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ORIGINAL ARTICLE

Protein phosphatase activity and sucrose-mediated induction of fructan synthesis in wheat

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Abstract In this work, we analyze protein phosphatase (PP) involvement in the sucrose-mediated induction of fructan metabolism in wheat (*Triticum aestivum*). The addition of okadaic acid (OA), a PP-inhibitor, to sucrose-fed leaves reduced fructosylsucrose-synthesizing activity (FSS) induction in a dose-dependent manner. The expression of the two enzymes that contribute to FSS activity, 1-SST (1-sucrose:sucrose fructosyltransferase, E.C. 2.4.1.99) and 6-SFT (6-sucrose:fructan fructosyltransferase, E.C. 2.4.1.10), was blocked by 1 μM OA. These results suggest the involvement of a PP type 2A in sucrose signaling leading to fructan synthesis. OA addition to the feeding medium impaired both sucrose accumulation in leaves and the expression of sucrose-H^+ symporter (SUT1). It is known that sucrose concentration must exceed a threshold for the induction of fructan metabolism; hence PP2A inhibition may result in lower sucrose levels than required for this induction. OA also induced the vacuolar acid invertase (acid INV) transcript levels suggesting that PP activity might play a role in carbon partitioning. Total extractable PP2A activity decreased during 24 h of treatment with sucrose, in parallel with declining sugar uptake into leaf tissues. In conclusion, our results suggest that PP2A is involved in sucrose-induction of fructan metabolism and may play a role in regulating sucrose uptake, but do not rule out that further steps in sucrose signaling pathway may be affected.

Keywords Fructan metabolism · Okadaic acid · Protein phosphatase · Sucrose signaling · Sucrose uptake · *Triticum*

Abbreviations

BA	Bis-(*o*-aminophenoxy)-*N,N,N′,N′*-tetraacetic acid
EtBr	Ethidium bromide
FSS	Fructosylsucrose-synthesizing activity
acid INV	Vacuolar acid invertase
La^{3+}	Lanthanum chloride
β-ME	β-Mercaptoethanol
OA	Okadaic acid
PK	Protein kinase
PMSF	Phenylmethylsulfonylfluoride
PP	Protein phosphatase
RR	Ruthenium red
6-SFT	6-Sucrose:fructan fructosyltransferase
1-SST	1-Sucrose:sucrose fructosyltransferase
ST	Staurosporine
SUT	Sucrose-H^+ symporter
Ubi	Ubiquitin
W7	*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide

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Introduction

In cool temperate grasses, environmental conditions that lead to the accumulation of photoassimilates (typically, low temperatures or other situations which impair plant growth) also induce sucrose polymerization into fructan. The first terms of fructan series are two distinct fructosyl-sucrose molecules: 1-kestose and 6-kestose, which are synthesized by the enzymes 1-SST (1-sucrose:sucrose fructosyltransferase, E.C. 2.4.1.99) and 6-SFT (6-sucrose:fructan fructosyltransferase, E.C. 2.4.1.10), respectively (Vijn and Smeekens 1999). These fructosyl-sucrose-synthesizing activities (FSS) are almost absent in plants growing actively (Koroleva et al. 1998), but are promptly induced when sucrose concentration exceeds a certain threshold. This can be experimentally produced by illuminating detached leaves or feeding them with exogenous sucrose (Wagner et al. 1986; Martínez-Noël et al. 2001).

Evidence has been presented indicating that sucrose plays a double role in fructan metabolism. It is not only the essential substrate used in fructan synthesis, but also it starts the signal transduction chain of events that results in 1-SST and 6-SFT induction. Protein kinases (PKs) are involved in the induction of fructan metabolism by sucrose, as demonstrated by using specific inhibitors of these activities (Martínez-Noël et al. 2001). It was shown that PK activity increases shortly after feeding wheat leaves with sucrose and that calcium ions are also involved, suggesting the participation of calcium-dependent PK activity in the signaling of FSS induction (Martínez-Noël et al. 2006, 2007). On the other hand, protein phosphatases (PPs) seem also to be involved in this process, since FSS activity was shown to be reduced by 1 μM okadaic acid (OA) in wheat leaves fed with 500 mM sucrose (Martínez-Noël et al. 2001), but the role of PPs in the fructan induction by sucrose has not been studied so far.

Protein phosphatase activities have been found to be required for the expression of several sugar-inducible genes, as for example. those encoding the sweet potato ß-amylase (Takeda et al. 1994), the ADP-glucose pyrophosphorylase small subunit in both sweet potato and *Arabidopsis* (Takeda et al. 1994; Siedlecka et al. 2003), the D-type cyclin (Riou-Khamlichi et al. 2000), the UDP-glucose pyrophosphorylase, the sucrose synthase (Ciereszko et al. 2001), and the ABA-responsive *rab*18 (Ciereszko and Kleczkowski 2002) in *Arabidopsis.* The way by which PPs exert this role is largely unknown. Takeda et al. (1994) suggested that the OA-mediated inhibition of sucrose-inducible expression of a ß-amylase:GUS fusion gene in transgenic tobacco leaves was due to its effect on the transduction of carbohydrate metabolic signals to the nucleus, and not to inhibition of the uptake and cleavage of sucrose. On the other hand, Roblin et al. (1998) reported that OA directly inhibited the activity of a sugar beet H^+-sucrose symporter of the plasma membrane, likely by maintaining it in a phosphorylated form, and without significantly affecting the amount of molecules of the sucrose symporter. However, Ransom-Hodgkins et al. (2003) found also in sugar beet that the expression of a gene encoding a phloem-specific H^+-sucrose symporter required PP activity, which, in turn, also affected the symporter activity.

Although the presence of PPs in wheat has been reported before (MacKintosh et al. 1991; Sun and Markwell 1992), very scarce information exists about PP activities in connection with physiological processes in this plant, except for the work of Xu et al. (2007) on the role of PP2Ac-1 in drought stress response. In the present work, we provide evidence of the requirement of PP activity in sucrose-mediated induction of fructan metabolism, together with insights about its possible role in this process.

Materials and methods

Plant material

Wheat seeds (*Triticum aestivum* L.) of winter type cv. PRO INTA Pincén (Instituto Nacional de Tecnología Agropecuaria, Argentina) were germinated and grown for 8 days in vermiculite under controlled conditions, at 27°C, 250 μmol photons (PAR) m^{-2} s^{-1} and a day/night regime of 16/8 h. Seedlings were watered daily with one-half strength Hoagland's solution. Blades from fully expanded primary leaves were excised and immediately placed in test tubes containing different solutions, and incubated for different time periods in darkness at a constant temperature of 25°C and 50% relative humidity. Sugars were supplied at 200 mM, as in previous works (Martínez-Noël et al. 2006, 2007). If inhibitors were used, leaves were pretreated with the inhibitor alone for 2 h before sucrose addition. Immediately after harvesting, leaf blades were frozen in liquid nitrogen and subsequently used for further analyses.

Chemical

Okadaic acid (Sigma, St Louis, MO, USA), inhibitor of PP2A/1 (concentration for 50% inhibition = 0.1–1 nM for PP2A and 10–15 nM for PP1, Cohen et al. 1990), was dissolved in dimethyl sulfoxide at 1 mM as stock solution. Stock solution of this inhibitor was diluted with water or with a solution of sucrose in water. BA (bis-*o*-aminophenoxy-*N,N,N′,N′*-tetraacetic acid, Sigma) was used as specific Ca^{2+} chelator, lanthanum chloride (La^{3+}, Sigma), and ruthenium red (RR, Sigma) as channel blockers, which inhibit Ca^{2+} uptake from extra- and intra-cellular stores,

respectively. Staurosporine (ST, Sigma) is an inhibitor of Ser/Thr PKs, and W7 [*N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide] (Sigma) is a calmodulin antagonist.

Carbohydrate analysis

Extraction of water-soluble carbohydrates and sugar analyses were done as described by Simmen et al. (1993). The samples were chromatographed on a CarboPac PA-100 anion exchange column, using a Dionex DX-300 gradient chromatography system (Dionex, Sunnyvale, CA, USA), coupled with pulse-amperometric detection. Sucrose accumulation rate was calculated from HPLC data of leaves fed with sucrose for 0, 0.5, 1, 3, 6, 8, 16, and 24 h.

Preparation of protein extracts and enzyme assays

Leaf blades were powdered in liquid nitrogen and ground with a mortar and pestle. Homogenates were prepared by extracting the powder in an ice-cold buffer (1.5 ml g FW^{-1}) containing 50 mM Tris–HCl (pH 7.5), 50 mM NaCl, 2 µg aprotinin, 0.5 µg leupeptin, 5 mM β-mercaptoethanol (β-ME), 0.5 mM phenylmethylsulfonylfluoride (PMSF), and 0.05% Triton X 100. Extracts were centrifuged at 10,000 *g* for 10 min and the supernatants were stored at −80°C.

Protein phosphatase activity from desalted crude extracts was measured by the non-radioactive Ser/Thr PP assay system (Promega, Madison, WI, USA), which estimates P_i liberated by spectrophotometric absorbance at 620 nm. The phosphothreonylpeptide used as substrate was RRA(pT)VA (0.1 mM), which is especially suited for differentiate PP2A, PP2B, and PP2C activities, according to the reaction buffer used. The reaction mixture also contained 50 mM imidazol buffer (pH 7.2) (for alkaline phosphatases inhibition), 0.2 mM EGTA (for chelating divalent cations which are required for PP2B and PP2C activity), 0.02% β-ME, 0.1 mg/ml bovine serum albumin and crude extract in a final volume of 50 µl. After 1.5 h of incubation the reaction was terminated by adding 50 µl molybdate dye/additive mixture.

Protein extractions and measurement of total FSS activity (1-SST and 6-SFT activities) were done as described by Puebla et al. (1999). Wheat leaf blades were powdered in liquid nitrogen and ground with a pestle in a mortar. Homogenates were prepared by extracting the powder in an ice-cold buffer (1.5 ml g FW^{-1}) containing 100 mM Hepes–NaOH (pH 7.5), 1 mM EDTA, 1 mM EGTA, 0.01% Triton, 20 mM $MgCl_2$, 20 mM β-ME, and 0.5 mM PMSF. Extracts were centrifuged at 10,000 *g* for 10 min and supernatants were desalted through Sephadex G-25. FSS activity was determined in a reaction mixture containing 100 mM sodium acetate buffer (pH 5.2), 200 mM sucrose, and protein extract in a total volume of 50 µl, by incubating for 2 h. Excess sucrose was eliminated by adding 0.23 U of a commercial sucrase (Megazyme International Ireland Ltd., Bray, Co. Wicklow, Ireland) in 100 mM sodium maleate buffer (pH 6.5), in a total volume of 400 µl, for 1.5 h. The monosaccharides produced were further destroyed with 0.4 N NaOH at 100°C, and fructans formed were quantified with the thiobarbituric acid procedure.

All enzyme assays were routinely conducted in duplicate at 30°C and activities were linear with time and the amount of enzyme. Soluble protein was determined according to the method of Bradford (1976).

Analysis of gene expression. Northern-blot hybridization

Total RNA was prepared from leaf tissues by the Trizol method and Northern-blot hybridization was carried out as described by Sambrook and Russell (2001). To demonstrate equivalent loadings, transferred ribosomal RNAs were stained with ethidium bromide (EtBr).

For RT-PCR and Real-time PCR experiments, RNA was isolated from leaf tissue using the RNeasy Plant Mini kit (Qiagen, Germantown, MD, USA). RNA extracts were treated with deoxyribonuclease I (Fermentas International Inc, Burlington, ON, Canada), subjected to phenol/chloroform extraction and quantified spectrophotometrically. The synthesis of cDNA was performed from 1 µg of RNA and the Reverse Transcription System (Promega). For the analysis of 1-SST, 6-SFT, SUT1, and acid INV gene expression, 1-SST-RTFor (5′-gtcgtcgattagactgatcact-3′), 1-SST-RTRev (5′-acatcatagccctgtcatcaac-3′), 6-SFT-RTFor (5′-ctctccaatatggacgatcact-3′), 6-SFT-RTRev (5′-gcaaaccacatcggttcactat-3′), SUT1For (5′-tcgtcggcatattcctcgtg-3′), SUT1Rev (5′-tccgctacacgcactgactt-3′), acidINVFor (5′-tatccgacggaggccatct-3′), and acidINVRev (5′-ggccaagagcttgattaattgc-3′) specific primer pairs were used. The SUT1 pair of primers amplifies the three SUT1 genes that have been described in wheat (Aoki et al. 2002). Ubiquitin (Ubi) gene expression was used as internal standard for gene expression analysis (UbiFor 5′-ccttcacttggtgctccgtct-3′ and UbiRev 5′-aacgaccaggacgacagacaca-3′ primers were used). For conventional RT-PCR, cDNAs were amplified with *Taq* polymerase (Promega) (2.5 U) in a reaction mixture containing 10 mM Tris–HCl buffer (pH 9), 10 ng of the template, 1.5 mM $MgCl_2$, 250 µM of each dNTP, and 20 pmol of each primer in a total volume of 10 µl. PCR parameters consisted of 30 s at 95°C for denaturing, 30 s at 55°C for annealing and 30 s at 72°C for extension for 30 cycles, and a final extension step of 7 min at 72°C. The PCR products were analyzed on 0.8% agarose gels and visualized with EtBr. Real-time PCR

assays were performed in a Gene Amp 5700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The thermal profile was 1 cycle 2 min at 50°C, 1 cycle 10 min at 95°C, 40 cycles 15 s at 95°C, 58°C 15 s and 1 min at 60°C. A 25-μl reaction volume consisted of 12.5 μl SYBR Green PCR master mix (Applied Biosystems), 8.5 μl water, 0.3 μM gene-specific forward primers, 0.3 μM gene-specific backward primers and 1 μl of cDNA preparation diluted 1:5. Ubi transcript levels in the different samples were used to normalize the amounts of 6-SFT.

Results

The incubation of excised wheat leaf blades with sucrose in the dark for 24 h led to more than tenfold increase in FSS activity, relative to water control. The addition of OA to the incubation medium reduced FSS induction in a dose-dependent manner: 1 and 2 μM OA inhibited FSS induction by more than 70 and 80%, respectively (Fig. 1).

The expression of 6-SFT, which is the enzyme involved in the most prevalent fructan biosynthetic pathways in grasses (Vijn and Smeekens 1999), was highly induced (about 25-fold) by 200 mM sucrose. The transcript induction by sucrose was drastically inhibited by the addition of 1 μM OA to the incubation medium, as revealed by Real-time PCR and Northern-blot analysis (Fig. 2a, b). Furthermore, 6-SFT transcripts, detectable after 6 h of sucrose feeding, reached the maximum at 16 h of treatment and started to decrease at 24 h (Fig. 2b). RT-PCR analysis of the expression of the other enzyme involved in fructan synthesis initiation, 1-SST, gave similar results than those

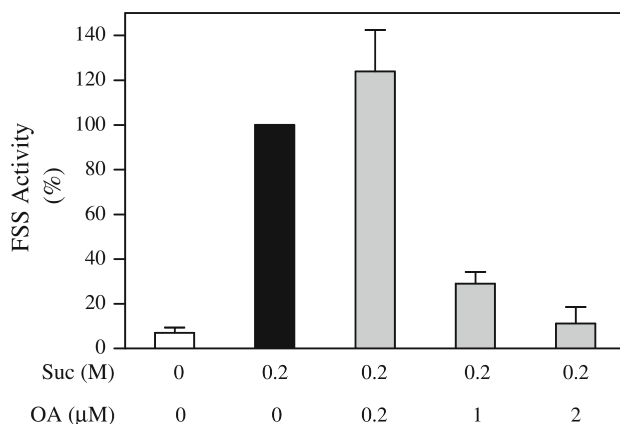

Fig. 1 Effect of OA treatment on the sugar-induced FSS activity. Wheat leaves were treated with 200 mM sucrose and different concentrations of OA for 24 h in darkness. Control leaves were water-fed for the same period. FSS activity is expressed as a percentage of controls. 100% FSS activity corresponds to 4.2 ± 0.8 μmol trisaccharide (g FW)$^{-1}$ h^{-1}. Data represent the mean ± SE of three different experiments

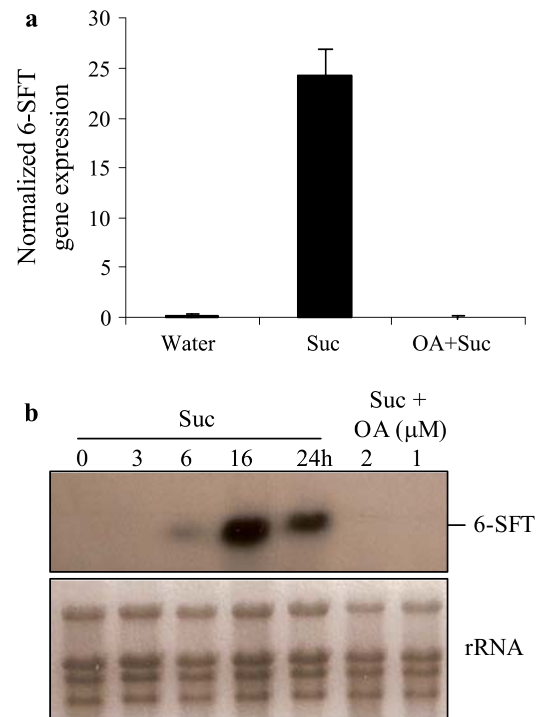

Fig. 2 Sucrose induction of FSS genes expression and its blockage by OA in wheat. Total RNA was isolated from wheat leaves treated with water or 200 mM sucrose in the presence or absence of 1 μM OA (unless otherwise indicated in the figure) for 24 h in darkness. **a** Real-time PCR analysis of 6-SFT. Relative expression corresponds to 6-SFT copy number relative to Ubi copy number. **b** Northern-blot analysis of 6-SFT. EtBr-stained gel is presented to show that similar amounts of total RNA (10 μg) were loaded in each track. Data represent the mean ± SE of three independent experiments

obtained for 6-SFT, i.e., its expression was induced by sucrose and this induction completely blocked by OA (not shown).

Sucrose-fed wheat leaves (200 mM, 24 h) accumulated this sugar over sevenfold as compared with water controls. When 1 μM OA was added to the sucrose feeding solution, a strong decrease of sucrose accumulation was observed (72.9% reduction), as shown by HPLC analysis of sugar leaf extracts (Fig. 3). Also, OA almost completely blocked fructan accumulation (Fig. 3a), which is consistent with the effect of this inhibitor on the gene expression of fructan synthesizing enzymes. The addition of the PP inhibitor to the sucrose feeding solution led to a decrease in monosaccharide content as well, which was similar to that found for sucrose (Fig. 3).

Reduced sugar accumulation in OA-treated leaf blades might be due to a decreased sucrose uptake into cells. Some reports have demonstrated that OA inhibits activity and/or expression of plasma membrane H^+-sucrose symporter in sugar beet (Roblin et al. 1998; Ransom-Hodgkins et al. 2003). The latter authors, working with sugar beet, suggested that SUT1 expression is controlled by a

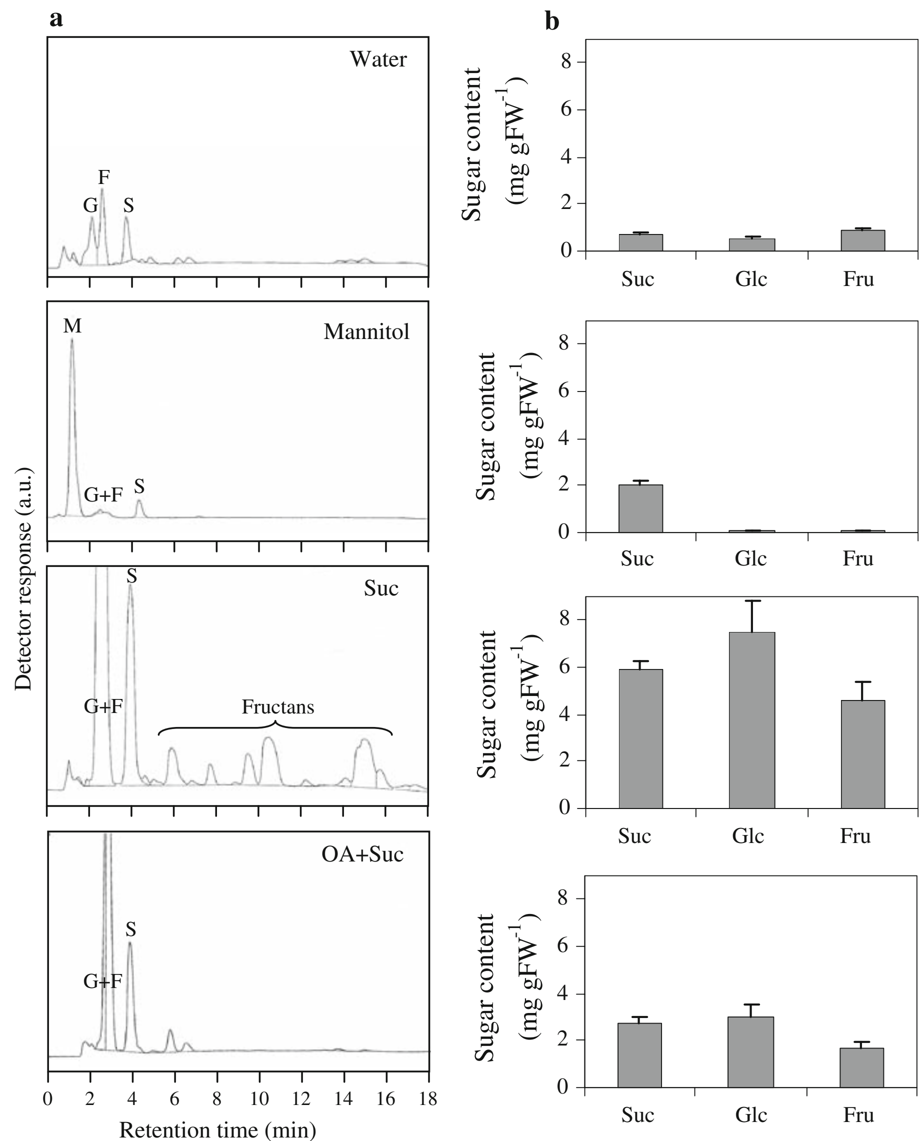

Fig. 3 Analysis of soluble carbohydrates present in sucrose-treated leaves. Wheat leaves were suspended in 200 mM sucrose in the presence or absence of 1 µM OA for 24 h in darkness. Control leaves were fed with water or mannitol for the same period. **a** HPLC profiles of soluble sugars. The detector response is given in arbitrary units (a.u.). Profiles from representative experiments are shown. Peaks are indicated as follows: glucose (*G*), fructose (*F*), sucrose (*S*), mannitol (*M*). **b** Sucrose, glucose, and fructose content in wheat treated-leaves. Data represent the mean ± SE of three different experiments

phosphorylation/dephosphorylation signaling pathway. We thus analyzed the effect of OA, in parallel with PK and calcium/calmodulin inhibitors, on the expression of the wheat SUT1 gene (encoding H^+-sucrose symporter protein of the plasma membrane, which is responsible for the loading of sucrose into cells) in sucrose-fed wheat leaves. As shown in Fig. 4, the expression of SUT1 in leaves treated with sucrose was completely blocked when 1 µM OA was added to the incubation medium, as revealed by RT-PCR analysis. On the other hand, ST, a Ser-Thr PK inhibitor, had no effect, while differential (but moderate) effects on symporter expression were observed with a calcium chelator (BA), calcium channel blockers (La^{3+}, RR), and a calmodulin antagonist (W7).

We also tested the expression of the gene coding for the vacuolar acid invertase (acid INV), whose activity appears to vary in an opposite manner than fructan accumulation (Tognetti et al. 1989). Sucrose decreased acid INV expression, while the combined addition of sucrose and OA led to a dramatic increase in the expression of this gene (Fig. 4). Small effects were found when PK inhibitor ST, calcium channel blockers or calmodulin antagonist were supplied together with sucrose (Fig. 4).

Possible changes of PP2A activity during the sucrose-mediated FSS induction process were studied. PP activity in sucrose-fed leaves drastically decreased after a brief initial lag period, while in water-fed leaves it also tended to decrease but at a slower rate (Fig. 5a). The effect of sucrose on PP2A activity seemed to be specific for this sugar since feeding with fructose or glucose at equimolar concentrations did not decrease PP2A activity after 24 h treatment, but instead a small increase was detected in relation to the initial value (t_0) (Fig. 5b). The reduction in PP2A activity along sucrose treatment occurred in parallel

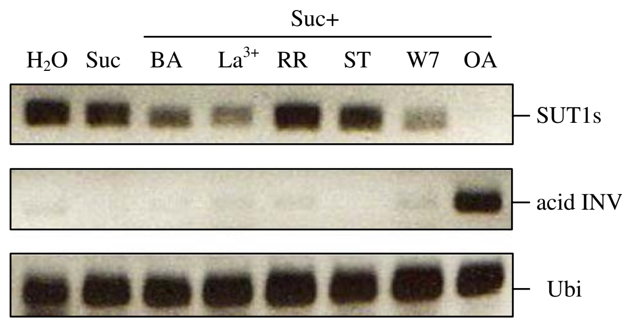

Fig. 4 Effect of OA in H^+-sucrose symporter of the plasma membrane and vacuolar acid invertase genes. Total RNA was isolated from sucrose-fed excised wheat leaf blades treated with or without 1 µM OA, 5 mM BA, 10 mM La^{3+}, 50 µM RR, 2 µM ST or 200 µM W7 after 24 h in darkness. RNA was subjected to reverse transcription, and the resulting cDNAs were amplified with specific primers for SUTs, acid INV, and Ubi by PCR. The PCR products were separated on agarose gels and stained with EtBr. Ubi gene expression was used as a loading control

with a decrease in the rate of sucrose accumulation in leaves (Fig. 5c).

Discussion

In wheat, fructan metabolism is induced when sucrose concentrations are high, such as during periods of low temperature or in general, when carbon fixation exceeds demand for growth. Sucrose then initiates intracellular events that lead to the induction of fructan synthesis. These events include an increase in cytosolic calcium levels and protein phosphorylation (Martínez-Noël et al. 2006, 2007). Although in plant tissues sucrose can be metabolized into fructose and glucose, hexoses do not produce the same induction of FSS activity as the disaccharide at equivalent molar concentrations. Also, mannose, which can be phosphorylated by hexokinase but is not metabolized, had no effect, thus suggesting that sucrose-sensing is independent of hexokinase sensor (Martínez-Noël et al. 2001). Therefore, sucrose seems to be the key molecule in this process.

This work focuses on the involvement of PP activity in the sucrose-mediated induction of enzymes leading to fructan synthesis in wheat leaves. We had previously shown that 1 µM OA inhibited FSS activity and 6-SFT expression (Martínez-Noël et al. 2001) in sucrose-fed wheat leaves. In the present study it was found that the external OA concentrations that caused a > 50% inhibition (Fig. 1) of FSS activity are within the range of those found in other systems to inhibit PP2A, but not PP1, which is 100-fold less sensitive to OA (Smith and Walker 1996; Rojo et al. 1998). Then, PP2A is most likely the PP involved in FSS induction by sucrose. OA blocked the sucrose induction of gene expression of both enzymes that

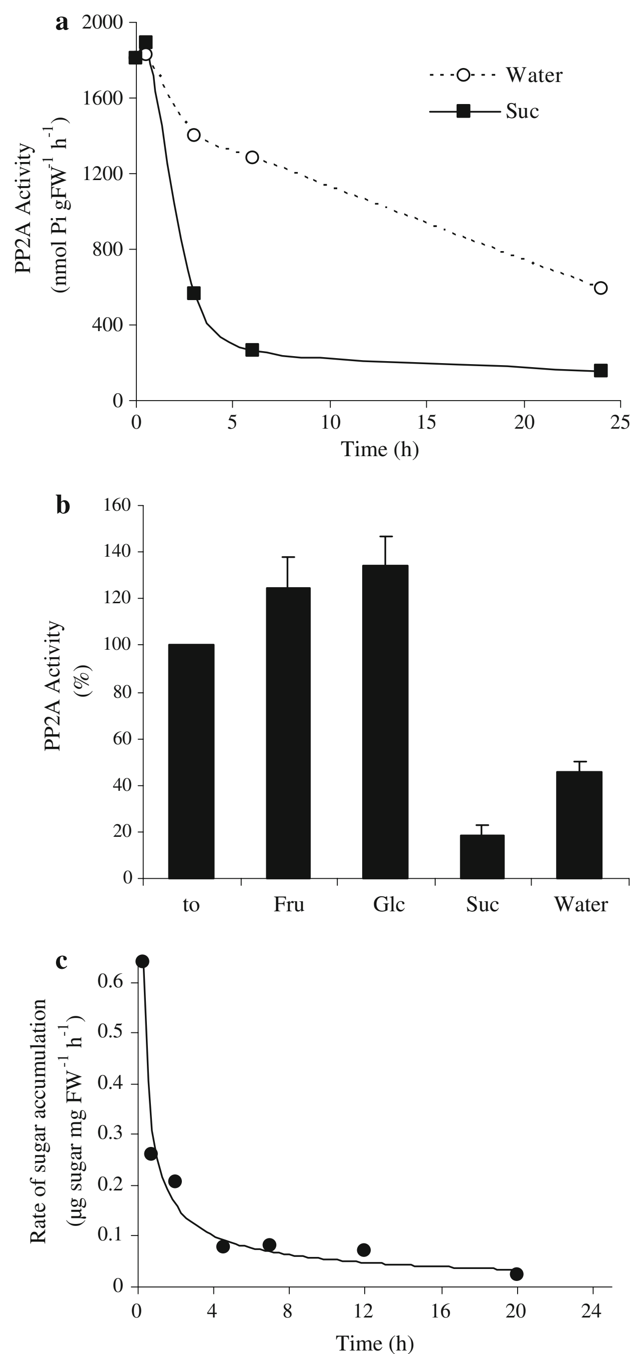

Fig. 5 Effect of sucrose on PP2A activity and sucrose accumulation rate in wheat leaves. **a** Time course of total PP2A activity extracted from wheat leaf blades fed with either 200 mM sucrose or water along 24 h of treatment. **b** Effect of different sugars (200 mM) on PP2A activity in wheat leaves treated for 24 h. **c** Sucrose accumulation rate in wheat leaves treated with the sugar for different periods

contribute to trisaccharide fructosyl sucrose synthesis (1-SST and 6-SFT; Fig. 2). In accordance with this, OA impaired fructan accumulation in sucrose-treated leaves (Fig. 3). These results show that PP2A activities are required for the sucrose signaling leading to fructan

synthesis induction. The participation of PP2A in this sugar-signaling process adds to the reported role of these enzymes in regulating the expression of other sugar-inducible genes in non-fructan-bearing species (Takeda et al. 1994; Siedlecka et al. 2003; Riou-Khamlichi et al. 2000; Ciereszko et al. 2001; Ciereszko and Kleczkowski 2002).

The PPs mode of action in sugar-inducible genes is largely unknown. Possible mechanisms include a direct effect in the transduction of sugar signal to the nucleus and/or an indirect effect, such us the sucrose uptake into the cells. The latter possibility is based on the fact that it has been reported that PPs regulate plasma membrane H^+-SUT activity and/or expression (Roblin et al. 1998; Ransom-Hodgkins et al. 2003). We tested if this could be the case in our system by adding OA to the sucrose-feeding solution. It was found that PP inhibitor led to a strong decrease in sugar accumulation of treated wheat leaves (Fig. 3). Low sucrose content in tissues treated with the PP inhibitor seems not to be due to cleavage of sucrose after its uptake into cells, since monosaccharide content of these leaves decreased in a similar proportion as sucrose. This suggests that PP2A activity could be required for sucrose uptake.

Sucrose uptake into cells is mediated by the H^+-symporting sucrose transporters (SUTs) (Sauer 2007). In wheat, three SUTs genes have been described, which belong to the SUT1 subfamily (Aoki et al. 2002, 2004). When we tested the effect of OA on SUT1 gene expression in sucrose-treated leaves, we showed that OA impaired SUT1 expression. This is in agreement with the results reported by Ransom-Hodgkins et al. (2003), who found that PP inhibitors decreased sugar beet sucrose symporter protein and mRNA abundance, as well as the transcription rate of the symporter gene. We also found an enhanced expression of vacuolar acid INV gene in wheat leaves treated with OA together with sucrose (Fig. 4). This finding, together with a reported OA-induced increase in Arabidopsis Sus1 gene expression (Ciereszko et al. 2001) and in oat starch degradation (Chang et al. 2001) suggest that PP activity might play a role in carbon partitioning to either storage or consumption. Evidence has been provided for a protein phosphorylation cascade operating between the sucrose-sensor and the transcriptional regulator that controls SUT1, and it has been reported that symporter message levels remained unchanged in sugar beet leaves fed with ST (Ransom-Hodgkins et al. 2003). Our data are in agreement with this effect (Fig. 4) and suggest that the model proposed by these authors may be also valid for grasses. Also, the involvement of calcium/calmodulin in this transduction pathway could not be discarded, but according to our results, a direct effect is not probable.

Taking together our results and results of previous studies, it appears that PP activity may be required for sucrose uptake into leaf tissues fed with this sugar. Ransom-Hodgkins et al. (2003) suggested a model where protein phosphorylation may mediate a key step in sucrose regulation of the sucrose symporter expression in *Beta vulgaris*. According to this, sucrose would interact with an unknown sucrose sensor which activates a PK that in turn acts as a negative regulator of a PP that is a positive regulator of *B. vulgaris* SUT1 transcription. Our results in wheat leaves are in accordance with this model, since we found that PP2A activity was reduced by sucrose (Fig. 5a), sucrose reduced the expression of SUT1 gene (Fig. 4), the inhibition of PP2A activity with OA also impaired sucrose accumulation (Fig. 3), and that decrease in PP2A activity along sucrose treatment occurred in parallel with a decreasing rate of sucrose accumulation in leaves (Fig. 5b). Alternatively, it is also possible that PP2A directly regulates SUT1 activity by maintaining it in a dephosphorylated active form, as proposed by Roblin et al. (1998). Following the proposed model, sucrose in the apoplast would rapidly enter the cells through the SUT1 symporter and then start the signaling pathway that regulates expression of fructan synthesizing enzymes (among many other genes). PP activity may be necessary for the expression of SUT1 gene and also probably to maintain the symporter in an active form. In a later phase, sucrose may inhibit PP2A activity, which could in turn lead to the inhibition of SUT gene expression and/or the inhibition of SUT activity. Because of this negative feedback, the rate of sucrose uptake would follow a decreasing trend and sucrose-mediated FSS induction would be reduced after an initial peak (in accordance with data of Fig. 2b). The decrease in PP2A activity with sucrose seems to be specific for this sugar since no similar effect was obtained with fructose and glucose. The reason why sucrose may lead to a decrease in PP2A activity is unknown, but we have shown previously that sucrose increases cytosolic calcium levels rapidly after feeding wheat leaves with this sugar (Martínez-Noël et al. 2006) and calcium has been suggested to inactivate PP2A activity in alfalfa cell suspensions (Monroy et al. 1998).

Okadaic acid-sensitive PP may also be involved in the intracellular sucrose-signaling process that leads to the induction of fructan synthesizing enzymes. Takeda et al. (1994) originally suggested such a role for PP in other processes regulated by sugars. As Rolland et al. (2002) stated, one of the most common mechanisms in signal transduction is protein phosphorylation and dephosphorylation, and the use of specific inhibitors has indicated the involvement of a variety of PKs and PPs in plant sugar signaling. In the case of fructan induction by sucrose, the involvement of PKs has been documented by previous work from our laboratory (Martínez-Noël et al. 2001, 2006, 2007). Besides, PP2A may be a general component of sucrose signaling pathway in plants and not just specific to

the regulation of fructan synthesis since it has been found that the wheat 6-SFT promoter in transgenic Arabidopsis (a plant that lacks fructan metabolism) was drastically inhibited by OA (G. Martínez-Noël et al., unpublished results). However, according to the present work, studies on the role of PPs should take into account the putative effect of OA on sucrose transport, which would render the pharmacological approach difficult.

The relationship between signal transduction and the induction of fructan metabolism by sucrose is undoubtedly very complex and there are many aspects which require further investigation. For instance, cytosolic calcium levels may be affected not only by sucrose but by OA-sensitive PPs as well, since calcium channel proteins at the plasma membrane may be regulated by reversible phosphorylation (Kuo et al. 1996). Also, calcium may take part in the induction of fructan metabolism by low temperatures. There are reports that show an increase in cytosolic calcium levels under cold stress (Monroy and Dhindsa 1995; Minorsky and Spanswick 2006).

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