The *rolB*-Like Part of the *Agrobacterium rhizogenes orf8* Gene Inhibits Sucrose Export in Tobacco

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Many *Agrobacterium* T-DNA genes belong to the highly diverse *rolB* family. The mode of action of most of these genes is still unknown. *rolB*-like sequences also are present at the 5' ends of the T-DNA-located *iaaM* genes and the *iaaM* homolog *orf8*, whereas *iaaM* genes from *Pseudomonas* and *Erwinia* spp. lack such sequences. *iaaM* genes encode tryptophan monooxygenases; these enzymes convert tryptophan into indole-3-acetamide, a precursor of indole-3-acetic acid. Tobacco plants expressing the *rolB*-like part of the A4 *orf8* gene (2x35S-A4-*Norf8* plants) accumulate glucose, fructose, sucrose, and starch and resemble sucrose transporter (NtSUT1) antisense plants. Different lines of evidence indicate that 2x35S-A4-*Norf8* plants export less sucrose from source leaves. Glucose, fructose, sucrose, and starch accumulate in source leaves during sink-source transition, whereas sink tissues like petioles and midveins contain lower levels than normal. Petiole exudation experiments demonstrate a significant decrease in export of label after ¹⁴C-sucrose infiltration and after ¹⁴CO₂ labeling. Grafting of stunted homozygous 2x35S-A4-*Norf8* plants onto wild-type rootstocks restores growth, indicating that unloading is not affected. Growth of 2x35S-A4-*Norf8* seedlings is inhibited on naphthalene acetic acid-containing media, suggesting a link between sucrose transport and auxin sensitivity.

A large number of *Agrobacterium* T-DNA genes have been grouped into the highly diverged *rolB* family (Levesque et al. 1988; Otten and Schmidt 1998). This gene family can be divided into 16 subfamilies represented by genes *rolB* and *rolC* (Schmülling et al. 1988); 5 (Körber et al. 1991); 6a (Messens et al. 1985); 6b (Hooykaas et al. 1988; Tinland et al. 1989, 1992; Wabiko and Minemura 1996); *orf13* and *orf14* (Hansen et al. 1993); *b*, *c*, *d*, *e*, *3'*, *4'*, and 7 (Otten et al. 1999); *rolB^{TR}* (Lemcke and Schmülling 1998a); and *Iso* (Otten and Schmidt 1998). The *rolB*-like genes encode proteins ranging in size from 180 to 260 amino acids; so far, no other homologs have been detected. Many induce morphological abnormalities in transgenic plants (Gaudin et al. 1994; Nilsson and Olsson 1997). Their modes of action are controversial or unknown but probably, in some way, related. *rolB*-like sequences have also been identified at the N-termini of all T-DNA-encoded *IaaM* (tryptophan 2-monooxygenase, six sequences) and *Orf8* (related to *IaaM*, two sequences) proteins (Levesque et al. 1988). *Pseudomonas savastanoi* and *Erwinia herbicola* *IaaM* proteins (Comai and Kosuge 1982; Gafni et al. 1997) lack *rolB*-like sequences. Tryptophan 2-monooxygenases catalyze the forma-

tion of indole-3-acetamide (IAM) from tryptophan (Van Onckelen et al. 1985, 1986). Indole-3-acetamide hydrolases (encoded by *iaaH* genes) convert IAM into the auxin indole-3-acetic acid (IAA) (Schröder et al. 1984; Thomashow et al. 1984, 1986). Whereas *iaaM* and *iaaH* genes always occur together, the *iaaM* homolog *orf8* lacks an accompanying *iaaH* gene, suggesting that it may have a different role. Indeed, *orf8* induces only low (Lemcke et al. 2000; Lemcke and Schmülling 1998b) or undetectable (Otten and Helfer 2001) levels of IAM. Because of their intimate connection with *IaaM* proteins, the *rolB*-like sequences may be involved in IAA metabolism, transport, or perception.

Previously (Otten and Helfer 2001), we investigated the biological activity of the *rolB*-like extensions of A-*iaaM* (from *A. tumefaciens* strain Ach5) and A4-*orf8* (from *A. rhizogenes* strain A4). Both were expressed in tobacco under control of the strong 2x35S promoter. Whereas the *rolB*-like sequence of A-*iaaM* did not cause any morphological changes, the *rolB*-like part of A4-*orf8* (A4-*Norf8*) induced stunted growth, intercostal chlorosis, and necrosis. A4-*Norf8* plants contained high levels of glucose, fructose, sucrose, and starch. Such high levels could be due to a number of different mechanisms: increased synthesis, decreased metabolism, or modifications in export or import into certain parts of the plant or within particular cell compartments. Here, we report a detailed analysis of two independent A4-*Norf8* lines and show that the *rolB*-like sequence of A4-*orf8* inhibits sucrose export from source leaves.

RESULTS

Growth of 2x35S-A4-*Norf8* tobacco plants.

In a preliminary analysis of primary tobacco regenerants carrying the 5' *rolB*-like part of A4-*orf8* from *A. rhizogenes* strain A4 and a GUS marker (2x35S-A4-*Norf8* plants) (Otten and Helfer 2001), we showed that such plants accumulated high levels of hexoses and starch. Further studies on the underlying mechanisms required the use of genetically well-characterized lines. GUS tests of F1 seedlings from a series of selfed primary 2x35S-A4-*Norf8* regenerants (results not shown) allowed us to identify two independent heterozygous single-copy lines, called 2.32 (weak phenotype) and 2.5 (strong phenotype). These lines were selfed, yielding four different genotypes (heterozygous 2.5-1x and 2.32-1x and homozygous 2.5-2x and 2.32-2x plants) showing different growth rates (Fig. 1A through E). Leaves of 2.5-1x, 2.32-1x, and 2.32-2x plants developed to normal sizes, whereas those of 2.5-2x seedlings remained very small. Stem growth decreased in the order 2.32-1x, 2.5-1x, 2.32-2x, and 2.5-2x (Fig. 2A); 2.5-2x stems did not elongate at all (Fig. 2B). At later stages, leaves of 2.5-1x, 2.5-2x, and 2.32-2x plants developed intercostal chlorosis starting

from the apical side of the leaf. Although 2.32-1x and 2.5-1x plants flowered, 2.32-2x and 2.5-2x plants did not (not shown). Growth inhibition of 2.32-1x, 2.5-1x, and 2.32-2x plants was correlated with 2x35S-A4-*Norf8* expression levels as shown by Northern analysis (Fig. 2C). The 2.5-2x plants were too abnormal to be analyzed for RNA levels. Accumulation of hexoses and starch in 2.5-1x and 2.5-2x plants was analyzed in more detail.

Accumulation of hexoses and starch in developing 2x35S-A4-*Norf8* tobacco plants.

At the four-leaf stage, 2.5-2x plants contained already high levels of glucose, fructose, sucrose, and starch with a strong gradient from young to old leaves. In 2.5-1x plants, levels at this stage were slightly higher compared with normal plants (Fig. 3A). Within mature 2.5-1x 19-leaf plants (Fig. 3B), levels of hexoses and starch varied according to leaf size: they were slightly less than normal in small leaves (length of less than 5 cm, leaf numbers 16 through 19) but higher than normal at later growth stages. Within a single leaf, a gradient devel-

oped, with highest concentrations for all compounds at the apical end (Fig. 3C). We also investigated sink tissues. Petioles of 2.5-1x plants contained lower levels of glucose, fructose, sucrose, and starch than normal (Fig. 3D). The same pattern was found for midribs (not shown). Another sink tissue, roots, showed reduced growth compared with normal plants (Fig. 4).

Anatomical analysis of 2.5-1x plants.

Anatomical sections from wild-type and 2.5-1x leaves showed that, in 2.5-1x leaves, starch accumulated in epidermal cells, palisade, and spongy mesophyll cells and in the bundle

Fig. 1. Phenotype of different 2x35S-A4-*Norf8* lines. **A**, Wild type, **B**, 2.32-1x, **C**, 2.5-1x, and **D**, 2.32-2x 55 days after sowing. Scale bar = 10 cm. **E**, 2.5-1x F1 population 30 days after sowing. Scale bar = 5 cm. Arrow indicates a 2.5-2x plant.

Fig. 2. Growth of different 2x35S-A4-*Norf8* lines and transgene expression. Length was measured and averaged for 10 plants per line. Vertical bars indicate standard errors. **A**, Stem growth. Closed squares = wild type, open squares = 2.32-1x, closed circles = 2.5-1x, open circles = 2.32-2x, and crosses = 2.5-2x. **B**, Leaf growth. Closed squares, continuous line = wild type, third leaf; closed squares, dashed line = wild type, fourth leaf; crosses, continuous line = 2.5-2x, third leaf; and crosses, dashed line = 2.5-2x, fourth leaf. (Growth of 2.32-1x, 2.5-1x, and 2.32-2x leaves was similar to that of wild-type leaves). **C**, Northern analysis of wild-type, 2.32-1x, 2.5-1x, and 2.32-2x plants. Top: bands hybridizing to *orf8*. Bottom: ethidium-stained 28S RNA as internal reference.

sheath cells, but not in companion cells, vascular parenchyma, or sieve elements (Fig. 5). This suggests that sucrose transport is blocked before entry in the vascular system.

Reduction of sucrose export as measured by petiole exudation.

The data presented above indicate a decrease in sucrose export from source to sink tissues in 2x35S-A4-*Norf8* plants. This was further tested by infiltration of leaves with ^{14}C -sucrose and monitoring export of label by petiole exudation in the dark over a period of 14 h. The 2.5-1x plants exported radioactive label at a rate of only 15% of that of wild-type plants (Fig. 6A). In a second approach, source leaves were labeled with $^{14}\text{CO}_2$ and, after a chase period in the light, export of label was measured by petiole exudation in the dark over a period of 19 h.

Leaves from control and transgenic plants incorporated between 2 and 5 mmol of $\text{CO}_2 \text{ m}^{-2} \text{ h}^{-1}$. No significant difference between control plants and transgenic plants in the incorporation of labeled CO_2 was observed during the pulse. During the 4-h light chase, only a small amount of the recently assimilated CO_2 was exported via the vasculature to the petiole in transgenic and control plants. During the following dark chase, however, control leaves exported approximately 6.7% of the total assimilated label, whereas the transgenic line 2.32-1x exported only 0.88% of the recently assimilated CO_2 . The exudation of label from the transgenic lines 2.5-1x and 2.32-2x during the dark chase was barely detectable and exhibited a more than 300-fold reduction compared with the wild type (Fig. 6B). Autoradiography of the labeled leaf from the transgenic line 2.5-1x after the dark chase revealed that the remaining label was not evenly distributed across the leaf blade but was concentrated along the vasculature. In the control line, how-

ever, the remaining label was almost evenly distributed over the whole leaf blade (Fig. 6C).

Restoration of growth of 2.5-2x plants by grafting.

The 2.5-2x seedlings stopped growing and became chlorotic when the leaves reached a length of approximately 3 cm. When grafted onto normal tobacco plants with three source leaves, these plantlets resumed growth, forming many leaves, which subsequently accumulated high levels of starch and stopped growth. The leaf surface of the grafted plants increased 100-fold compared with nongrafted plants. No flowers were formed (not shown). Grafting of the less stunted 2.32-2x plants onto wild-type tobacco also restored growth and, unlike the 2.5-2x plants, allowed flowering and seed production (not shown).

Growth of 2x35S-A4-*Norf8* seedlings on medium with naphthalene acetic acid (NAA).

Lemcke and Schmölling (1998b) reported that seed of tobacco plants expressing the intact A4-*orf8* gene developed normally on 5 μM NAA, unlike wild-type seed, which formed callus, suggesting an increase in auxin sensitivity. The *rolB* gene increases auxin sensitivity (Nilsson and Olsson 1997); therefore, it was of interest to determine the effect of the *rolB*-like part of the A4-*orf8* gene on auxin sensitivity. F1 seed from the 2.5-1x line were germinated on Murashige-Skoog (MS) medium with 0, 0.5, 1, 5, 10, and 20 μM NAA. Shoot inhibition became evident at 5 μM and was essentially complete at 10 and 20 μM . At 5 μM NAA, 111 seeds formed a callus with a shoot and 112 developed calli without shoots. GUS tests showed that 54 calli with shoots and 103 shootless calli were transformed. These values differ significantly from those expected in cases where A4-*Norf8* has no effect on auxin sensitivity (83 and 84; P values of

Fig. 3. Hexose and starch analysis of 2x35S-A4-*Norf8* plants. **A**, Young four-leaf plants. True leaves (excluding cotyledons) are numbered in order of appearance. **B**, Mature 19-leaf plants. **C**, Gradients of hexoses and starch in the second full-grown leaf of 12-leaf plants (start of the scale at the base of the leaf). **D**, Hexoses and starch in petioles of 12-leaf plants. Symbols: squares, continuous line = wild type; squares, dashed line = 2.5-1x; and triangles = 2.5-2x. Vertical bars indicate standard errors.

0.1 and 5%, respectively). Thus, the A4-*Norf8* gene increases the sensitivity of tobacco seedlings to NAA.

DISCUSSION

The 5' *rolB*-like part of the *A. rhizogenes* T-DNA gene *orf8* induces a "high starch" phenotype in transgenic tobacco (Otten and Helfer 2001). Different mechanisms could lead to such a phenotype. The evidence presented here suggests that the accumulation of starch is caused by the inhibition of sucrose export from source leaves (Oparka and Turgeon 1999). The resulting deficiency of sucrose in sink tissues reduces stem size and, in more severe cases, leaf growth. Transgene expression levels correlate with levels of growth reduction.

The morphological and physiological properties of the 2x35S-A4-*Norf8* plants are similar to those of plants with antisense constructs derived from the H⁺-sucrose transporter encoding gene *SUT1*. Such plants are impaired in the loading of sucrose into the phloem of source leaves (Bürkle et al. 1998; Kühn et al. 1996; Riesmeier et al. 1994). During maturation, leaves of NtSUT1 antisense tobacco plants become first chlorotic in a basipetal way and necrotic at later stages, whereas sink leaves remain normal. In severely affected plants, leaf and root development are reduced and plants remain stunted without flowering. Concentrations of soluble sugars and starch increase and excess starch is not mobilized during the night (Bürkle et al. 1998). Inhibition of carbon export from leaves through the expression of a yeast-derived invertase in tobacco (Sonnewald et al. 1991; von Schaewen et al. 1990) and phloem-specific expression of an *Escherichia coli*-derived pyrophosphatase (Lerchl et al. 1995) also lead to accumulation of soluble sugars and starch. In 2x35S-A4-*Norf8* plants, hexoses and starch levels increase with a clear developmental pattern. In mature 2.5-1x plants, levels in young leaves are slightly below normal but increase progressively in older leaves, after which intercostal chlorosis develops in a basipetal pattern. Sink tissues like midribs and petioles contain significantly less soluble sugars and starch compared with wild-type plants. Root growth is reduced.

These data suggest that A4-*Norf8* overexpression leads to a block in the export of sucrose from source to sink tissues. Experimental data support these conclusions. Sucrose export measured by monitoring export of label from ¹⁴C-sucrose-fed leaves is reduced by 85% in 2.5-1x leaves. Pulse labeling of assimilates followed by dark chase clearly demonstrated that the transgenic 2x35S-A4-*Norf8* plants display a dramatic reduction in the export of recently generated assimilates. This could be either due to (i) a restriction of symplastic transport between mesophyll cells, (ii) a block of export of sucrose from the mesophyll cells to the apoplast, (iii) a restriction in loading of sucrose into companion cells, or (iv) a hampered translocation of sucrose into sieve elements. Infiltration of sucrose into leaves mainly leads to

accumulation of sucrose in the apoplastic space, and we observed that source leaves of the 2x35S-A4-*Norf8* plants did not efficiently translocate infiltrated sucrose to the sink tissues; therefore, we assume that either the uptake of sucrose from the apoplastic space into companion cells is limiting in the transgenic lines or the symplastic translocation of sucrose from companion cells to the sieve elements via branched type plasmodesmata is restricted. In addition, our observation that assimilates accumulate in the regions surrounding the vasculature in transgenic line 2.5-1x strongly suggests that overexpression of A4-*Norf8* leads to restriction in either assimilate uptake into the phloem or phloem translocation itself. In the light of the presented data, an effect of A4-*Norf8* overexpression on symplastic transport between mesophyll cells or on the unloading of assimilates to the apoplast from mesophyll cells seems unlikely. Growth of 2.5-2x and 2.32-2x plants can be restored by grafting, suggesting that the unloading of sucrose by sink tissues is not impaired.

Further studies should aim at an understanding of the mechanism by which A4-*Norf8* inhibits sucrose transport. Protein localization studies and tissue-specific expression experiments may help to solve this question. Subsequently, such studies could be extended to plants that express the closest homologs of A4-*Norf8*: *rolB* and *rolB*^{TR}.

It will also be of interest to determine the function of the C-terminal part of the A4-Orf8 protein (A4-COrf8) and the way A4-COrf8 and A4-Norf8 interact. In spite of the fact that A4-COrf8 shows considerable homology to C-IaamMs and the Iaam

Fig. 4. Root growth of A, 2.5-1x and B, wild-type plants. Scale bar = 3 cm.

Fig. 5. Transverse sections of leaves from a mature 19-leaf wild-type and 2.5-1x tobacco plants, stained for starch with iodine and viewed with phase contrast optics. A, Wild-type, overview, magnification $\times 13,500$; B, 2.5-1x, overview, magnification $\times 13,500$; C, wild-type, magnification $\times 65,000$; D, 2.5-1x, magnification $\times 65,000$. Scale bars: A and B = 25 μ m, C and D = 10 μ m. Symbols: se = sieve element, bs = bundle sheath cell, x = xylem, cc = companion cell, and vp = vascular parenchyma.

proteins from *Pseudomonas* and *Erwinia* spp., it does not seem to encode high levels of IAM synthesis (Lemcke et al. 2000; Lemcke and Schmülling 1998b), if at all (Otten and Helfer 2001). The situation is further complicated by the fact that tobacco plants carrying the A4-*orf8* gene under control of a tetracycline-dependent 35S promoter were morphologically normal (Lemcke and Schmülling 1998b), which is rather unexpected in view of the "high starch" phenotype encoded by the

5' part of this gene. Possibly, the C-terminal part of Orf8 reverses or counteracts the activity of the RolB-like part. Comparative studies with 2x35S-A4-*orf8* and 2x35S-A4-*Corf8* plants may clarify this point. It also would be of interest to combine 2x35S-A4-*Corf8* and 2x35S-A4-*Norf8* constructs in one plant and compare the resulting phenotype with that of 2x35S-A4-*orf8* plants.

Lemcke and Schmülling (1998b) reported that A4-*orf8* over-expressing seedlings germinated at a normally inhibitory NAA concentration of 5 μ M, and concluded that *orf8* decreased auxin sensitivity. We found that 2x35S-A4-*Norf8* plants germinated less well under these conditions. Thus, the decrease in auxin sensitivity of A4-*orf8* seedlings does not appear to be due to the 5' part of the gene. It is not clear how the supposed IAM synthesis activity of A4-*orf8* could lead to decreased auxin sensitivity.

It remains to be established whether sucrose export inhibition and increased auxin sensitivity in 2x35S-A4-*Norf8* plants are causally related. Auxin gradients are known to be essential for the development of the leaf vascular system (Berleth and Sachs 2001; Dengler and Kang 2001). Increased auxin sensitivity may lead to a modification of the vascular system and subsequent changes in sucrose transport. Our anatomical studies did not show gross alterations of the vascular system. However, it is conceivable that minor changes in anatomy could lead to cumulative effects on sucrose transport. The RolB-like extension of the Orf8 protein is not only an interesting model to study other RolB-like proteins, but also can be used as a new tool to study sucrose transport.

MATERIALS AND METHODS

Hexoses and starch measurements.

Hexoses and starch were measured as described using enzymatic assays yielding NADH (Otten and Helfer 2001). All experiments were done with plant batches that had been grown in a greenhouse under controlled temperature (22 to 24°C during the day, 18 to 19°C during the night, 16 h of light, and 8 h of dark) and humidity (60 to 80%) conditions and watered daily with a standard nutrient solution. Samples were always taken 4 to 5 h after sunrise. The standard error of the data was less than 5%. All experiments were repeated at least twice with different plant batches and yielded essentially the same results.

Northern analysis.

Total RNA was extracted according to Verwoerd and associates (1989) and run on a 1% agarose gel. RNA (15 mg) was loaded per lane and, after transfer to nitrocellulose, hybridized with the 32 P-labeled *orf8* coding sequence. Amounts were standardized using ethidium bromide staining of ribosomal 28S RNA. Extraction of sufficiently pure RNA from 2.5-2x plants proved to be difficult due to the very high starch levels in these plants.

Petiole exudation after infiltration with 14 C-sucrose.

Petiole exudation experiments were carried out according to King and Zeevaart (1974). The youngest fully grown leaves (length 20 cm) of mature plants with 20 leaves were infiltrated with 0.1 ml of a 1-mM 14 C(U)-sucrose solution (3.7 MBq). After 30 min, individual leaves were detached and placed in 10-ml plastic tubes filled with 4.5 ml of a 20-mM EDTA solution. The tubes were placed in racks in the dark in large plastic boxes saturated with water vapor to avoid evaporation from the leaves. Duplicate samples of 0.1 ml were taken at 2-h intervals and counted with a Beckman LS6500 scintillation counter (Fullerton, CA, U.S.A.). Experiments were repeated three times. Standard error of the data was less than 5%.

Fig. 6. Export of label from 14 C-sucrose-infiltrated leaves and from leaves labeled with 14 CO₂. **A**, Exudation of 14 C label from 14 C-sucrose-infiltrated leaves. Export was calculated as a percentage of infiltrated label. Closed squares = wild-type, open squares = 2.5-1x plants. The 2x35S-A4-*Norf8* plants show a strong decrease in export of label. **B**, Exudation of 14 C label from leaves after 40 min of labeling, a 4-h chase period in the light, and a 19-h chase period in the dark. Export was calculated as a percentage of total incorporation during the pulse. The three 2x35S-A4-*Norf8* plants show a strong decrease in export of label. **C**, Autoradiographs of wild-type and 2.5-1x leaves after the dark chase. Whereas wild-type leaves show a low and homogeneous labeling pattern, the 2.5-1x leaves show a more intense labeling concentrated around the minor veins. Scale bars = 2 cm.

Petiole exudation after $^{14}\text{CO}_2$ labeling.

Before the $^{14}\text{CO}_2$ feeding, plants were subjected to prolonged darkness of 20 h and light-adapted for 15 min before single leaves of comparative whorl positions were harvested and fixed in a chamber. Detached leaves were preilluminated for 5 min at $200\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$ before a 40-min pulse with $80\ \mu\text{Ci } ^{14}\text{CO}_2$ ($0.4\ \text{Bq per nmol of HCO}_3^-$) was applied. At the end of the pulse, leaf disks of $2.5\ \text{cm}^2$ were extracted in 80% ethanol and processed as described by Häusler and associates (1998) to determine the incorporation of ^{14}C into the EtOH-soluble and insoluble fraction. To collect the exudates, the petioles of the leaves were transferred to microcentrifuge tubes containing 10 mM HEPES, pH 7.5, and 0.5 mM EDTA (Hansen and Cohen 1985). The leaves were placed into transparent incubation chambers saturated with humidity and the buffer was exchanged after 4 h at $100\ \mu\text{mol m}^{-2}\ \text{s}^{-1}$. The chambers were darkened and the exudates were collected after 19 h in the dark. The ^{14}C in the samples was quantified by scintillation counting. The export of labeled compounds during the light and the dark chases was calculated as percentage of total incorporation during the pulse.

autoradiography of labeled leaves.

At the end of the dark chase, the leaves were incubated on phosphostorage screens at 4°C for 14 h. The image was recorded with a phospho-imager (Storm 860; Molecular Dynamics, Sunnyvale, CA, U.S.A.).

Anatomical studies.

Leaves from wild-type tobacco plants and 2.5-1x plants were fixed in 5% glutaraldehyde, 0.1 M phosphate, pH 7.2 for 7 h at 4°C , washed overnight at 4°C with phosphate buffer, fixed for 2 h at 4°C with 1% osmium tetroxide, washed in water, dehydrated in a graded ethanol series, and embedded in LR White embedding medium. Sections of $0.35\ \mu\text{m}$ were cut with a diamond knife on a Reichert-Jung Ultracut microtome (Vienna, Austria), mounted on glass slides, and stained for starch with iodine. Sections were viewed and photographed on a Nikon Eclipse E800 microscope (Nikon France, Paris) equipped with a Sony DXC950 camera (Tokyo) and image analysis software (VisioLab2000; Biocom, Paris).

Germination of seedlings in vitro.

F1 seedlings from a 2.5-1x plant were sterilized and placed on MS medium (Murashige and Skoog 1962) with different NAA concentrations. Results were scored 7 weeks later.

Root growth analysis.

Plants were grown in large pots (15 cm in diameter) up to the 20-leaf stage. Stems and leaves were cut off and the soil clump was carefully removed and photographed.

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