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Antisense Repression of Vacuolar and Cell Wall Invertase in Transgenic Carrot Alters Early Plant Development and Sucrose Partitioning

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To unravel the functions of cell wall and vacuolar invertases in carrot, we used an antisense technique to generate transgenic carrot plants with reduced enzyme activity. Phenotypic alterations appeared at very early stages of development; indeed, the morphology of cotyledon-stage embryos was markedly changed. At the stage at which control plantlets had two to three leaves and one primary root, shoots of transgenic plantlets did not separate into individual leaves but consisted of stunted, interconnected green structures. When transgenic plantlets were grown on media containing a mixture of sucrose, glucose, and fructose rather than sucrose alone, the malformation was alleviated, and plantlets looked normal. Plantlets from hexose-containing media produced mature plants when transferred to soil. Plants expressing antisense mRNA for cell wall invertase had a bushy appearance due to the development of extra leaves, which accumulated elevated levels of sucrose and starch. Simultaneously, tap root development was markedly reduced, and the resulting smaller organs contained lower levels of carbohydrates. Compared with control plants, the dry weight leaf-to-root ratio of cell wall invertase antisense plants was shifted from 1:3 to 17:1. Plants expressing antisense mRNA for vacuolar invertase also had more leaves than did control plants, but tap roots developed normally, although they were smaller, and the leaf-to-root ratio was 1.5:1. Again, the carbohydrate content of leaves was elevated, and that of roots was reduced. Our data suggest that acid invertases play an important role in early plant development, most likely via control of sugar composition and metabolic fluxes. Later in plant development, both isoenzymes seem to have important functions in sucrose partitioning.

INTRODUCTION

In most plants, carbon assimilated in leaf mesophyll cells (source cells) is transported to the heterotrophic organs (sink organs) as sucrose. Utilization of sucrose as a source of carbon and energy requires cleavage by either invertase or sucrose synthase (Kruger, 1990). Invertase, which hydrolyzes sucrose into glucose and fructose, exists in several isoforms with different biochemical characteristics and distinct subcellular localizations. Invertases with acidic pH optima (acid invertases) either are ionically bound to the cell wall (cell wall invertase) or accumulate as soluble proteins in the vacuole (vacuolar invertase). Invertases with neutral or slightly alkaline pH optima (neutral and alkaline invertases) are thought to be cytoplasmic proteins.

Several different functions have been proposed for invertases. They cleave sucrose to (1) provide growing tissues with hexoses as a source of energy and carbon (Ap Rees, 1974); (2) generate a sucrose concentration gradient between source and sink tissues to aid sucrose transport (Eschrich, 1980); (3) regulate cell turgor, for example, for cell expansion (Meyer and Boyer, 1981; Wyse et al., 1986; Perry et al., 1987); and (4) control sugar composition in storage organs, such as fruits (Klann et al., 1993). Furthermore, some of the invertases seem to be involved in the responses of plants to environmental factors, such as wounding and infection (Sturm and Chrispeels, 1990; Benhamou et al., 1991).

The specific roles of the invertase isoforms in the different subcellular compartments are largely unknown. Expression of yeast invertase in the cytosol, vacuole, or apoplast of transgenic tobacco led to stunted plant growth and reduced root formation (Sonnewald et al., 1991). Analysis of soluble sugars and starch indicated that sucrose distribution was impaired in all cases. Thus, invertase expression at the wrong place and time had profound consequences for the physiology and development of plants. In developing tomato fruit (Ohyama et al., 1995; Klann et al., 1996) and mature potato tubers (Zrenner et al., 1996), downregulation of vacuolar invertase activity by gene supression or an antisense mRNA approach altered the hexose-to-sucrose ratio without major effects on plant development. Lack of invertase activity in a natural mutant of maize (*miniature1*) (Miller and Chourey,

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1992) causes an early degeneration and withdrawal of maternal cells from the endosperm and thereby an interruption of the transport of photoassimilates into the developing kernel. As a result, the seeds have only one-fifth the normal weight.

The genes for the invertases described above are expressed only at a late stage in plant development. The functions of invertases expressed at earlier stages are less clear, and this was the subject of our study. We previously characterized the different isoforms of invertase from carrot by using molecular, biochemical, and physiological methods (Sturm, 1996). In addition, we established an efficient protocol for the transformation of carrot by agrobacteria and the regeneration of transgenic plants (Hardegger and Sturm, 1998). These tools were used subsequently to study isoform functions in transgenic carrot plants by taking a molecular genetics approach.

RESULTS

Antisense Repression of Vacuolar and Cell Wall Invertases in Transgenic Carrot

To study the effects of a reduction of invertase activity in transgenic carrot plants, we downregulated invertase expression by using an antisense approach (Stitt and Sonnewald, 1995). For this purpose, the full-length cDNA clones for isoform I of vacuolar invertase (Unger et al., 1994) or cell wall invertase (Sturm and Chrispeels, 1990) were cloned into the binary vector pBI121 in an antisense orientation behind the 35S promoter of the cauliflower mosaic virus (CaMV) in place of the reporter gene β-glucuronidase (*GUS*). The binary vectors were introduced into Agrobacterium strain LBA4404. Kanamycin-resistant agrobacteria then were used for the transformation of hypocotyl segments of 7-day-old seedlings of carrot cultivar Nantaise (Hardegger and Sturm, 1998). Transformed plant cells were selected by applying the kanamycin-derivate geneticin (G418), and ~15% of the hypocotyl segments developed viable calli. Homogeneously growing callus cultures were obtained after several rounds of subculture. Soluble and cell wall proteins of geneticin-resistant calli were extracted and analyzed on protein gel blots with antibodies raised against vacuolar invertase (Unger et al., 1992; Figure 1A) or cell wall invertase (Laurière et al., 1988; Figure 1B).

Approximately 15% of the independent callus lines showed a marked reduction in invertase polypeptide levels (Figures 1A and 1B, asterisks), and only these lines were used for plant regeneration. For this purpose, we induced fast-growing suspension cultures from the selected callus cultures. To initiate the development of somatic embryos, single cells and small cell clusters were collected on a 100-μm mesh and diluted into a hormone-free medium. As a non-antisense transgenic control, we used a carrot cell line harboring 549 bp of the promoter of the gene for cell wall invertase fused to *GUS* (Ramloch-Lorenz et al., 1993).

Plantlets obtained from 10 of 13 independent antisense vacuolar invertase (Avi) lines had a similar phenotype, and so did the plantlets of 11 of 17 antisense cell wall invertase (Acwi) lines (data not shown). This high conformity between the phenotypes from independent cell lines strongly suggested that the phenotypes were related to the transgene effects. For this reason and because it is extremely time- and labor-consuming to generate T_2 progeny and T_2 seeds (carrot plants are biennials and cannot easily be selfed; Peterson and Simon, 1986), mature transgenic plants were analyzed as primary transformants.

Mature plants were grown from three independent lines of cells expressing either antisense mRNA for vacuolar invertase (lines Avi-34, Avi-47, and Avi-65) or cell wall invertase (lines Acwi-59, Acwi-65, and Acwi-76). The lines chosen clearly had reduced enzyme protein levels (comparable with the cell lines labeled with asterisks in Figure 1) and belonged to the lines sharing a similar phenotype at the plantlet level.

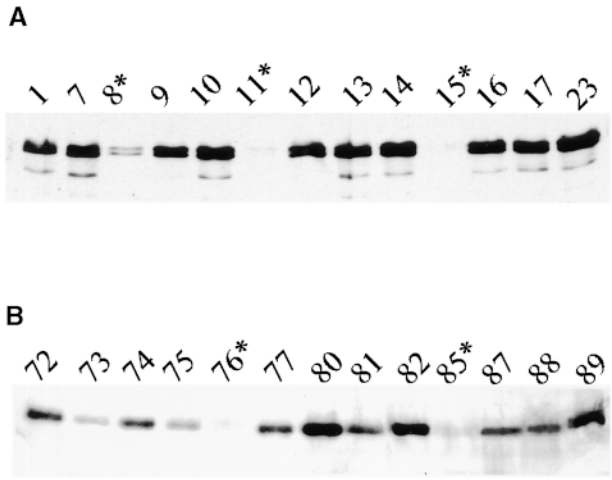

Figure 1. Gel Blot Analysis of Proteins from Selected Transgenic Callus Lines.

Soluble proteins were extracted from Avi callus lines and cell wall proteins from Acwi lines. Subsequently, the proteins were separated by SDS-PAGE, transferred to nitrocellulose membranes, and probed with antibodies raised against purified and deglycosylated carrot vacuolar or cell wall invertases. The numbers above the lanes indicate the identification numbers of independently cultured plant cell lines. Cell lines with reduced enzyme protein levels are marked by asterisks and were used for plant regeneration. The plant lines used in this study (Avi-34, Avi-47, and Avi-65, and Acwi-59, Acwi-65, and Acwi-76) are not shown on these blots; however, comparable reductions in enzyme protein levels were found.

(A) The blot was probed with an antibody raised against vacuolar invertase.

(B) The blot was probed with an antibody raised against cell wall invertase.

Antisense Repression of Acid Invertases Alters the Phenotype of Somatic Embryos and Small Plantlets

Approximately 2 weeks after culture initiation, different developmental stages of somatic embryos were selected manually. Globular-, heart-, torpedo-, and late-cotyledon-stage embryos derived from transgenic cell lines with reduced invertase protein levels were compared with embryos derived from transgenic control cells (Figures 2A to 2C, only cotyledon-stage embryos of one cell line per antisense construct are shown). Clear differences were visible at the cotyledon stage, and the phenotypes described below were shared by all of the selected cell lines. In embryos expressing antisense mRNA for vacuolar invertase, the cotyledons were large, and the hypocotyls and roots were stunted (Figure 2C). In contrast, in embryos expressing antisense mRNA for cell wall invertase, the cotyledons were not well separated (Figure 2B).

Late-stage embryos of several independent cell lines were placed on Murashige and Skoog medium (Murashige and Skoog, 1962) with 3% sucrose. Within 4 to 6 weeks, embryos of control cell lines developed into small plantlets with several leaves (Figures 3A and 3B), whereas embryos of cell lines expressing antisense mRNA for vacuolar invertase (Figure 3B) or cell wall invertase (Figure 3A) developed into stunted green structures. The structures derived from all three cell lines per antisense construct were phenotypically very similar.

Growth on Different Sugars Markedly Affects the Phenotypes of Transgenic Plantlets

Sugars function not only as nutrients for plants but also as signals regulating expression of genes (Koch, 1996). Therefore, a reduced invertase level most likely will affect both carbon nutrition and sugar signaling. To circumvent the sugar signaling problem, we supplied developing embryos with a mixture of sucrose, glucose, and fructose (1:2:2) rather than only with sucrose. This change in sugar composition drastically altered the development of both Acwi (Figures 4A to 4C) and Avi (Figures 4D to 4F) plantlets. The malformation clearly was alleviated, and most of the transgenic plantlets looked more or less normal.

The Leaf-to-Root Ratio Is Altered Drastically in Antisense Plants

When plantlets from hexose-containing media were transferred to soil, mature plants were obtained. After ~3 months, control plants had a well-developed tap root and five to seven leaves. The dry weight ratio of leaves to roots was 1:3 (Figure 5). Due to the development of approximately threefold more leaves than control plants, Acwi plants had a

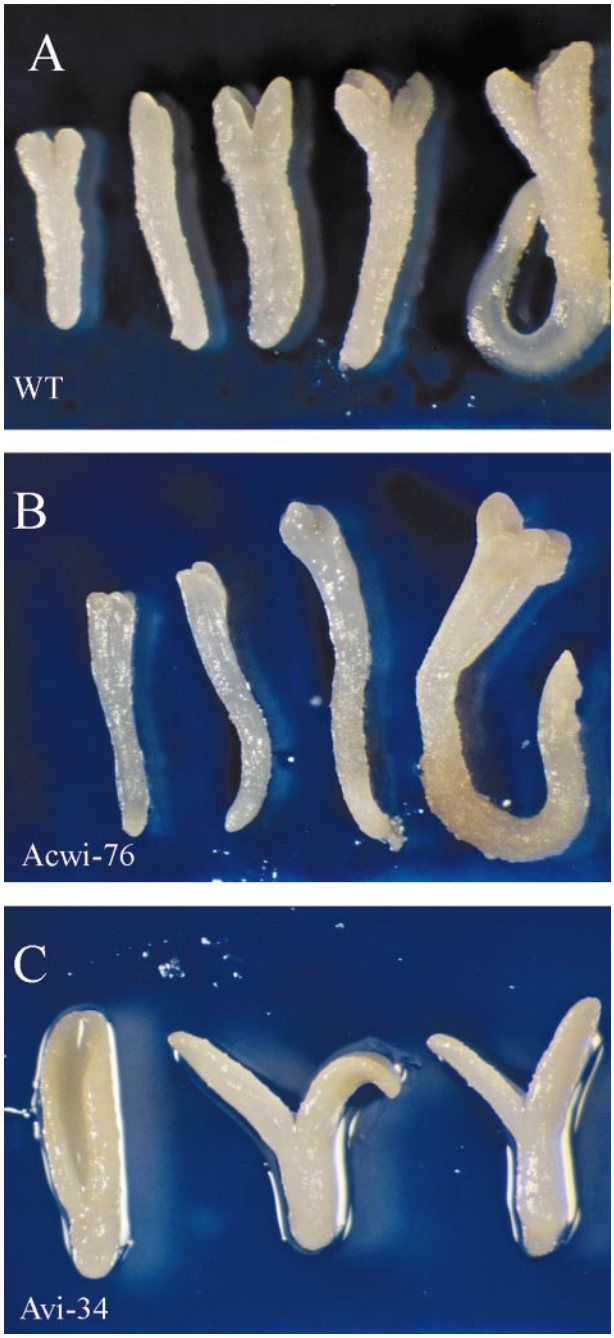

Figure 2. Morphology of Somatic Embryos at the Cotyledon Stage.

Somatic embryos were induced from single cells or small cell clusters and kept for 2 weeks in half-strength Murashige and Skoog medium containing only macroelements and microelements and 3% sucrose.

(A) Cotyledon-stage embryos from a wild-type control cell line (WT).

(B) Cotyledon-stage embryos from a cell line expressing antisense mRNA for cell wall invertase (Acwi-76).

(C) Cotyledon-stage embryos from a cell line expressing antisense mRNA for vacuolar invertase (Avi-34).

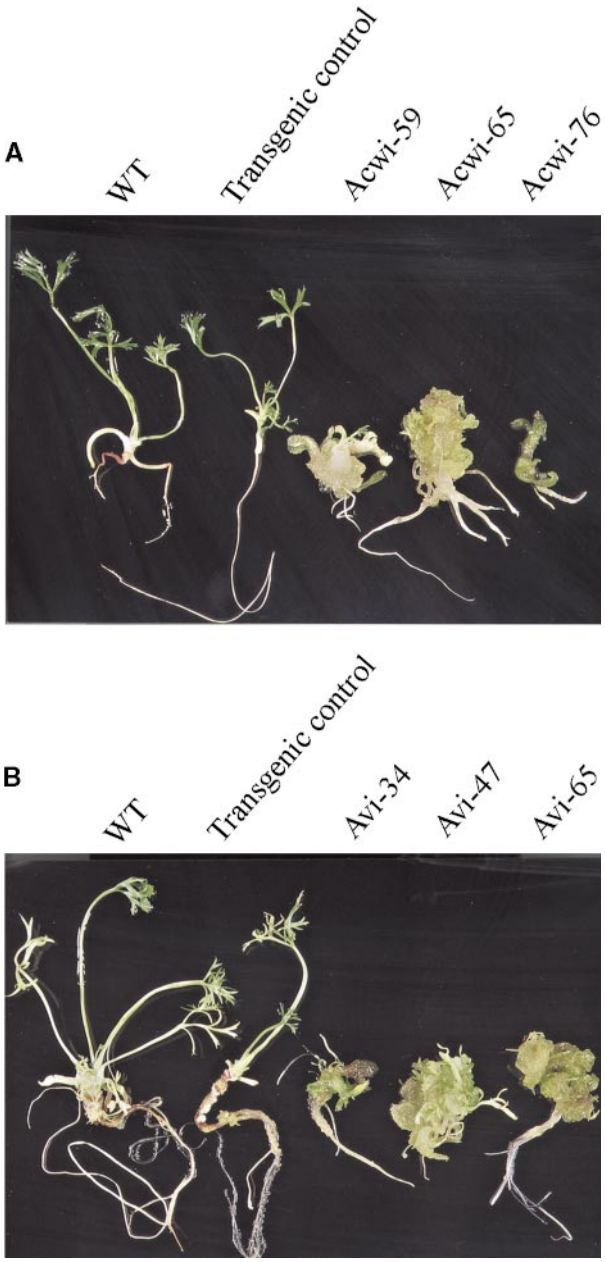

Figure 3. Phenotypic Changes in Transgenic Plantlets Expressing Antisense mRNA for Vacuolar Invertase or Cell Wall Invertase.

All plantlets were grown on half-strength Murashige and Skoog medium containing only macroelements and microelements and 3% sucrose. The transgenic control plantlets harbor the promoter of the gene for carrot cell wall invertase fused to the *GUS* reporter gene.
(A) Antisense plantlets for cell wall invertase (Acwi-59, Acwi-65, and Acwi-76) and wild-type (WT) and transgenic control plantlets.
(B) Antisense plantlets for vacuolar invertase (Avi-34, Avi-47, and Avi-65) and wild-type (WT) and transgenic control plantlets.

bushy appearance. At the same time, tap root development was markedly reduced (Figure 6A). The dry weight leaf-to-root ratio was 17:1 (Figure 5). On average, Avi plants also had more leaves than did control plants (up to twofold more). Tap roots developed normally but remained small (Figure 6B). In these plants, the leaf-to-root ratio was ~1.5:1 (Figure 5).

Close inspection of the leaves of the antisense plants revealed phenotypic alterations. Leaves of Avi (Figure 7B) or Acwi (Figure 7A) plants had narrower lamina than did leaves of control plants. This phenotype was more pronounced in Acwi plants than in Avi plants.

The Amounts of Sugar and Starch Are Markedly Altered in Mature Leaves and Tap Roots of Antisense Plants

To analyze the influence of reduced acid invertase activity on carbohydrate metabolism in transgenic carrot plants, we analyzed extracts of wild-type and antisense plants for their carbohydrate content. Mature leaves of plants of the three Acwi lines (Figure 8A) and the three Avi lines (Figure 8B) had up to twofold higher sucrose levels, whereas the levels of glucose and fructose were not markedly changed. Furthermore, in mature leaves, the levels of starch were elevated up to fivefold (Figures 9A and 9B). Tap roots of plants of two of the three Acwi lines (Figure 8C, lines Acwi-59 and Acwi-76) had lower sucrose levels (~50% that of wild-type tap roots) and markedly reduced levels of glucose and fructose (~25% that of wild-type tap roots). Tap roots of plants from the three Avi lines (Figure 8D) had slightly lower sucrose levels (~70% that of wild-type plants) and clearly reduced levels of glucose and fructose (~25% that of wild-type plants). On average, tap roots of Avi and Acwi plants also had reduced levels of starch (<30% that of wild-type plants; Figures 9C and 9D, respectively).

Molecular and Biochemical Analyses of Antisense Plants

To test for the presence and integrity of transgenes, we conducted DNA gel blot analysis on DNA isolated from transgenic plants. The CaMV 35S promoter was used as a probe. A different T-DNA integration pattern was found for each independent transformant (Figure 10). On average, three or four copies of the transgene were present in the genome of each individual cell line.

Plant acid invertases are encoded by a small family of related genes. The amino acid sequences of the different isoforms share several short, highly conserved domains but otherwise are quite different (Unger et al., 1994); for example, isoform I of carrot vacuolar invertase and cell wall invertase are ~43% identical (52% similar). To test whether downregulation of the activity of one isoform by an antisense approach affected other invertase isoenzymes, we tested protein extracts of leaves of young Avi or Acwi plantlets on

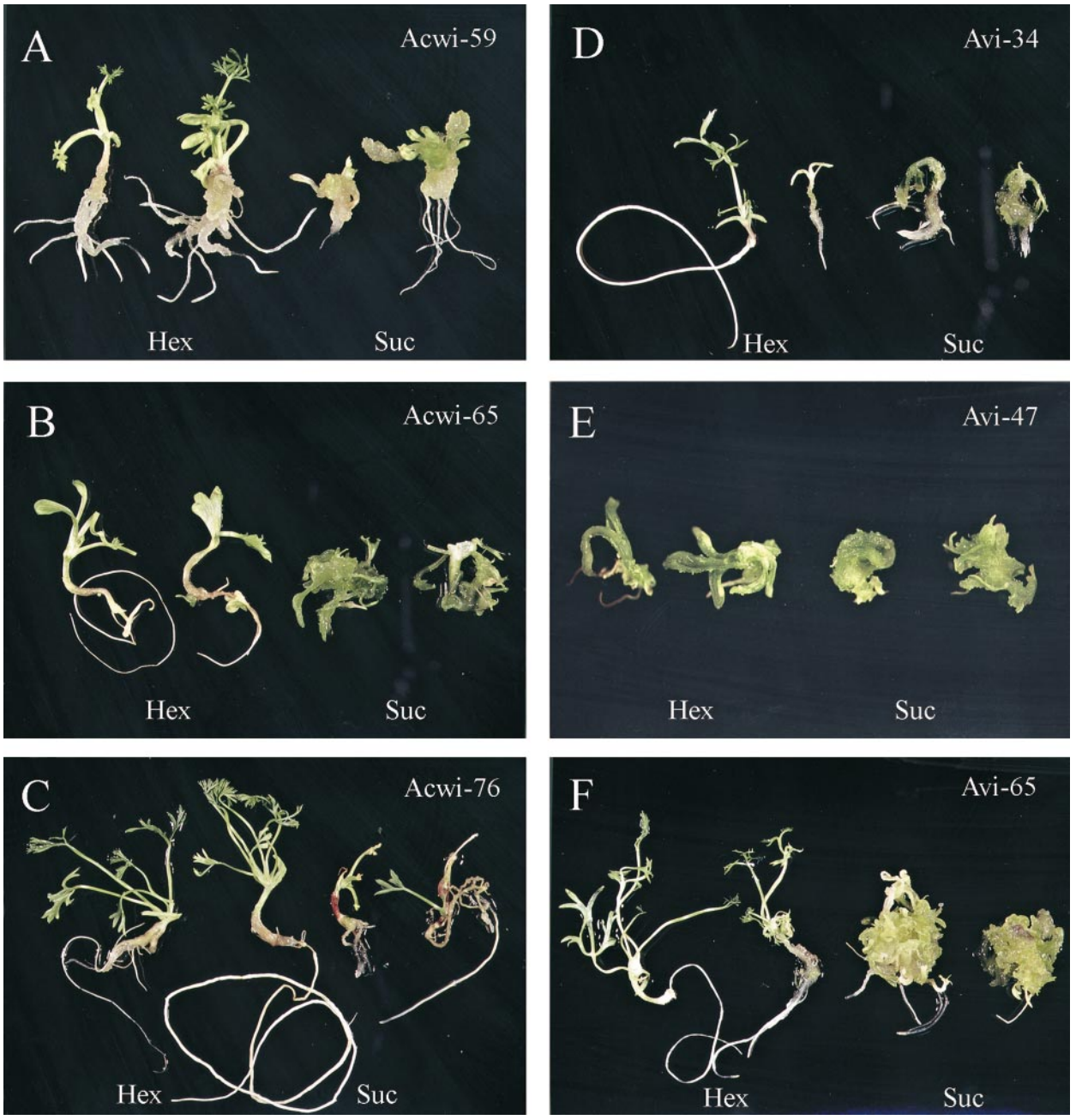

Figure 4. Phenotypic Changes in Transgenic Plantlets Grown on Media Containing Different Sugars.

Transgenic plantlets were grown on half-strength Murashige and Skoog medium containing only macroelements and microelements and a mixture of sucrose, glucose, and fructose (Hex, 1:2:2, 3% total; the two plantlets at left in **[A]** to **[F]**) or 3% sucrose alone (Suc; the two plantlets at right).

(A) to **(C)** Acwi plantlets grown on media containing Hex or Suc.

(D) to **(F)** Avi plantlets grown on media containing Hex or Suc.

protein gel blots with antibodies raised against both isoforms (Figures 11A and 11B). Leaves with clearly reduced levels of cell wall invertase protein had fairly normal levels of vacuolar invertase (Figure 11A), whereas leaves of Avi plants had slightly reduced levels of cell wall invertase protein (Figure 11B).

The determination of enzyme protein levels in leaves of young plantlets was complemented by measurements of invertase activity in tap roots of mature Avi and Acwi plants (Figures 12A to 12D). On average, storage roots of Acwi plants had ~30% of the cell wall invertase activity of roots of control plants (Figure 12A), whereas vacuolar invertase activity was still 87% of the activity of control tap roots (Figure 12B). On average, storage roots of Avi plants had ~19% of the vacuolar invertase activity of roots of control plants (Figure 12C), whereas cell wall invertase activity was still 76% of the activity of roots of control plants (Figure 12D).

DISCUSSION

Antisense Repression of Acid Invertases Is Isoform Specific

To unravel the functions of acid invertases expressed early during plant development, we downregulated their expression by an antisense mRNA approach. We chose carrot as a model plant because we have studied extensively the different acid invertase isoforms of this plant (Sturm, 1996) and can easily transform it with Agrobacterium (Hardegger and Sturm, 1998). Carrot acid invertases are encoded by a small family of genes with similar structure but very different 5′ upstream sequences (Sturm, 1996). Although the amino acid sequences of the invertase isoforms also are fairly dissimilar (Unger et al., 1994), no major differences were found in the biochemical characteristics of the isoenzymes. All acid invertases seem to be β-fructofuranosidases, and cleavage of sucrose, the preferred substrate, is optimal between pH 4.5 and 5.5. Thus, the different carrot invertase genes encode very similar enzymes with markedly different properties at the level of gene regulation.

An important prerequisite of our study was that the reduction of invertase activity by expression of antisense mRNA is isoform specific. On DNA gel blots, the different cDNA probes bound to different DNA fragments, showing that the sequences of the cDNAs are diverse enough to prevent cross-hybridization (Unger et al., 1994). Analysis of enzyme protein levels in the antisense plants was in agreement with this finding, and only small effects of a given antisense construct on the expression of other invertase isoforms were found. Furthermore, the three different isoforms of, for example, cell wall invertase (Sturm, 1996), are expressed at very different times and places during plant development, and only the main form used in our study seems to have a function during vegetative plant growth (Lorenz et al., 1995). Thus, even if we could affect the expression of, for example, the flower bud–specific cell wall invertase by using antisense mRNA for the main cell wall enzyme, we would not see it because in this study, we are not looking at the development of reproductive organs.

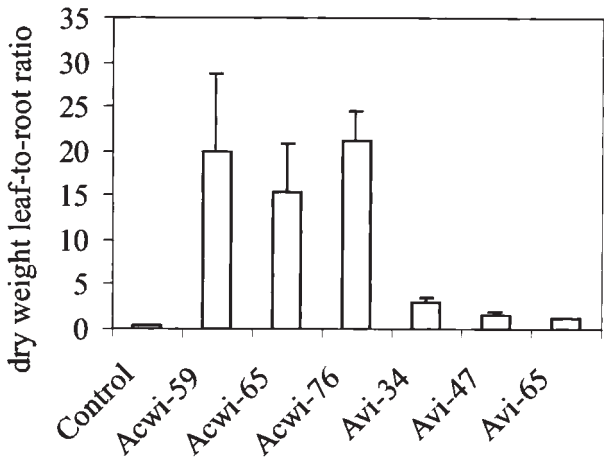

Figure 5. Ratio of Leaves and Roots of Mature Avi and Acwi Plants.

Leaves and roots were harvested separately from representative plants of each of the three independent Avi or Acwi lines. The organs were chopped into small pieces and immediately freeze-dried. All values represent the mean ±SD of dry weight leaf-to-root ratios of three to six plants.

Reduced Acid Invertase Activity Alters Sucrose Partitioning in Carrot Plants with Developing Tap Roots

The expression patterns of the acid invertase isoforms differ widely, suggesting isoform-specific functions. We found transcripts for carrot cell wall invertase in all plant organs from germinating seeds to plants with developing tap roots (Sturm et al., 1995). Because different physiological processes take place in source (sucrose biosynthesis) and sink (sucrose utilization and storage), it has been suggested that the function of cell wall invertase is different in each organ. In leaves of plants with developing tap roots, cell wall invertase may control the exit of assimilated carbon by cleaving sucrose to support leaf growth. In roots, cell wall invertase may cleave sucrose directly after phloem unloading, thereby creating the sucrose concentration gradient required for long-distance transport of photoassimilates. Because the CaMV 35S promoter used in our studies is expressed poorly only in source leaves compared with tap roots (Hardegger and Sturm, 1998), the expression of antisense mRNA for cell

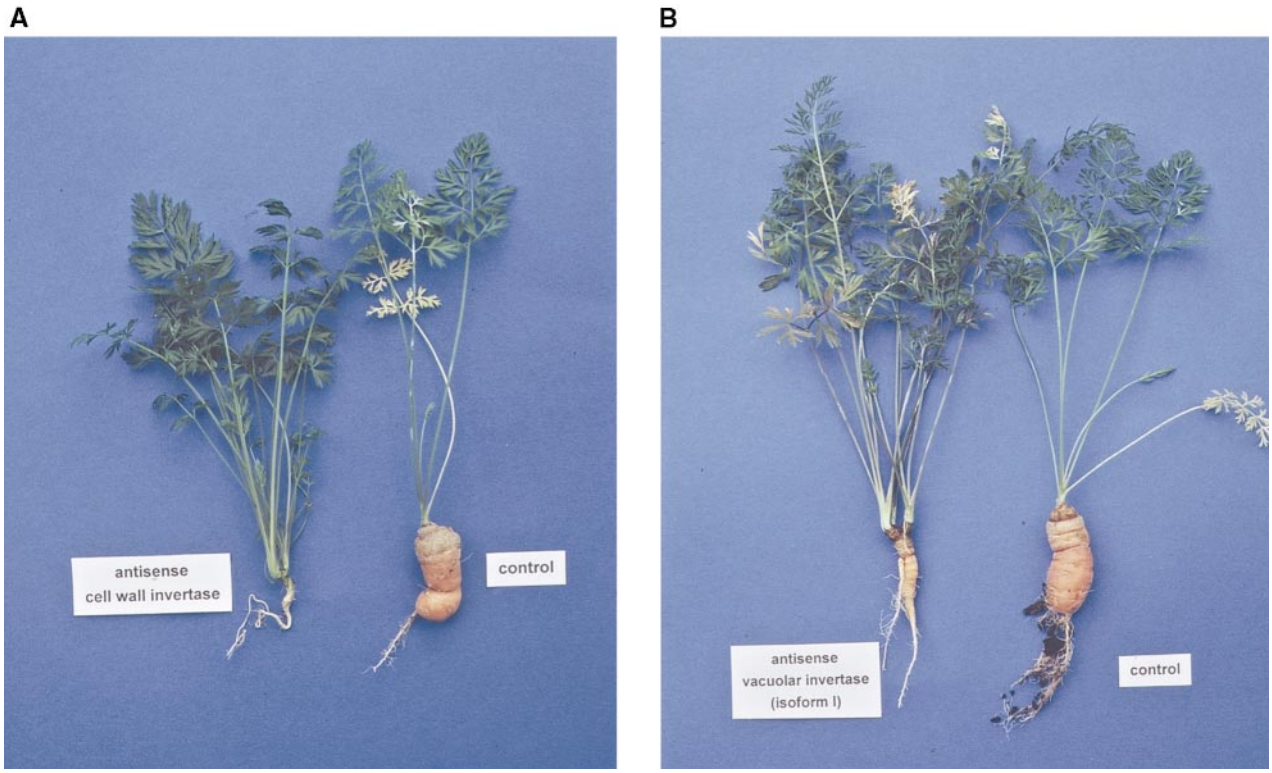

Figure 6. Phenotypic Changes in Mature Transgenic Plants.

Plants were grown in soil in a growth chamber. The control plants are transgenic and harbor the promoter of the gene for carrot cell wall invertase fused to the *GUS* reporter gene.
(A) An Acwi plant and a control plant.
(B) An Avi plant and a control plant.

wall invertase may preferentially affect its root function. The phenotype of the transgenic plants obtained corresponds very well with this hypothesis. The plants did not develop tap roots within the first 16 weeks and apparently used the surplus of assimilated carbon in the growth of additional leaves. Furthermore, these leaves accumulated up to twofold higher levels of sucrose and up to fivefold higher levels of starch than did leaves of nontransformed plants. After $>$3 months, some of the Acwi plants finally developed small storage roots, and this process coincided with the downregulation of the natural cell wall invertase expression (Sturm et al., 1995).

In young carrot seedlings, we previously found transcripts for isoforms I and II of vacuolar invertase in both leaves and roots. In older plants, their expression was restricted to roots, with isoform I being highly expressed in primary roots and isoform II in developing tap roots (Sturm et al., 1995). During the first weeks of plant development, when the organs rapidly grow and elongate, vacuolar invertase may be a key regulator of cell expansion by doubling the osmotic potential of cells when sucrose is converted into glucose and fructose. Later, when sugars are stored in tap roots, vacuolar invertase may cleave sucrose to maintain the sucrose concentration gradient between leaves and roots (Sturm, 1996). Again, because the CaMV 35S promoter is expressed poorly only in carrot source leaves compared with tap roots, we expected that the expression of antisense mRNA for vacuolar invertase in developing plants would mainly affect the root functions of the enzyme. The phenotypes of the transgenic plants obtained are in good agreement with this prediction. Presumably due to a reduced osmotic potential, root development was delayed, and at a time when control plants already had large tap roots, those of the Avi plants were still very small. Our hypothesis is supported by the finding that these roots have lower sucrose levels in addition to clearly reduced levels of glucose and fructose. Because in these plants carbon assimilation was not impaired, the excess of sucrose apparently was invested in the growth of more leaves, which also accumulated higher levels of sucrose and starch than did the wild type.

Antisense Repression of Acid Invertases Alters Plant Development

A novel finding of our study is that a reduction of acid invertase expression alters carrot development at a stage as early as embryo formation. In contrast, expression of antisense mRNA for acid invertase in tomato (Ohyama et al., 1995; Klann et al., 1996) and potato (Zrenner et al., 1996) did not lead to phenotypic changes in transgenic plants. A possible explanation for this difference is that the tomato and potato genes downregulated by antisense mRNA expression are expressed only at a late stage of plant development, for example, in tomato during fruit ripening and in potato during cold sweetening of stored tubers. In contrast, in carrot, the genes for vacuolar invertase and cell wall invertase already are expressed at a very early stage of plant development (Sturm et al., 1995).

During further growth of the invertase antisense embryos on sucrose-containing media, proper organ formation was impaired and led to poorly differentiated but green struc-

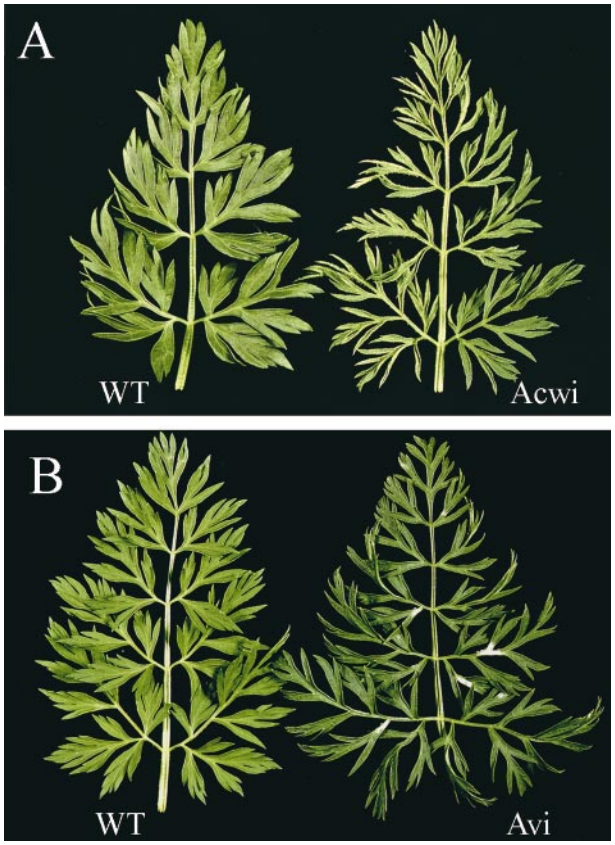

Figure 7. Phenotypic Changes in Transgenic Leaves.

(A) Leaf from an Acwi plant and a wild-type control leaf (WT).
(B) Leaf from an Avi plant and a wild-type control leaf (WT).

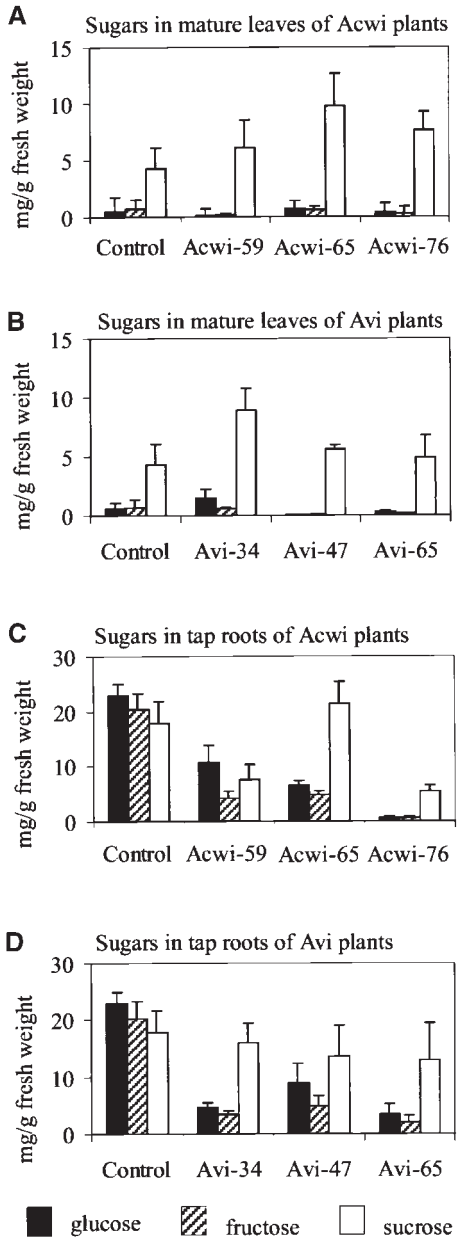

Figure 8. Influence of Antisense Inhibition of Vacuolar Invertase or Cell Wall Invertase on the Sucrose, Glucose, and Fructose Content of Source Leaves and Tap Roots of Transgenic Carrot Plants.

Leaves and tap roots were harvested from mature Avi or Acwi plants, and soluble sugars were extracted and measured as described in Methods. All values are given as milligrams of sugar per gram fresh weight of tissue and represent the mean ±SD of the results from the analysis of three to six plants.
(A) and **(B)** Sugar content of mature leaves of Acwi or Avi plants.
(C) and **(D)** Sugar content of tap roots of Acwi or Avi plants.

tures. The most likely cause of the undifferentiated growth is the altered sucrose-to-hexose level in these plantlets. This hypothesis is in line with the finding that sugars not only function as substrates for growth but also regulate genes (Koch, 1996). Glucose and fructose especially seem to be direct signal molecules, and sucrose must be converted into its hexose components before it can function as a signal (Jang and Sheen, 1997). Furthermore, to function as a signal, the hexose concentration must be above a certain threshold level (Herbers et al., 1996). In the invertase antisense plantlets, the markedly reduced enzyme activity may have kept the hexose level below such a threshhold. This may occur not in whole tissues but only in a small but important number of cells, such as those of the maize kernel pedicel (Miller and Chourey, 1992) or the thin-walled parenchyma of the seed coat of developing bean seeds (Weber et al., 1995). Numerous genes whose expression is either upregulated or downregulated by sugars have been identified. Many of them code for enzymes of primary metabolism (Koch, 1996), but sugars also affect plant development throughout the life cycle, from germination to flowering to senescence (Jang et al., 1997). Prominent examples of the latter type are the phytochrome A signaling pathway in Arabidopsis (Dijkwel et al., 1997) required for photomorphogenesis and the genes for δ2 and δ3 cyclins (Soni et al., 1995) involved in cell cycle control.

Acwi and Avi plantlets grown on sucrose have fairly similar abnormal phenotypes, suggesting that sugar signaling from the apoplast and the endomembrane system feeds into the same developmental pathway. Similar conclusions were drawn when yeast invertase was overexpressed in the apoplast or the vacuole of tobacco cells (Sonnewald et al., 1991). The resulting plants with invertase in different subcellular compartments had similar phenotypes (Sonnewald et al., 1991). Furthermore, different transgenic lines also shared a marked increase in defense-related gene transcripts and elevated levels of salicylic acid, both signs of an induced systemic acquired resistance (Herbers et al., 1996). The authors suggested that sugar signaling occurs at any place in the secretory system.

In the Acwi and Avi carrot plantlets, the assumed missignaling brought about by an altered sucrose-to-hexose ratio was compensated by the addition of glucose and fructose, the products of the invertase reaction, and plants grown under these conditions developed normally. Again, the different antisense lines responded very similarly, supporting the idea that sugar signaling from the apoplast and the endomembrane system feed into the same signaling pathways.

In conclusion, our data indicate that invertases have multiple functions. Novel here is that during early plant development, invertase levels seem to play an important role, most likely via control of sugar composition and metabolic fluxes. Later in plant development, when reduction of cell wall invertase or vacuolar invertase activity shifts the leaf-to-root ratio toward the development of leaves, both isoenzymes appear to have important functions in sucrose partitioning.

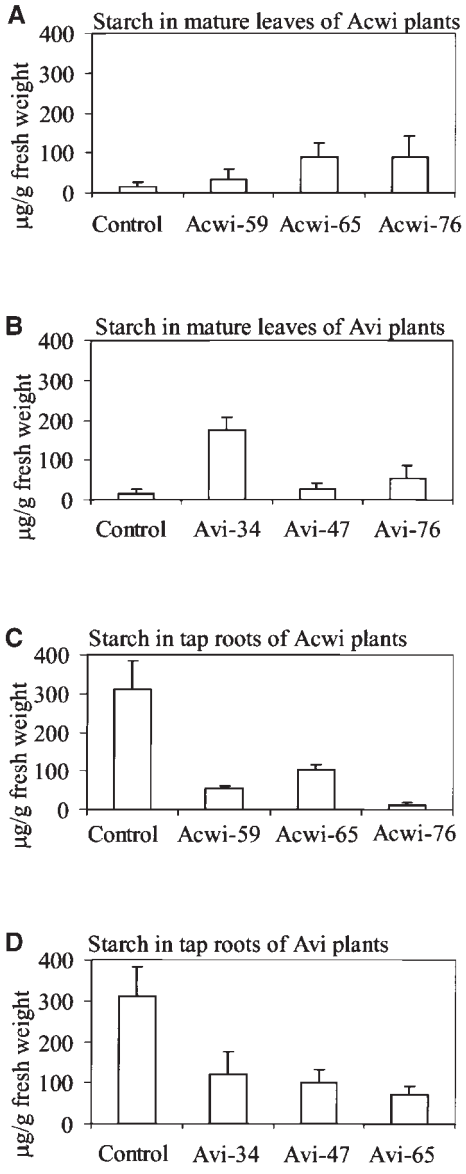

Figure 9. Influence of Antisense Inhibition of Vacuolar Invertase or Cell Wall Invertase on the Starch Content of Source Leaves and Tap Roots of Transgenic Carrot Plants.

Leaves and tap roots were harvested from mature Avi or Acwi plants, and starch was extracted and measured as described in Methods. All values are given as micrograms of starch per gram fresh weight of tissue and represent the mean ±SD of results from the analysis of three to six plants.

(A) and **(B)** Starch content of mature leaves of Acwi or Avi plants.

(C) and **(D)** Starch content of tap roots of Acwi or Avi plants.

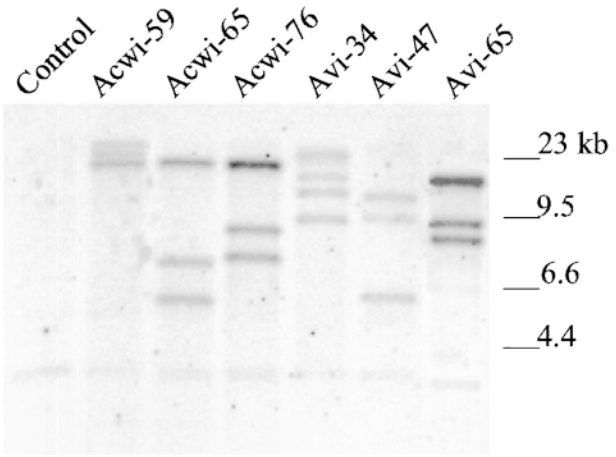

Figure 10. Gel Blot Analysis of DNA Isolated from Independent Transgenic Plants.

DNA was isolated from leaves of plant lines Acwi-59, Acwi-65, and Acwi-76 expressing antisense mRNA for cell wall invertase, from plant lines Avi-34, Avi-47, and Avi-65 expressing antisense mRNA for vacuolar invertase, or from a transgenic control plant harboring the promoter of the gene for carrot cell wall invertase fused to the *GUS* reporter gene. The DNA (10 μg per lane) was restricted with EcoRI, and the blot was probed with the ^{32}P-labeled CaMV 35S promoter.

METHODS

Plant Material

Seeds of carrot (*Daucus carota* cv Nantaise) were purchased from Hild (Mambach, Germany) and sterilized consecutively for 15 min in 70% ethanol and 10% sodium hypochlorite. After extensive washing in sterile water, the seeds were germinated on 0.8% agar in the dark at 24°C. After 7 days, hypocotyls were harvested and cut into segments of ~0.5 cm for transformation with agrobacteria.

Recombinant DNA Technology

Restriction sites for SmaI (5′) and SacI (3′) were added to the ends of the carrot cDNAs for cell wall invertase (1816 bp; Sturm and Chrispeels, 1990) and isoform I of vacuolar invertase (2024 bp; Unger et al., 1994). Individually, the polymerase chain reaction fragments were ligated in reverse orientation into the SmaI and SacI sites of the binary vector pBI121 (Clonetech, Palo Alto, CA) behind the 35S promoter of califlower mosaic virus (CaMV) and instead of the gene for β-glucuronidase (*GUS*). The constructs were introduced into *Agrobacterium tumefaciens* LBA4404 (Hoekma et al., 1983) via direct DNA transformation (Hofgen and Willmitzer, 1988).

Plant Transformation and Regeneration

Transformation of hypocotyl segments and regeneration of transgenic plants were conducted as described by Hardegger and Sturm (1998). Transgenic plants were grown in soil in a growth chamber at 22°C with a day and night cycle of 16 and 8 hr, respectively.

Protein Gel Blot Analysis

Soluble proteins and cell wall–bound proteins were extracted by following the procedure of Laurière et al. (1988). Briefly, 200 to 300 mg

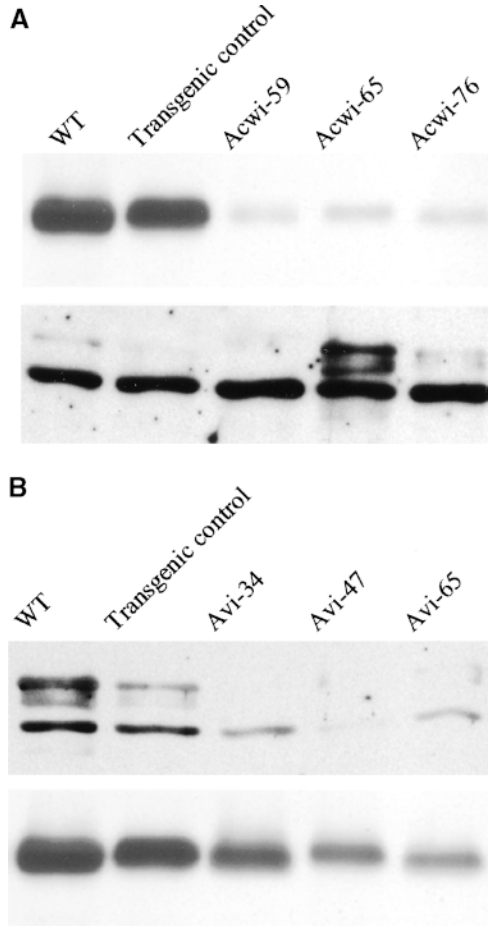

Figure 11. Protein Gel Blot Analysis of Vacuolar Invertase and Cell Wall Invertase in Leaves of Young Transgenic Plantlets.

Soluble proteins or cell wall proteins were extracted from leaves of small transgenic Acwi or Avi plantlets. After separation by SDS-PAGE and transfer to nitrocellulose membranes, the blots were probed with antibodies raised against vacuolar invertase (**[A]**, bottom blot; **[B]**, top blot) or antibodies raised against cell wall invertase (**[A]**, top blot; **[B]**, bottom blot). Leaf proteins from wild-type plants (WT) or transgenic plants harboring the promoter of the gene for carrot cell wall invertase fused to the *GUS* reporter gene were blotted as controls.

(A) Cell wall proteins (top) or soluble proteins (bottom) from Acwi or control plantlets.

(B) Cell wall proteins (bottom) or soluble proteins (top) from Avi or control plantlets.

of tissue (callus or leaf) was homogenized with a Polytron homogenizer in 1 mL of ice-cold 25 mM sodium acetate buffer, pH 5.2, containing 10 mM 2-mercaptoethanol, 10 mM lysine, and 1 mM sodium EDTA. After centrifugation for 10 min at 10,000 rpm and 4°C, the supernatant containing the soluble proteins was collected. The pellet was washed four times with ice-cold water and subsequently extracted with 5 volumes of 1 M NaCl overnight at 4°C. The supernatant containing the cell wall–bound proteins was harvested by centrifugation.

Protein samples (10 μg per lane) were separated on a 12.5% SDS–polyacrylamide gel and blotted onto nitrocellulose membranes. Subsequently, immunological detection of cell wall invertase or isoform I of vacuolar invertase was performed by using an electrochemiluminescence-based detection system, as described by the manufacturer (Amersham). The antibodies against cell wall invertase (Laurière et al., 1988) and isoform I of vacuolar invertase (Unger et al., 1992) were diluted 1:3000 and 1:2000, respectively.

DNA Gel Blot Analysis

DNA was isolated from carrot leaves, as described by Murray and Thompson (1980). DNA was digested with EcoRI, fractioned on an 0.8% agarose gel, and transferred onto a nylon membrane (Hybond N; Amersham). The CaMV 35S DNA fragment was labeled with phosphorus-32 by following the manufacturer's protocol (Amersham) for the random primer labeling kit. Hybridization and washing of the blots were performed using standard procedures (Sambrook et al.,1989).

Determination of Soluble Sugars and Starch

Leaf or tap root samples of mature plants (0.5 g) were ground in liquid nitrogen, and the powder was extracted twice with 60% ethanol (1 mL each) at 60°C for 15 min. Subsequently, the extracts were cleared by centrifugation. Aliquots of the supernatants (50 μL) were rapidly dried, and the residues were redisolved in 250 μL of water and kept at 100°C for 10 min. After centrifugation, 25 μL of each sample was loaded onto a Dionex CarboPac PA-100 column (4 mm × 250 mm) attached to a Dionex DX-300 HPLC system and a pulsed amperometric detector (Dionex, Sunnyvale, CA). Sugars were eluted with 120 mM NaOH at room temperature and a flow rate of 1 mL/min.

After sugar extraction, the pellets were washed with ice-cold water and dried. Determination of starch was conducted with a TC Starch Kit from Boehringer Mannheim (No. 207 748), according to the manufacturer's instructions.

Assay of Acid Invertase Activity

Leaves or tap roots of mature plants (2 g) were homogenized in 5 volumes of ice-cold 25 mM sodium acetate buffer, pH 5.0, containing 0.5% β-mercaptoethanol, 10 mM lysine, 1 mM EDTA, and 0.1 mM phenylmethanesulfonyl fluoride. The homogenates were centrifuged at 10,000*g* for 30 min, and the supernatants were used for the determination of acid-soluble invertase activity. The pellets were washed extensively with ice-cold water, and cell wall proteins were extracted with 5 volumes of 1 M NaCl overnight at 4°C.

Invertase activity was assayed at 37°C on 50 mM sucrose in 13.5 mM citric acid and 26.5 mM disodium phosphate, pH 4.6, for cell wall invertase, and 10.5 mM citric acid and 29 mM disodium phosphate,

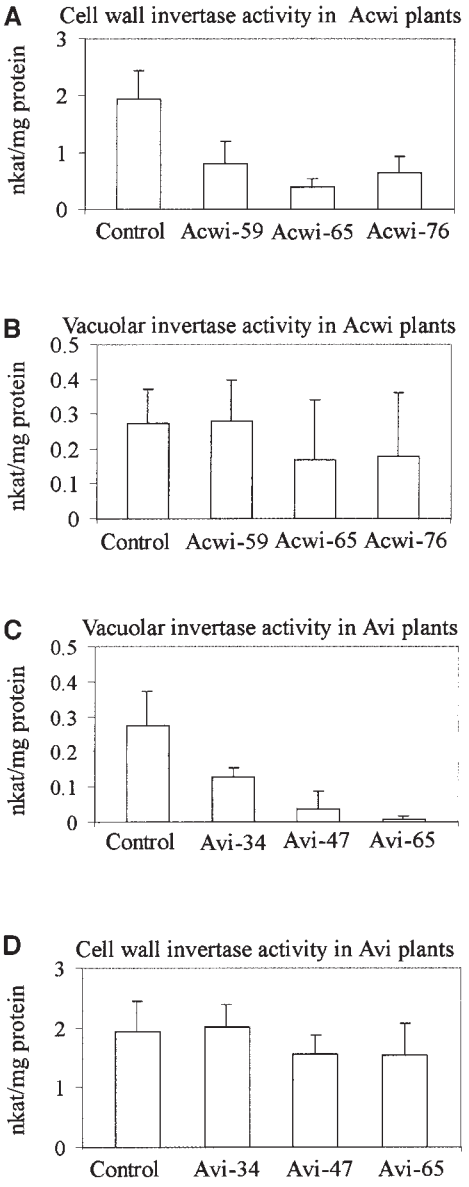

Figure 12. Activities of Vacuolar Invertase and Cell Wall Invertase in Tap Roots of Mature Transgenic Carrot Plants.

Tap roots were harvested from mature Avi or Acwi plants, and enzymes were extracted and measured as described in Methods. All values are given as nanokatals per milligram protein and represent the mean ±SD of results from the analysis of three to six plants.
(A) and **(B)** Activities of cell wall invertase or vacuolar invertase in Acwi or control plantlets.
(C) and **(D)** Activities of cell wall invertase or vacuolar invertase in Avi or control plantlets.

pH 5.4, for the soluble enzyme. The reaction was stopped with alkaline copper reagent, and the liberated reducing sugars were measured according to Somogyi (1952).

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REFERENCES

Ap Rees, T. (1974). Pathways of carbohydrate breakdown in higher plants. In Biochemistry, Series I, Vol. XI, Plant Biochemistry, D.H. Northcote, ed (London: Butterworth), pp. 89–127.

Benhamou, N., Genier, J., and Chrispeels, M.J. (1991). Accumulation of β-fructosidase in the cell walls of tomato roots following infection with a fungal wild pathogen. Plant Physiol. **97,** 739–750.

Dijkwel, P.P., Huijser, C., Weisbeek, P.J., Chua, N.-H., and Smeekens, S.C.M. (1997). Sucrose control of phytochrome A signaling in Arabidopsis. Plant Cell **9,** 583–595.

Eschrich, W. (1980). Free space invertase, its posible role in phloem unloading. Ber. Dtsch. Bot. Ges. **93,** 363–378.

Hardegger, M., and Sturm, A. (1998). Transformation and regeneration of carrot (*Daucus carota* L.). Mol. Breeding **4,** 119–127.

Herbers, K., Meuwly, P., Frommer, W.B., Métraux, J.-P., and Sonnewald, U. (1996). Systemic acquired resistance mediated by the ectopic expression of invertase: Possible hexose sensing in the secretory pathway. Plant Cell **8,** 793–803.

Hoekma, A., Hirsch, P.R., Hooykass, P.J.J., and Schilperoort, R.A. (1983). A binary plant vector strategy based on separation of the vir- and T-region of *Agrobacterium tumefaciens* Ti plasmid. Nature **303,** 179–180.

Hofgen, R., and Willmitzer, L. (1988). Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res. **16,** 9877.

Jang, J.-C., and Sheen, J. (1997). Sugar sensing in plants. Trends Plant Sci. **2,** 208–214.

Jang, J.-C., León, P., Zhou, L., and Sheen, J. (1997). Hexokinase as a sugar sensor in higher plants. Plant Cell **9,** 5–19.

Klann, E.M., Chetelat, R.T., and Bennett, A.B. (1993). Expression of acid invertase gene controls sugar composition in tomato (*Lycopersicon*) fruit. Plant Physiol. **103,** 863–870.

Klann, E.M., Hall, B., and Bennett, A.B. (1996). Antisense acid invertase (*TIV1*) gene alters soluble sugar composition and size in transgenic tomato fruit. Plant Physiol. **112,** 1321–1330.

Koch, K.E. (1996). Carbohydrate-modulated gene expression in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **47,** 509–540.

Kruger, J.N. (1990). Carbohydrate synthesis and degradation. In Plant Physiology, Biochemistry, and Molecular Biology, D.T. Dennis and D.H. Turpin, eds (Harlow, UK: Longman Scientific and Technical Publishers), pp. 59–76.

Laurière, C., Laurière, M., Sturm, A., Faye, L., and Chrispeels, M.J. (1988). Characterization of β-fructosidase, an extracellular glycoprotein of carrot cells. Biochemie **70,** 1483–1491.

Lorenz, K., Lienhard, S., and Sturm, A. (1995). Structural organization and differential expression of carrot β-fructofuranosidase genes: Identification of a flower bud–specific isozyme. Plant Mol. Biol. **28,** 189–194.

Meyer, R.F., and Boyer, J.S. (1981). Osmoregulation, solute distribution and growth in soybean seedlings having low water potentials. Planta **151,** 482–489.

Miller, M.E., and Chourey, P.S. (1992). The maize invertase-deficient *miniature-1* seed mutation is associated with aberrant pedicel and endosperm development. Plant Cell **4,** 297–305.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bioassays with *Camellia japonica* pollen. Phytochemistry **19,** 205–209.

Murray, M.G., and Thompson, W.F. (1980). Rapid isolation of high molecular weight plant DNA. Nucleic Acids Res. **8,** 4321–4325.

Ohyama, A., Ito, H., Sato, T., Nishimura, S., Imai, T., and Hirai, M. (1995). Suppression of acid invertase activity by antisense RNA modifies the sugar composition of tomato fruit. Plant Cell Physiol. **36,** 369–376.

Perry, C.A., Leigh, R.A., Tomos, A.D., Wyse, R.E., and Hall, J.L. (1987). The regulation of turgor pressure during sucrose mobilization and salt accumulation by excised storage-root tissue of red beet. Planta **170,** 353–361.

Peterson, C.E., and Simon, P.W. (1986). Carrot breeding. In Breeding Vegetable Crops, M.J. Bassett, ed (Westport, CT: AVI), pp. 321–356.

Ramloch-Lorenz, K., Knudsen, S., and Sturm, A. (1993). Molecular characterization of the gene for carrot cell wall β-fructosidase. Plant J. **4,** 545–554.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). Molecular Cloning: A Laboratory Manual, 2nd ed. (Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press).

Somogyi, M. (1952). Notes on sugar determination. J. Biol. Chem. **195,** 19–23.

Soni, R., Carmichael, J.P., Shah, Z.H., and Murray, J.A.H. (1995). A family of cyclin D homologs from plants differentially controlled by growth regulators and containing the conserved retinoblastoma protein interaction motif. Plant Cell **7,** 85–103.

Sonnewald, U., Brauer, M., von Schaewen, A., Stitt, M., and Willmitzer, L. (1991). Transgenic tobacco plants expressing yeast-derived invertase in either the cytosol, vacuole or the apoplast: A powerful tool for studying sucrose metabolism and sink/source interactions. Plant J. **1,** 95–106.

Stitt, M., and Sonnewald, U. (1995). Regulation of metabolism in transgenic plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **46,** 341–368.

Sturm, A. (1996). Molecular characterization and functional analysis of sucrose-cleaving enzymes in carrot (*Daucus carota* L.). Eur. J. Bot. **47,** 1187–1192.

Sturm, A., and Chrispeels, M.J. (1990). cDNA cloning of carrot extracellular β-fructosidase and its expression in response to wounding and bacterial infection. Plant Cell **2,** 1107–1119.

Sturm, A., Šebková, V., Lorenz, K., Hardegger, M., Lienhard, S., and Unger, C. (1995). Development- and organ-specific expression of the genes for sucrose synthase and three isoenzymes of acid β-fructofuranosidase in carrot. Planta **195,** 601–610.

Unger, C., Hofsteenge, J., and Sturm, A. (1992). Purification and characterization of a soluble β-fructofuranosidase from *Daucus carota.* Eur. J. Biochem. **204,** 915–921.

Unger, C., Hardegger, M., Lienhard, S., and Sturm, A. (1994). cDNA cloning of carrot (*Daucus carota*) soluble acid β-fructofuranosidase and comparison with the cell wall isoenzyme. Plant Physiol. **104,** 1351–1357.

Weber, H., Borisjuk, L., Heim, U., Buchner, P., and Wobus, U. (1995). Seed coat–associated invertases of fava bean control both unloading and storage functions: Cloning of cDNAs and cell type–specific expression. Plant Cell **7,** 1835–1846.

Wyse, R.E., Zamski, E., and Tomos, A.D. (1986). Turgor regulation of sucrose transport in sugar beet taproot tissue. Plant Physiol. **81,** 478–481.

Zrenner, R., Schüler, K., and Sonnewald, U. (1996). Soluble acid invertase determines the hexose-to-sucrose ratio in cold-stored potato tubers. Planta **198,** 246–252.