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Growth habit and sugar accumulation in sugarbeet (*Beta vulgaris* L.) transformed with a cytokinin biosynthesis gene

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Abstract Expression of a bacterial cytokinin biosynthesis gene fused to a patatin gene promoter was studied in sugarbeet (*Beta vulgaris* L.). Two independent transformants, Pat-*ipt* 1 and 2, exhibited a number of distinguishable morphological alterations commonly induced by cytokinins, i.e. less root growth, reduced leaf surface area, and increased axillary shoot development. Concentrations of the cytokinins zeatin and zeatin riboside were increased by twofold in taproots and 7- to 18-fold in leaves. Leaf sucrose and glucose concentrations were not significantly different from those in control plants except in Pat-*ipt* 2 where glucose levels were elevated ninefold. Since normal taproot development was severely inhibited, sucrose concentrations in the taproots were significantly reduced.

Keywords *Beta vulgaris* · Isopentenyl transferase (*ipt*) · Sugarbeet · Sucrose

Abbreviations *BAP*: N^6-Benzylaminopurine · *BHT*: Butylated hydroxytoluene · *IBA*: Indole-3-butyric acid · *ipt*: Isopentenyl transferase · *NAA*: α-Naphthaleneacetic acid · *Pat*: Patatin · *Z*: Zeatin · *ZR*: Zeatin riboside

Introduction

Cytokinins have been shown to alter phloem unloading as well as sink initiation, strength, and capacity (Thomas 1986). A broad mobilizing effect of cytokinins has been demonstrated using cytokinin applications to organs or tissues; these cause increased photosynthate transport to the site of cytokinin application. In sugarbeet, high endogenous cytokinin levels have been correlated with cambial initiation and rapid cell division in developing taproots, the sucrose-storing organs of sugarbeet (Elliott et al. 1983). Similarly, high cytokinin levels have been reported in synchronized taproot cell suspension cultures prior to cytokinesis (Elliott et al. 1986). Based on these findings, Elliott and Weston (l993) suggested that higher cytokinin levels might increase the cell division rate, vascular ring number, and sucrose accumulation in taproots.

To study the effects of cytokinin on various aspects of growth and sugar accumulation, Snyder et al. (1999) transformed sugarbeet cells with the isopentenyl transferase gene that catalyzes the rate-limiting step of the cytokinin biosynthetic pathway, i.e., the condensation of isopentenyl pyrophosphate and adenosine monophosphate to form isopentenyladenosine monophosphate (Akiyoshi et al. 1984). In the study reported here, transgenic plants were analyzed for expression of the *ipt* gene, phenotypic effects, cytokinin concentration, and sugar content. A number of morphological alterations commonly observed in *ipt* transformants were noted. Leaf and taproot cytokinin levels were elevated, but leaf sucrose concentrations were comparable to those of the untransformed controls. Normal growth and development of the transgenic taproots were inhibited, resulting in a decreased accumulation of sucrose.

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Materials and methods

Plant material

Transgenic sugarbeet plants, Pat-*ipt* 1 and Pat-*ipt* 2, were generated by *Agrobacterium*-mediated transformation of cotyledons (Snyder et al. 1999). These Pat-*ipt* transformants carried the gene for cytokinin synthesis (*ipt*) under the control of a tuber-specific, patatin gene promoter (Pat) from potato. Transgenic and untransformed (REL-1) plants were maintained in tissue culture on B0.25

medium (Snyder et al. 1999) at 25°C under a 16/8-h (light/dark) photoperiod and fluorescent lighting (cool-white, 30 μmol/m^2 s). Shoots were subcultured at 4-week intervals. For root induction, shoots (10 mm in height) were cultured on MSB medium (Snyder et al. 1999) with 30 g/l glucose and 5.0 g/l agar gel (Sigma, St. Louis, Mo.) and supplemented with either IBA or with IBA and NAA at 25°C under continuous light. Shoots were exposed to 50 mg/l IBA for a 24-h period and then transferred to MSB medium without plant growth regulators or they were cultured on medium with 3 mg/l IBA and 2 mg/l NAA. Rooted plantlets were transferred to potting soil mix (Jiffy Products, Batavia, Ill.), covered with plastic bags, and acclimatized for 2 weeks in a growth chamber. The plants were then potted in soil (Conrad Fafard, Agawam, Mass.) and grown in a growth chamber at temperatures of 22°C (day) and 19°C (night) under a 16/8-h (light/dark) photoperiod and fluorescent lighting (cool-white, 270 μmol/m^2 s) for 4 weeks, followed by growth in the greenhouse at 25°C (day) and 22°C (night) temperatures under natural light. The plants were fertilized once a month with a 20/20/20 nutrient mix (Spectrum Group, St. Louis, Mo.).

Northern blot analyses

Total RNA was extracted from plant tissues, fractionated on formaldehyde gels and hybridized as previously reported (Smigocki et al. 1993). Hybridization reactions were carried out at 65°C for 24 h with a 0.5-kb *ipt* probe labeled with α-[^{32}P]-dCTP using a random primer kit (Boehringer Mannheim). Blots were washed at 65°C with 40 m*M* sodium phosphate buffer (pH 7.2), 1 m*M* EDTA and 5% (w/v) SDS and exposed to X-omat AR film (Kodak, Rochester, N.Y.) at –80°C.

Cytokinin analysis

Cytokinins were extracted in 80% methanol (10 ml/g of tissue) containing 20 mg/l BHT at –80°C for 16 h. The extracts were centrifuged (2,000 *g*, 10 min, 4°C) and filtered through Whatman No. 1 filter paper. Pellets were briefly resuspended in the same volume of 80% methanol and BHT, centrifuged, and filtered as before. Combined filtrates were evaporated to an aqueous phase under vacuum at 35°C and partitioned three times against *n*-pentane. The aqueous phase was then applied to a Sep-Pack C_{18} column (Waters, Milford, Mass.) prewashed with 2 ml methanol and 5 ml of H_2O. Cartridges were flushed with 5 ml H_2O and cytokinins eluted with 7 ml methanol. This fraction was dried under vacuum at 35°C, redissolved in Tris-buffered saline, and purified on columns packed with monoclonal anti-zeatin riboside antibodies. The eluted cytokinins were quantified by ELISA using an analytical kit (Phytodetek-t-ZR, Idetek, San Bruno, Calif.). The anti-ZR antibodies provided in the kit cross-reacted most strongly with *trans*-ZR, ZR-5′-monophosphate, and *trans*-zeatin. To determine the percentage recovery, we spiked control samples with 2,000 pmol of *trans*-ZR. Analysis was done in triplicate for each of two 8-month-old greenhouse-grown Pat-*ipt* 1, 2, and REL-1 plants.

Carbohydrate and chlorophyll analysis

Tissue samples were collected from 8- to 12-month-old fully expanded source leaves and taproots of the greenhouse-grown plants. Two leaf discs (3.5 cm^2) and two taproot cores (0.1–0.3 gFW) were taken from each plant between 4 h and 5 h after the start of the photoperiod and immediately frozen in liquid N_2 to stop metabolism. Samples were extracted with 2–4 ml methanol/chloroform/water (5:3:1) in a ground glass tissue homogenizer at 4°C. Homogenates were centrifuged at 4,000 *g* for 5 min at 4°C, and the resultant pellets were re-extracted with 1 ml of 80% methanol. The supernatant and wash fractions were combined and partitioned with 1 ml chloroform. Total chlorophyll ($a + b$) content in the organic phase was measured in 80% acetone (Lichtenthaler 1987). The alcohol fraction was evaporated to a minimum volume under a stream of N_2 at 37°C and diluted to 1 ml with deionized H_2O. The soluble carbohydrates sucrose and glucose were determined in coupled enzyme assays (Bergmeyer et al. 1974). Taproots were placed in a forced-air oven at 80°C for 72 h prior to dry matter determinations. Significant differences were estimated at the 5% level using a one-tailed Student's *t*-test assuming equal variances.

Results and discussion

Growth characteristics of *ipt* plants

Pat-*ipt* 1 and 2 shoots were propagated in tissue culture and transferred to auxin-containing media for rooting. Pat-*ipt* 1 transformants rooted at a frequency of 65% after a 24-h exposure to 50 mg/l IBA in comparison to 86% of the untransformed control shoots. Roots were induced in 4–8 weeks on the *ipt* shoots and in 2 weeks on the controls. Of the more than 100 Pat-*ipt* 2 shoots, only 4 shoots rooted on media containing both 2 mg/l NAA and 3 mg/l IBA. Attempts to root Pat-*ipt* 2 plants on higher concentrations of auxin (25, 50, 100, 150, or 200 mg/l IBA or NAA) with longer times of exposure (1, 2, 4, 5 days; 2, 3, 4 weeks) were not successful. Unlike the untransformed controls, rooted Pat-*ipt* 1 and 2 plantlets had a very low survival rate when transferred to soil.

All greenhouse-grown transgenic sugarbeets exhibited phenotypic alterations that have been previously reported for *ipt*-transformed plants (Medford et al. 1989; Li et al. 1992; Smigocki 1995). Pat-*ipt* 1 plants developed wrinkled leaves (Fig. 1) similar to ones observed in transgenic HS-*ipt* tobacco plants following heat shock treatment (Ainley et al. 1993). Pat-*ipt* 2 plants had small, thick leaves and excessive axillary shoot development on a large, proliferative crown (Fig. 1). Reduced leaf size has also been reported in *ipt*-transgenic tobacco (Medford et al. 1989), tomato (Groot et al. 1995), and potato plants (Mahácková et al. 1997). Total chlorophyll levels in Pat*ipt* 1 and 2 leaves were similar to those in untransformed plants (Table 1), in contrast to the mostly elevated (Beinsberger et al. 1991) or, in few cases, reduced (Groot et al. 1995; Smigocki 1995) levels reported in other *ipt*-transformed plants.

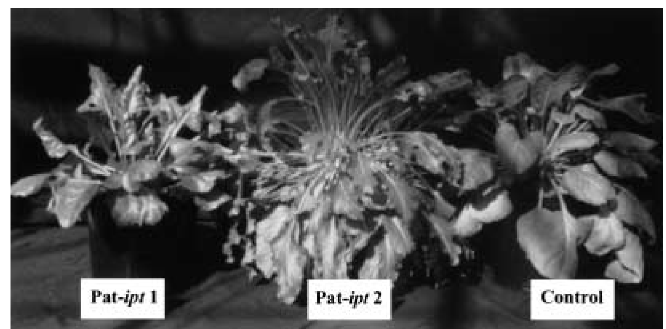

Fig. 1 Phenotypic characteristics of transgenic sugarbeets carrying the *ipt* gene. From *left to right*: 6-month-old Pat-*ipt* 1, Pat-*ipt* 2, and REL-1 (control) untransformed plant

Table 1 Cytokinin, carbohydrate, and chlorophyll concentrations and taproot dry weights of sugarbeet transformants, Pat-*ipt* 1 and -2 and untransformed REL-1 plants (*FW* fresh weight)

Genotype	Z/ZR content[a] (pmol ZR equiv/gFW)	Sucrose[b] (μmol/gFW)	Glucose (μmol/gFW)	Chlorophyll (mg/gFW)	Dry weight (g)
Leaves					
Pat-*ipt* 1 (4)	141±25.1*	3.1±0.3	4.1±1.2	0.63±0.05	ND[c]
Pat-*ipt* 2 (3)	61±10.2*	4.7±2.2	68.3±15.5*	0.82±0.16	ND
REL-1 (5)	8±1.6	8.1±3.2	5.6±2.5	0.83±0.08	ND
Taproots					
Pat-*ipt* 1 (4)	65±5.0*	147.2±24.1*	1.00±0.32	ND	20.4±7.1*
Pat-*ipt* 2 (2)	64±11.3*	13.5±9.5*	10.25±9.7	ND	1.3±0.3*
REL-1 (5)	33±3.6	389.5±54.2	0.64±0.3	ND	111.3±46.3

* Different from the control (REL-1), P=0.05
[a] Samples were taken from 8-month-old plants. Each value represents a mean of two experiments done in triplicate ± standard error. The results are corrected for 100% recovery
[b] Values for carbohydrates and chlorophyll concentrations are means ± standard error of 8- to 12-month-old plants
[c] Not done

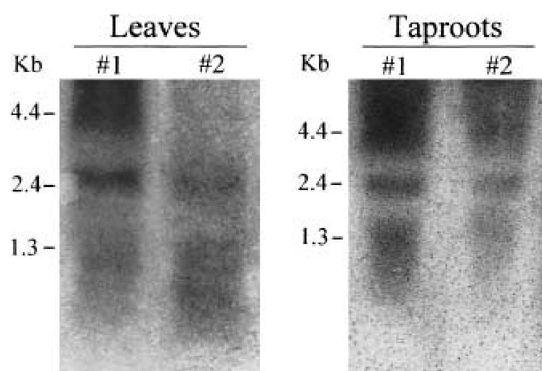

Fig. 2 Analysis of *ipt* transcripts (1.3 kb) in leaves and taproots of greenhouse-grown Pat-*ipt* 1 (*#1*) and Pat-*ipt* 2 (*#2*) plants

All plants derived from independent transformants Pat-*ipt* 1 and 2 had smaller taproots than the control plants. The reduced size of the taproot can be compared to the decrease in root growth reported for other cytokinin-overproducing transgenic plants (Li et al. 1992; Binns 1994). The average taproot dry weight of 8- to 12-month-old Pat-*ipt* 1 and 2 plants was 18% and 1.2% of the untransformed control, respectively (Table 1).

Molecular analyses

Southern blot analysis of Pat-*ipt* 1 and 2 plants had revealed the expected 2.1-kb fragment corresponding to the Pat-*ipt* gene construct (Snyder et al. 1999). The class-I patatin gene promoter is normally active in potato tubers but can be induced in leaves and roots of potato (Rocha-Sosa et al. 1989) or tobacco (Wenzler et al. 1989). In sugarbeet Pat-*ipt* transformants, presence of the *ipt* transcript (1.3 kb) was confirmed in both the leaves and taproots (Fig. 2). In addition, a larger transcript (2.4 kb) was detected that is likely the result of a read through the transcription stop codon at the end of the *ipt* gene. No *ipt* message was detected in the leaves and taproots of control plants (data not shown).

Cytokinin concentrations

Leaves and taproots of greenhouse-grown Pat-*ipt* 1 and 2 plants were analyzed for the cytokinins zeatin and zeatin riboside since both of these have been reported to be elevated in other plants transformed with the *ipt* gene (Medford et al. 1989; Smigocki and Owens 1989). Leaf cytokinin levels in Pat-*ipt* 1 and 2 increased 8- and 18-fold, respectively, relative to the controls (Table 1). In the taproots, only a twofold increase was observed, but the control concentrations were about fourfold higher than in the leaves. Presence of the *ipt* message and elevated cytokinin levels indicate that the Pat-*ipt* gene construct was active in the leaves and taproots of the transformants. The observed phenotypes of the Pat-*ipt* 1 and 2 plants correspond to those previously noted for *ipt* transformants and are likely a response to the increased cytokinin levels. It is unlikely that these phenotypes were induced by somaclonal variations since tissue culture-derived control plants did not exhibit any morphological changes, even after 5 years of micropropagation.

Carbohydrate content

The concentrations of sucrose in the leaves of Pat-*ipt* 1 and 2 plants were not significantly different from those in control plants (Table 1). Glucose concentrations were highly elevated in Pat-*ipt* 2 leaves (68.3±15.5 μmol/gFW) but not in Pat-*ipt* 1 leaves (Table 1). Sucrose hydrolysis during carbohydrate extraction did not appear to be a major source of the high glucose content since leaf fructose concentrations in Pat-*ipt* 2 plants were low (15.5±1.5 μmol/gFW; data not shown). Sucrose content in the taproots was significantly lower in Pat-*ipt* 1 and 2 plants than in the controls (Table 1). Efficient sucrose accumulation in sugarbeet taproots has been shown to be dependent on the relative proportion of young, small cells close to the cambia (Milford 1973). The reduced taproot sizes of the Pat-*ipt* 1 and 2 transformants suggest

that the activity of secondary cambia responsible for the production of efficient sucrose-accumulating cells was decreased, resulting in lower sugar content. Root glucose levels, however, were not significantly different from those found in the control plants.

The effects on photosynthesis and assimilate partitioning that are induced by changes in the endogenous cytokinin content due to the expression of a cytokinin biosynthesis gene have not been extensively studied (Li et al. 1992; Catsky et al. 1993; Martineau et al. 1995). We did not observe bigger taproots or an increased sucrose storage capacity in *ipt*-transformed sugarbeets as hypothesized by Elliot and Weston (1993). Different approaches, such as a modulation of the activities of enzymes that control the cell division cycle or sucrose synthesis and partitioning, may prove to be more fruitful for the development of high-sucrose sugarbeet.

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References

Ainley WM, McNeil KJ, Hill J, Lingle WL, Simpson RB, Brenner ML, Nagao RT, Key JL (1993) Regulatable endogenous production of cytokinin up to “toxic” levels in transgenic plants and plant tissues. Plant Mol Biol 22:13–23

Akiyoshi DE, Klee H, Amasino R, Nester EW, Gordon MP (l984) T-DNA of *Agrobacterium tumefaciens* encodes an enzyme of cytokinin biosynthesis. Proc Natl Acad Sci USA 81:5994–5998

Beinsberger SEI, Valcke RLM, Van Onckelen HA, Clijsters HMM (1991) Effect of the introduction of *Agrobacterium tumefaciens* T-DNA *ipt* gene in *Nicotiana tabacum* L. cv. Petit Havana SR1 plant cells. Plant Cell Physiol 32:489–496

Bergmeyer HU, Brent E, Schmidt F, Stock H (1974) D-Glucose. Determination with hexokinase and glucose 6-phosphate dehydrogenase. In: Bergmeyer HU (ed) Methods of enzymatic analysis, 2nd edn, vol 3. Academic Press, New York, pp 1196–1198

Binns AN (1994) Cytokinin accumulation and action: biochemical, genetic, and molecular approaches. Annu Rev Plant Physiol 45:173–196

Catsky J, Pospísilová J, Machácková I, Synková H, Wilhelmová N, Sesták Z (1993) High level of endogenous cytokinin in transgenic potato plantlets limits photosynthesis. Biol Plant 35:191–198

Elliot MC, Weston GD (1993) Biology and physiology of the sugarbeet plant. In: Cooke DA, Scott RK (eds) The sugar beet crop: science into practice. Chapman and Hall, London, pp 37–66

Elliott MC, Hosford DJ, Lenton JR, Milford GFJ, Pocock TO, Smith JI, Lawrence DK, Firby DJ (1983) Hormonal control of storage root growth. In: Jackson MB, Stead T (eds) Growth regulators in root development. Monograph no.10. British Plant Growth Regulator Group, Wantage, pp 25–35

Elliott MC, Hosford DJ, Smith JI, Lawrence DK (1986) Opportunities for regulation of sugar beet storage root growth. Biol Plant 28:1–8

Groot SPC, Bouvier R, Busscher M, Lindhout P, Dons HJ (1995) Increase of endogenous zeatin riboside by introduction of the *ipt* gene in wild type and the *lateral suppressor* mutant of tomato. Plant Growth Regul 16:27–36

Li Y, Hagen G, Guilfoyle TJ (l992) Altered morphology in transgenic tobacco plants that overproduce cytokinins in specific tissues and organs. Dev Biol l53:386–395

Lichtenthaler HK (1987) Chlorophyll and carotenoids: pigments and photosynthetic biomembranes. Methods Enzymol 148: 350–382

Mahácková I, Segeeva L, Ondrej M, Zaltsman O, Konstantinova T, Eder J, Ovesná J, Golyanovskaya S, Rakitin Y, Yaksenova N (1997) Growth pattern, tuber formation and hormonal balance in in vitro potato plants carrying *ipt* gene. Plant Growth Regul 21:27–36

Martineau B, Summerfelt KR, Adams DF, DeVerna JW (1995) Production of high solids tomatoes through molecular modification of levels of the plant growth regulator cytokinin. Biotechnology 13:250–254

Medford JI, Horgan R, El-Savi Z, Klee HJ (1989) Alterations in endogenous cytokinins in transgenic plants using a chimeric isopentenyl transferase gene. Plant Cell 1:403–413

Milford GFJ (1973) The growth and development of the storage root of sugarbeet. Ann Appl Biol 75: 427–38

Rocha-Sosa M, Sonnewald U, Frommer W, Stratmann M, Schell J, Willmitzer L (l989) Both developmental and metabolic signals activate the promoter of class I patatin gene. EMBO J 8:23–29

Smigocki AC (l995) Expression of a wound-inducible cytokinin biosynthesis gene in transgenic tobacco: correlation of root expression with induction of cytokinin effects. Plant Sci l09: l53-l63

Smigocki AC, Owens LD (1989) Cytokinin-to-auxin ratios and morphology of shoots and tissues transformed by a chimeric isopentenyl transferase gene. Plant Physiol 91:808–811

Smigocki AC, Neal JW, Mc Canna IJ, Douglass L (1993) Cytokinin-mediated insect resistance in *Nicotiana* plants transformed with the *ipt* gene. Plant Mol Biol 2:335–350

Snyder GW, Ingersoll JC, Smigocki AC, Owens LD (1999) Introduction of pathogen defense genes and a cytokinin biosynthetic gene into sugarbeet (*Beta vulgaris* L.) by *Agrobacterium* or particle bombardment. Plant Cell Rep 18:829–834

Thomas TH (1986) Hormonal control of assimilate movement and compartmentation. In: Bopp M (ed) Plant growth substances. Springer, Berlin Heidelberg New York, pp 350–359

Wenzler HC, Mignery GA, Fisher LM, Park WD (1989) Sucrose-regulated expression of a chimeric potato tuber gene in leaves of transgenic tobacco plants. Plant Mol Biol 13:347–354