Biological Activity of the *rolB*-like 5' End of the A4-*orf8* Gene from the *Agrobacterium rhizogenes* TL-DNA

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The *iaaM* gene from different plant-associated bacteria encodes a tryptophan monooxygenase (IaaM) that catalyzes the synthesis of indole-3-acetamide (IAM), a precursor of indole-3-acetic acid (IAA). Unlike the IaaM proteins from other bacteria, *Agrobacterium* spp. T-DNA-encoded IaaM proteins carry a 200 amino acid N-terminal extension with low homology to various members of the RolB protein family. This family is composed of 18 highly divergent T-DNA-encoded proteins, the basic functions of which are still largely undetermined. Deletion of the 5' *rolB*-like extension of the *iaaM* gene from *Agrobacterium tumefaciens* strain Ach5 did not lead to a reduction in IAM synthesis in plants. When expressed in tobacco, the *rolB*-like fragment did not affect growth or morphology. An *iaaM* homolog (A4-*orf8*) from the TL-DNA of *Agrobacterium rhizogenes* strain A4 also was investigated. Neither the full-size A4-*orf8* gene nor the 5'-truncated form induced detectable IAM synthesis. Plants expressing the *rolB*-like part of the A4-*orf8* gene, however, were dwarfed and mottled to various extents and synthesized abnormally high amounts of glucose, fructose, sucrose, and starch.

Additional keywords: crown gall tumors, hairy roots, starch synthesis, T-DNA evolution.

On susceptible plants, *Agrobacterium* spp. induces crown gall tumors or hairy roots in which large amounts of small molecules are synthesized. These substances, called opines, are a source of energy and metabolic intermediates for the bacterium. Plant cell growth and opine synthesis are encoded by one or more fragments of bacterial DNA (T-DNA), transferred to the plant cell during infection (Zupan and Zambryski 1997). For many opine synthesis genes, a biochemical mechanism has been established, but for most of the plant growth inducing genes only phenotypes have been described (Gaudin et al. 1994; Nilsson and Olsson 1997). Over ten years ago, Levesque et al. (1988) reported weak homologies between a number of T-DNA-encoded proteins. More recent homology searches (Otten and Schmidt 1998; Otten et al. 1999) revealed a group of at least 18 highly divergent T-DNA-encoded proteins, which we called the RolB family. Most of these proteins

(encoded by genes *rolB*, *rolB*^{TR}, *rolC*, *iso*, 3', 5, 6a, 6b, 7, b, c', d, e, *orf8*, *orf13*, *orf14*, *orf18*, and *iaaM*) range from 180 to 260 amino acids. Some of the *rolB*-like genes induce tumor-like growth on a limited range of plant species: *iso* (Otten and Schmidt 1998), *6b* (Hooykaas et al. 1988; Tinland et al. 1989), and 3' (Otten et al. 1999), whereas others modify the growth of transgenic plants carrying them: *rolB* and *rolC* (Schmülling et al. 1988), *rolB*^{TR} (Lemcke and Schmülling 1998a), gene 5 (Körber et al. 1991) and *6b* (Tinland et al. 1992; Wabiko and Minemura 1996), and *orf13* (Hansen et al. 1993). Gene *6a* facilitates transport of nopaline and octopine (Messens et al. 1985). No phenotype has been found for genes 7, b, c', d, e, and *orf14*. Two unusual members of the RolB family are located at the N-terminal side of the tryptophan monooxygenase (IaaM) and the related pOrf8 proteins from *Agrobacterium tumefaciens* and *Agrobacterium rhizogenes*, respectively (Levesque et al. 1988). These RolB-like sequences are linked to a 550 amino acid C-terminal fragment with strong homology to the IaaM proteins of *Pseudomonas savastanoi* and *Erwinia herbicola*. The latter proteins lack RolB-like sequences are active in the bacteria and catalyze the conversion of tryptophan into indole-3-acetamide (IAM). Conversion of IAM by an indole-3-acetamide hydrolase (IaaH) leads to the synthesis of large amounts of indole-3-acetic acid, which is secreted and induces galls on susceptible hosts (Comai and Kosuge 1982). The *iaaM* and *iaaH* genes from the TL-DNA of *A. tumefaciens* strain Ach5 (also known as *tms1-tms2*, *aux1-aux2*, and gene 1–gene 2) encode similar biosynthetic activities as their *Pseudomonas* spp. counterparts (Schröder et al. 1984; Thomashow et al. 1984; Thomashow et al. 1986; Van Onckelen et al. 1985; Van Onckelen et al. 1986). The *orf8* gene from *A. rhizogenes* strain A4 (Slightom et al. 1986) encodes weak IAM synthesis activity (Lemcke et al. 2000).

The different members of the RolB family may have related functions. If the precise role of one of its members could be elucidated, this may yield clues to the functions of the other family members. In this respect, the *rolB*-like extensions of the T-DNA *iaaM* and *orf8* genes are interesting for two reasons. First, their association with a sequence that encodes IAM synthesis in *Pseudomonas* spp. suggests a functional link with this enzymatic activity. Secondly, because the function of the IaaM proteins is well known, it should be possible to establish whether the RolB-like part plays a role in IAM synthesis or has an independent function, e.g., growth modification as is found for other RolB-like proteins. We tested these two possibilities by deleting the *rolB*-like parts of an *iaaM* and an

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orf8 gene and measuring the enzymatic activity of the remaining fragments. We also expressed the *rolB*-like fragments in tobacco plants.

RESULTS

Boundary between RolB-like extension and bacterial IaaM part.

Levesque et al. (1988) showed that the N-terminal parts of the Ach5 IaaM (A-IaaM) and A4 pOrf8 (A4-pOrf8) proteins are weakly similar to other T-DNA proteins. Since this observation, five new IaaM sequences and one additional pOrf8 sequence were reported. All contain the N-terminal extension, and homology searches with the PSI-BLAST program (Altschul et al. 1997) confirmed the homology of this part to other members of the RolB family. Figure 1 shows an alignment of the N-terminal parts of the IaaM and pOrf8 sequences with their closest relatives, the RolB proteins. The alignment defines the putative boundary between the RolB-like sequences and the *Pseudomonas* and *Erwinia* spp. IaaM proteins, which we call bacterial IaaM proteins. Residues M197 and F225 of the A-IaaM and A4-pOrf8 proteins correspond to the first methionine of the bacterial IaaM proteins. Phylogenetic trees for the N- and C-terminal fragments of the IaaM

and pOrf8 proteins are shown in Figure 2. The N-terminal fragments of pOrf8 cluster with the RolB sequences rather than with the N-terminal parts of the IaaM sequences. The two trees have a similar overall structure, although the N-terminal parts evolve faster than the C-terminal parts. Having established the most likely boundaries between the RolB-like parts and the bacterial IaaM proteins, we deleted the *rolB*-like sequences of the A-*iaaM* and A4-*orf8* genes and tested whether the truncated genes would still direct IAM synthesis in plants and induce roots in the presence of an *iaaH* gene.

IAM synthesis and root induction by full-size and 5'-truncated A-*iaaM* and A4-*orf8* genes.

Complete and 5'-truncated versions of the coding sequences of the A-*iaaM* and A4-*orf8* genes (A-*iaaM*, A-*CiaaM*, A4-*orf8*, and A4-*Corf8*, respectively) were constructed by polymerase chain reaction (PCR) with appropriate primers, inserted into the pCK expression cassette containing a 2× 35S promoter and a 35S terminator (Carrington et al. 1991) and recloned into the binary vector pBI121.2, which carries a kanamycin resistance gene and a β-glucuronidase (GUS) marker gene (Jefferson et al. 1987). The resulting vectors (pPM2014, pPM2012, pPM2020, and pPM2018 carrying the 2x35S-A-*iaaM*, 2x35S-A-*CiaaM*, 2x35S-A4-*orf8*, and 2x35S-A4-*Corf8*

Fig. 1. Alignment of N-terminal parts of tryptophan monooxygenase (IaaM), pOrf8, and RolB sequences. IaaM from Ach5 (CAA25167), C58 (AAD30489), TB-DNA of Tm4 (AAD30493 + CAA79149), TA-DNA of Tm4 (CAA39646), S4 (S30105), A4 (I39708), *Pseudomonas syringae* pv. *sa-vastanoi* (P06617), and *Erwinia herbicola* (Q47861). pOrf8 from 2659 (CAB65893), A4 (translated from DNA sequence K03313). RolB from TL-DNA of A4 (P20402), 8196 (I39721), and 2659 (S52639). RolB^{TR} from TR-DNA of A4(P15397). For clarity, RolB sequences were truncated at the fusion site.

genes, respectively) were introduced into strain LBA4404 (Hoekema et al. 1983) and tested for IAM synthesis by infiltration of *Nicotiana tabacum* leaves. IAM synthesis from endogenous tryptophan, tryptophan infiltrated into the leaves, and tryptophan added to crude enzymatic extracts was measured in a semiquantitative way (see below). In addition, IAM synthesis was tested indirectly by root induction on *Kalanchoe daigremontiana* and *Kalanchoe tubiflora* stems in coinfection experiments with a strain carrying the *A-iaaH* gene (see below).

Neither the 2x35S-A4-*orf8* nor the 2x35S-A4-*Corf8* construct led to measurable IAM synthesis. IAM levels from endogenous tryptophan were less than 5%, whereas those from tryptophan infiltrated into leaves or from enzymatic extracts were less than 0.2% as compared with the levels obtained with the 2x35S-A-*iaaM* construct. IAM levels obtained in the enzymatic assay are shown in Figure 3. The A4-*orf8* constructs did not induce roots in coinfection experiments with the *A-iaaH* gene on *Kalanchoe* spp. The 2x35S-A-*iaaM* and 2x35S-A-*CiaaM* constructs led to comparable, high IAM levels in all three assays and induced similar abundant root formation on *Kalanchoe* spp. in the presence of an *iaaH* gene (not shown). Thus, the N-terminal extension of the A-*IaaM* protein is not essential for efficient IAM synthesis. Because several *rolB*-like proteins influence plant growth strongly when expressed under the control of a 35S promoter (Hansen et al. 1993; Lemcke and Schmülling 1998a; Lemcke and Schmülling 1998b; Schmülling et al. 1988), we tested the possible effects of the *rolB*-like 5' sequences of the *A-iaaM* and A4-*orf8* genes in tobacco plants.

Expression of *rolB*-like sequences of *A-iaaM* and A4-*orf8* in tobacco.

The *rolB*-like parts of the *A-iaaM* and A4-*orf8* genes (*A-NiaaM* and A4-*Norf8*) were placed under the control of the 2x35S promoter in the pCK expression cassette and transferred into pBI121.2. The resulting constructs (pPM2041 and

pPM2039) were introduced in LBA4404 (see below). Tobacco plants transgenic for each of the two constructs were selected on kanamycin and regenerated in parallel under standard conditions. Sixteen independent kanamycin-resistant and GUS-positive 2x35S-A-*NiaaM* plants had a normal phenotype. Of the 12 kanamycin-resistant, GUS-positive 2x35S-A4-*Norf8* plants, however, six (2.1, 2.2, 2.3, 2.5, 2.6, and 2.10) had chlorotic leaves and were stunted to various degrees as a result of reduced internodal growth (Fig. 4A to C). Microscopic observation of chlorotic leaves after staining with iodine showed large numbers of starch granules (not shown). Chlorosis developed in the intercostal regions in a basipetal manner when leaves had reached about 50% of their final size (Fig. 4D). Subsequently, the leaves became fully chlorotic, leaving only some chlorophyll close to the major veins. The presence of starch did not coincide with the chlorosis pattern (Fig. 4E). Necrosis did develop in older leaves. Contrary to the control leaves, 2x35S-A4-*Norf8* leaves stained strongly for starch after a dark period (Fig. 4F). Of the six phenotypically normal 2x35S-A4-*Norf8* regenerants, three (2.30, 2.31, and 2.33) retained significant amounts of starch after a dark period. Thus the *rolB*-like part of the A4-pOrf8 protein interferes with starch metabolism.

Starch, glucose, fructose and sucrose levels in 2x35S-A4-*Norf8* plant 2.5.

Starch, glucose, fructose, and sucrose levels were measured in the mottled transgenic 2x35S-A4-*Norf8* plant 2.5 after a dark and light period. Wild-type tobacco was used as a control. Results (Table 1) show strongly increased levels of each of the measured compounds in the 2x35S-A4-*Norf8* plant after a dark period and after 5 h of light.

DISCUSSION

In this study, we investigated the possible role of the *rolB*-like 5' extensions of the *iaaM* and *orf8* genes. These exten-

Fig. 2. Average distance trees for the N-terminal parts of the tryptophan monooxygenase (*IaaM*), *pOrf8*, and *RolB* sequences and the C-terminal parts of the *IaaM* and *pOrf8* sequences. *pOrf8* sequences are more closely related to the *RolB* sequences than to the *IaaM* sequences. Divergence patterns of the two trees are similar.

sions are found at each of the eight *Agrobacterium* spp. *iaaM* and *orf8* genes reported so far, but are lacking in the *Pseudomonas* and *Erwinia* spp. *iaaM* genes. It seems likely that at one point in evolution the *rolB*-like extension was fused to an ancestor *iaaM* gene that was similar in size to the present-day *Pseudomonas* and *Erwinia* spp. *iaaM* genes. Because the *Agrobacterium* spp. IaaM protein sequences are colinear and show the same divergence pattern for the N- and C-terminal parts, we deduce that this happened at an early stage of the *Agrobacterium* spp. T-DNA evolution. Although the RolB-like parts of the IaaM proteins diverge faster than the bacterial IaaM parts, suggesting that they are less important, their retention over time indicates that they do play some role. Deletion of the *rolB*-like part of the *A-iaaM* gene, however, did not affect its capacity to encode a functional IaaM protein. Even when placed under the control of a strong promoter, the *rolB*-like sequence from *A-iaaM* did not modify the morphology of transgenic tobacco plants. Thus, the role of this sequence remains unclear. It may be that the original function was lost or that it is needed only on certain hosts or under certain physical or physiological conditions.

The N-terminal parts of the pOrf8 proteins are related more closely to the RolB proteins than to the N-terminal parts of the IaaM sequences. This and the fact that the pOrf8 proteins have six additional amino acids (HDELDC-HNALHQ) at the putative RolB-IaaM junction suggests that the *orf8* genes result from a different fusion event. The *A4-orf8* gene of *A. rhizogenes* strain A4 did not encode IAM synthesis in our hands, despite the fact that it was placed under the control of a strong 2x35S promoter. In addition, the gene was unable to induce roots in the presence of an *iaaH* gene. It seems, therefore, that its main role is not the synthesis of IAM. This also is suggested by the fact that the *orf8* genes of strain A4 (Slightom et al. 1986), 8196 (Hansen et al. 1991), and 2659 (Combard and Baucher 1988) are not accompanied by an *iaaH* gene on the same T-DNA as are other *iaaM* genes. Surprisingly, the *rolB*-like extension of the *A4-orf8* gene has a strong effect on the growth of tobacco. The most striking visible modifications are stunted growth, intercostal chlorosis, and necrosis. Microscopic observation showed large amounts of starch granules.

Fig. 3. Indole-3-acetamide (IAM) synthesis by tobacco leaves infiltrated with LBA4404 (pPM2020) (full size *A4-orf8*, lanes 1 and 2), LBA4404 (pPM2014) (full size *A-iaaM*, lanes 3 and 4), and LBA4404 (pPM2012) (*A-CiaaM*, lanes 5 and 6). Enzymatic extracts were incubated with tryptophan, extracted with methanol, and redissolved in one-fourth (1, 3, 5) or one-twentieth (2, 4, 6) of the enzyme assay volume. Two microliters of extracts were run on thin-layer chromatography and stained with Van Urk-Salkowski reagent. Lanes 7 to 13, Reconstitution experiment to estimate IAM levels. IAM was added to enzyme extracts of tobacco leaves infiltrated with the empty vector construct LBA4404 (pBI121.2) at concentrations of 1.7, 1.2, 0.7, 0.35, 0.17, 0.07 and 0.02 mM, respectively.

Mottling occurred in a basipetal pattern in leaves of intermediate size, suggesting that it is linked to the sink-source transition. The morphological modifications are accompanied by strong increases in glucose, fructose, sucrose, and starch concentrations within the leaves. Similar morphological modifications and increases in carbohydrate contents have been reported for plants with increased invertase activity (Sonnewald et al. 1991) or expressing viral movement proteins (Lucas and Wolf 1999). The closest relatives of the N-terminal part of pOrf8 are RolB and RolB^{TR}. Intriguingly, plants expressing a 35S-A4-*rolB* gene showed intercostal necrosis (Schmülling et al. 1988), whereas plants expressing a 35S-A4-*rolB*^{TR} gene did not show necrosis but had a reduced size, offshoots at the stem base, and wrinkled leaves with epinastic growth (Lemcke and Schmülling 1998a).

Lemcke and Schmülling (1998b; 2000) also obtained tobacco plants carrying a full-size 35S-A4-*orf8* construct. These plants were phenotypically normal, which is surprising in light of our results. It may be that the bacterial *iaaM*-like part compensates for the effects of the *rolB*-like part. Further studies with regenerants carrying intact, 5'-truncated, or 3'-truncated versions of the *A4-orf8* gene may yield more insight into this problem. The same authors noted that seedlings from 35S-A4-*orf8* plants germinated in the presence of otherwise inhibitory levels of auxin, whereas normal and *iaaM* transgenic seedlings did not. Our results suggest that this property might be a result of the *rolB*-like part. The 35S-A4-*rolB*, 35S-A4-*rolB*^{TR}, and 35S-A4-*orf8* plants were not analyzed for carbohydrates. The present report is the first, to our knowledge, to investigate levels of general metabolites in plants carrying a member of the *rolB* family and shows the potential of such studies. Further work is needed to determine the primary cause of the strong increases in carbohydrate concentrations in 2x35S-A4-*Norf8* plants. Whichever mechanism is involved, it is of interest to study whether such modifications also occur in the case of other *rolB*-like genes and whether high carbohydrate levels in transformed tissues could be of benefit to the bacterium. They may be used directly, but also could contribute to the synthesis of opines like agropine, mannopine, or agrocinopine, which contain glucose and sucrose moieties. Apart from their use in studies on RolB-like proteins, the 2x35S-A4-*Norf8* plants could become interesting models for starch synthesis and source-sink relations in plants and provide applications for biotechnology.

MATERIALS AND METHODS

Homology searches and sequence comparisons.

Homology searches were carried out with the PSI-BLAST program (Altschul et al. 1997), and sequences were aligned with the CLUSTAL-W program (Corpet 1988; Thompson et al. 1994) with CLUSTAL-W default groups to highlight conserved residues (default threshold: 51%). Bootstrap analysis was performed with 32,000 repeats.

Gene constructs.

Gene constructs were obtained by PCR amplification of coding regions with pPM1023 (Huss et al. 1990) carrying the *A-iaaM* gene of Ach5 and pLJ1 (Jouanin 1984) carrying the *A4-orf8* gene of A4 as targets. Primers adding an *NcoI* site, indicated in bold (start codons are underlined) to the 5' ends

were **CCCGCCATGGCAGCTTCACCTCTCCTTGAT**, **CCC GCCATGGTGAAGGGTTCGGCAGGTTCC**, **CCCGCCAT GGATCTTCCATATCAGCGCCA**, **CCCGCCATGGCTCA TGATGAACTGGATTGCCCAG** for the full-size *A-iaaM*, 5'-truncated *A-CiaaM*, full-size *A4-orf8*, and 5'-truncated *A4-Corf8* genes, respectively. Primers adding a *Bam*HI or *Xba*I site (sites in bold) at the 3' ends after the stop codon (underlined) were **GCGTCTAGACTAATTTCTAGTGCGGT AGTTATA**, **GCGGGATCCTTAGATCCGTCCACTATCGG AACA**, **CCCGGGATCCCTATTTTTTATTCCAGTCC**, **GC**

GGGATCCTTACACACCATCATGGGCATCGCA (for the full-size *A-iaaM*, *rolB*-like part of *A-iaaM*, full-size *A4-orf8*, and *rolB*-like part of *A4-orf8*, respectively).

*Nco*I-*Bam*HI and *Nco*I-*Xba*I fragments were cloned into the pCK expression cassette (Carrington et al. 1991), placing them under the control of a strong 2x35S promoter and a 35S terminator sequence. The constructs were sequenced and re-cloned as *Hind*III fragments into the binary vector pBI121.2, which carries an *nptII* (kanamycin resistance) and an *uidA* (GUS) gene (Jefferson et al. 1987). The resulting constructs

Fig. 4. *A4-Norf8* regenerants showing mottled leaves, necrosis, and stunted growth. **A**, Regenerant 2.5 (in front) compared with a normal tobacco plant (in rear). Regenerant 2.5 is stunted, and lower leaves are mottled. **B**, Regenerant 2.6, severely stunted and mottled; oldest leaves nearly fully white. **C**, Regenerant 2.2, very small and severely stunted; nearly all leaves chlorotic. **D**, Regenerant 2.5, mottling starts at the tip of the leaf (arrow); older leaf below is completely mottled. **E**, Starch in mottled leaf of regenerating 2.5. Top: intercostal chlorosis pattern; bottom: iodine staining of same area. Chlorosis does not correlate with starch content. **F**, Starch revealed by iodine staining after a dark period. Normal leaves (top): no starch present; leaves from regenerating 2.5 (bottom): high levels of starch.

Table 1. Carbohydrate levels in leaves of *A4-Norf8* transformant 2.5^a

Plant	Dark, light	Glucose	Fructose	Sucrose	Starch
Control	Dark	0.37 ± 0.08	0.14 ± 0.08	0.49 ± 0.07	< 1.0
2.5	Dark	4.83 ± 0.89	4.42 ± 0.70	5.03 ± 0.45	127 ± 26.0
Control	Light	0.72 ± 0.08	0.12 ± 0.06	0.49 ± 0.19	6.8 ± 1.1
2.5	Light	7.94 ± 0.85	3.03 ± 1.37	5.69 ± 0.93	127 ± 25.0

^a Leaf samples were harvested from plants after one night in a dark room or after 5 h light in the greenhouse. Four samples from one leaf were analyzed and the values were averaged. Values are given as mean ± standard error and expressed as mmol m⁻².

were introduced into the disarmed vector strain LBA4404 (Hoekema et al. 1983) by electroporation. Standard molecular biology techniques were performed as described in Sambrook et al. 1989.

Plant infections.

Stems of *K. daigremontiana* and *K. tubiflora* were inoculated with overnight *Agrobacterium* cultures as described (Huss et al. 1990). Strains carrying full-size and 5'-truncated *A-iaaM* and *A4-orf8* constructs were coinoculated with strain GV3101(pPM1023-39), which contains the *A-iaaH* gene from strain Ach5 under control of its own promoter (Huss et al. 1990). Root growth was scored after 6 weeks.

To obtain transgenic plants, *N. tabacum* cv. Samsun NN leaf fragments were dipped in a fivefold diluted *Agrobacterium* overnight suspension in 10 mM MgSO₄ and placed on MS medium (Murashige and Skoog 1962) with 0.05 mg of 1-naphthalene acetic acid per liter and 2 mg of 6-benzylaminopurine (MSH medium) per liter. Two days later, the fragments were washed with liquid MSH medium containing 500 mg of claforan (MSHC) per liter to eliminate the bacteria and placed on solid MSHC with 150 mg of kanamycin per liter at 24°C (12-h light, 12-h dark). Kanamycin-resistant plantlets were transferred to the greenhouse.

Transient infections were carried out by leaf infiltration (Schöb et al. 1997) as follows: *agrobacteria* were grown overnight in yeast extract broth (Leemans et al. 1981) to an optical density (OD) at 600 nm of 2.5. They were then washed with 10 mM MgSO₄ and resuspended at an OD of 0.5. Tobacco leaves were infiltrated with 50 µl of this suspension and extracts were prepared 4 days after infiltration.

IAM synthesis.

Four days after infiltration of tobacco leaves with *Agrobacterium*, IAM levels were determined directly or after overnight infiltration with 50 µl of an aqueous solution of 10 mM tryptophan. Fifty milligrams of *Agrobacterium*-infiltrated tobacco leaves were ground with 100 µl of 100 mM Tris-HCl, pH 7.0, in a mortar on ice. The extract was centrifuged for 10 min at 10,000 g and 9 µl of 1 N HCl were added to the supernatant. Tryptophan and IAM were extracted with 300 µl of water-saturated butanol. After centrifugation for 10 min × 10,000 g, the butanol phase was recovered and dried with a SpeedVac (Kaiser & Kaiser, Tostedt, Germany). Residues were dissolved in 10 µl of methanol. Two µl were spotted on a Silica gel 60 thin-layer chromatography (TLC) plate (Merck, Darmstadt, Germany). The chromatogram was developed with a chloroform-methanol-acetic acid mixture (80:15:5 vol/vol/vol). After the run, the TLC plate was developed with Van Urk-Salkowski reagent (Ehmann 1977). R_f values for tryptophan and IAM were 0.17 and 0.73, respectively. IAM amounts were estimated by comparison to standards with image analysis. The detection level was 0.02 mM IAM.

Enzyme tests for tryptophan monooxygenase were carried out according to Emanuele et al. (1995). Briefly, 0.3 mg of leaf tissues infiltrated for 4 days with an *Agrobacterium* suspension was ground in a mortar with 1 ml of extraction buffer (25 mM Tris-HCl, pH 7.5; 15 mM MgCl₂; 15 mM ethylene glycol bis[β-aminoethyl ether]-N,N,N',N'-tetraacetic acid; 100 mM NaCl; 0.1% Tween-20; 1 mM dithiothreitol [DTT]; and 1% [vol/vol] Sigma plant proteinase inhibitor cocktail [St.

Louis, MO, U.S.A.]). The extract was cleared by centrifugation (10 min × 10,000 g at 4°C) and proteins were precipitated with 50% ammonium sulfate for 4 h on ice. After centrifugation for 10 min × 10,000 g at 4°C, pellets were dissolved in 0.2 ml of extraction buffer. The crude enzyme samples containing 10.7 ± 0.5 mg of protein per ml, as determined by a Bradford assay (Bradford 1976), were incubated with tryptophan: 0.2 ml of enzyme extract was added to 0.2 ml 100 mM Tris-HCl (pH 8.3), 2 mM EDTA, 1 mM DTT, 1 mM tryptophan, and 0.5 mM flavin adenine dinucleotide. Samples were incubated for 24 h at 28°C, and butanol extracted, dried, and redissolved in 20 µl of methanol and analyzed by TLC, as described above.

Starch, sucrose, glucose, and fructose measurements.

Starch, sucrose, glucose, and fructose were determined enzymatically with the GAHK-20, FA-20, SCA-20, and SA-20 kits (Sigma), respectively, following the supplier's instructions and adapted for microtiter plates. OD₃₄₀ values were determined with a Dynatech MR700 microplate reader (PolyLabo, Paris, France). Results were expressed in mmol m⁻².

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