Effects of Altered Fructose 2,6-Bisphosphate Levels on Carbohydrate Metabolism in Carnation

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Abstract. The aim of this work was to examine the role of fructose 2,6-bisphosphate (fru 2,6P₂) in the carbohydrate metabolism in carnation (*Dianthus caryophyllus* L.). For this purpose, transgenic plants harboring two modified bifunctional enzyme complementary DNAs of rat liver origin (6-phosphofructo-2-kinase/fructose 2,6-bisphosphatase) were generated. Transformation with the kinase construct resulted in a 45% to 85% increase in fru 2,6P₂ concentrations compared with the wild type. Transformation with the phosphatase construct reduced the fru 2,6P₂ contents by 45% and 70%. These alterations in fru 2,6P₂ amounts affected the key enzyme activities of sucrose and starch metabolism. Accordingly, plants with elevated fru 2,6P₂ concentrations had high levels of starch, fructose, and triose phosphates, and low levels of sucrose, glucose, and hexose phosphates. In plants with reduced amounts of fru 2,6P₂ different results could be observed in major carbohydrate compounds.

In all eukaryotic cells, fructose 2,6-bisphosphate (fru 2,6P₂) is an important regulator of carbohydrate metabolism (Okar and Lange, 1999). In plants, this molecule coordinates the rate of CO₂ assimilation and carbon partitioning between sucrose and starch synthesis during photosynthesis (Stitt, 1990; Stitt et al., 1987). As a signal metabolite it regulates the interconversion between fructose 1,6-bisphosphate and fructose 6-phosphate, the crucial step of carbon partitioning (Nielsen et al., 2004) (Fig. 1). The process is based on the following:

- Fructose 2,6-bisphosphate inhibits the cytosolic fructose 1,6-bisphosphatase (FBPase), the key regulator in sucrose synthesis (Stitt et al., 1985, 1987).
- Fructose 2,6-bisphosphate is a potent activator of pyrophosphate:fructose 6-phosphate-1-phosphotransferase (PFP) (Kruger and Scott, 1994) and activates in an indirect way the chloroplast ADP-

glucose pyrophosphorylase (AGPase), which is a regulatory enzyme in starch synthesis (Smith et al., 1997).

The level of fru 2,6P₂ is determined by a bifunctional enzyme, the relative activities of 6-phosphofructo-2-kinase (6PF2K) and fructose 2,6-bisphosphatase (fru 2,6P₂ase). In most plants this bifunctional enzyme is encoded by a single gene (Draborg et al., 1999; Markham and Kruger, 2002; Villadsen et al., 2000), although monofunctional fru 2,6P₂ase was also found in spinach (Macdonald et al., 1989).

Transgenic plants provide a powerful means to analyze the function of genes. Recently there have been two potential strategies developed to change the levels of fru 2,6P₂ in plants. The first one involves the transformation of plants with a plant endogenous bifunctional enzyme gene in sense or antisense orientation (Draborg et al., 2001; Rung et al., 2004); the other is the transformation carried out with a modified bifunctional enzyme gene of rat liver origin (Scott et al., 1995, 2000; Truesdale et al., 1999). The second approach enables the kinase and the phosphatase activity of the enzyme to be separated.

Carnation (*Dianthus caryophyllus* L.) is one of the most important cut flowers in the world. Understanding the fundamental metabolic pathways and physiological processes (e.g., photosynthesis, carbohydrate metabolism, flower senescence and pigmentation, host-pathogen interaction) enables us to improve the economic and aesthetic traits of carnation (Savin et al., 1995; Zuker et al., 2001). To investigate the regulatory role of fru 2,6P₂ in carnation carbohydrate metabolism, plants were transformed with two modified rat liver bifunctional enzyme complementary DNAs (cDNAs): the first one encoding a polypeptide with kinase activity and the second one encoding a polypeptide with bisphosphatase activity. Transgenic plants showed a 45% to 85% higher and a 45% to 70% lower fru 2,6P₂ level compared with wild-type plants, respectively. In the current study we discuss the effects of altered fru 2,6P₂ concentrations on several enzyme activities and the carbohydrate metabolism in carnation.

Materials and Methods

Vector construction and plant transformation. Two different modified rat liver bifunctional enzyme cDNAs coding a polypeptide with fru 2,6P₂ase activity (Li et al., 1992) and a polypeptide with only 6PF2K activity (Kurland et al., 1992; Tauler et al., 1990) were used. These sequences were inserted via subcloning steps into pBIN19 under the control of CaMV 35S promoter and introduced into *Agrobacterium tumefaciens* strain LBA 4404 (Scott et al., 1995, 2000). Carnation leaves were transformed as described by Van Altvoorst et al. (1995).

Plant material and growth conditions. Carnation (*D. caryophyllus*, L.) variety Improved White Sim was obtained from the Obuda Horticultural Laboratory (Budapest, Hungary). Transgenic shoots were potted in soil and grown in a climate chamber in a 16-h light/8-h dark regime with 200 W·m⁻² light intensity at 20 °C. Leaves were harvested at different time intervals from the start of the illumination period (0, 1, 2, 4, 6, and 8 h). The plants were sampled 3 months after potting.

Measurement of enzyme activities and metabolites. Enzyme extraction and activity assays were performed as described by Hatzfeld et al. (1990), Scott et al. (1995), Sweetlove et al. (1996) and Veramendi et al. (2002). Fructose 2,6-bisphosphate was extracted with NaOH according to the method of Ball and Rees (1988) and assayed by the method of Van Schaftingen et al. (1982).

Extraction and measurement of sucrose, starch, glucose, and fructose were performed by using Boehringer Mannheim test combination kits according to the manufacturer's instructions. Phosphorylated intermediates were assayed as described by Michal (1984a, b).

Statistical analysis. The Student *t* test was performed using Microsoft Office Excel 2003 (Microsoft Corporation, Seattle). Significant differences between two sets of data were assessed at *P* < 0.05.

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Results and Discussion

Fructose 2,6-bisphosphate content. To investigate the effect of altered fru 2,6P₂ content on carbohydrate metabolism, transgenic carnation harboring two types of modified rat liver bifunctional enzyme cDNAs (6PF2K/fru 2,6P₂ase) was produced (Szöke et al., 2006). The most substantial difference in the amounts of fru 2,6P₂ was experienced 4 h after the start of illumination (data not shown). Therefore, the samples were taken from plants at this time of the photoperiod to assess the consequences of altered fru 2,6P₂ contents on enzyme activities and to determine how these changes in enzyme activities affect the important carbohydrate and phosphorylated intermediate concentrations. Four independent transgenic lines (F406 and F407, and P207 and P228) were characterized in our experiment. In plants transformed with the phosphatase construct (F406 and F407), the fru 2,6P₂ levels decreased from 45% to 70% compared with the wild type. Conversely, transformation with the kinase construct (P207 and P228) caused a 45% to 85% increase in fru 2,6P₂ content (Table 1). In comparison with transgenic tobacco plants, expressing the same genes of rat liver origin with only phosphatase activity, the fru 2,6P₂ content decreased by 54% to 75% (Scott et al., 2000). At the same time, tobacco plants harboring the gene with kinase activity alone contained 130% and 214% more fru 2,6P₂ than the wild-type plants (Scott et al., 1995). This increase in *Kalanchoe* ranged from

228% to 350% (Truesdale et al., 1999). More efficient reduction in fru 2,6P₂ levels was obtained in *Arabidopsis* (up to 5% of wild type) and potato (up to 10% of wild type) transformed with an endogenous bifunctional enzyme gene in sense or antisense orientation (Draborg et al., 2001; Rung et al., 2004).

Enzyme activities. There were significant changes in enzyme activities influenced by fru 2,6P₂ content (Table 1). Elevated fru 2,6P₂ levels inhibited the activity of FBPase and stimulated that of PFP. Fructose 1,6-bisphosphatase catalyzes the hydrolysis of fru 1,6P₂ to fructose 6-phosphate (fru6P), and it is an essential enzyme in sucrose biosynthesis. Pyrophosphate:fructose 6-phosphate-1-phosphotransferase has a different role to play. It determines the rate-limiting step of glycolysis and gluconeogenesis, the reversible conversion between fru 1,6P₂ and fru6P. The direction of the reaction depends on the concentration of substrates and products (Stitt, 1990). The decreased fru 2,6P₂ concentrations resulted in higher FBPase activities in both transgenic lines. The PFP activity decreased significantly in the F406 line, but increased in the F407 plant (Table 1). Thus, the effect of the reduced amount of fru 2,6P₂ on PFP activities was not clear. In tobacco and *Arabidopsis*, reduced fru 2,6P₂ levels did not cause any significant differences in the enzyme activities between transgenic and wild-type plants (Draborg et al., 2001; Scott et al., 2000), but elevated fru 2,6P₂ levels in tobacco affected key enzyme activities (Scott et al., 1995). These varia-

tions may reflect differences between plant species. The enzyme 6-phosphofructo-1-kinase (PFK) in nonplant organisms—as a catalyst of the irreversible conversion of fru6P to fru 1,6P₂—was found to be activated by fru 2,6P₂ (Van Schaftingen, 1987). In plants it was determined that PFK is insensitive to fru 2,6P₂ concentrations (Stitt, 1990). On the other hand, decreased fru 2,6P₂ contents lowered PFK activities by 10% to 54% (F406 and F407 plants), at the same time the elevated fru 2,6P₂ levels caused a slight, 8% activation of PFK in the P207 plant, whereas in the P228 line the PFK activity decreased by 11% compared with the wild type (Table 1). This means that there is no unambiguous evidence for the effect of elevated fru 2,6P₂ levels on PFK activity. Hexokinase activities—the catalyst of the phosphorylation of hexoses to hexose monophosphates—were not altered significantly in any transgenic plant lines compared with the wild types (Table 1).

Carbohydrates and phosphorylated intermediates. Reduced fru 2,6P₂ levels stimulated the activity of FBPase (key enzyme of sucrose synthesis) causing a two- to threefold increase in the amount of sucrose in plants expressing bisphosphatase (F406 and F407; Fig. 2A). Experiments with radioactively labeled ¹⁴CO₂ feeding in transgenic *Arabidopsis* and tobacco also verified that the decreased fru 2,6P₂ levels favor sucrose synthesis (Draborg et al., 2001; Scott et al., 2000). In tobacco and potato this abundant sucrose during the light period was cleaved and accumulated as glucose and fructose (Rung et al., 2004; Scott et al., 2000). We observed an increase in hexose phosphate [fru6P, glucose 6-phosphate (glu6P)] pools (Fig. 3A, B). The 20% to 38% increase in the amounts of these metabolites is not the result of elevated hexokinase activity. There can be two possible explanations: First, when the reduction in fru 2,6P₂ levels releases the inhibition of FBPase, the accumulation of fru6P and glu6P proceeds (Rung et al., 2004); and second, the superoptimal activation of FBPase raises the export of triose phosphates from the chloroplast to the cytosol (Scott et al., 2000), and this can accelerate the conversion of triose phosphate into hexose phosphate.

Glucose 6-phosphate is an activator of sucrose phosphate synthase (Doehlert and Huber, 1983). Therefore, it can be assumed that the increased glu6P levels can also cause additional stimulation of sucrose synthesis. In contrast, in transgenic *Arabidopsis*, the decreased fru 2,6P₂ concentrations did not result in glu6P accumulation, indicating the upregulation of sucrose synthesis (Draborg et al., 2001). In agreement with these data, decreased levels of fru 2,6P₂ led to reduced amounts of 3-phosphoglycerate (3PGA) and dihydroxyacetone phosphate as well (Fig. 3C, D). Declines in triose phosphates may stimulate the export of carbon from the chloroplast through the triose phosphate translocator, thus depleting the substrate for starch synthesis (Scott et al., 2000). Accordingly, starch accumulation was 14% to 37%

Fig. 1. The crucial step of carbon partitioning influenced by fructose 2,6-bisphosphate (fru 2,6P₂).

Table 1. Effect of fructose 2,6-bisphosphate content on enzyme activities in leaves of wild-type and transgenic plant lines (F406 and F407, plants harboring phosphatase construct; P207 and P228, plants transformed with kinase construct).

Plant lines	Fru 2,6P ₂ (pmol·g ⁻¹ FW)	Enzyme activity (nmol·min ⁻¹ ·g ⁻¹ FW)				
		FBPase	PFP	AGPase	PFK	Hexokinase
WT	27.64 ± 1.26	425.5 ± 23.7	95.2 ± 2.5	10.7 ± 1.3	69.4 ± 2.1	20.7 ± 1.8
F406	12.56 ± 1.25*	740.5 ± 31.6*	79.4 ± 1.8*	3.1 ± 1.1*	62.2 ± 1.9*	20.9 ± 1.5
F407	19.60 ± 2.97*	697.5 ± 21.7*	106.9 ± 5.2	6.1 ± 0.9*	61.5 ± 2.7*	24.1 ± 1.8
P207	51.19 ± 5.03*	398.5 ± 11.6	119.5 ± 5.5*	18.6 ± 0.9*	74.4 ± 1.2*	19.3 ± 1.5
P228	40.20 ± 3.19*	257.3 ± 8.5*	140.6 ± 12.9*	15.7 ± 0.5*	61.5 ± 1.9	21.4 ± 0.8

Leaves were harvested 4 h after the onset of light period from 3-month-old plants. Each value represents the mean value ±SE of three separate plants.

*Significant at *P* < 0.05.

WT, wild type; Fru 2,6P₂, fructose 2,6-bisphosphate; FW, fresh weight; FBPase, fructose 1,6-bisphosphatase; PFP, pyrophosphate:fructose 6-phosphate-1-phosphotransferase; AGPase, ADP-glucose pyrophosphorylase; PFK, 6-phosphofructo-1-kinase.

Fig. 2. (A–D) Effects of decreased and increased fructose 2,6-bisphosphate (fru 2,6P₂) on sucrose (A), starch (B), glucose (C), and fructose (D) content of leaves in wild-type (WT) and transgenic lines (F406 and F407, plants harboring phosphatase construct; P207 and P228, plants transformed with kinase construct) 4 h after the onset of light period from 3-month-old plants. Each value represents the mean value $\pm$ SE of three separate plants. Significant differences ($P < 0.05$) are indicated by asterisks.

Fig. 3. (A–D) The level of glucose 6-phosphate (A), fructose 6-phosphate (B), 3-phosphoglycerate (C), and dihydroxyacetone phosphate (D) of leaves in wild-type (WT) and transgenic lines (F406 and F407, plants harboring phosphatase construct; P207 and P228, plants transformed with kinase construct) 4 h after the onset of light period from 3-month-old plants. Each value represents the mean value $\pm$ SE of three separate plants. Significant differences ($P < 0.05$) are indicated by asterisks. FW, fresh weight.

lower than in the wild-type plants, but this difference was not as dramatic as the sucrose overproduction (Fig. 2B).

As for glucose concentration, similar to our data obtained in carnation (Fig. 2C), a considerable increase was also observed in glucose concentration in transgenic tobacco with decreased amounts of fru 2,6P₂. This accumulation can be explained by the hydrolysis of starch in light. Because decreased levels of triose phosphates restricted the activity of AGPase, plants were not able to recycle these glucose surpluses into starch (Scott et al., 2000). In other transgenic plants, stimulation of sucrose synthesis by the expression of yeast acid invertase caused similar glucose accumulation as well

(Sonnwald et al., 1991, 1997). In these cases, the hydrolysis of sucrose was also increased and exceeded the glucose phosphorylation capacity of plants. In contrast with these studies (except the potato), significant fructose overproduction was measured in F406 and F407 carnation transgenic lines (Fig. 2D).

In transgenic plants expressing kinase resulting from elevated fru 2,6P₂ levels (P207 and P228) generally different results were obtained with regard to carbohydrates and phosphorylated intermediate concentrations than in plants expressing bisphosphatase (Fig. 2, 3). In these transgenic plants an increase in the content of triose phosphates was observed (Fig. 3C, D). This could be accompanied by a decrease in the inorganic

phosphate (Pi) levels and will result in an increase in the 3PGA-to-Pi ratio (Stitt, 1990), activating AGPase and stimulating starch synthesis in the chloroplast (Scott and Kruger, 1995). In the P207 and P228 lines, AGPase activity was stimulated, which led to a 32% to 40% increase in starch accumulation (Fig. 2B). In transgenic tobacco containing elevated fru 2,6P₂ levels, the higher rate of accumulation of starch was attributed to a decrease in the rate of net starch degradation in the dark period rather than to an increase in starch synthesis during the light period (Scott and Kruger, 1995). We did not examine this possibility in carnation.

The amounts of sucrose, glucose, and hexose phosphates decreased compared with wild-type plants (Figs 2A, C and Fig. 3A, B). Elevated fru 2,6P₂ levels have a direct inhibitory effect on FBPase activity, which prevents the conversion of triose phosphates to hexose phosphates. As a result, sucrose synthesis was restricted to 32% to 40% of that of the wild-type plants. There can be another explanation, too. The increased amounts of fru 2,6P₂ activate PFP, resulting in an enhanced rate of recycling of triose phosphates to hexose phosphates (Fornie et al., 2001). Unexpectedly, the fructose content—similarly to the F406 and F407 transgenic lines—was elevated (Fig. 2D). The reason for this is unknown and requires further investigation.

Knowledge of the role of fru 2,6P₂ in sink tissues is still rather limited. Comparative studies in transgenic potato revealed the different role of fru 2,6P₂ in sink-source tissues. The influence of fru 2,6P₂ in tuber carbohydrate metabolism was less pronounced and had no effect on carbohydrate contents in intact tubers (Rung et al., 2004). In heterotrophic tobacco cells, elevated fru 2,6P₂ levels showed increased rates of cycling of triose phosphates and hexose phosphates (Fornie et al., 2001).

In transgenic plants, bifunctional enzyme genes (6PF2K/fru 2,6P₂ase) of different origin were expressed under the control of the constitutive 35S CaMV promoter (Draborg et al., 2001; Rung et al., 2004; Scott et al., 1995, 2000; Truesdale et al., 1999). Additional understanding of the sink source regulation by fru 2,6P₂ and the application of tissue and organ-specific promoters may enable us to enhance the amounts of different carbohydrates in special tissues and organs in agronomically important species.

In summary, in the current study the effects of down-/upregulated fru 2,6P₂ contents on the levels of several enzymes and carbohydrate compounds were described. A decrease in fru 2,6P₂ concentrations favored sucrose synthesis, whereas elevated fru 2,6P₂ contents supported starch accumulation. These results also indicate the key role of fru 2,6P₂ in regulating carbohydrate metabolism of photosynthetic tissues, which is in agreement with the results in transgenic plants presented by Scott et al. (1995, 2000), Draborg et al. (2001), and Rung et al. (2004). Modification of this signal

metabolite in carnation provides a powerful tool to modify carbohydrate composition in a complex manner.

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