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Decreased amount of reducing sugars in transgenic potato tubers and its influence on yield characteristics

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Abstract

This work focuses on the comparison of field characteristics and amounts of reducing sugars in cold-stored tubers of transgenic plants derived from two potato cultivars. The bacterial gene coding for phosphofructokinase under the tuber-specific promoter was used to support the glycolysis in stored tubers. While the tubers from untransformed control plants steadily accumulated reducing sugars during cold storage, the tubers from transformed plants regardless the genotype were characterized by subsequent decrease in the sugar content. After long period of cold storage the greatest reduction in the reducing sugar content was by more than 60 % compared to control. Before the storage, however, the content of reducing sugars was in 80 % of transgenic lines higher than in control ones. The plants evaluated in field trials for their appearance showed any changes in growth characteristics in about 25 % of the transgenic lines. Despite the introduced modification of sugar metabolism the yield of transgenic plants with normal appearance did not differ significantly from the yield of control plants.

Additional key words: *Lactobacillus bulgaricus*, low temperature sweetening, phosphofructokinase.

Introduction

Potato (*Solanum tuberosum* L.) is ranked fourth in production of all agricultural commodities in the world. Due to the efficient cold storage harvested potato tubers are processed all the year round. Storage at low temperature excludes the wide application of sprout suppressants, which could affect the quality of final products. However, the cold storage is accompanied by acceleration of the conversion of starch to reducing sugars (glucose and fructose), the phenomenon known as low temperature sweetening (LTS). The sugar content can exceed 2 % of the tuber fresh mass (Isherwood 1973) with the variability in glucose content greater than 100-fold depending on the potato cultivar (reviewed in Sowokinos 2001). As the reducing sugars affect flavour and colour of fried potato products (Roe and Faulks 1991) the acceptable sugar content in tubers of so-called chipping cultivars should not exceed 0.33 % of the fresh mass (Duplessis *et al.* 1996).

At least five pathways of sugar metabolism could contribute to LTS, *i.e.* starch synthesis, starch breakdown, glycolysis, hexogenesis and respiration. Despite of many sophisticated experiments involving the gene manipulations in potatoes, it is still not clear which of these biological processes are indeed involved in initiating and/or controlling LTS (for review see Sowokinos 2001). A cold-induced block of glycolysis is suggested to be the reason for the accumulation of sugars arising from starch cleavage. Both potato phosphofructokinases localized either in plastids or in cytosol are cold sensitive (Dixon *et al.* 1981, Hammond *et al.* 1990). For example the activity of the cytosolic form from cv. Bintje drops down already at temperatures below +14.9 °C. Phosphofructo-

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Abbreviations: FM - fresh mass; LbPFK - phosphofructokinase from *Lactobacillus bulgaricus*; LTS - low temperature sweetening; MS medium - Murashige and Skoog medium; PFK - phosphofructokinase.
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kinase from chloroplasts is only slightly less sensitive and its inactivation substantially increases below temperature +12.5 °C (Bredemeijer *et al.* 1991). The cytosolic form requires the pH value around 6.5 and is sensitive to the low pH values, which are commonly achieved in tubers in one week of cold storage (Bredemeijer *et al.* 1991).

Introduction of cold-stable 6-phosphofructokinase into potato tubers could help to support the glycolysis and reduce the hexose accumulation. The gene encoding such enzyme was cloned from *Lactobacillus bulgaricus* by Branny *et al.* (1993). The purified enzyme (LbPFK) differs substantially from other known bacterial phosphofructokinases because of the lack of an allosteric inhibition (Le Bras *et al.* 1991).

Here we present the results of the field-trial evaluation of two Czech potato cultivars carrying the *LbPFK* gene under the control of a tuber specific promoter.

Materials and methods

Plants: Potato (*Solanum tuberosum* L.) cultivars Kamýk (breeder Selekta Pacov, Czech Republic) and Korela (breeder Sativa Keřkov, Czech Republic) were grown *in vitro* on MS media supplemented with 3 % of sucrose. Harvested tubers from plants with unaltered growth characteristics in field conditions were used for biochemical analysis.

Transformation and verification of transgenic plants: The gene encoding 6-phosphofructokinase from *Lactobacillus bulgaricus* (*LbPFK*; kindly provided by Dr. P. Branny, Institute of Microbiology, CAS, Prague) was fused with class I patatin promoter *B33* (kindly provided by prof. L. Wilmitzer; Rocha-Sosa *et al.* 1989) and inserted into the binary vector pBin19 (Bevan 1984). The final construct was transformed into *Agrobacterium tumefaciens* using electroporator *BioRad* (USA) *E. coli* pulser (Shen and Forde 1989). The leaves from 4 to 5 weeks old *in vitro* grown plants were used for *Agrobacterium tumefaciens* (strain C58C1 with plasmid pGV2260; Deblaere *et al.* 1985) mediated transformation as described by Dietze *et al.* (1995).

The genomic DNA was isolated from 3 to 5 leaves of *in vitro* grown plants (Štorchová *et al.* 2000). The transgenic plants were verified by polymerase chain reaction (PCR) with primers *PFK1*: CCA CGA TCG GCT ATG ACA CG and *PFK2*: AAT ATC GTC TGA AGC TGG CC yielding the fragment 646 bp long. The transgene copy number was estimated in selected lines by genomic Southern hybridization (Sambrook *et al.* 1989) with *LbPFK* sequence as a probe. Expression of *LbPFK* was confirmed by Northern hybridization (Sambrook *et al.* 1989). Total RNA was isolated (Stiekema *et al.* 1988) from matured *in vitro* grown microtubers or tubers grown in the greenhouse.

Field trials: Ten to 40 tubers per transgenic line were planted. The evaluated growth characteristics were following: plant type (the normal upright habit or prostrate habit, the high or stunted growth), time of flowering (early or late), inflorescence (the abundance and the colour), leaves (the shape and the colour).

Sugar content of harvested tubers was determined before storage and in tubers stored at +4 °C. One portion of tubers was analyzed directly after the storage and the other after the additional period of reconditioning (as established in food industry practice) at +16 °C for 18 d. The determination of reducing sugars was performed by the Luff-Schoorl method using the portion of homogenate from the middle sections of tubers (4 to 7 tubers per sample). Tubers of transgenic lines with the lowest content of reducing sugars after 72-d storage were subjected to the additional analysis after 160-d cold storage.

Results

Molecular characterization of transgenic plants: The transformation of plants was confirmed by PCR of the coding part of the *LbPFK* gene. The total number of verified independent transformed lines reached 48 of cv. Kamýk and 22 of cv. Korela. The majority of the transgenic Kamýk plants had only one copy of T-DNA per genome (Southern hybridization; data not shown). Three transformants (out of 24) were shown to have two copies and one line had at least 12 copies of T-DNA. However, the phenotype of this line was strongly affected. Tubers and leaves of selected transformants were subjected to mRNA analysis and transcript was detectable only in tubers (Fig. 1*A*). Higher amount of *LbPFK* mRNA could be seen particularly in the transgenic line with 12 copies of T-DNA (line 142 4/1) and in the transgenic lines with two T-DNA copies (lines 142 3/2 and 151 16/2). Analysis of transcript level in 12 transgenic lines of cv. Korela demonstrated satisfactory level of *LbPFK* transcript in about half of them and perceptible signal in almost all lines (Fig. 1*B*).

Field trials: The growth characteristics of the field-grown transgenic lines of Kamýk and Korela were monitored for 7 and 3 years, respectively. The appearance of transgenic plants was mostly normal. Changes in growth characteristics were observed in 27.5 and 21.4 % of Kamýk and Korela transgenic lines, respectively. These plants gave also lower yield of tubers. The highest

yield of Kamýk transgenic lines with changed habit was 977 g per plant in the 1st year and 857 g per plant in the 2nd year. Korela transformants with some changes in their habit gave the maximal yield 887 g per plant in the 1st year and 617 g per plant in the 2nd year. The lines with the normal appearance gave usually the same yield as the control plants or even higher with the highest yield for Kamýk transgenic lines 1780 g and 1483 g per plant and for Korela transformants 1321 g and 1521 g per plant in the 1st and 2nd year, respectively.

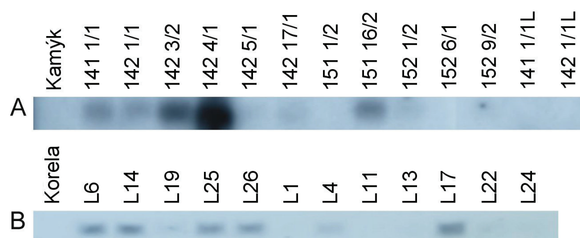

Fig. 1. Northern analysis of *LbPFK* expression in leaves (141 1/1L and 142 1/1L) and matured tubers (all other samples) of transgenic plants of potato (*S. tuberosum*) cvs. Kamýk (*A*) and Korela (*B*). The DIG labelled hybridisation probe covered the whole sequence of the *LbPFK* gene. The equal loading of RNA on the gel (15 µg of total RNA in each lane) was confirmed by ethidium bromide staining.

Biochemical analysis: When compared to control plants the content of reducing sugars before storage was higher in tubers of all tested Korela transformants (Table 1). However, after 72-d cold storage the difference was much less pronounced and after additional 88 d (160 d together) it was evident that the amount of reducing sugars was steadily decreasing in all lines selected for further analysis by the result of the latest measurement. The content of reducing sugars in control tubers was steadily rising at the same conditions. Finally, the amount of reducing sugars in transgenic tubers (0.70 % of FM in average of the selected lines) dropped below the content determined in control ones (0.94 % of FM). The situation was slightly different in case of Kamýk. Unlike Korela transformants, the variance in the values from individual transgenic lines of Kamýk was much higher. The amounts of sugars of few lines were lower than in control already at the time of harvest and on the contrary, the sugar content of few other lines exceeded that of control even after prolonged period of cold storage (Table 1). Despite the variability described, the tendency of gradual decrease in sugar content during cold storage was significant in the majority of transformed lines. The lowest final sugar content reached 0.17 % of tuber FM, the value representing 38 % of that determined in the control Kamýk tubers (0.45 % of tuber FM).

Table 1. Reducing sugar content [% FM] in tubers of untransformed (NT) potato cvs. Kamýk and Korela and transgenic lines carring *LbPFK* gene before and after storage at 4 °C for 72 and 160 d. The measurement in cold stored tuber was done immediately after storage (I) and after a period of reconditioning at 16 °C for 18 d (R). (* - sugar content in tubers of transgenic lines lower than that of untransformed lines, n.d. - not determined).

Cultivars	Lines	After harvest	Storage 72 d I	R	Storage 160 d I	R
Korela	NT	0.35	1.10	0.81	1.20	0.94
	L7	0.55	1.07*	0.84	n.d.	n.d.
	L13	0.49	1.03*	0.89	n.d.	n.d.
	L14	0.71	1.42	0.93	n.d.	n.d.
	L17	1.19	0.96*	0.85	1.05*	0.73*
	L18	0.64	1.60	0.98	n.d.	n.d.
	L22	0.55	1.01*	0.85	0.81*	0.75*
	L25	0.63	1.07*	0.95	n.d.	n.d.
	L26	0.72	1.06*	0.84	0.86*	0.63*
Kamýk	NT	0.21	0.79	0.42	0.75	0.45
	141 1/1	0.18*	0.80	0.71	n.d.	n.d.
	142 2/3	0.26	0.51*	0.32*	0.40*	0.21*
	142 3/2	0.21	0.65*	0.45	0.54*	0.25*
	142 4/1	0.27	0.50*	0.40*	0.65*	0.29*
	142 5/1	0.26	0.86	0.82	0.77	0.44*
	142 20/1	0.16*	0.40*	0.38*	0.32*	0.20*
	142 20/2	0.16*	0.41*	0.35*	0.35*	0.17*
	151 1/5	0.37	0.77*	0.61	0.86	0.50
	151 5/2	0.16*	0.61*	0.49	n.d.	n.d.
	151 9/5	0.20*	0.65*	0.54	n.d.	n.d.
	151 10/1	0.25	1.02	0.70	n.d.	n.d.
	151 16/2	0.33	0.97	0.72	n.d.	n.d.
	151 16/3	0.25	0.88	0.60	n.d.	n.d.
	152 1/2	0.34	0.91	0.65	n.d.	n.d.
	152 4/1	0.30	0.86	0.60	n.d.	n.d.
	152 6/1	0.21	0.89	0.51	n.d.	n.d.
	152 9/3	0.22	0.93	0.66	n.d.	n.d.

Discussion

The number of T-DNA copies per genome of transgenic plants was in good agreement with the previous findings that the *Agrobacterium* infection of potato prevalently results in plants with one T-DNA insertion (Gelvin 2000). *LbPFK* mRNA detected by Northern hybridization was found only in the tubers (not in the leaves) of transgenic plants as expected considering the fact, that the promoter *B33* ensures 99 % of patatin mRNA in tubers (Blundy *et al.* 1991). The activity of the patatin promoter *B33* is mainly localized in the phloem of developing tubers (Kühn *et al.* 2003) and decreases with tuber development (Blundy *et al.* 1991). However, it is still measurable in the mature tubers (Borgmann *et al.* 1994) what could assure sufficient content of the bacterial enzyme in cold-stored tubers.

The active LbPFK in tubers of transgenic plants is

believed to diminish the LTS effect. This assumption is based on two prerequisites. The first one, the increased rate of glycolysis in tubers should reduce the content of soluble sugars and the second, the activity of introduced phosphofructokinase should be able to accelerate the net flux through glycolytic pathway. Validity of the first assumption is supported by Gupta and Sowokinos (2003), who studied the role of UDP-Glc pyrophosphorylase in LTS. The change in the isozyme spectrum in LTS resistant cultivar successively led to strengthening of the glycolysis. The key role of glycolysis in LTS is proved in the paper of Blenkinsop *et al*. (2003), summarizing the results from four-year study of the role of glycolysis, anaerobic respiration, oxidative pentose phosphate pathway, and mitochondrial respiration in LTS.

As concern the role of the PFK in glycolysis, there are papers demonstrating that the considerable increase in the PFK activity in transgenic potatoes does not adequately change the net flux through glycolysis (Thomas *et al.* 1997a,b). However, the conditions of their experiments with *PFK* gene from *Escherichia coli* were not designed to study the possible effects of introduced PFK on LTS, *i.e*. the measurements were done at +20 °C ensuring high activity of both bacterial and plant PFKs in aged tuber discs. The same gene under the control of patatin promoter was used also in the experimental work of Burrell *et al*. (1994), who evaluated PFK activity and sugar content in tubers of transgenic plants after short-term storage (two weeks) at +20 °C. The sugar contents were not sufficiently affected in spite of several fold elevated PFK activity. An assumed reason could be the strong inhibition of phosphofructokinase from *Escherichia coli* by phosphoenol pyruvate, the intermediate of glycolysis sitting downstream the reaction catalyzed by PFK (Burrell *et al.* 1994). In our work this bottleneck in the metabolic pathway should be overcome because the phosphofructokinase from *Lactobacillus bulgaricus* is phosphoenol pyruvate insensitive (Le Bras *et al.* 1991), thus preventing the feed-back inhibition. The results obtained at the temperature permissive for the plant PFKs cannot be easily extrapolated for the temperature and conditions appropriate for LTS. This can be demonstrated even on the changes of sugar contents in our transgenic plants. At the permissive temperature (after harvest) the content of reducing sugars was in the majority of transgenic plants surprisingly higher when compared to the original cultivar. This change originates presumably from the LbPFK activity. *LbPFK* gene is actively transcribed from patatin promoter during tuber maturation (Blundy *et al.* 1991). The activity of the bacterial enzyme in tubers is probably compensated by complex mechanisms assuring the overall sugar balance, which might result in the reverse reaction concerning the reducing sugar content. Later on, in the same tubers stored for at least three months at +4 °C, the sugar levels started to decrease and the relations between transgenic lines and control changed significantly. During cold storage plant PFKs loose nearly all their activity (Hammond *et al.* 1990) and even other metabolic processes are decelerated, what corresponds with the general finding that the activity of common enzymes belonging to organisms characterized as mesophyles (*e.g. S. tuberosum*) drops 5 to 100-times at +5 °C (More *et al.* 1995). In such conditions the flux through glycolysis could be driven mainly by the introduced LbPFK and even minor change in the PFK activity could finally result in decrease in the amount of reducing sugars.

The changes in sugar content were more obvious in the transgenic lines of Kamýk compared to Korela. After 160 d of cold storage the sugar content in tubers of the best performing Kamýk transgenic line decreased 2.6-times compared to control. The best Korela transgenic line had the sugar content only 1.5-times lower. This tendency was confirmed also in other years when only low number of lines was tested (data not shown). The greatest registered reduction in sugar content was 5.5-times for Kamýk transgenic line and 2.7-times for Korela. The difference between the cultivars could be related to the different genetic background and original sugar content in the wild type tubers - the content of reducing sugars in Kamýk and Korela is 0.21 % FM and 0.35 % FM, respectively.

Initial active transcription of transgene detected in the majority of analyzed transgenic plants might result in the gene silencing. The appearance of this phenomenon is usually enhanced with prolonged cultivation (Wolters *et al.* 1998). This fact could explain relatively high heterogeneity in the sugar content found in Kamýk transgenic lines growing in the field conditions since 1997. Korela transgenic lines transferred to the field conditions in 2000 express much lower diversity. The other explanation could be in the higher variance resulting from the higher number of transformed lines of Kamýk.

Except for the differences among individual transgenic lines, there is an apparent variability in the reducing sugar content of individual tubers (Roessner *et al.* 2000). That is why the mixed samples of minimally four to seven randomly selected tubers from each transgenic line or wild-type plants were analyzed. The values should then represent the average sugar content typical for each transgenic line.

Our results obtained from the field trials might show the real difference between transformed and untransformed plants. As was demonstrated on the values of different biochemical markers for transgenic and wild type soil-grown tubers formed independent clusters, while the values of the same plants grown *in vitro* formed clusters very close each other (Roessner *et al.* 2002).

Conclusion: The plants evaluated in our study were grown under actual field conditions and, therefore, these results are not only of scientific interest but also highly relevant to both growers and the potato-processing industry. The usage of the transgenic approach to support the glycolytic pathway in cold-stored potato tubers led to the stable performance of some lines during several years of cultivation. The majority of transgenic lines regardless

the cultivar showed decreased sugar content in tubers after long period of cold storage. Our further investigations will lead through the improvement of the *LbPFK* gene expression and direct analysis of the LbPFK content in tubers by the monoclonal antibody, which has been already prepared.

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