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ORIGINAL PAPER

Role of fructose in the adaptation of plants to cold-induced oxidative stress

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Abstract This work presents findings, which indicate important role of fructose, fructose 6-phosphate (F6P), and fructose 1,6-bisphosphate (FBP) in preservation of homeostasis in plants under low temperature. Cold combined with light is known to incite increased generation of superoxide in chloroplasts leading to photoinhibition, but also an increased level of soluble sugars. In the present study, oxidative stress in pea leaves provoked by cold/light regime was asserted by the observed decrease of the level of oxidized form of PSI pigment P700 ($P700^+$). Alongside, the increased antioxidative status and the accumulation of fructose were observed. The antioxidative properties of fructose and its phosphorylated forms were evaluated to appraise their potential protective role in plants exposed to chilling stress. Fructose, and particularly F6P and FBP exhibited high capacities for scavenging superoxide and showed to be involved in antioxidative protection in pea leaves. These results combined with previously established links implicate that the increase in level of fructose sugars through various pathways intercalated into physiological mechanisms of homeostasis represents important non-enzymatic antioxidative defense in plants under cold-related stress.

Keywords Fructose · Oxidative stress · Superoxide · Low temperature · Photoinhibition

Abbreviations

F6P	Fructose 6-phosphate
FBP	Fructose 1,6-bisphosphate
ROS	Reactive oxygen species
OPP	Oxidative pentose-phosphate pathway
PSI	Photosystem I
SOD	Superoxide dismutase
ABTS	2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid)

Introduction

Higher plants are rarely afforded the luxury of perfect growing environment. Under such conditions, photosynthetic electron transport is geared to minimize the possibility of potentially damaging side reactions that are capable of generating superoxide ($O_2^{\cdot -}$), hydrogen peroxide, and other reactive oxygen species (ROS) (Wise 1995; Sonoike 1996). In chilling, however, the flux through these side reactions is enhanced leading to the oxidative damage in plants (Foyer et al. 1994; Sonoike 1996; Foyer et al. 2002). Cold is known to slow the energy-consuming Calvin cycle enzymes more than the energy-transducing light reactions, thus causing the leakage of energy to oxygen (Wise 1995). So, in chilling combined with moderate or even weak light (Sonoike 1995; Tjus et al. 1998), photosystem I (PSI) reduces oxygen to hazardous $O_2^{\cdot -}$ (Hodgson and Raison 1991; Havaux and Davaud 1994; Foyer et al. 1994;

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Sonoike 1996). Since chilling also inactivates mechanisms of PSI protection (Tjus et al. 1998), superoxide attacks iron–sulfur centers (Inoue et al. 1986; Sonoike et al. 1995; Tjus et al. 1998) and decreases the level of photooxidizeble primary electron donor of PSI-P700, so the photoinhibition occurs (Terashima et al. 1994; Havaux and Davaud 1994; Sonoike 1996; Ivanov et al. 1998). Superoxide represents a ground for generation of other ROS (e.g., hydrogen peroxide (Pastori et al. 2000)), which causes a cascade of oxidative reactions provoking cellular damage (Wise 1995; Sonoike 1996).

Pea (*Pisum sativum* L.), as some other plants, is known to poses antioxidative system, which is competent for removal of $O_2^{\cdot -}$. However, it is not known whether it is enzymatic or non-enzymatic (Wise 1995). Since chilling promotes both, photooxidative damage, and the accumulation of sugars (Levitt 1980; Streb et al. 2003), soluble sugars were proposed to act as cold-stress protectants (Couée et al. 2006). They have been observed to participate in signaling pathways and the regulation of expression of genes related to the control of oxidative stress in plants (Ciereszko et al. 2001; Couée et al. 2006). The ability of soluble sugars to combine hydroxyl radical has been speculated to be one of the mechanisms of antioxidative protection in plants (Aver'yanov and Lapikova 1989). Plants transformed in order to pose higher activity of invertase, which cleaves sucrose into fructose and glucose, showed to be less subjective to lipid peroxidation during the cold-related stress (Deryabin et al. 2005). However, the concrete role of soluble sugars, such as glucose and fructose, in the redox metabolism is still an unresolved issue. While the increased level of glucose in cells is associated with ROS production through glucose autooxidation, glucose feeding of the oxidative pentose-phosphate pathway (OPP) pathway which is activated in chilling (Maciejewska and Bogatek 2002; Crecelius et al. 2003) can enhance NADPH and fructose 6-phosphate (F6P) production (Couée et al. 2006). In fact, F6P has been reported to accumulate in pea leaves exposed to chilling temperatures combined with light (Streb et al. 2003). Chilling in the light was observed to lower redox potential in chloroplast stroma to such a degree that reductively activated enzyme fructose 1,6-bisphosphatase (FBP) is oxidatively inhibited, which leads to the accumulation of FBP in the Calvin cycle (Sassenrath et al. 1990; Wise 1995). Fructose and FBP have been speculated to poses good ROS scavenging performances (Lazzarino et al. 1987; Girard et al. 2005), as we have observed recently (Maksimović et al. 2006), however, antioxidative capacity of fructose was not evaluated nor was it compared to related sugars (glucose).

Low temperatures induce phase changes in energy production, subsequent ROS production, and provoke physiological responses. The involvement of ROS and role of antioxidative defense system (ADS) in organisms subjected to low temperatures, are features intercalated into physiological mechanisms of homeostasis (Andjus 1964; Blagojević 2007). Detailed examinations in homeotherms and heterotherms gave lot of valuable data about that problem, but in plants there are not enough data yet. Here, we present results, which more closely describe the role of fructose, F6P, and FBP in the preservation of homeostasis in plants under low temperatures.

Experimental

Plant material and growing condition

Pisum sativum seeds were washed for 5 h under the tap water and sown on (and covered with) moistened filter paper. Seeds were germinated in darkness at 25°C, for 4 days. Pea seedlings, 4 days old, were grown in tap water for 10 days. The temperature in growth chamber was 22°C (night/day), photoperiod (16 h)/(8 h) light/darkness and irradiance of 70 μmol m^{-2} s^{-1} which was provided by white fluorescent tubes. After night, at the beginning of the light period, 10 days old plants were transferred to 2°C/70 μmol m^{-2} s^{-1} regime for 3 h, or maintained at 22°C/70 μmol m^{-2} s^{-1} (non-chilled controls). Leaves were collected at the start of 3-h period and after 0.5, 1, 2, and 3 h, and immediately frozen in liquid N_2.

Extraction of sugars

Frozen leaves were ground with liquid N_2 using a mortar and pestle. The powdered leaves were extracted with 80% ethanol in a 1:10 ratio (w/v). Extracts were stored on ice for 30 min prior to centrifugation for 10 min at 10,000 g and 4°C. The solid residue was stored at −80°C and used for HPLC measurements and 2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) assay.

EPR study of the level of $P700^+$

The amount of $P700^+$ was determined by the amplitude of the associated light-induced EPR signal. EPR spectra were recorded at −100°C, using a Varian E104-A EPR spectrometer operating at X-band (9.51 GHz) using the following settings: modulation amplitude, 2 G; modulation frequency, 100 kHz; microwave power, 10 mW. Similar signal, but of less intensity, was obtained at room temperature. Spectra were recorded using EW software (Scientific Software, Bloomington, IL, USA). Powdered leaves were placed in quartz capillaries, frozen in liquid N_2, and positioned in the cavity of the EPR spectrometer equipped with

the temperature regulator. Amplitudes of obtained signals were normalized to the mass of the sample.

Determination of sugars by HPLC technique

Separations were performed on a Waters Breeze chromatographic system (Waters, Milford, MA) containing binary pumps system, a thermostated column compartment and a model 2465 Waters electrochemical detector. Separation of sugars was performed on CarboPac PA1 (Dionex, Sunnyvale, CA, USA) 250 × 4 mm column equipped with corresponding CarboPac PA1 guard column. Sugars were eluted with 200 mM NaOH for 20 min at a flow rate of 1.0 mL min^{-1} at a constant temperature of 30°C.

2,2′-Azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) assay

We used ABTS method to determine total antioxidant activity (Cano et al. 1998). The reaction mixture contained 2 mM ABTS, 15 μM hydrogen peroxide and 0.25 μM horseradish peroxidase in 50 mM phosphate buffer (pH 7.5), in a total volume of 1 ml at 25°C. The reaction was monitored at 730 nm in order to estimate the formation of the ABTS radical. Different amounts of L-ascorbic acid, sugars and plant extracts were added and the decrease in absorbance was determined. Total antioxidant activities of sugars and extracts were calculated relative to ascorbate, and expressed as equivalents of ascorbic acid which produced the same antioxidant effect as the sample. Different concentrations of sugars: 1 M (glucose and fructose) and 50 mM (F6P and FBP) were used to accommodate the detection limits of the assay.

Spectrophotometric assay of $O_2^{\cdot -}$ scavenging capacity of sugars

Superoxide scavenging capacity of sugars was determined spectrophotometrically at 550 nm in 50 mM sodium phosphate buffer at pH 7.8 by measuring the inhibition of the reduction of cytochrome c using xanthine/xanthine oxidase system as the source of $^{\cdot}O_2^-$ as previously described by McCord and Fridovich (McCord and Fridovich 1968). Results are presented as inhibition of cytochrome c reduction (%), compared to the control system with no inhibitor. Different concentrations of sugars: 60 mM (glucose), 30 mM (fructose), and 6 mM (F6P and FBP) were used to accomodate the detection limits of the assay.

All experiments were performed at least in triplicate. Data are presented as the mean ± the standard deviation. Statistical analysis of data employed the Student's *t* test. Significance was assumed if $P < 0.05$.

Results

Decrease of the level of $P700^+$

Figure 1 shows light inducible EPR signal, which has been recognized to reflect the oxidized form of PSI pigment P700 ($P700^+$) (Ivanov et al. 1998). Typical spectrum of homogenates of leaves of plants exposed to 2°C/70 μmol m^{-2} s^{-1} temperature/irradiance regime for 3 h (dark trace), and the spectrum of control plants (pale trace), are presented. The level of $P700^+$ is lower in plants exposed to cold by 28 ± 3%, showing that chilling conditions led to the damage of P700 and photoinhibition of PSI. In addition, the decrease of the level of $P700^+$ was observed even after 30 min of treatment, however it was determined not to be statistically significant (data not shown).

Antioxidative status during cold-related stress

Antioxidative status in leaves of pea exposed to cold stress was evaluated using ABTS test, and expressed in the equivalents of L-ascorbic acid (Fig. 2). It does not significantly change during the first hour of exposure, when compared to the antioxidative status of leaves of untreated plants (0 h). However, it exhibits significant increase during the further exposure, reaching the value 50% higher than at the start of the treatment. During the third hour of treatment, there were not significant changes in antioxidative status. Antioxidative status of control plants did not significantly varied during the 3-hour monitoring.

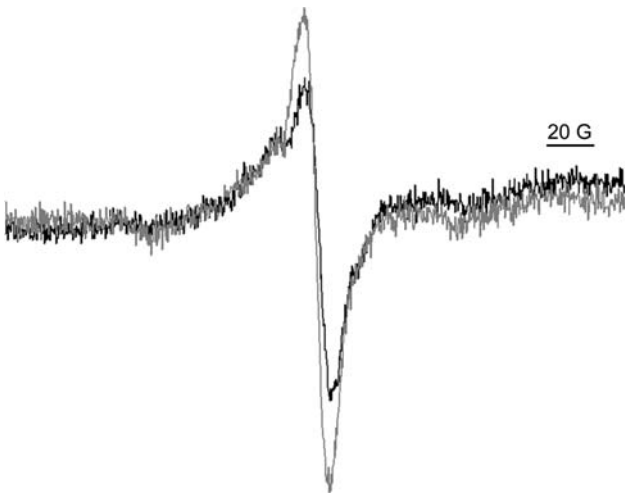

Fig. 1 EPR spectra of light-induced EPR active specie—oxidized PSI pigment ($P700^+$). Pale trace—samples from leaves of control plants exposed to 22°C/70 μmol m^{-2} s^{-1} temperature/light conditions; dark trace—samples of leaves of peas under 2°C/70 μmol m^{-2} s^{-1} regime. Spectra were recorded at −100°C

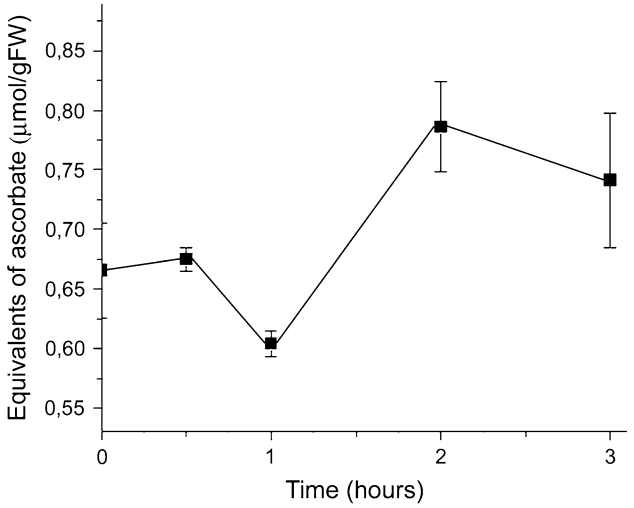

Fig. 2 Antioxidative status in pea leaves under 2°C/70 µmol m^{-2} s^{-1} regime determined by the ABTS assay. Capacities are presented relative to the ascorbic acid activity in removing ABTS radical

Levels of fructose and glucose during cold-related stress

Figure 3 shows changes of the level of fructose in pea leaves during 3-hour exposure to cold. It can be observed that the level of fructose alters in similar manner as the antioxidative status (Fig. 2). After the first hour of treatment the concentration of fructose exhibited rapid increase, and reached, after the second hour of exposure to cold, almost double value compared to the fructose level in untreated plants (0 h). During the third hour of treatment, there were no statistically significant changes of the fructose level in leaves of treated plants.

In contrast to fructose, the glucose level presented in Fig. 4 using the same concentration scale as in Fig. 3, showed only slight increase during the second hour of the cold treatment. In leaves of control plants kept under 22°C/70 µmol m^{-2} s^{-1} regime concentrations of fructose and glucose did not show statistically significant variations during the 3-hour monitoring (data not shown).

Antioxidative capacities of sugars

Table 1 shows antioxidative properties of sugars obtained by ABTS assay based on the evaluation of capacity to scavenge ABTS radical, an unspecific model-compound of reactive species in biosystems. Capacities are presented relative to the activity of ascorbic acid evaluated under same experimental settings. It can be observed that fructose poses antioxidative capacity which is 2 times higher than glucose. F6P and FBP showed even more exalted capacity for removal of ABTS radical—6 and 10 times higher then glucose.

Capacities of sugars for removal of $O_2^{\cdot -}$

Table 2 shows capacities of sugars for removal of $O_2^{\cdot -}$ presented as percentages of inhibition of cytochrome *c* reduction. Similarly to the results acquired by the ABTS assay (Table 1), fructose showed two times higher ability for removal of $O_2^{\cdot -}$relative to the glucose. F6P and FBP exposed significantly higher $O_2^{\cdot -}$ scavenging activity—16 and 8 times more pronounced when compared to glucose. In contrast to the results obtained using ABTS assay, F6P showed higher antioxidative activity then FBP. This indicates that F6P is specifically active in removing $O_2^{\cdot -}$. Obtained results also point to an additional issue. High capacity of sugars for removal of $O_2^{\cdot -}$ should be taken into

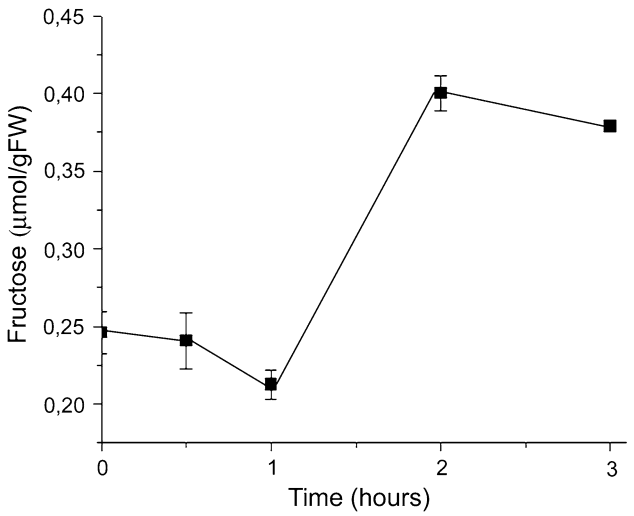

Fig. 3 Level of fructose in pea leaves under 2°C/70 µmol m^{-2} s^{-1} regime. Concentrations are normalized to mass and presented as µmol of sugar per g of fresh mass (FW—fresh weight) of leaves

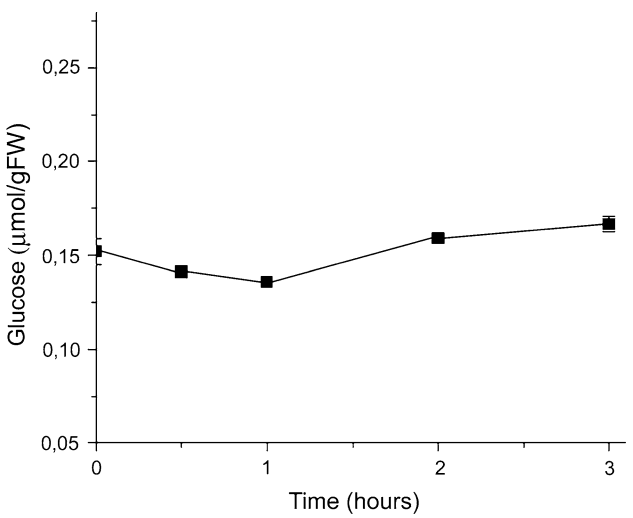

Fig. 4 Level of glucose in pea leaves under 2°C/70 µmol m^{-2} s^{-1} regime. Concentrations are normalized to mass and presented as µmol of sugar per g of fresh mass (FW—fresh weight) of leaves

Table 1 Antioxidative capacities of sugars obtained by the ABTS assay

Sugar	L-Ascorbic acid equivalents (μM)
Glucose (1 M)	47 ± 1
Fructose (1 M)	88 ± 2
F6P (50 mM)	15 ± 1
FBP (50 mM)	24 ± 1

Capacities are presented relative to the ascorbic acid activity in the removal of ABTS radical. Different concentrations of sugars were used to accomodate the detection limits of the assay

Table 2 Capacities of sugars for scavenging $O_2^{\cdot -}$

Sugar	Inhibition of cytochrome *c* reduction (%)
Glucose (60 mM)	8 ± 1
Fructose (30 mM)	9 ± 2
F6P (6 mM)	13 ± 1
FBP (6 mM)	6 ± 1

Results are presented as inhibition of cytochrome c reduction (%). As much as 50% of inhibition equals 1 unit of SOD activity (McCord and Fridovich 1968). Different concentrations of sugars were used to accomodate the detection limits of the assay

concern in studies interested in the activity of superoxide dismutase (SOD) in biological samples. Investigated sugars participate in various physiological processes in cells, so if any of these should be present in the sample, additional precaution (e.g., gel filtration of samples) ought to be made in order to avoid misjudgment of SOD activity.

Discussion

In the present study exposure of pea to low temperature/weak irradiance regime led to the decrease of the level of oxidized form of P700. Experimental setup and observed decrease of the $P700^+$ level were very similar to some previous studies on cucumber and spinach exposed to cold/weak light regime (Terashima et al. 1994; Sonoike 1995). This result combined with the activation of the antioxidative defense disclosed by ABTS test, confirms that cold/light treatment provokes oxidative stress in pea leaves. Low temperatures are known to inactivate energy consuming Calvin cycle, thus limiting the supply of $NADP^+$ for reduction and ADP and P_i for phosphorylation (Wise 1995). Incoming light energy continues to be funneled into electron transport, which is now shunted towards oxygen, leading to the generation of $O_2^{\cdot -}$ (Foyer et al. 1994; Havaux and Davaud 1994; Foyer et al. 2002), and other ROS (Wise 1995; Sonoike 1996). The main site of production of $O_2^{\cdot -}$ is PSI at both, stromal and luminal side of the complex (Wise 1995). Increased generation of reactive species leads to the PSI photoinhibition by decreasing the level of $P700^+$ (Terashima et al. 1994; Sonoike 1996; Tjus et al. 1998).

Although sugars have been proposed to act as protectants from the cold-related stress (Couée et al. 2006), the direct antioxidative role of soluble sugars in plants has not been investigated, excluding one report on the capacity for removal of hydroxyl radical (Aver'yanov and Lapikova 1989). Similarly to several previous studies of the effects of low temperature/light regime on plants (Sassenrath et al. 1990; Streb et al. 2003), we observed the accumulation of fructose in treated pea leaves. The level of glucose did not change significantly, so the principal cause of the increase of the level of fructose could be the increase of glucose feeding of the OPP pathway. Using ABTS test, we showed that fructose poses higher antioxidative capacity than glucose. In addition, F6P and FBP showed to be three and five times more active radical scavengers compared to fructose, which indicates that these sugars could play a major role in plant defense against cold-provoked oxidative stress.

The important role of $O_2^{\cdot -}$ in cold-related damage of PSI is upheld by several reports. The photoinhibition of PSI was only observed at chilling temperatures in the presence of oxygen (Terashima et al. 1994; Havaux and Davaud 1994). Chilling tolerance has been observed to increase in maize plants overproducing chloroplast-targeted MnSOD (Van Breusegem et al. 1999), and to decrease when Cu,ZnSOD was inhibited in barley (Tjus et al. 1998). Finally, it has been reported that the generation of $O_2^{\cdot -}$ by xanthine/xanthine oxidase in darkness results in a decrease in PSI activity in isolated thylakoid membranes (Inoue et al. 1986). Obviously, it is imperative to the survival of photosynthetic organisms to prevent damaging effects of $O_2^{\cdot -}$ (Wise 1995; Sonoike 1996). Therefore, in order to further explore antioxidative role of sugars in cold-related stress, the capacity of sugars for removal of $O_2^{\cdot -}$ was investigated. Fructose showed to be two times more active against $O_2^{\cdot -}$ than glucose. F6P and FBP, which are known to accumulate in plants under chilling (Sassenrath et al. 1990; Streb et al. 2003), showed eight and four times higher superoxide scavenging capacity than fructose. In addition, we have recently observed very high efficiency of fructose (Maksimović et al. 2006), for scavenging hydroxyl radical, which is also involved in cold-related damage in plants (Wise 1995; Sonoike 1996).

Presented results combined with previously established links show that fructose and its phosphorylated forms could play a significant role as one of non-enzymatic defense mechanisms in chilling stress. It should be stressed out that the accumulation of fructose, F6P, and FBP is not the sole defense against cold-related damage and photoinhibition. However, since ROS exhibit non-selective damaging effects, the corresponding protection mechanisms ought to

be pleiotropic and intercalated into physiological mechanisms of homeostasis. Fructose, F6P, and FBP, which represent ubiquitous species produced under normal conditions through various metabolic pathways, seem to satisfy postulated principles.

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