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ARTICLE

Pyrophosphate:fructose 6-phosphate 1-phosphotransferase operates in net gluconeogenic direction in taproots of cold and drought stressed carrot plants

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ABSTRACT The purpose of this work was to further investigate the regulatory interplay between pyrophosphate:fructose 6-phosphate 1-phosphotransferase (PFP) and its positive effector fuctose 2,6-bisphosphate (Fru-2,6-P_2) in heterotrophic tissues. Transformation of carrot plants (*Daucus carota* L. cv. Nantes Duke) with mutated mammalian 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene (6-PF-2-K/ Fru 2,6-P_2ase) produced carrot taproots which possessed between 163% and 410% of the Fru-2,6-P_2 levels observed in wild-type taproots. Besides Fru-2,6-P_2, the levels of 3-phosphoglycerate (3PGA) and hexose phosphates (hexose-P) showed the most significant alterations. Transgenic taproots possessed a marked increase in PFP activity that was accompanied by high 3PGA / hexose-P ratios under normal physiological conditions. Interestingly, 3PGA / hexose-P ratios became significantly lower in taproots exposed to drough or cold without any decrease in PFP activity. We suggested that changes in 3PGA / hexose-P ratios are a direct result of stimulation of PFP activity by the elevated Fru-2,6-P_2 levels. The Fru-2,6-P_2-stimulated PFP operates in the glyconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction dominates in the non-stressed controls. This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situations and such, PFP is an important sensor of the environmental changes. Likely that PFP is also involved in mobilisation of energy reserves upon unfavourable environmental changes by promoting the re-synthesis of transportable sucrose through gluconeogenesis from accumulated starch in taproots.
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KEY WORDS

PFP
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glycolysis
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taproot

Fru-2,6-P_2 is an important regulator of photosynthetic carbon metabolism (for review see Stitt 1990 and Nielsen et al. 2004). In leaves this signal metabolite contributes both to the coordination of sucrose synthesis with the rate of carbon dioxide fixation, and to the control of partitioning of photosynthate between sucrose and starch (Scott et al. 1995; Truesdale et al. 1999; Toldi et al. 2002). The allosteric inhibition of cytosolic fructose-1,6-bisphosphatase (FBPase) by Fru-2,6-P_2 is central to the proposed mechanism by which this effector influences both of these processes (Stitt 1997).

In contrast, the role of Fru-2,6-P_2 in non-photosynthetic plant tissues is poorly understood (Fernie et al. 2001). By analogy with animal and fungal systems it is frequently suggested that Fru-2,6-P_2 may contribute to the regulation of glycolytic flux (Stitt 1990). The basis of the this proposal is that non-gluconeogenic plant tissues often lack a detectable cytosolic FBPase activity (Entwistle and ap Rees 1990). Therefore, any influence of Fru-2,6-P_2 on metabolism must be attributed to the modulation of the glycolytic pathway and such the respiration through the activity of PFP in non-photosynthetic tissues of plants. The reaction catalysed by PFP in the cytosol of plant cells is close to equilibrium *in vivo* under normal physiological conditions (Weiner et al. 1987) meaning that PFP contributes equally both to the gluconeogenic and glycolytic flux. However, when plants are subjected to environmental stressors this equilibrium can be modulated.

It is known that Fru-2,6-P_2 provides adaptive abilities by metabolic fine tuning that are advantageous under suboptimal growth conditions (Okar et al. 2001; Nielsen et al. 2004). The Fru-2,6-P_2 signalling system sensitively responds to salt, drought, cold and osmotic stress by adjusting the fuel homeostasis according to the changing demand for survival (Reddy 1996, 2000, Banzai et al. 2003, Villadsen et al. 2005). Storage organs like potato tubers and carrot taproots function as fuel reserves providing mobilisable energy sources under stress conditions. Fru-2,6-P_2 is involved in the regulation of diurnal turnover of starch, which is the most important mobilisable energy source in higher plants, and has pivotal role is stress adaptation. At the same time, PFP can substitute 6-phosphofructo-1-kinase (PFK) in maintaining glycolytic flux under ATP-limited stress situations by using PPi as phosphoryl

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donor. Since PFP is stimulated by Fru-2,6-P_2 allosterically, it is not an exaggregation to suppose that the functions of PFP and Fru-2,6-P_2 overlap in plant stress responses.

To validate this assumption, transgenic carrot plants with elevated levels of Fru-2,6-P_2 were produced and analysed. Taproots of these transgenic plants have been used to analyse whether (i) PFP activity is altered as a result of elevated Fru-2,6-P_2 levels and (ii) is there a change in the direction of the net activity of PFP when taproots are subjected to different abiotic stressors (drought, cold).

Materials and Methods

Plant material and growth conditions

In vitro grown carrot plantlets (*Daucus carota* L. cv. Nantes Duke) were obtained from dr. Seppo Sorvari (MTT Agrifood Research, Piikkio, Finland). After micropropagation and genetic transformation, transgenic and control plants were potted and grown in peat-soil in a growth chamber with supplemetary lighting of 12 h / 150 mmol photons m^{-2} s^{-1} at 24°C, and 70% relative humidity. Healthy, young taproots of 2 month old plants were used for physiological measurements. Stressed lines were cultured in two ways prior to analysis. While half of the potts were grown at 10°C for 10 days, the other half of the potts were grown without further irrigation also for 10 days. Plants were then re-watered and samples were taken for physilogical measurements at noon 1 day after re-watering.

Plasmid constructs

Functional 6-PF-2-K was provided by a modified coding region of the rat liver 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene (6-PF-2-K/ Fru 2,6-P_2ase). The gene contained point mutations which changed serine-32 and histidine-258 to alanine (Tauler at al. 1991; Kurland et al. 1992). These modifications result in a functional enzyme which possesses no Fru 2,6-P_2ase activity, but can still make Fru-2,6-P_2 (Scott et al. 1995). This construct was identical to that described by Scott et al. (1995). A *Hind* III fragment containing 6-PF-2-K/ Fru 2,6-P_2ase was inserted between a 35S CaMV promoter and polyadenilation signal in the vector pJIT62. The final construct was digested with *Kpn* I and *Eco* RV, then filled in, and cloned into pBIN19 (Bevan 1984). The pBIN19::6-PF-2-K construct was introduced into *Agrobacterium tumefaciens* strain LBA 4404, containing pAL4404, by direct transformation (Höfgen and Willmitzer 1988).

Plant transformation

Somatic embryos from cell suspension cultures of carrot plants (*Daucus carota* L. cv. Nantes Duke) were infected with the transforming *Agrobacterium*. Co-culture took place by immersing 2-3 mm long embryo segments into 40 ml *Agrobacterium* suspension that contained bacterial cells at OD_{660} = 0.5 density, one third-strength MS macro and microelements (Murashige and Skoog 1962), half-strength MS vitamins, 1.0 g l^{-1} casein hydrolysate, 100 µM acetosyringone and 10 g l^{-1} glucose (pH 5.0) for 20-30 minutes at 22°C under dim light. After infection, embryo segments were shortly dried and then placed to growth regulator-free MS medium solidified with 7 g l^{-1} plant agar for the following 2-3 days. After the 2-3 days co-culture, plant explants were transferred into selective callus induction medium that contained 1.0 mg l^{-1} 2,4-D, 100 mg l^{-1} kanamycin and 500 mg l^{-1} cefotaxime. The selection took 6-8 weeks and required subculturing in every 2 weeks. Calluses were transferred into solid regeneration media that contained MS salts and vitamins, 1.0 mg l^{-1} zeatin, 50 mg l^{-1} kanamycin and 300 mg l^{-1} cefotaxime. The plant regeneration required 16 h daylength condition at 25°C. When *de novo* developed shoots reached 8-10 mm in length they were transferred onto solid MS media containing 1 mg l^{-1} IBA for rooting. Viable plants with well developed root system were potted in the greenhouse and were cultivated for further examinations.

Analysis of gene expression

In order to establish whether the transgene was present and expressed in putative transgenic carrot plants northern hybridisation was used. RNA was extracted from taproots using the methods described by Lichenstein and Draper (1985). Northern hybridisation was carried out as described by Sambrook et al. (1989).

Extraction and measurement on enzyme activity

For experimental material we concentrated on the non-photosynthetic taproots from the carrots. For measurements separate taproots were used for each experimental sample. Each of these taproots originated from separate clonal carrot plants.

Slices (ø = 10 mm x 2 mm thick) were cut from the centre of the taproot with a razor blade and immediately frozen in liquid nitrogen. A sample of about 500 mg FW^{-1} were then homogenised to a fine powder at 4°C in a mortar and pestle in the presence of 5 ml of extraction medium, 100 mM Hepes, 4 mM $MgCl_2$, 1 mM EDTA, 1 mM EGTA, and 5 mM β-mercaptoethanol (Hajirezaei and Stitt 1991). The homogenate was first centrifuged at 14000 rpm for 5 min, then the supernatant was assayed for 6-PF-2-K as described by Scott et al. (1995). These assay conditions were optimal for both the mammalian and the carrot enzyme. Other enzyme activities were measured as described in the following references: cytosolic FBPase (Hatzfeld et al. 1990; Hajirezaei and Stitt 1991) and ADPglucose pyrophosphorylase (Hajirezaei et al. 1994) PFP activites were measured first in glycolytic and gluconeogenic directions separately as described by Theodorou and Kruger (2001) and then total PFP activities were calculated by the addition of related data.

To check the reliability of the extraction and measurement

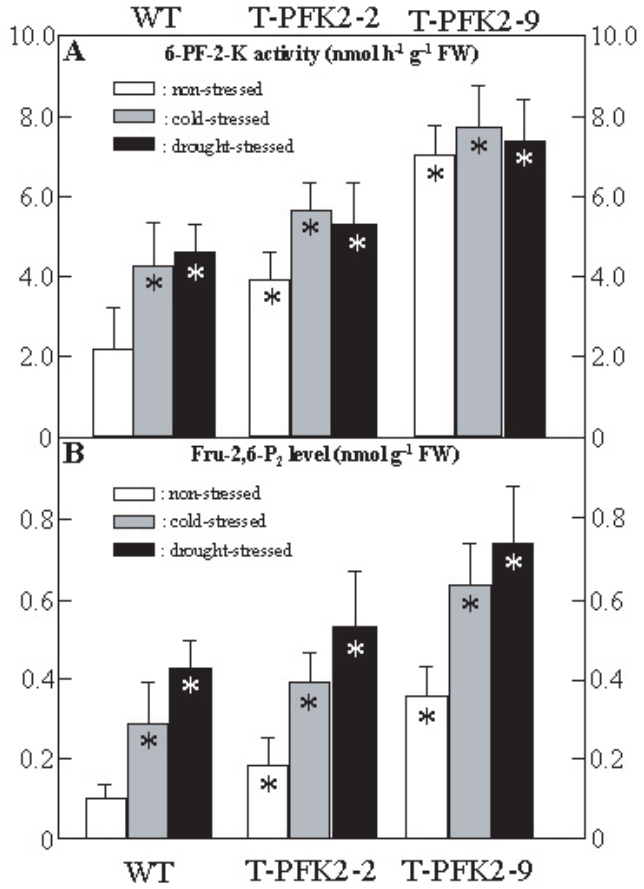

Figure 1. (i) Total activites of 6-phosphofructo-2-kinase (6-PF-2-K), responsible for synthetising Fru-2,6-P_2, and levels of Fru-2,6-P_2 in taproots of transgenic plant lines (T-PFK2-2 and T-PFK2-9) relative to WTs. (ii) The impact of cold and drought stress on the above data. Samples were harvested at noon from taproots of non-stressed control, as well as cold and drought stressed carrot plants. All data are the mean ± SD of replicate measurements of three separate taproots. Values labelled by asterisks are different significantly (Student's *t* test $P < 0.05$) from the corresponding data of non-stressed WT controls.

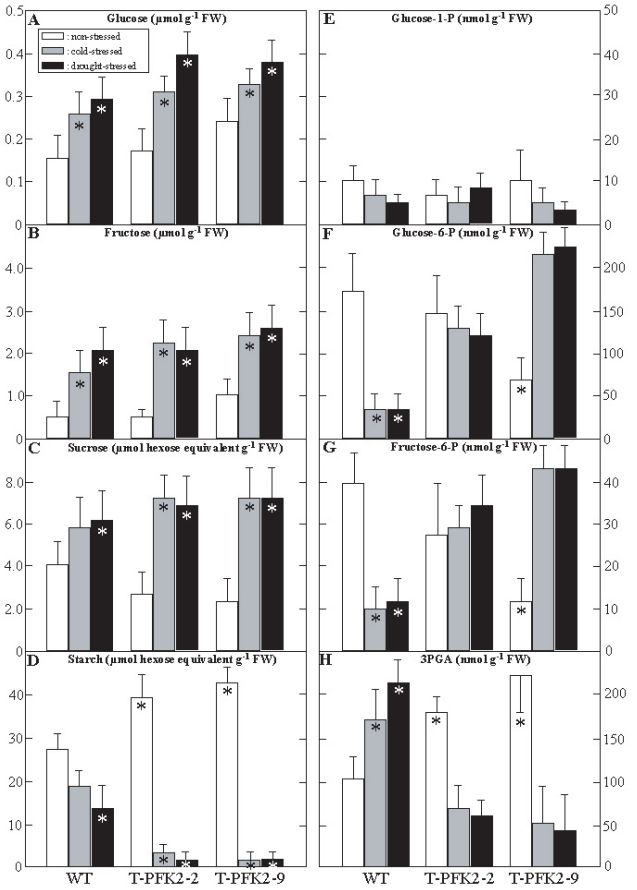

Figure 2. The content of soluble sugars, phosphorylated intermediates and starch in non-stressed (white bars), cold-stressed (grey bars) and drought-stressed carrot taproots with elevated levels of Fru-2,6-P_2 (lines T-PFK2-2 and T-PFK2-9) compared to WTs. Samples were harvested at noon from taproots. All data are the mean ± SD of replicate measurements of three separate taproots. Values labelled by asterisks are different significantly (Student's *t* test $P < 0.05$) from the corresponding data of non-stressed WT controls.

of 6-PF-2-K activity we performed recombination experiments. Spinach leaf extracts, containing approximately the same activity of 6-PF-2-K as was expected to be present in the taproots, were added to the samples prior to homogenisation.

Extraction and measurement of metabolites

Cores of tissue (10 mm x 10 mm, approximately) were removed from the centre of the taproots, and slices (2 mm thick) were cut and instantly frozen in liquid nitrogen. Samples (1 g FW^{-1}) were homogenised in liquid nitrogen in a mortar and pestle. For all substrates except Fru 2,6-P, the homogenate was immediately suspended in 2 ml of 1.4 M perchloric acid and left on ice for 2 h. Then the extract was neutralised with 5 M K_2CO_3, and the insoluble debris removed by centrifugation at 10000 g twice. Starch in the insoluble fraction was determined according to Morrell and ap Rees (1986). Sucrose and phosphorylated intermediates (3PGA, Glc-1-P, Glc-6-P, and Fru-6-P) were assayed in the soluble fraction by following enzyme-linked reduction of NAD^+ or oxidation of NADH spectrophotometrically at 340 nm according to Scott and Kruger (1995). Fru-2,6-P_2 was extracted from taproots and assayed as described by Scott and Kruger (1994). To confirm the reliability of the extraction and measurement of the metabolic intermediates we performed recovery experiments. This was done by adding to the sample, prior to extraction, an amount of the metabolite similar to that present in taproots. The recovery of metabolites added to the carrot tissue was 90.7 ± 5.0% for Suc; 88.7 ± 6.6% for Glc; 89.8 + 9.4% for Glc-6-P; 80.5 ± 4.5% for Glc-1-P; 69.2 + 8.7% for Fru-6-P;

89.1 + 3.6% for 3PGA and 83.2 ± 2.5% for Fru-2,6-P_2 (mean + SE, where n = 3).

Statistical analysis

Significance of differences between treatment groups was determined using Student's t-test followed by Fisher's LSD test as appropriate. If differences were considered significant for $P < 0.05$, means were separated by LSD at $P = 0.05$. Results for continuous variables are expressed as means ± SD.

Results and Discussion

In contrast to our clear understanding of the role of Fru-2,6-P_2 in the regulation of carbon partitioning during photosynthesis (Stitt 1990; Nielsen et al. 2004), the function of this signal metabolite in non-photosynthetic metabolism is equivocal (Fernie et al. 2001; Theodorou and Kruger 2001). Using transgenic tobacco plants containing Fru-2,6-P_2 levels which are up to double that measured in wild-type plants, evidence could be provided that this signal metabolite has an important role during starch mobilisation in tobacco levels in the dark (Scott and Kruger 1995). The rate of starch degradation in darkened tobacco leaves - used to model heterotrophic metabolism - is lower in the transgenic plants than in the wild-type plants. Through a combination of inhibition of the cytosolic FBPase and stimulation of PFP by increased Fru-2,6-P_2 levels, the amount of 3PGA in leaves is raised in the dark in the transgenic compared to the wild-type leaves (Scott and Kruger 1995). This rise correlates with an increase in the rate of unidirectional starch synthesis in the leaves. These data suggest that the net rate of starch degradation is reduced in the transgenic plants, because the elevated 3PGA levels stimulate starch synthesis through ADPglucose pyrophosphorylase. While such starch accumulation has not been observed in callus cultures of the above transgenic tobacco lines, the marked increase of 3PGA/hexose-P ratio in the same samples indicates a similar response to elevated Fru-2,6-P_2 levels in a different type of heterotrophic tissues (Fernie et al. 2001). On the contrary, no alterations in the 3PGA / hexose-P ratio were detected when the carbohydrate metabolism of potato tubers containing elevated levels of Fru-2,6-P_2 was compared to wild type controls (Rung et al. 2004).

As it can be seen from the above controversial conclusions, our understanding of the role of Fru-2,6-P_2 in heterotrophic plant tissues is still poor. More reliable observations can be made, if the regulatory role of Fru-2,6-P_2 in such tissues is evaluted considering the environmental aspects (Reddy 1996; 2000; Banzai et al. 2003; Villadsen et al. 2005). PFP is belived to be a typical sensor enzyme catalysing a near equlibrium reaction at the entering point of glycolysis (Stitt 1990). The metabolic consequence of being such sensor enzyme is that PFP activity is multimodulated by numerous positive and negative effectors. Environmental changes can be mirrored by dynamic changes in relative concentrations of these effectors and such, PFP becomes an indicator of optimal or suboptimal growth conditions.

The purpose of our work was dual. First, the regulatory interplay between PFP and its positive effector Fru-2,6-P_2 has been investigated in heterotrophic tissues. Second, we wished to check wether the integrated action of Fru-2,6-P_2 and PFP has any role in mobilizing energy from such storage organs like carrot taproots upon stress. Carrot somatic embryo explants were transformed with T-DNA containing the *npt* II and the 6-PF-2-K / Fru 2,6-P_2ase genes under the control of a CaMV 35S promoter. Kanamycin resistant plants which formed were then tested to confirm expression of the 6-PF-2-K / Fru 2,6-P_2ase gene. RNA was isolated and purified from leaves of putative transgenic lines. A radioactive probe for the 6-PF-2-K / Fru 2,6-P_2ase gene hybridized to the anticipated 1400 nucleotid transcript in all of the transformed plants, but did not hybridized to any message from wild-type plants (data not shown). In total, out of the twelve

Table 1. (i) Interrelation of the 3PGA/hexose-P ratio with the gluconeogenic and the glycolytic activity of PFP in taproots with elevated levels of Fru-2,6-P_2 (transgenic plant lines T-PFK2-2 and T-PFK2-9) relative to WTs. (ii) The impact of cold and drought stress on the above interrelation. 3PGA/hexose-P ratio was considered to be 1.0 in the case of non-stressed WT control. Samples were harvested at noon from taproots of non-stressed control, as well as cold and drought stressed carrot plants. All data are the mean ± SD of replicate measurements of three separate taproots.

Plant lines	Treatments	3PGA/hexose-P ratio	Enzyme activity [µmol min^{-1} g^{-1} FW] PFP gluconeogenic	PFP glycolytic	PFP total
WT	Non-stressed	1.00	0.09±0.02	0.11±0.02	0.20±0.04
	Drought-stressed	0.65	0.25±0.03	0.17±0.01	0.42±0.04
	Cold-stressed	0.70	0.26±0.04	0.14±0.02	0.40±0.06
T-PFK2-2	Non-stressed	1.25	0.17±0.01	0.21±0.02	0.38±0.03
	Drought-stressed	0.43	0.38±0.04	0.13±0.01	0.52±0.05
	Cold-stressed	0.26	0.41±0.02	0.10±0.01	0.51±0.03
T-PFK2-9	Non-stressed	1.29	0.19±0.03	0.24±0.02	0.43±0.05
	Drought-stressed	0.29	0.62±0.05	0.25±0.02	0.87±0.07
	Cold-stressed	0.31	0.67±0.04	0.15±0.02	0.82±0.06

kanamycin resistant plants tested, seven independent lines of transgenic plants possessed detectable levels of the appropriate transcript. As the result of successfull transformation, transgenic taproots possessed between 163% and 410% of the Fru-2,6-P_2 levels observed in wild-type taproots (Fig. 1). Besides Fru-2,6-P_2, the concentrations of 3-phosphoglycerate (3PGA) and hexose phosphates (hexose-P) showed the most significant alterations (Fig. 2). Transgenic taproots possessed a marked increase in PFP activity that was accompanied by high 3PGA / hexose-P ratios under normal physiological conditions (Table 1). Interestingly, 3PGA / hexose-P ratios became significantly lower in taproots exposed to drough or cold without any decrease in PFP activity. We suggested that changes in 3PGA / hexose-P ratios are a direct result of stimulation of PFP activity by the elevated Fru-2,6-P_2 levels and by cold and drought stress (Fig. 1, Table 1). The Fru-2,6-P_2-stimulated PFP operated in the glyconeogenic direction in the taproots of stressed carrot plants, whereas the glycolytic direction dominated in the non-stressed controls or in other heterotrophic tissues like darkened leaves (Scott and Kruger 1995) and tobacco calluses (Fernie et al. 2001). This suggests that the metabolic status determining the net activity of PFP depends on the physiological stress situations and such, PFP is an important sensor of the environmental changes. Likely that PFP is also involved in mobilisation of energy reservoires upon unfavourable environmental changes by promoting the re-synthesis of transportable sucrose through gluconeogenesis from accumulated starch in taproots (Fig. 2).

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Abbreviations

6-PF-2-K/ Fru 2,6-P_2ase = 6-phosphofructo-2-kinase / fructose 2,6-bisphosphatase gene; PFP = pyrophosphate:fructose 6-phosphate 1-phosphotransferase; PFK = 6-phosphofructo-2-kinase; Fru-2,6-P_2 = fuctose 2,6-bisphosphate; 3PGA - 3-phosphoglycerate; hexose-P = hexose phosphate; FBPase = cytosolic fructose-1,6-bisphosphatase; Suc = sucrose; Glc = glucose; Glc-1-P = glucose 1-phosphate; Glc-6-P = glucose 6-phosphate; Fru-6-P = fructose 6-phosphate; PPi = pyrophosphate

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