ^d

^a The program Clustal W (Higgins et al. 1994) was used for alignments. For source of sequence information see text.

^b Homology of the N-terminus to *rolB* of *A. rhizogenes* TL-DNA (Slightom et al. 1986).

^c Homology range of the C terminus to all other tryptophan 2-monoxygenase homologs (see text).

^d Homology range of the C terminus to all other t2m homologs except ORF8.

Table 3. In vitro indole-3-acetamide (IAM) formation by plant protein extracts

Plant ^a	IAM ^b [pmol × h ⁻¹ × mg protein ⁻¹ ± SE]
SR1	90 ± 10
35S-ORF8	194 ± 15
35S- <i>iaaM</i>	289 ± 18

^a Protein from 2 g of 5-week-old tobacco seedlings was extracted and determination of tryptophan 2-monoxygenase activity was performed according to Van Onckelen et al. (1986).

^b Tryptophan (4 mM) was used as substrate. Incubation time was 3 h at 37°C. Controls with 2 mM IAM, 2 mM indole-3-acetic acid (IAA), without substrate or without enzyme extract were assayed in parallel (data not shown). After incubation, 100 ng of ¹³C-IAM and an equal volume of 0.1 N HCl were added. Samples were purified on an RP-C18 (500 mg; Varian, Middleburg, The Netherlands) cartridge. IAM was eluted with 3 ml of diethylether. IAM was analyzed by micro liquid chromatography with column switch configuration coupled to positive ion electrospray tandem mass spectrometry with multiple reactant monitoring as described in Table 2. Data are average of eight replicates.

Fig. 1. Western blot (immunoblot) analysis of bacterial protein extracts. Lanes 1 and 2, pQE-aux1; lanes 3 and 4, pQE30-ORF8; lanes 5 and 6, pQE30; lane 7, size marker. Samples in lanes 1, 3, 5 are from bacteria grown at 37°C; samples in lanes 2, 4, 6 are from bacteria grown at 28°C. Upper arrow, His-tagged ORF8 protein in lanes 3 and 4; lower arrow, aux1 protein in lanes 1 and 2. Growth of bacteria and induction of gene expression by IPTG (isopropyl-β-D-thiogalactopyranoside) were carried out according to the Qiagen protocol (Qiagen, Hilden, Germany). Proteins were separated in a 12.5% polyacrylamide gel, 0.2% sodium dodecyl sulfate. After electrophoretic transfer of proteins to a polyvinylidene difluoride (PVDF) membrane the N-terminal HIS-tag of the protein was detected with the Qiaexpress^{MRGS}His-antibody, mouse IgG1 (Qiagen). Peroxidase coupled anti mouse IgG antibody (Fab specific) from goat was used as a secondary antibody (A 2304; Sigma, Deisenhofen, Germany). Peroxidase activity was detected by the ECL method according to the supplier's protocol (Amersham, Freiburg, Germany). The film was exposed for 3 min.

tion into the *KpnI* and *SalI* sites of vector pQE30. After induction by IPTG (isopropyl-β-D-thiogalactopyranoside) the production of ORF8 and aux1 protein in the bacteria was demonstrated by Western blot (immunoblot) analysis with His-tag-specific antibodies (Fig. 1). Bands of lower molecular weight detected on the gel for ORF8 could be degradation products. Table 4 shows that ORF8 and aux1 protein expressing bacteria produced 57 and 110% more IAM, respectively, than bacteria harboring the empty vector.

Taken together, the data strongly indicate that the ORF8 gene product has t2m activity. The experimental results obtained from plants and bacteria seem to indicate that the ORF8 gene product has a somewhat lower activity than the *iaaM* and *aux1* proteins. However, it should be noted that the activities cannot be compared directly. For example, differences in the optimal conditions for the various enzymes could account for the different turnover rates of ORF8, *iaaM*, and *aux1* proteins.

The fact that the TL-DNA of *A. rhizogenes* harbors a *t2m* gene is interesting. In some hosts the activity of the principal oncogene of the TL-DNA, *rolB*, is dependent on auxin (White et al. 1985). The ability to produce the auxin precursor IAM by a gene product encoded by the same T-DNA as *rolB* could be relevant for the success of the infection in host plants that possess IAM hydrolase activity and are thus able to convert IAM to IAA. In this context it would be interesting to determine whether the native *ORF8* promoter shares expression domains with the *rolB* promoter. In addition, localization of

Table 4. In vivo indole-3-acetamide (IAM) formation by bacteria

Clone ^a	IAM ^b [pmol × h ⁻¹ × mg protein ⁻¹ ± SE]
pQE30	389 ± 64
pQE30-ORF8	611 ± 72
pQE-aux1	817 ± 133

^a The pQE30, pQE30-ORF8, and pQE30-aux1 transgenic *Escherichia coli* strains (TG1) were grown over night in minimal A medium at 28°C and 200 rpm (Innova 2000 shaker, Merck Eurolab, Darmstadt, Germany). A 100-ml culture was inoculated with 5 ml of the overnight culture and grown further under the same conditions. After 3 h (OD₆₀₀ = 0.3) 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) was added; 2 h later (OD₆₀₀ = 0.7) tryptophan was added to a final concentration of 4 mM. Bacteria were harvested after 3 h of further growth.

^b Analysis of IAM in the supernatant was carried out as described in Table 2.

the second auxin-synthesizing gene *aux2* on the TR-DNA and the physical separation from *rolB* that is achieved in this way could provide protection from toxic auxin overproduction in *rolB*-infected cells.

Interestingly, ORF8 overproducing tobacco seedlings were protected against auxin in a seedling germination assay (Lemcke and Schmülling 1998). Increased auxin resistance, as judged by the morphology and survival of seedlings, has also been demonstrated in plants that overexpress the *ORF8* of the *A. rhizogenes* cucumopine plasmid pRi2659 (A. Meyer, *personal communication*). This ORF8 protein contains the *rolB* homologous N-terminal extension. In contrast, *iaaM* overproducing plants lacked the auxin protective phenotype (Lemcke and Schmülling 1998). Therefore, it could be that the protective activity is located in the N-terminal extension, which is most likely not needed for t2m activity. It would be interesting to investigate whether removal of the N-terminal part would alleviate the increased auxin resistance of seedlings or whether expression of the N-terminal part alone is sufficient to confer the protective effect. In summary, domain shuffling may have contributed to host range of the bacterium and fine-tuning of the auxin effects.

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