Abnormal Accumulation of Sugars and Phenolics in Tobacco Roots Expressing the *Agrobacterium* T-6*b* Oncogene and the Role of These Compounds in 6*b*-Induced Growth

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The *Agrobacterium* T-DNA oncogene 6*b* induces tumors and modifies the growth of transgenic plants by an unknown mechanism. We have investigated changes in roots of tobacco seedlings that express a dexamethasone-inducible T-6*b* (dex-T-6*b*) gene. On induction medium with sucrose, intact or isolated dex-T-6*b* roots accumulated sucrose, glucose, and fructose and changed their growth, contrary to noninduced roots. Root fragments bridging agar blocks with or without sucrose accumulated sugars at the site of sucrose uptake, resulting in local growth. Induced root fragments showed enhanced uptake of ¹⁴C-labeled sucrose, glucose, and fructose. When seedlings were placed on sucrose-free induction medium, sugar levels strongly decreased in roots and increased in cotyledons. Collectively, these results demonstrate that 6*b* stimulates sugar uptake and retention with drastic effects on growth. Apart from sugars, phenolic compounds also have been found to accumulate in 6*b* tissues and have been proposed earlier to play a role in 6*b*-induced growth. Induced dex-T-6*b* roots accumulated high levels of 5-caffeoylquinic acid (or chlorogenic acid [CGA]), but only under conditions where endogenous sugars increased. Inhibition of phenylalanine ammonia-lyase with the competitive inhibitor 2-aminooindan-2-phosphonic acid (AIP) abolished CGA accumulation without modifying sugar accumulation or affecting the 6*b* phenotype. We conclude that the absorption, retention, and abnormal accumulation of sugars are essential factors in 6*b*-induced growth changes, whereas phenylpropanoids only marginally contribute to the 6*b* seedling phenotype.

Additional keywords: crown gall, *plast* family.

Many *Agrobacterium* T-DNA genes belong to the family of so-called developmental plasticity (*plast*) genes (Levesque et al. 1988). This family includes genes 3', 5, 6*a*, 6*b*, *rolB*, *rolC*, *orf8*, *orf13*, *Iso*, and various other T-DNA genes (Helfer et al. 2002). No homologues have been found outside this group and, more than 20 years after their discovery, the basic functions and roles of *plast* genes in crown gall and hairy root disease remain largely unknown (Nilsson and Olsson 1997). Among the *plast* genes, the 6*b* oncogenes have particularly interesting effects on

plant growth. They induce tumors on stems of *Nicotiana glauca* (Hooykaas et al. 1988; Tinland et al. 1989), grapevine (Huss et al. 1990), and *N. rustica* (Paulus et al. 1991) and modify the effects of cotransferred cytokinin and auxin biosynthesis genes (Bonnard et al. 1989; Gális et al. 1999; Paulus et al. 1991; Spanier et al. 1989; Tinland et al. 1989). Stably introduced, they produce spectacular growth changes in *N. rustica* (Tinland et al. 1992), *N. tabacum* (Grémillon et al. 2004; Helfer et al. 2003; Wabiko et al. 1996), and *Arabidopsis thaliana* (Helfer et al. 2003). Morphological and anatomical changes in AB-6*b*-, T-6*b*-, and AK-6*b*-expressing tobacco plants are complex. Enations (leaf blade outgrowths on the abaxial leaf side) are among the most striking features of 6*b* plants. Enations also are encountered in several viral plant diseases, such as cotton leaf curl disease (CLCuD) or those caused by *Pea enation virus* (PEV) or *Beet curly top virus* (BCTV) (Helfer et al. 2003). Enations are due to extra cell divisions on the lower leaf side (Helfer et al. 2003; Terakura et al. 2006). Cotyledons, flowers, petioles, stems, and roots also are modified by 6*b* gene activity; the collective set of modifications has been called the 6*b* enation syndrome (Helfer et al. 2003). The enation syndrome can be transmitted from 6*b* plants to nontransformed rootstocks or scions by grafting, showing that 6*b* plants synthesize a phloem-mobile growth factor (enation factor) (Helfer et al. 2003). Nuclear localization of the 6*b* protein is essential for its activity (Kitakura et al. 2002; Terakura et al. 2006); however, the 6*b* protein also can traffic in leaves and, therefore, may be the enation factor (Grémillon et al. 2004).

In this work, we characterize the effects of T-6*b* expression on root growth. In earlier studies, we showed that T-6*b* activity shortens and thickens *rolABC* roots on *Daucus carota* and *N. rustica* (Tinland et al. 1990). Roots of heat-shock-inducible hsp-T-6*b* *N. rustica* seedlings (Tinland et al. 1992) and dexamethasone (dex)-inducible dex-T-6*b* *N. tabacum* seedlings (Grémillon et al. 2004) showed radial expansion and became brown upon induction. Abnormal cell division occurred in the stele, affecting pericycle and vascular cells. Analysis of phenolic compounds in C58-6*b* (Gális et al. 2002), 35S-AK-6*b* (Gális et al. 2004), and dex-AK-6*b* (Kakiuchi et al. 2006) tobacco plants revealed large differences compared with control plants. On medium with cytokinin and auxin, 5-caffeoylquinic acid (chlorogenic acid [CGA]), caffeoyl putrescine (CaPu), rutin, and kaempferol-3-rutinoside (K-3-R) levels increased, whereas those of scopolin and scopoletin (SCT) were lower. On hormone-free medium,

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only SCT levels were affected and lower than normal. In induced dex-AK-6*b* tobacco seedlings, CaPu, SCT, CGA, and K-3-R accumulated in leaves and roots and their levels were correlated with the degree of abnormality of the different transgenic lines. Polar auxin transport was modified in dex-AK-6*b* hypocotyls; this was attributed to kaempferol (Kakiuchi et al. 2006), a well-known inhibitor of auxin transport (Jacobs and Rubery

1988). The authors proposed a model in which AK-6*b* modifies phenylpropanoid metabolism, thereby leading to K-3-R accumulation and a block in auxin transport. This then would result in growth modifications, such as aberrant leaf venation (Kakiuchi et al. 2006). Phenylpropanoid changes may result from 6*b*-induced changes in gene expression, as suggested by the nuclear localization of the 6*b* protein (Kitakura et al. 2002, Terakura et al. 2006), but this has not been demonstrated so far. Recently, we found that dex-T-6*b* induction in leaves led to a rapid increase in sugar concentrations, with corresponding changes in osmolarity and cell expansion. We postulated that these are essential factors in 6*b*-induced growth changes. The mechanism of the sugar changes remained to be established. An increase in leaf sugars also was found in tobacco plants that expressed *Norf8*, another member of the *plast* family (Otten and Helfer 2001; Umber et al. 2002, 2005), suggesting that sugar accumulation could be a general feature of *plast* gene-expressing plants. Thus, a provisional sugar accumulation model (Clément et al. 2006) and a phenylpropanoid-auxin model (Kakiuchi et al. 2006) have been proposed to explain 6*b* growth effects. These models are not mutually exclusive and could describe different elements of the enation syndrome. Here, we report on early effects of dex-T-6*b* expression on sugar and phenylpropanoid levels in roots, investigate the role of these compounds in 6*b*-induced growth changes, and analyze the mechanism underlying the increase in sugars in 6*b* tissues.

RESULTS

Induction of dex-T-6*b* seedlings and early growth changes in roots.

One of the most striking features of 6*b*-expressing roots is their strong radial expansion (Gális et al. 1999; Grémillon et al. 2004; Kakiuchi et al. 2006; Tinland et al. 1990, 1992). The expansion of 6*b*-expressing leaf discs is proportional to the sucrose (Suc) concentration in the medium (Clément et al. 2006); therefore, we investigated the role of Suc in 6*b* root expansion. For all experiments reported here, the single-copy homozygous dex-T-6*b* line D6Nt17 was used (Clément et al. 2006). A special root growth assay was developed to study morphological and biochemical changes due to 6*b* induction. D6Nt17 seeds were germinated for 11 days on horizontal agar plates with Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with 30 mM Suc (Ms30). At day 11, seedlings were placed on nylon filters on vertical Ms30 agar plates. Three days later, filters were transferred to inducing plates containing 1 μ M dex (Ms30D; discussed below) or to noninducing control plates (Ms30). At transfer, either seedlings were left intact or roots were separated from the shoots. On Ms30, roots of intact D6Nt17 seedlings continued their growth whereas, on Ms30D, they slowed down. Isolated roots behaved in the opposite way; they stopped growth on Ms30 but continued on Ms30D (Fig. 1A). On Ms30D, D6Nt17 root morphology changed. Two days after transfer to Ms30D, new root hairs failed to develop (Fig. 1B and C); at day four,

Fig. 1. Growth of roots of intact D6Nt17 seedlings (not cut: NC) and isolated D6Nt17 roots (cut: C) on noninducing (Ms30) and inducing medium (Ms30D). Growth at time (t) = x was expressed as a fraction of length increase between $t = 0$ (transfer to first vertical plate) to $t = x$ h relative to the length increase between $t = 0$ and $t = 62$ h (transfer to second vertical plate). **A**, Upon induction, roots of intact seedlings slowed down, whereas isolated roots grew faster than noninduced controls. **B**, through **G**, Early changes in D6Nt17 roots after transfer to inducing Ms30D medium. **B**, **D**, and **F**, Control roots on Ms30. **C**, **E**, and **G**, Induced roots on Ms30D. Scales: **A**, 1 mm and **B**, 1 cm. **C**, Loss of root hairs after 2 days. **E**, Root curling at the apex (arrows) after 3 days. **G**, Root thickening and browning after 6 days.

roots started to curl at the apex (Fig. 1D and E). At day six, root diameter increased and roots turned brown (Fig. 1F and G). Isolated roots showed the same morphological modifications as roots of intact seedlings (not shown), showing that these changes were independent of changes in the aerial parts.

We next tested whether the remarkable autonomous growth of isolated induced D6Nt17 roots depended on exogenous Suc. D6Nt17 seedlings (11 days old) first were grown for 3 days on Suc-free medium (Ms0) in order to avoid the build-up of sugar reserves. Roots were isolated and placed on noninducing or inducing media with different Suc concentrations. On Suc-free medium, no growth occurred; however, on medium with as little as 5 mM Suc, *6b* expression stimulated growth (Fig. 2). Thus, *6b*-induced growth of isolated root fragments is fully dependent on external Suc.

Root growth on media with other carbon sources.

In order to test whether *6b* also allowed growth of isolated roots on media with other carbon sources than Suc, intact seedlings first were grown on Ms0 for 3 days. Subsequently, roots were isolated and transferred to noninducing and inducing plates with Suc, glucose (Glc), fructose (Fru), maltose, raffinose, α -methylglucose, α -methylmannose, mannitol, xylose, lactose, arabinose, rhamnose, salicin, and sorbitol, all at 30 mM. Under noninducing conditions, galactose led to rapid root-tip necrosis and mannose reduced growth twofold (not shown); therefore, these compounds were not included. Four days later, growth was measured. Only Suc led to significant growth stimulation on inducing medium. Glc, Fru, maltose, and raffinose induced very little growth, and the other compounds none at all (results not shown).

T-*6b* induction modifies root sugar levels.

In maize, Suc is imported into the roots via the phloem and found at the root apex, whereas Glc and Fru accumulate in more basal regions (Muller et al. 1998; Walter et al. 2003). Suc is hydrolyzed to Glc and Fru by invertase activity in the elongation zone (Toko et al. 1987) and Glc levels are correlated with growth rate (Freixes et al. 2002). Glc, Fru, and Suc (GFS) levels were measured in different D6Nt17 root zones. In the first series of experiments, D6Nt17 seedlings were grown on Ms30 and transferred to Ms30 or Ms30D. Four days later, GFS values were measured in 3-mm-long root sections, 0 to 3, 3 to 6, 6 to 9, and 9 to 12 mm from the apex (in tobacco, the 0-to-3-mm apical fragment contains the meristematic region and

Fig. 2. Growth of isolated D6Nt17 roots on different sucrose concentrations on noninducing (no DEX) and inducing (DEX) medium. Growth stimulation by *6b* gene expression is evident from 5 mM sucrose onward.

the elongation zone, and the remaining sections represent mature root zones). In noninduced seedlings, Glc and Fru values were highest in the apical region and decreased in the subapical regions, whereas Suc values were low and did not vary much along the roots (Fig. 3A). In induced roots, Glc and Fru levels were lower in the apex but higher in the subapical 3-to-9-mm regions, whereas Suc levels were higher in all regions (Fig. 3B). Although these results suggested Suc uptake by roots, sugar changes also could be caused by increased Suc import from the shoot. Therefore, we analyzed sugar changes in isolated roots, separated from the shoot at the moment of transfer to Ms30 or Ms30D medium. Data for the apical (0 to 3 mm) and subapical (3 to 6 mm) are presented separately (Fig. 4A and B). In the apical region, Glc and Fru remained relatively stable on Ms30; however, on Ms30D, GFS strongly increased. The subapical region showed a decrease in GFS on Ms30 whereas, on Ms30D, a strong increase was found in Suc and a smaller increase in Glc and Fru, after an initial GFS decrease at day one. The lower Suc/Glc+Fru ratio in the apical region probably reflects invertase activity in the elongation zone. Root GFS levels could be increased further on media with high Suc concentrations. Cumulated GFS values for the apical and subapical region 3 days after induction are shown in Figure 4C and D. On Ms90D and Ms120D, GFS values in the subapical region reached 200 mM (i.e., 15 times more than in noninduced roots) and Suc/Glc+Fru ratios again were lower in the apical region. Suc accumulation in certain root regions may result from entry at these sites or from transport after entry elsewhere. This was tested as follows: apical root fragments (11 mm) from Ms0-grown seedlings were placed over two agar blocks separated by a 7-mm gap. One block contained Ms60 (or Ms60D), the other Ms0 (or Ms0D) medium. Both root orientations (apex on Suc-containing or on Suc-free medium) were tested. Longitudinal and radial growth remained largely limited to blocks with Suc and was enhanced on medium with dex (Fig. 5A). Root GFS values were measured in a parallel experiment 4 days after the start of induction. Sugar levels were highest in root parts in contact with Suc under both inducing and noninducing conditions, and high sugar levels corresponded to growth zones (Fig. 5B). We conclude that exogenous Suc accumulates at the site of uptake and that local growth depends on this accumulation.

Fig. 3. Sugar changes along the roots (0 mm: apex) of intact D6Nt17 seedlings on noninducing and inducing medium with 30 mM sucrose (Suc). **A**, On noninducing Ms30 medium, glucose (Glc) and fructose (Fru) concentrations sharply decreased toward the root base, and Suc levels remained stable. **B**, On inducing Ms30D medium, Glc and Fru levels in the apical 0-to-3-mm region were reduced compared with noninduced seedlings but increased in the subapical region, whereas Suc levels were higher in all regions.

Because induced roots showed also some growth on Glc and Fru (see above), we tested whether roots of intact seedlings would be able to accumulate sugars when grown on these compounds. Seed were germinated on MS medium without sugars, or with 30 mM Glc, Fru, or Suc (Ms0, Mg30, Mf30, or Ms30). They were transferred to the same media or to corresponding inducing media (Ms0D, Mg30D, Mf30D, or Ms30D) 14 days later, and root GFS levels were measured 3 days later. On non-inducing media (Fig. 6A), sugar levels did not differ much between roots from seedlings grown on sugar-free, Glc, or Fru medium; on Suc medium, they were higher. On inducing medium (Fig. 6B), seedlings on sugar-free, Glc, or Fru medium had strongly diminished root sugar levels (compare with Fig.

6A), whereas seedlings on Suc medium had much higher GFS levels. On all inducing media, GFS levels in cotyledons were increased compared with noninducing media, suggesting Suc retention and (partial) conversion to Glc and Fru. Sugar depletion in roots of intact seedlings grown on sugar-free inducing medium occurred between 24 and 48 h after the start of induction (Fig. 6C and D).

Uptake of radioactive Suc by root fragments.

Isolated D6Nt17 roots cultivated on medium with Suc accumulated high Suc levels, especially in the subapical regions. To obtain a more direct proof of Suc uptake, D6Nt17 seedlings or isolated roots were grown for 4 days on Ms30 or Ms30D, after

Fig. 4. Sugar levels in isolated D6Nt17 roots on noninducing and inducing medium. Glucose, fructose, and sucrose (GFS) levels were determined in the **A** and **C**, apical (0 to 3 mm) and **B** and **D**, subapical (3 to 6 mm) regions. **A** and **B**, GFS levels on four successive days after transfer; **C** and **D**, GFS levels reached after 3 days of culture on different sucrose (Suc) concentrations. **A**, In the apical region, sugar levels remained relatively stable on Ms30 and increased on Ms30D. **B**, In the subapical region, sugars decreased on Ms30 but increased on Ms30D after a short initial decrease between day 0 and 1. **C** and **D**, In the apical and subapical regions, GFS levels increased with the concentration of Suc in the medium under both noninducing (no DEX) and inducing (DEX) conditions. Under inducing conditions, the glucose and fructose increase in the apical region was higher, as in the subapical region, whereas the reverse was true for Suc.

which Suc uptake was measured by placing 3-mm-long root fragments on 15- μ l droplets of liquid MS medium (discussed below) containing radioactive ^{14}C -Suc at 1 mM (Eksittikul et al. 2001; Lin et al. 1984; Peiter and Schubert 2003). Apical fragments (0 to 3 mm) of noninduced seedlings absorbed approximately 40% of the radioactive Suc in 24 h, but mature subapical fragments (9 to 12 mm) absorbed less than 10%. Roots of induced seedlings showed high uptake in the apical and subapical region (Fig. 7A). Uptake activity of apical fragments from isolated, noninduced roots was reduced, implying loss of this activity after root cutting. Apical and subapical fragments of induced, isolated roots maintained uptake activity (Fig. 7B). Thus, *6b* stimulated Suc uptake in fragments of intact and isolated roots.

Characteristics of Suc uptake in normal and *6b*-induced roots.

Suc uptake by *6b*-expressing roots might differ from normal uptake. In order to compare both, we measured uptake of radioactive Suc (at 1 mM) in apical fragments (0 to 3 mm) of noninduced seedlings and in subapical (9 to 12 mm) fragments of induced seedlings in the presence of one of the following

compounds at a concentration of 10 mM: Suc, Glc, Fru, maltose, raffinose, salicin, xylose, L-arabinose, and 3-O-methylglucose. Two well-known inhibitors of Suc uptake, carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP, 0.05 mM) and N-ethylmaleimide (NEM, 0.1 mM) also were tested. Galactose, mannose (see above), 2-deoxyglucose, paranitrophenyl- α -glucoside, and paranitrophenyl- β -glucoside (often used in Suc

Fig. 5. Local feeding of D6Nt17 root fragments with sucrose (Suc) leads to local sugar accumulation and growth. **A**, Isolated root fragments (apex to the right) were placed on two agar blocks separated by a 7-mm gap; one block contained no Suc and the other block 60 mM Suc. Roots were induced (DEX) or not (no DEX). Only those parts of the roots in contact with Suc showed growth. **B**, Four days after the start of an experiment similar to **A**, GFS values were measured. Sugars accumulated in the root parts that were in contact with Suc, and sugar levels were correlated with growth.

Fig. 6. Sugar accumulation in roots and cotyledons of intact D6Nt17 seedlings grown on Murashige and Skoog (MS) media without sugar (Ms0 and Ms0D), and with 30 mM glucose (Glc) (Mg30 and Mg30D), fructose (Fru) (Mf30 and Mf30D), or sucrose (Suc) (Ms30 and Ms30D). Sugars were measured in four successive root fragments (0 to 3, 3 to 6, 6 to 9, and 9 to 12 mm) and cotyledons 4 days after transfer. **A**, Sugar levels on non-inducing media. On Ms30, Glc and Fru levels were higher than on the other media. **B**, Sugar levels on inducing media. Root sugar levels strongly decreased after induction on Ms0D and somewhat less on Mg30D and Mf30D, whereas Glc and Fru remained high on Ms30D and Suc levels increased. On all inducing media, sugar levels strongly increased in the cotyledons. **C** and **D**, Kinetics of root sugar changes in intact seedlings grown on Ms0 and Ms0D. **C**, On noninducing Suc-free medium (Ms0), Glc, Fru, and Suc levels remained stable for 3 days. **D**, On inducing Suc-free medium (Ms0D), Glc, Fru, and Suc levels strongly decreased between 24 and 48 h after the start of *6b* induction.

transport inhibition assays) were found to inhibit normal root growth (*unpublished observations*) and excluded. Results are shown in Figure 7C. Maltose, raffinose, and Glc competed strongly with Suc, as noted by others (Baskin et al. 2001; Chandran et al. 2003). Fru, xylose, and 3-O-methylglucose also inhibited Suc uptake. Salicin and arabinose only slightly influenced Suc uptake. Two-factor analysis of variance showed no significant differences between normal and *6b*-induced Suc uptake. Uptake of radioactive Glc and Fru also was stimulated by *6b* expression (Fig. 7D) and inhibited by 0.05 mM CCCP (not shown). Radioactive dextran (molecular weight [MW] 70,000) was not taken up (results not shown).

Accumulation of phenylpropanoids.

dex-AK-*6b* tobacco roots accumulate high levels of phenylpropanoids when germinated on inducing medium (Gális et al. 2002, 2004; Kakiuchi et al. 2006). One of these, K-3-R, has been proposed to block auxin transport and thereby cause (at least part of) the *6b* phenotype (Kakiuchi et al. 2006). However, the strong *6b*-induced accumulation of sugars reported here (up to 200 mM GFS on Ms60D) also can be expected to influence root growth. Therefore, we determined the relative importance of sugar and phenylpropanoid accumulation in *6b*-induced growth changes. D6Nt17 seedlings were germinated on inducing Ms30D medium. At 18 days after the start of the culture, D6Nt17 seedlings were highly abnormal (Grémillon et al. 2004). High-performance liquid chromatography (HPLC) analysis showed that roots had accumulated one major phenylpropanoid compound, CGA, to a level of 3.8 mM, compared with 0.1 mM in noninduced seedlings. The specific accumulation of CGA in roots is noteworthy and contrasts with the complex array of phenylpropanoids in tobacco leaves (La Camera et al. 2004). This 40-fold CGA increase confirms the dex-AK-*6b* results, obtained under similar conditions; thus, CGA accumulation in roots appears to be a reliable marker of *6b* activity. However, whereas dex-AK-*6b* seedling roots contained 35 μ M K-3-R, considered to be sufficient to cause auxin transport inhibition (Kakiuchi et al. 2006), roots of induced D6Nt17 seedlings contained less than 0.06 μ M K-3-R (our detection limit) in spite of the highly abnormal *6b* seedling phenotype.

In order to compare CGA and sugar accumulation at early phases of *6b* induction, seedlings were grown on noninducing Ms30 medium for 14 days. Roots then were isolated and transferred to Ms30 or Ms30D. CGA and GFS values remained low on noninducing Ms30 medium. On inducing medium, both CGA and sugar values strongly increased (Fig. 8).

Inhibition of CGA accumulation

does not modify sugar accumulation or *6b* phenotype.

CGA synthesis requires phenylalanine ammonia-lyase (PAL) activity. PAL can be inhibited by various substrate analogs, one of which, 2-aminoindan-2-phosphonic acid (AIP), is a particularly efficient and specific competitive inhibitor (Appert et al. 2003; Zon and Amrhein 1992). Therefore, we tested the effect of a range of AIP concentrations (0.2 to 125 μ M) on CGA and GFS accumulation in subapical fragments (6 to 9 mm) of isolated roots on Ms60 and Ms60D. On noninducing

Fig. 7. Uptake of ¹⁴C-sucrose (¹⁴C-Suc), ¹⁴C-glucose (¹⁴C-Glc), and ¹⁴C-fructose (¹⁴C-Fru) by isolated D6Nt17 root fragments. **A**, Intact D6Nt17 seedlings were grown for 4 days on noninducing (Ms30) or inducing (Ms30D) medium. Then, 3-mm root fragments were isolated and tested for ¹⁴C-Suc uptake by incubation on drops of liquid medium. Uptake was expressed as the fraction of label absorbed after 24 h. Noninduced seedlings showed high uptake in the apex (0 to 3 mm) but low uptake in the subapical (9 to 12 mm) region. **B**, Uptake by fragments prepared from roots that were isolated 4 days earlier and grown on noninducing (Ms30) or inducing (Ms30D) medium. Whereas apical and subapical fragments of noninduced roots showed low uptake, those of induced roots showed high uptake. **C**, Uptake of ¹⁴C-Suc in the presence of different inhibitors (at 10 mM). Abbreviations: no, no inhibitor; sali, salicin; ara, arabinose; xyl, xylose; 3-O, 3-O-methylglucose; raf, raffinose; and malt, maltose. White bars: noninduced apical fragments. Black bars: induced subapical (9 to 12 mm) fragments. None of the differences was significant as determined by two-factor analysis of variance. **D**, Uptake of radioactive Glc, Fru, and Suc by isolated root fragments. Seedlings were induced on Ms30D and uptake by apical (0 to 3 mm) and subapical (9 to 12 mm) root fragments was tested on 1 mM radioactive Glc, Fru, or Suc. Root fragments of induced plants showed induced Glc and Fru uptake.

medium, no CGA was detected, and AIP treatment led to only a small increase in Glc and Fru (Fig. 9A). On inducing medium without AIP, CGA accumulated as before; however, in the presence of AIP, its levels were dramatically reduced without affecting GFS levels (Fig. 9B). Root growth was modified only slightly by AIP, mainly by shortening of root hairs, from 5 μ M AIP onward (results not shown). This might be due to effects of PAL inhibition on lignin synthesis but was not further tested. Reduction of CGA levels by 1 μ M AIP could be reversed by including 1 mM phenylalanine in the medium (not shown), demonstrating that AIP indeed inhibited PAL activity. We also tested long-term effects of AIP on CGA, sugars, and growth of *6b*-expressing seedlings. D6Nt17 seed were germinated and grown for 18 days on Ms30 or Ms30D with 0, 1, or 5 μ M AIP. On Ms30 with 1 μ M AIP, seedlings developed similarly as on medium without AIP; on 5 μ M AIP, seedling leaves were smaller. On Ms30D, CGA values decreased from 3.5 mM in the absence of AIP to 0.2 and 0.05 mM on 1 and 5 μ M AIP, respectively. GFS values were not modified (Fig. 9C). Seedlings retained the same typical *6b* phenotype on 1 and 5 μ M AIP as on medium without AIP: they had short and thick roots, small and thick cotyledons, and failed to develop primary leaves (Fig. 9D).

DISCUSSION

Sugars and phenylpropanoids both have been proposed to play major roles in *6b*-induced growth changes. Dex-induced D6Nt17 leaves accumulated Glu, Fru, and (to a lesser extent) Suc on Suc-containing media, resulting in osmotic changes and cell expansion (Clément et al. 2006). In the present work, it could be shown that abnormal sugar changes and enhanced growth also occurred in the roots of induced D6Nt17 plants. These changes strictly depended on the presence of Suc in the medium, showing the essential role of the accumulation of this compound in *6b*-induced growth modifications. Sugar changes could result from modifications in metabolic pathways or from uptake. Our results clearly showed that the sugar changes were due to increased Suc uptake. First, in subapical root zones, Suc was the major accumulating sugar. High Glc and Fru levels in the apical root zone were most likely due to cleavage of Suc by invertase in the elongation zone. Second, local Suc feeding caused sugar accumulation and growth at the site of absorption. Third, in uptake experiments with radioactive Suc, *6b* induction stimulated Suc uptake in both apical and basal root

Fig. 8. Sugar and chlorogenic acid (CGA) changes in apical regions (0 to 3 mm) of isolated D6Nt17 roots. **A**, On noninducing Ms30 medium, CGA concentrations and the cumulated glucose, fructose, and sucrose concentrations remained low. **B**, On inducing Ms30D medium, CGA values increased from day two onward.

Fig. 9. 2-Aminoindan-2-phosphonic acid (AIP) blocks *6b*-induced chlorogenic acid (CGA) accumulation but does not affect sugar accumulation or the typical *6b* phenotype. **A**, Effect of different AIP concentrations on sugar accumulation in isolated roots growing on noninducing medium. A small increase in sugar levels is seen at high AIP levels. **B**, On inducing medium, AIP inhibited CGA accumulation but did not diminish sugar accumulation. **C**, Long-term effects of AIP on CGA and sugar accumulation. Seed were germinated on Ms30D with 0, 1, or 5 μ M AIP and sugars were measured 18 days later. AIP strongly reduced CGA levels without modifying sugar concentrations. **D**, Phenotypes of seedlings germinated on Ms30 or Ms30D with 0, 1, or 5 μ M AIP. On noninducing Ms30, seedling growth was not affected by 1 μ M AIP and slightly inhibited by 5 μ M AIP. On inducing Ms30D, neither 1 nor 5 μ M AIP significantly modified the typical *6b* phenotype.

zones. Inhibition studies with various compounds showed that *6b*-induced uptake did not measurably differ from normal Suc uptake.

Induction of intact seedlings on Suc-free media resulted in a remarkable decrease in root sugars and a corresponding increase in cotyledons. The earlier reported accumulation of Glc and Fru in dex-infiltrated D6Nt17 leaves in situ (Clément et al. 2006) suggested a block in Suc export from source leaves to sink tissues and cleavage of the accumulating Suc by extracellular invertase. Effects on Suc transport from source leaves have been reported for other plant-pathogen interactions. In the incompatible interaction between tobacco and *Phytophthora nicotianae*, callose formation blocks Suc export from leaves and increases sugar levels (Scharte et al. 2005). Suc retention in *6b* leaves and cotyledons probably is not due to a physical barrier but to Suc retrieval from the apoplast before entry into the phloem, as indicated by our Suc uptake studies with induced roots. It is intriguing that infection of *N. benthamiana* by the enation-causing BCTV leads to retardation of Suc export from source leaves, dramatic Suc accumulation in leaves, enlargement of mesophyll cells, and enations. The reason for Suc transport inhibition by BCTV is as yet unknown (Swiech et al. 2001); its role in the induction of enations and the possible similarity to *6b*-induced changes requires further investigation.

Fig. 10. Model showing relation between *6b* expression and sugar uptake, retention, and accumulation in plants germinated on medium without sucrose (Suc) (Ms0 and Ms0D) or with Suc (Ms30 and Ms30D). Three types of induction are shown: **A**, long-term induction: seed placed on inducing medium and left for 18 days; **B**, short-term (3-day) induction of intact seedlings grown for 14 days on noninducing medium; and **C**, short-term (3-day) induction of isolated roots from 14-day-old seedlings grown on noninducing medium. **A** and **B**, On Ms0D medium, cotyledons of intact seedlings retain Suc. This leads to sugar depletion in roots and root growth arrest. On Ms30D medium, sugars accumulate in the cotyledons but, in addition, Suc is taken up from the medium and sugars accumulate in the roots leading to strong radial growth. **C**, In isolated roots, sugars are rapidly depleted in the absence of exogenous Suc. On inducing media with Suc, Suc absorption is stimulated and longitudinal and radial growth is observed. Local contact with Suc leads to local absorption and local growth. White, gray, and black areas indicate regions of low, medium, and high sugar levels, respectively. White, vertical arrows represent root growth and black, horizontal arrows indicate enhanced Suc uptake from the medium. Asterisk indicates Suc retention in cotyledons.

Induced D6Nt17 roots showed increased uptake of not only Suc but also Glc and Fru. Thus, *6b* may affect a process that enhances the activity of different transporters. In spite of this, growth of isolated roots on Glc or Fru clearly was less efficient than on Suc. It is interesting to note that reabsorption of apoplastic sugars and their local accumulation in *6b*-expressing plants would lead to phenotypes that reflect apoplastic localization patterns of such compounds. This could be an important aspect of the enation syndrome. We hypothesize that, in some tissues, Suc accumulation induces cell division. This may lead to tumor formation (obtained by infection of stems with *Agrobacterium* strains carrying a *6b* gene) and to growth around leaf veins, to enations, catacorollas, and multiplication of primordia and ectopic meristems on seedling hypocotyls (Grémillon et al. 2004; Helfer et al. 2003). Sugar accumulation also will induce sugar signaling (Lalonde et al. 1999) and osmotic stress reactions. More research is needed to test this. Our results suggest a model (Fig. 10) that explains the growth pattern of *6b*-expressing seedlings on Suc-free medium as a result of Suc retention in cotyledons and Suc depletion in roots. On medium with Suc, *6b* seedling growth patterns result not only from retention of endogenous Suc in the cotyledons but also from uptake and local accumulation of exogenous Suc in roots.

The pTiBo542 *6b* gene is preferentially expressed in roots (Bagyan et al. 1995), suggesting that *6b*-induced root sugar changes might be relevant under natural infection conditions. Expression of the *6b* gene in tumor cells might stimulate sugar uptake from surrounding cells, accumulation, and conversion into opines. Gális and associates (2002, 2004) and Kakiuchi and associates (2006) noted phenylpropanoid changes in *6b*-expressing tobacco plants and proposed a role for these compounds in *6b*-modified growth. The present study confirms CGA accumulation in *6b* tobacco roots. It is interesting to note that CGA is the only phenolic compound that accumulates to high levels upon induction of T-*6b* roots. Normal tobacco leaves contain large amounts of different phenylpropanoids (La Camera et al. 2004), thereby complicating the analysis of phenylpropanoid changes. To our knowledge, phenylpropanoid metabolism has not been studied previously in isolated roots. Clearly, D6Nt17 roots constitute an interesting experimental system for studying the regulation of phenylpropanoid synthesis because only one major compound accumulates and its levels can be controlled by induction with dex. In spite of high CGA levels, we failed to detect significant amounts of CaPu, SCT, or K-3-R in D6Nt17 seedlings, even after 18 days of growth on dex. This may be due to differences between *6b* gene constructs or to differences in plant-growth conditions. Whatever the reasons, the dramatic phenotypic changes in induced D6Nt17 seedlings do not seem to require high K-3-R levels. The PAL inhibitor AIP blocked CGA accumulation without affecting the abnormal sugar levels or the *6b* phenotype. On Suc-free inducing media, no CGA accumulated. When Suc was added, CGA levels increased in parallel with endogenous sugar concentrations, suggesting that CGA synthesis results from osmotic stress. From our data, it appears that CGA starts to accumulate beyond 50 mM GFS. On noninducing medium, root GFS values remained below 50 mM, even at exogenous Suc concentrations of 90 mM Suc or more. Only *6b* expression led to sufficient Suc uptake to increase GFS levels beyond 50 mM. CGA is oxidized easily by polyphenol oxidases, generating reactive quinone species. These reactive species can cross-link cell wall components and thereby limit microbial growth and tissue damage (Niggeweg et al. 2004). Therefore, CGA accumulation may favor crown gall growth by blocking microbial infections; however, this interesting possibility remains to be studied.

MATERIALS AND METHODS

Root growth assays.

In order to transfer dex-T-6b roots to different media and analyze their metabolites, a root growth system was developed that avoided contamination of roots with agar and allowed rapid changes of culture medium with minimal disturbance. Seedlings were grown for 11 days on horizontal agar plates with MS medium (Duchefa, M222) with 30 mM Suc (Ms30) or other compounds. Sugars and sugar alcohols were filter sterilized and added after autoclaving to avoid breakdown. At day 11, seedlings were carefully extracted from the agar and placed on nylon filters (mesh 60 by 60 µm; SEFAR NITEX 03-60/42, Heiden, Switzerland) on vertical plates with noninducing Ms30 agar medium. When seeds were placed directly on nylon, germination was irregular. At day 14, filters were transferred to noninducing Ms30 plates or to inducing plates with 1 µM dex in Ms30 (Ms30D). In case isolated roots were studied, roots were separated from the shoot with a razor blade and left in place on the nylon filters. Growth was measured by marking root apex positions. Sealing of petri dishes with Parafilm leads to ethylene accumulation, slowing down root growth (Buer et al. 2003); therefore, petri dishes were taped with Urgopore (URGO, Chenove, France).

Sugar measurements.

Seed were germinated for 11 days on noninducing horizontal plates and placed on nylon filters on noninducing vertical plates. Three days later, seedlings or isolated roots were transferred to inducing or noninducing vertical plates. At different times, 3-mm-long root fragments were cut and carefully washed in 0.1 ml of Ms0 medium. After one additional wash, fragments were placed in 80% ethanol (0.4 ml per 10 fragments) and sugars were extracted for 30 min at 65°C. Extracts were dried by SpeedVac (Savant Instruments, Holbrook, NY, U.S.A.) and dissolved in distilled water. Glc, Fru, and Suc were measured with enzymatic assays as described in Otten and Helfer (2001).

Uptake studies.

Seed were germinated for 11 days on noninducing Ms30 medium. They were placed on nylon filters on noninducing vertical Ms30 plates for 3 days. Seedlings or isolated roots then were transferred to inducing (Ms30D) or noninducing (Ms30) medium for 4 days. Subsequently, root fragments (3 mm) were cut with a razor blade and placed on a drop of 15 µl of liquid medium (Ms0) on microtiter plates. After two changes with fresh Ms0 medium, Ms0 medium containing labeled and cold compounds was added. Five fragments were tested per assay. Plates were sealed and incubated at 21 to 23°C at 100% humidity. Samples of 3 µl were taken at 0 and 24 h and counted with a liquid scintillation counter (Beckman LS6500, Fullerton, CA, U.S.A.). Evaporation did not exceed 5% per 24 h. D-(U-¹⁴C) Suc, D-(U-¹⁴C) Glc, D-(U-¹⁴C) Fru (specific activities 21.8, 10.7, and 11.1 GBq/mmol, with radioactive concentrations of 3.7, 7.4, and 7.4 MBq/ml respectively) (Amersham, Buckinghamshire, U.K.) and carboxyl-¹⁴C-dextran (48 MBq/g, MW 70,000) (ARC, St. Louis, U.S.A.) were diluted 100-fold in the test solutions. Radioactive compounds were tested at 1 mM, unless otherwise indicated. Uptake was expressed as percent label removed from the medium in 24 h.

HPLC analysis of root extracts.

Root fragments of 3 mm were washed twice with 0.1 ml of Ms0 medium, extracted with 80% ethanol (0.4 ml per 10 fragments), and dried by SpeedVac. The dried material was dis-

solved in methanol and analyzed by HPLC according to Hoffmann and associates (2004).

Statistical analysis.

Each experiment was done at least three times using different plant batches. For each set of experiments, averages were calculated. Error bars indicate standard errors of the means. Two-factor analysis of variance was carried out using MINITAB software (Minitab Inc., State College, PA, U.S.A.).

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