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Changes in stomatal function and water use efficiency in potato plants with altered sucrolytic activity

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ABSTRACT

As water availability for agriculture decreases, breeding or engineering of crops with improved water use efficiency (WUE) will be necessary. As stomata are responsible for controlling gas exchange across the plant epidermis, metabolic processes influencing solute accumulation in guard cells are potential targets for engineering. In addition to its role as an osmoticum, sucrose breakdown may be required for synthesis of other osmotica or generation of the ATP needed for solute uptake. Thus, alterations in partitioning of sucrose between storage and breakdown may affect stomatal function. In agreement with this hypothesis, potato (*Solanum tuberosum*) plants expressing an antisense construct targeted against sucrose synthase 3 (SuSy3) exhibited decreased stomatal conductance, a slight reduction in CO_2 fixation and increased WUE. Conversely, plants with increased guard cell acid invertase activity caused by the introduction of the *SUC2* gene from yeast had increased stomatal conductance, increased CO_2 fixation and decreased WUE. $^{14}CO_2$ feeding experiments indicated that these effects cannot be attributed to alterations in photosynthetic capacity, and most likely reflect alterations in stomatal function. These results highlight the important role that sucrose breakdown may play in guard cell function and indicate the feasibility of manipulating plant WUE through engineering of guard cell sucrose metabolism.

Key-words: guard cell; invertase; sucrose synthase.

INTRODUCTION

Water stress and drought are currently one of the major causes of low yield and crop loss in worldwide agriculture (Boyer 1982; Valliyodan & Nguyen 2006). As water availability decreases, and the need to use additional drought-prone land for agriculture increases (Morison *et al*. 2008; Habash, Kehel & Nachit 2009) over the coming years, the breeding or engineering of crop species for increased water use efficiency (WUE) will be required (Condon *et al*. 2004). Stomata control the flow of water vapour and CO_2 into and out of the leaf (Kim *et al*. 2010), and thus stomatal function plays an important role in determining both the rate of net CO_2 fixation and WUE during photosynthesis (Condon *et al*. 2004). In C3 and C4 plants, stomata open during the day as the guard cells that form these pores accumulate solutes and consequently expand as they take up water by osmosis (Lawson 2009). Thus, understanding which solutes accumulate, the pathways by which they are accumulated and the mechanisms that regulate their accumulation are important if strategies for improving WUE of plants are to be developed.

Guard cells possess a number of routes by which they may accumulate the solutes necessary for stomatal opening, and the relative importance of these different routes varies throughout the day (Talbott & Zeiger 1998; Schroeder *et al*. 2001; Outlaw 2003), between species (Schnabl & Raschke 1980) and in different climates (Talbott & Zeiger 1996). Potassium (K^+), and its counter ions malate^{2-} and chloride, are typically considered the major osmolytes in the guard cells of open stomata (Outlaw 1983, 2003; Zeiger 1983). The uptake of K^+ through plasma membrane channels (Schroeder, Raschke & Neher 1987; Szyroki *et al*. 2001; Lebaudy *et al*. 2008) and the synthesis of malate are thought to be triggered by blue light in the morning (Assmann, Simoncini & Schroeder 1985; Shimazaki, Iino & Zeiger 1986). However, evidence exists to suggest that the changes observed in vacuolar ion content are too small to account for the osmotic changes required for stomatal opening (MacRobbie & Lettau 1980a,b; MacRobbie 1981). In agreement with this, sucrose has also been shown to accumulate in guard cells and make a significant contribution to total osmotic content. For example, in *Vicia faba* guard cells, K^+/malate is the major osmolyte in the morning, while sucrose reaches high levels in the afternoon as K^+/malate levels decline (Talbott & Zeiger 1996). At present, it is unclear how widespread this pattern is among plant species, and indeed whether sucrose may have other important functions within guard cells.

Several sources of carbon for the accumulation of these osmolytes have been identified. Starch is known to be broken down during the day in guard cells (Kang *et al*. 2007), but the resulting sugars seem to contribute mainly to malate synthesis (Outlaw & Lowry 1977; Outlaw & Manchester 1979). In addition, guard cells are able to take up sucrose produced by mesophyll cell photosynthesis from the apoplast (Dittrich & Raschke 1977), and this process is likely to be a significant contributor to guard cell carbon

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supply under certain conditions (Ritte *et al*. 1999). While guard cells contain chloroplasts, there is conflicting evidence regarding their ability to support sufficient rates of photosynthetic CO_2 fixation for this to make a significant contribution to carbon supply for stomatal opening (Lawson 2009).

The uptake and accumulation of sucrose and K^+, which require the operation of the plasma membrane ATPase to generate a H^+ gradient (Shimazaki *et al*. 1986; Serrano, Zeiger & Hagiwara 1988; Schwartz, Illan & Assmann 1991), place a significant energetic burden upon guard cells (Vavasseur & Raghavendra 2005). This demand is likely to be met in part by photosynthesis; it appears that guard cell chloroplasts help to maintain ATP (Tominaga, Kinoshita & Shimazaki 2001) and NADPH levels during solute accumulation (Zeiger *et al*. 2002). However, it is also clear that respiratory metabolism involving catabolism of sugars plays an important role in generating the energy required for stomatal opening. Statistical comparison of guard cell and mesophyll cell proteomes showed specific enrichment of respiratory enzymes within guard cells (Zhu *et al*. 2009), and furthermore the application of cyanide, an inhibitor of mitochondrial cytochrome oxidase, prevented stomatal opening in *V. faba* and *Commelina communis* (Schwartz & Zeiger 1984).

Thus, the fate of sucrose within guard cells, whether it is stored as osmoticum or broken down to meet energetic demands or produce other osmolytes, may impact upon stomatal function and ultimately affect whole plant WUE and drought tolerance. The enzymes sucrose synthase (SuSy), which catalyzes the reversible conversion of sucrose and UDP to UDP-glucose and fructose, and invertase, which catalyzes the irreversible breakdown of sucrose to glucose and fructose, are most likely to be responsible for sucrose breakdown within guard cells. The activities of these enzymes may therefore affect the partitioning of sucrose between storage and breakdown and hence influence guard cell osmoregulation. Indeed, isoform 3 of sucrose synthase (SuSy3) has previously been implicated in leaf responses to drought (Kopka, Provart & Müller-Röber 1997). This isoform represents up to 70% of total leaf SuSy activity (Meyer & Loureiro, unpublished results, 2003), is preferentially expressed in guard cells relative to other leaf cell types (Hite, Outlaw & Tarczynski 1993) and its expression is known to increase under drought conditions (Kopka *et al*. 1997). The function of SuSy in leaf metabolism is currently unclear, as the majority of sucrose synthesis is believed to be mediated by sucrose phosphate synthase (SPS), and no role for sucrose degradation in mesophyll cells is known. One possibility is the involvement of SuSy in a futile cycle of sucrose synthesis and degradation in leaves (Geigenberger & Stitt 1991; Nguyen-Quoc & Foyer 2001), but the physiological significance of this process has not been demonstrated.

To test whether altered sucrolytic activity affects stomatal function, and to further investigate the function of SuSy3 in leaves, transgenic potato plants expressing an antisense construct targeted against SuSy3, or expressing yeast acid invertase specifically within guard cells, were generated. The effects of these modifications on gas exchange, leaf biochemistry and WUE were then determined.

MATERIALS AND METHODS

Plant growth and transformation

For all plant transformation, leaf explants (1 cm^2) of *Solanum tuberosum* L. (cv. Désirée) from plants grown under aseptic conditions were inoculated with transformed *Agrobacterium tumefaciens* (Strain LBA 4404). For antisense repression of sucrose synthase isoform 3 (SS3), *A. tumefaciens* carried a pBinAR-Kan vector containing a 1.5 kb fragment of the *SS3* gene (STU24088) in the antisense orientation under the control of the CaMV 35S promoter. For guard cell-specific expression of the yeast *SUC2* (acid invertase) gene, the *A. tumefaciens* carried a pBin19 vector, where the CaMV 35S promoter was excised and replaced with a 0.3 kb promoter fragment (S1Δ4) from a potato *AGPase* gene. This promoter has previously been demonstrated to drive guard cell-specific gene expression (Müller-Röber *et al*. 1994). Regenerated (T0) plants were selected in MS medium supplemented with 50 mg L^{-1} kanamycin sulfate. Rooted, transformed (confirmed by PCR, data not shown) plants were transferred to soil. For all experiments, potato plants originating from tubers of around 20 g were grown in pots containing 5 dm^3 of a mixture of soil, sand and cattle manure (3:2:1), and with fertilizer as necessary. Irrigation was carried out to maintain the moisture of the soil close to the capacity of the pot. Control plants in all experiments were untransformed [wild type (WT)] potato plants of the same cultivar (Désirée) as the transgenic lines.

Gas exchange measurements

Gas exchange measurements were made on young, completely expanded leaves from 25-, 35- and 55-day-old plants using an infrared gas analyser (LI 6400, Li-Cor, Lincoln, NE, USA) equipped with a source of photosynthetically active radiation (PAR; 1000 μmol m^{-2} s^{-1} for measurements made during the daytime). Temperature was kept constant and close to the appropriate ambient temperature for each time point. All measurements were made under an air flow of 500 cm^3 min^{-1} and ambient CO_2 and relative humidity levels.

To measure the response of photosynthetic carbon assimilation to light, a decreasing level of PAR (1000, 800, 500, 100, 50, 10 and 0 μmol m^{-2} s^{-1}) was applied, while the temperature was maintained at 27 °C and the concentration of CO_2 at 360 μL L^{-1}. To measure the response of photosynthetic CO_2 assimilation rate to CO_2 levels, leaves were exposed to a sequence of CO_2 concentrations (400, 300, 200, 100, 50, 400, 600, 800 and 1000 μL L^{-1}) at constant PAR (1000 μmol m^{-2} s^{-1}) and temperature (27 °C).

Radiolabelling

Analysis of uptake and redistribution of radioactivity from $^{14}CO_2$ by leaf discs was performed according to DaMatta *et al*. (2008). Four hundred μL of $NaH^{14}CO_3$ (100 Bq μL^{-1}) with 10 μL carbonic anhydrase (0.5 U μL^{-1}) was incubated with a leaf disc in a Clark-type oxygen electrode at 30 °C and with a PAR level of 1300 μmol m^{-2} s^{-1}. After 30 min incubation, samples were frozen in liquid N_2. Soluble metabolites were extracted using hot ethanol and fractionated into neutral (sugars), basic (amino acids) and acidic (organic acids) components using ion exchange chromatography. Radioactivity in these fractions, and in the ethanol insoluble fraction containing starch and cell wall, was measured by liquid scintillation counting.

Epidermal fragment preparation and analysis

Epidermal fragments were prepared from leaves of potato plants according to the method of Kopka *et al*. (1997). This approach is known to produce samples in which more than 95% of living cells are guard cells (Kopka *et al*. 1997). Soluble sugars were extracted using ethanol, and quantified according to the method of Stitt *et al*. (1989). Enzymes were extracted and assayed as described previously.

Determination of enzyme activity in leaves

For determination of enzyme activities in leaves and epidermal fragments, previously frozen leaf discs or fragments were extracted and desalted using gel filtration chromatography according to Stitt *et al*. (1989). Protein concentration in extracts was measured using the method of Bradford (1976), with a standard curve constructed using bovine serum albumin (BSA). SuSy (EC 2.4.1.13) activity was measured in the direction of sucrose degradation according to the method of Zrenner *et al*. (1995). Acid invertase (EC 3.2.1.26) activity was determined in the direction of sucrose degradation based on the quantity of glucose liberated (Geigenberger & Stitt 1993; Zrenner *et al*. 1995). The maximum activity of SPS (EC 2.4.1.14) was determined according to Huber, Huber & Nielsen (1989) in the direction of formation of sucrose phosphate. Frutose-1,6-P_2 bisphosphatase (FBPase; 3.1.3.11) activity was determined in the direction of F6P formation according to Kruger & Beevers (1984), and ADP glucose pyrophosphorylase (AGPase; 2.7.7.27) activity was measured in the direction of G1P formation using a method based on Müller-Röber *et al*. (1992).

Determination of invertase activity in epidermal fragments

For determination of invertase activity in epidermal fragments, epidermal fragments were homogenized in liquid nitrogen, and 5× extraction buffer (250 mM Hepes-NaOH pH 7.4, 25 mM $MgCl_2$, 5 mM EDTA, 5 mM EGTA, 2.5 mM PMSF, 50% glycerol, 0.5% Triton X-100) was added to the frozen powder at a 1:4 (v/v) ratio. Samples were thawed on ice, centrifuged at 15 000 *g* for 10 min at 4 °C and protein concentration in the supernatant was assayed after Bradford (1976). Samples were concentrated approximately 10-fold using Centricon-3 concentrators (Millipore, Billerica, MA, USA) according to the manufacturer's instructions. 'Half-native' gel electrophoresis was carried out using around 400 μg protein; sample loading buffer (250 mM Tris-HCl pH 6.8, 0.8% SDS, 20% v/v β-mercaptoethanol, 40% v/v glycerol and 0.01% v/v Bromophenol Blue) was added to samples at a 4:1 (v/v) ratio and protein separated using a 7.5% (w/v) SDS polyacrylamide gel. Gels were stained for total protein using Coomassie Blue. Invertase activity was then assessed in gel according to von Schaewen *et al*. (1990). Briefly, gels were incubated for 30 min at 30 °C in a solution of 0.1 M sucrose, 0.1 M sodium acetate pH 5.0, washed three times with distilled water and incubated in a solution of 0.1% (w/v) TPTC and 0.5 M NaOH preheated to approximately 95 °C. Gels were heated further in this solution using a microwave oven until bands appeared, at which point the reaction was stopped by incubation of the gel in 10% acetic acid.

Metabolite assays

Leaf discs were sampled at regular intervals throughout the day from young and completely expanded leaves, immediately frozen in liquid nitrogen and stored at –80 °C until further analysis. Soluble sugars were extracted according to Trethewey *et al*. (1998) from around 120 mg fresh weight (FW) of tissue using 80% (v/v) ethanol. Starch was then extracted from the washed ethanol insoluble pellet by heating to 95 °C for 90 min with 400 μL 0.2 M KOH. Seventy μL 1 M acetic acid was then added and the mixture was centrifuged, the supernatant then being retained for starch analysis. Glucose, fructose and sucrose levels were determined enzymatically according to Stitt *et al*. (1989) using an enzyme-linked immunosorbent assay (ELISA) plate reader. Levels of starch were measured by firstly incubating an aliquot of starch extract at 55 °C for 1 h with 30 μL 300 mM citrate buffer (pH 4.6) and 2 U amyloglucosidase in a final volume of 300 μL. This mixture was then assayed enzymatically for glucose released from starch.

Glycolytic intermediates were extracted according to Jelitto *et al*. (1992) from around 500 mgFW leaf material using trichloroacetic acid (TCA). Levels of glucose 6-phosphate, fructose 6-phosphate and glucose 1-phosphate were determined using cycling assays as described in Gibon *et al*. (2002). 3-PGA was quantified according to the method of Stitt *et al*. (1989) and Pi using the method of Penney (1976). Levels of ATP, ADP, UTP, UDP and UDP-glucose were determined using high-resolution liquid chromatography (Regierer *et al*. 2002).

^{13}C composition measurements

Two expanding leaves from each plant ($n = 5$) were used for ^{13}C determination. The leaves were dried at 70 °C and then ground to powder. ^{13}C determination was performed

according to DaMatta *et al*. (2003) in a specialized laboratory, with Pee Dee Belemnite as a standard.

Statistical analysis

Statistical analysis was carried out using the software Statistica (StatSoft Inc., Tulsa, OK, USA). Differences between groups were considered significant based on a P value of <0.05 (Duncan's test) except where indicated otherwise.

RESULTS

Generation of plants with altered leaf sucrolytic activity

In order to investigate the effect of altered sucrolytic (enzymatic sucrose cleavage) activity on stomatal function, two transgenic strategies were adopted. Firstly, transgenic lines with decreased sucrolytic activity were generated by expressing a 35S::SuSy3 antisense construct targeting the SuSy3 isoform of SuSy. Seven lines were confirmed to have lower levels of *SS3* mRNA, and clear expression of the antisense transgene, in leaves and tubers (data not shown). Lines SS3 10, SS3 30, SS3 54 and SS3 57 were then selected for further study. Measurements of enzyme activity in epidermal fragments isolated from leaves revealed significant decreases in SuSy activity in three of the transgenic lines (Table 1) relative to WT, while acid invertase activity was unchanged.

Secondly, lines with increased guard cell sucrolytic activity were generated by expressing the *SUC2* (acid invertase) gene from yeast under the control of the guard cell-specific AGPase promoter S1Δ4 (Müller-Röber *et al*. 1994). Lines INV 4, INV 7 and INV 72 were selected for further study on the basis of invertase activity gel analysis of protein extracts from whole leaves of tissue-cultured plants (data not shown). The guard cell specificity of *SUC2* gene expression was tested using enzyme activity assays. Activity gels indicated an increase in acid invertase activity within epidermal fragments isolated from the transgenic lines (Supporting Information Fig. S1), while no change in whole leaf invertase activity relative to WT plants was detected using activity gels (data not shown) or using standard enzyme assays (Supporting Information Fig. S2a), confirming the guard cell specificity of the S1Δ4 promoter.

Gas exchange parameters in transgenic plants

In order to determine whether alterations in leaf/guard cell sucrolytic activity affected stomatal function, gas exchange parameters were measured for both the SUC2 expression lines and SuSy3 antisense lines.

In all SuSy3 antisense lines, stomatal conductance was significantly reduced, by a maximum of around 60%, at 0800 and 1200 h in 35-day-old plants grown under greenhouse conditions (Fig. 1a). Similar results were obtained for the same plants at 25 and 55 d growth (Supporting Information Fig. S3a,c). This decrease in stomatal conductance was accompanied by a small but significant decrease in net CO_2 assimilation rate in lines SS3 10, SS3 30 and SS3 54 at 0800 h and in lines SS3 10 and SS3 54 at 1200 h for 35-day-old plants (Fig. 1c) under greenhouse conditions. A similar pattern was observed at 25 and 55 d growth (Supporting Information Fig. S3b,d). This decrease in net CO_2 assimilation rate relative to WT plants was also seen in lines SS3 10, SS3 30 and SS3 57 when leaves were subjected to a range of different light intensities (Fig. 1e). Transpiration rate at 1200 h was also reduced in the transgenic plants, with a significant decrease (maximum, 35%) in lines SS3 10 and SS3 54 in 25- and 35-day-old plants and in lines SS3 10 and SS3 30 in 55-day-old plants (Supporting Information Fig. S4).

In plants with expression of yeast acid invertase in guard cells (SUC2 lines), the opposite effects on gas exchange parameters to those in SuSy3 antisense plants were observed. Stomatal conductance was increased by around 35% relative to WT plants in line INV 4, the line showing higher invertase activity of the two (Supporting Information Fig. S1), at 0800 and 1200 h (Fig. 1b). These changes were paralleled by an increase in net CO_2 assimilation rate in line INV4 at 0800 h and in lines INV4 and INV 72 at 1600 h under greenhouse conditions (Fig. 1d). The same effect on net CO_2 assimilation rate was also observed in both lines under a range of different light intensities (Fig. 1f). Measurements of the response of net CO_2 assimilation rate to altered leaf internal CO_2 concentration revealed no difference in CO_2 assimilation rate between WT plants and SUC2 expression lines (Supporting Information Fig. S5).

WUE of transgenic plants

Altered stomatal conductance, transpiration rate or net CO_2 assimilation rate may result in changes in WUE, and this was therefore tested in both SuSy3 antisense lines and SUC2 lines. Increases in instantaneous WUE of between 25 and 50%, calculated from the ratio of net CO_2 assimilation rate to transpiration rate, were observed for all SuSy3 antisense lines at 0800 h (Fig. 2), the time at which stomatal conductance also reached its maximum (Fig. 1a), and

Table 1. Invertase and SuSy activity in epidermal fragments isolated from SuSy3 antisense and wild-type (WT) potato plants

	WT	SS3 10	SS3 30	SS3 54	SS3 57
SuSy	42.61 ± 2.59	43.57 ± 1.40	**23.39 ± 2.43**	**22.82 ± 1.64**	**22.73 ± 3.44**
Invertase	284.1 ± 34.0	214.2 ± 22.7	247.7 ± 26.8	256.8 ± 58.5	314.0 ± 36.6

Values are mean (nmol min^{-1} mg^{-1} protein) and standard error of the mean ($n = 5$). Statistically significant differences ($P < 0.05$, Duncan's test) are indicated in bold.

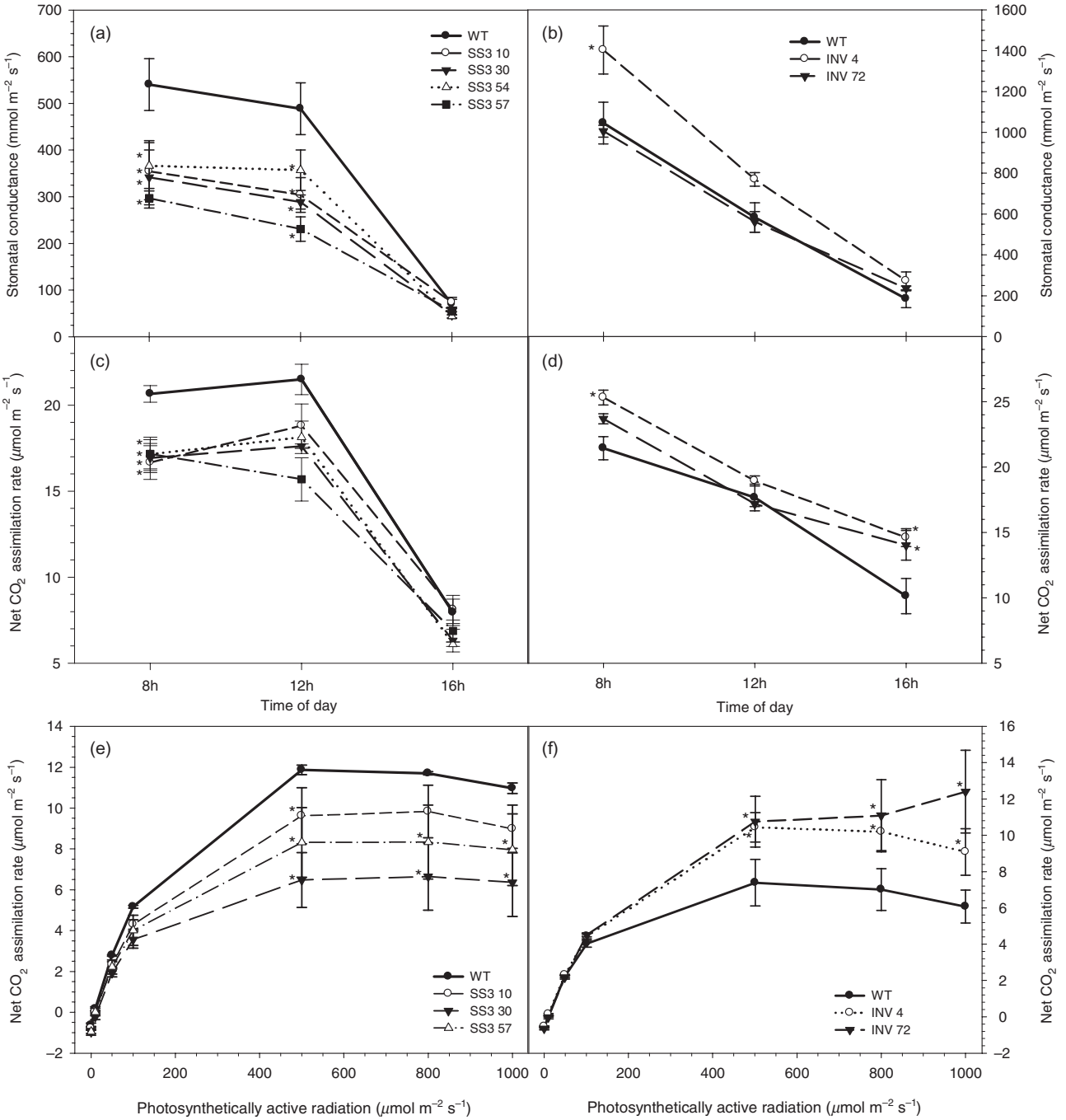

Figure 1. Gas exchange parameters in wild-type (WT), SuSy3 antisense (Lines SS3 10, 30, 54, and 57) and SUC2 overexpressing (lines INV 4 and INV 72) plants. (a) Stomatal conductance in WT and SuSy3 antisense lines throughout the day. (b) Stomatal conductance in WT and SUC2 expressing lines throughout the day. (c) Net CO_2 assimilation rate in WT and SuSy3 antisense lines throughout the day. (d) Net CO_2 assimilation rate in WT and SUC2 expressing lines throughout the day. (e) Net CO_2 assimilation rate in WT and SuSy3 antisense lines under different light conditions. (f) Net CO_2 assimilation rate in WT and SUC2 expressing lines under different light conditions. All measurements were made on approximately 35-day-old plants. Values represent the mean and standard error of the mean ($n = 5$). Asterisks indicate statistically significant differences between transgenic lines and WT plants ($P < 0.05$, Duncan's test).

the mean WUE calculated over the entire day was also significantly higher in the transgenic lines (Fig. 2, inset).

^{13}C isotope composition, an indicator of long-term WUE and stomatal conductance, was significantly increased (i.e. became less negative) in SuSy antisense lines SS10, SS30 and SS57 (Fig. 3a). The opposite trend was observed in the SUC2 lines, with decreased ^{13}C isotope composition in INV 4 and INV 72 (Fig. 3b). These changes indicate increased WUE in the SuSy antisense (i.e. reduced stomatal

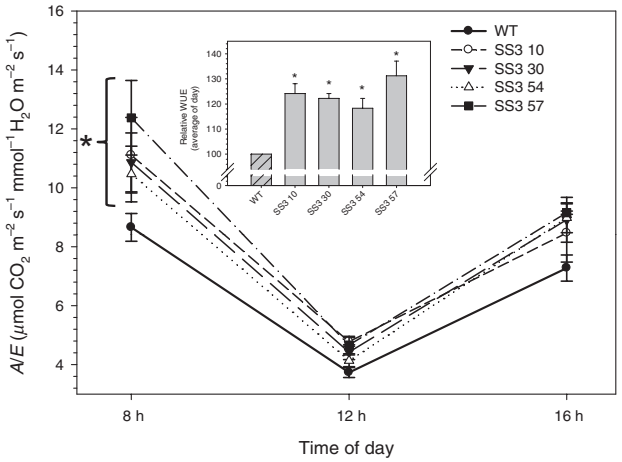

Figure 2. Instantaneous water use efficiency (WUE) in wild-type (WT) and SuSy3 antisense plants throughout the day. All measurements were made on 35-day-old plants. Values represent the mean and standard error of the mean ($n = 5$). Inset, the mean WUE over the entire day, relative (%) to control plants (WT). At each time point, average points followed by asterisks differ significantly from the control ($P < 0.05$; Dunnett's test). Line SS3 10 at 12 h and line SS3 57 at 16 h also showed significant differences from control plants; asterisks are omitted for clarity.

conductance) lines and decreased WUE in the SUC2 (i.e. increased stomatal conductance) lines.

Biochemical characterization of transgenic plants

Enzyme activities, metabolite abundances, and metabolic fluxes in SuSy3 antisense and SUC2 plants were measured as a means to relate any biochemical changes to the effects observed on gas exchange and WUE.

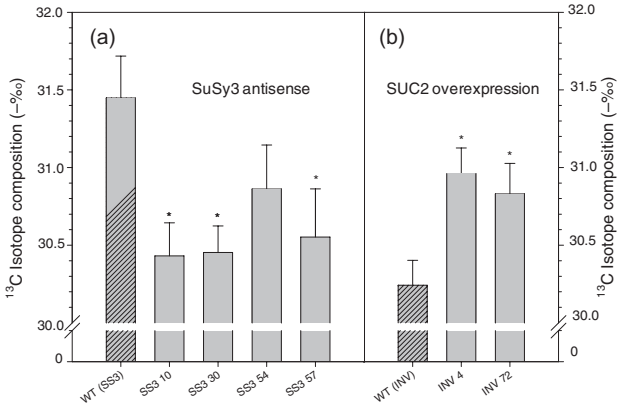

Figure 3. ^{13}C isotope composition of wild-type (WT), SuSy3 antisense (a) and SUC2 expressing lines (b). Values represent the mean and standard error of the mean ($n = 5$). Asterisks indicate transgenic lines differing significantly from WT ($P < 0.05$; Dunnett's test). $n = 5 \pm$ SE.

Measurements of enzyme activity in leaves revealed a 20–70% reduction in total SuSy activity in all SuSy3 antisense lines relative to WT plants at 0800, 1200 and 1600 h (Fig. 4a). In addition, total leaf acid invertase activity was significantly reduced in all SuSy3 lines by 20 to 55% at 0800, 1200 and 1600 h (Fig. 4b). Activities of AGPase, SPS and FBPase exhibited fewer and less dramatic alterations in SuSy3 antisense plants relative to WT plants (Supporting Information Table S1). The significant changes ($P < 0.05$, $n = 5$, Duncan's test) detected were (1) an increase in AGPase of around 22% in line SS3 10 at 1600 h; (2) a decrease in FBPase activity of around 15% in lines SS3 30 and SS3 57 at 1600 h; and (3) increases in SPS activity of around 30% in line SS3 54 at 0800, 1200 and 1600 h and in line SS3 30 at 0800 and 1600 h (Supporting Information Table S1).

The abundance of several metabolites was also altered in leaves from SuSy3 antisense plants. Sucrose levels were slightly increased in several of the SuSy3 antisense lines relative to WT at several points during the day (Fig. 5a). Levels of starch in leaves of SuSy3 antisense plants were increased relative to WT plants towards the end of the day

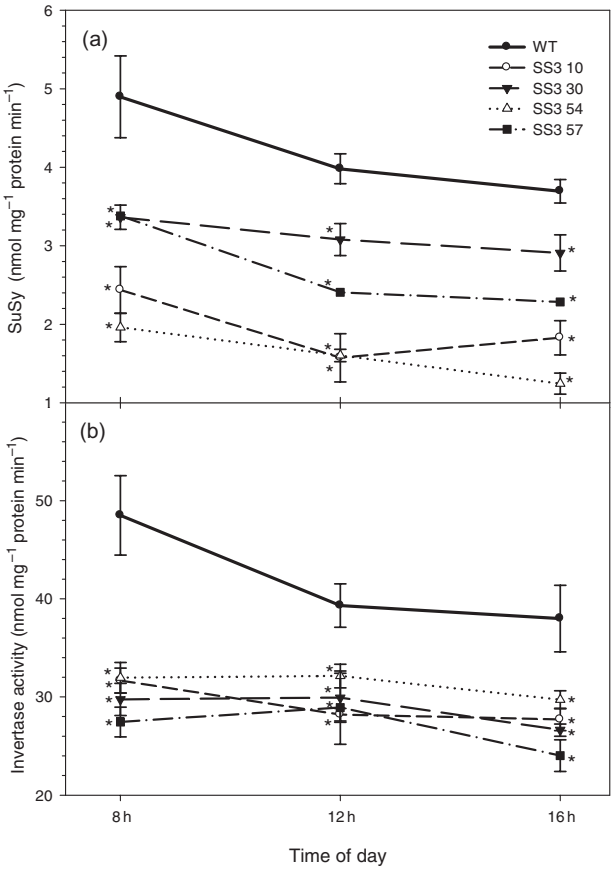

Figure 4. (a) SuSy and (b) invertase activity in whole leaves of SuSy3 antisense and wild-type (WT) plants throughout the day. Values are mean and standard error of the mean ($n = 5$). Asterisks indicate statistically significant differences between the SuSy3 antisense line and wild-type plants at the indicated time ($P < 0.05$, Duncan's test).

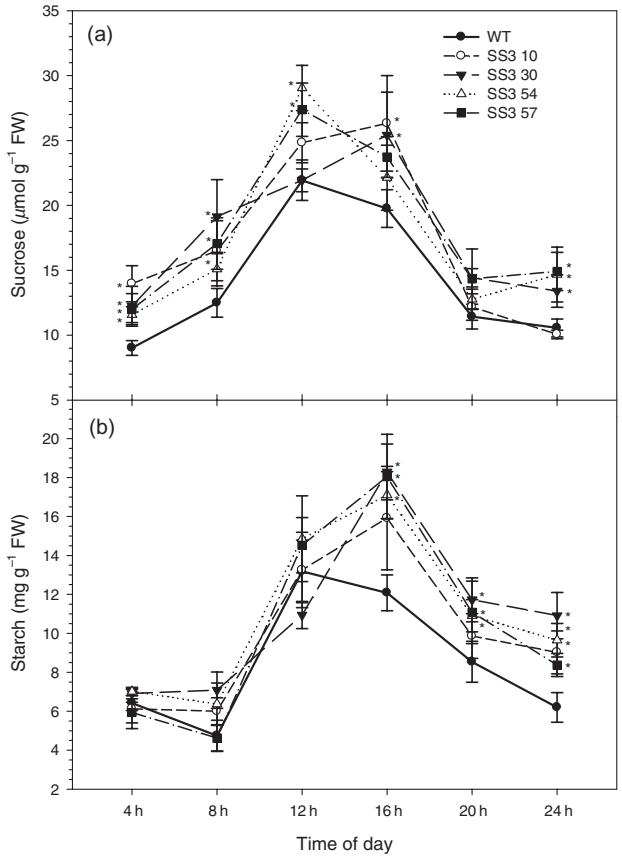

Figure 5. (a) Sucrose and (b) starch abundance in leaves of SuSy3 antisense and wild-type (WT) plants throughout the day. Values are mean and standard error of the mean ($n = 5$). Asterisks indicate statistically significant differences between the SuSy3 antisense line and WT plants at the indicated time ($P < 0.05$, Duncan's test).

(Fig. 5b); all lines showed a significant increase in starch content at 1600 and 2400 h, while levels were also increased at 2000 h in lines SS3 10, SS3 54 and SS3 57. Glucose and fructose levels in leaves were unchanged relative to WT throughout the day (data not shown) apart from in line SS3 30 where decreases in both these sugars were observed at 1200 h that may be related to an elevated rate of respiration detected in this line (data not shown). Measurements of adenylate, uridenylate and 3-PGA levels in leaves at midday revealed no differences between WT and SuSy3 antisense lines (Supporting Information Table S2), while levels of Pi were significantly reduced ($n = 5$, $P < 0.05$, Duncan's test) in lines SS3 30, SS3 54 and SS3 57 by 22, 26 and 24% respectively (Supporting Information Table S2).

In order to determine whether these changes in whole leaf enzyme activities and metabolite abundances were accompanied by changes in metabolic flux, uptake and redistribution of label from $^{14}CO_2$ into metabolites and biomass components were investigated in leaf discs of WT and SuSy3 antisense plants. These experiments revealed no significant changes in ^{14}C incorporation, with the exception of a 7% increase in incorporation into starch/cell wall in line SS3 30 (Table 2a).

In contrast to SuSy3 antisense plants, total leaf activity of SuSy and invertase (Supporting Information Fig. S2a,b) was unaltered in the SUC2 (increased guard cell acid invertase) lines, reflecting the guard cell specificity of the promoter used. FBPase and AGPase activity throughout the day was also unchanged (data not shown). Measurement of glucose, fructose, sucrose and starch in whole leaves at 1200 h revealed only a small increase in fructose levels in line INV 72 (data not shown). Measurement of phosphorylated metabolites in whole leaves revealed significantly increased total hexose-phosphate levels in lines INV 4 and INV 72 (391.8 and 334.9 nmol·gFW^{-1} respectively) compared with WT plants (235.2 nmol·gFW^{-1}, $P < 0.05$, $n = 5$, Duncan's test). Additionally the ratio of 3-PGA/Pi was

Table 2. Incorporation of $^{14}CO_2$ and redistribution of label in leaf discs from wild-type and SuSy3 antisense (a) or wild-type and SUC2 expressing (B) plants

	Total incorporation	Redistribution (%)			
Line	Bq cm^{-2}	Neutral/soluble sugars	Acid/organic acids	Basic/amino acids	Insoluble/starch and cell wall
(a) 35S:SuSy3					
WT	57.8 ± 3.7	45.7 ± 0.7	15.9 ± 0.3	0.7 ± 0.1	37.6 ± 0.4
SS3 10	52.9 ± 4.3	43.7 ± 1.8	17.4 ± 1.5	0.7 ± 0.1	38.2 ± 1.1
SS3 30	55.6 ± 2.0	41.6 ± 1.1	17.9 ± 1.8	0.8 ± 0.1	38.6 ± 0.5
SS3 54	60.2 ± 2.5	42.8 ± 1.3	16.2 ± 1.5	0.7 ± 0.1	**40.3 ± 1.0**
SS3 57	56.1 ± 4.8	45.3 ± 2.2	14.1 ± 2.9	0.6 ± 0.0	38.6 ± 0.5
(b) S1Δ4:SUC2					
WT	207.6 ± 10.7	46.4 ± 1.4	13.5 ± 1.0	1.8 ± 0.3	38.3 ± 0.5
INV 4	198.6 ± 21.6	**41.9 ± 1.0**	14.4 ± 0.9	1.9 ± 0.2	**41.8 ± 1.3**
INV 72	196.0 ± 20.9	45.2 ± 0.6	14.6 ± 1.1	2.3 ± 0.2	37.9 ± 0.8

Values are mean ± standard error of the mean ($n = 5$). Significant differences between wild type and transgenics are indicated in bold and underlined ($P < 0.05$, Duncan's test).

significantly higher in leaves of line INV 72 (0.207) compared with WT (0.124, $P < 0.05$, $n = 5$, Duncan's test).

Given the guard cell specific nature of SUC2 expression in these lines, metabolite abundances in epidermal fragments prepared from leaves at 1200 h were also determined (Supporting Information Fig. S6). Levels of sucrose were unaltered in fragments extracted from lines INV 72 and INV 4 relative to those from WT plants. However, glucose levels in epidermal fragments were increased in lines INV 4 ($P < 0.1$, *t*-test, $n = 6$) and INV 72 ($P < 0.05$, *t*-test, $n = 6$), and fructose levels were increased in line INV 72 ($P < 0.1$, *t*-test, $n = 6$). This led to increases in the ratio of total hexoses to sucrose in lines INV 4 (0.61 ± 0.17) and INV 72 (0.39 ± 0.07) relative to WT (0.28 ± 0.03, significant at $P < 0.1$, *t*-test, $n = 6$).

Uptake and incorporation of radiolabel from $^{14}CO_2$ was measured in leaf discs of WT and SUC2 expressing lines (Table 2b). This experiment revealed no change in $^{14}CO_2$ uptake or in label incorporation into organic acids or amino acids in lines INV 4 and INV 72. However, in line INV 4, two significant changes were observed; a 10% decrease in label incorporation into total soluble sugars which was paralleled by a 9% increase in incorporation into starch and cell wall.

DISCUSSION

Stomatal function is affected in plants with altered sucrolytic activity

In order to investigate the role of sucrose as osmoticum and substrate in guard cells, and to further elucidate the function of SuSy3, an isoform that may play a specific role in stomatal function (Hite *et al*. 1993; Kopka *et al*. 1997), two transgenic approaches were adopted. Firstly, potato (*S. tuberosum*) plants were generated with decreased SuSy3 activity using an antisense approach (Table 1, Fig. 4a). Secondly, guard cell acid invertase activity was increased by expression of the yeast *SUC2* gene under control of a guard cell specific promoter (Supporting Information Figs S1 & S2).

Gas exchange parameters followed a diurnal pattern typically observed in a tropical climate (Chaves *et al*. 2008) but also revealed significant differences between the transgenic lines and WT plants. Decreases in stomatal conductance, net CO_2 assimilation rate and transpiration were observed in SuSy3 antisense lines (Fig. 1a,c; Supporting Information Figs S3 & S4). The converse was recorded for SUC2 lines, with increased stomatal conductance and net CO_2 assimilation rate (Fig. 1b,d). In both cases, the effects on stomatal conductance were detected under a range of light conditions (Fig. 1e,f). These data strongly suggest alterations in stomatal function in the SuSy3 antisense and SUC2 expressing lines that might in turn be caused by altered sucrolytic activity in guard cells.

The changes described previously might also plausibly be explained by alterations in total capacity for mesophyll cell CO_2 assimilation in the transgenic lines, perhaps associated with the changes in leaf enzyme activities and metabolite abundances discussed next. However, $^{14}CO_2$ feeding experiments, carried out under saturating levels of CO_2 where stomata present no barrier to CO_2 assimilation, revealed no change in CO_2 uptake or its partitioning into different metabolites in the SuSy3 antisense lines (Table 2a). Similarly, there were no changes in $^{14}CO_2$ incorporation in the SUC2 expressing lines, though partitioning between soluble sugars, cell wall and starch was slightly altered in line INV 4 (Table 2b). Furthermore, the response of net CO_2 assimilation rate to changes in internal leaf CO_2 was unaltered in these lines (Supporting Information Fig. S5). These results indicate that there was no change in mesophyll capacity for CO_2 assimilation in either the SuSy3 antisense or in the SUC2 expressing lines. Alterations in mesophyll conductance between WT and transgenic lines could also potentially contribute to the differences in rates of CO_2 assimilation that were detected under greenhouse conditions (Fig. 1c,d). However, given that these alterations were also accompanied by significant changes in stomatal conductance (Fig. 1a,b), and that the response of CO_2 assimilation rate to alterations in light level at low light levels (Fig. 1e,f, linear portion) is identical in the WT and transgenic plants, this appears an unlikely explanation. We therefore conclude that the measured changes in gas exchange in both the SuSy3 antisense plants and SUC2 plants are highly likely to arise from altered stomatal function.

Further evidence for a specific effect on stomatal function in the SuSy3 antisense and SUC2 expressing lines derives from the measurements of ^{13}C composition (Fig. 3a). A lower (more negative) percentage ^{13}C, as occurred in the SUC2 expressing lines, is indicative of increased leaf internal CO_2 (Fig. 3b; Condon *et al*. 2002), while increased percentage ^{13}C indicates a decreased CO_2 concentration. As alterations in mesophyll conductance in the transgenics appear unlikely, these changes in leaf CO_2 are likely to arise from decreased stomatal aperture in the case of the SuSy3 antisense plants and increased aperture in the SUC2 expressing plants (Condon *et al*. 2004; Roussel *et al*. 2009).

Plants with altered sucrolytic activity show changes in WUE

The changes in gas exchange parameters observed, most likely arising from altered stomatal function, in the SuSy3 antisense lines and SUC2 lines appear in turn to have altered the instantaneous and/or long term WUE of these lines. Instantaneous (or short-term) WUE is the ratio of CO_2 assimilated to H_2O transpired as determined at a single time point though gas exchange measurements and SuSy3 antisense lines showed an increase in instantaneous WUE compared with WT plants (Fig. 2). Long-term WUE, the ratio of CO_2 assimilated to H_2O transpired over the period of growth of the tissue, is indicated by ^{13}C isotope composition. If alterations in mesophyll conductance can be excluded (see above) for a given rate of CO_2 fixation,

decreased stomatal conductance (i.e. conditions typically associated with increased WUE) will tend to lead to decreased internal CO_2 levels. Due to the slight preference of Rubisco for $^{12}CO_2$ over $^{13}CO_2$ at high internal CO_2 levels, this will lead to increased $^{13}CO_2$ incorporation and increased total ^{13}C (Condon *et al*. 2004). Consistent with instantaneous WUE measurements, leaf ^{13}C composition measurements indicate increased long-term WUE in the SuSy3 antisense lines and decreased long-term WUE in the SUC2 expressing lines (Fig. 3a,b; Impa *et al*. 2005). These changes in WUE are also consistent with the short-term measurements of net CO_2 fixation rate and stomatal conductance. For example, in the SuSy3 antisense lines, stomatal conductance was decreased by a greater extent than net CO_2 fixation rate (Fig. 1a,c), suggesting that WUE should be increased.

These results raise the possibility of engineering plants for increased WUE, and hence perhaps improved drought tolerance, through manipulation of sugar metabolism in guard cells. Such manipulations would not be limited to enzymes catalyzing sucrose cleavage or synthesis directly, and could target pathways responsible for sucrose import into guard cells, its transport into the vacuole or the demand for its breakdown. However, while it has been possible to breed plants for increased long-term WUE on the basis of decreased ^{13}C isotope discrimination, this trait was also often linked to reduced photosynthesis or productivity (Condon & Richards 1993; Centritto *et al*. 2009). Furthermore, drought tolerance is a quantitative trait that is strongly affected by the environment (Condon *et al*. 2004). Consequently, whether the transgenic lines described here that exhibited increased WUE also exhibit improved tolerance to this abiotic stress needs to be tested under field conditions, in different environments and across multiple growing seasons.

The effect of decreased SuSy activity on leaf metabolism

Decreased SuSy activity in the SuSy3 antisense plants (Table 1) was accompanied by additional changes in enzyme activities and metabolite abundances (Figs 4 & 5, Supporting Information Table S1) that may shed some light upon the role of SuSy3 in leaves. Both guard cell (Table 1, SS30, SS54, SS57) and whole leaf SuSy activities were reduced in the SuSy3 antisense plants (Fig. 4a). This is expected given the non-cell-specific nature of the antisense construct and the fact that SuSy3 is known to be the dominant isoform of SuSy in leaves (Meyer & Loureiro, unpublished observations, 2003). However, invertase activity was also significantly lower in leaves from SuSy3 antisense plants than from WT plants throughout the day (Fig. 4b); the reasons for this are unclear at present. This decrease in total leaf sucrolytic activity, and the slight increase detected in activity of leaf SPS, may have led to the increases in sucrose concentration that were measured in the SuSy3 antisense lines (Fig. 5a), and also supports the idea that SuSy3 normally operates in the direction of sucrose cleavage in the leaf (Bieniawska *et al*. 2007). Levels of starch were also higher in leaves from SuSy3 antisense compared with WT towards the end of the day (Fig. 5b). Increased starch levels are consistent with the slight increases in AGPase activity measured in leaves of lines SS3 10 and SS3 54 at 1600 h, and also with the increase in 3-PGA/Pi ratio which was detected (Supporting Information Table S2) that has previously been shown to activate starch synthesis (Preiss 1982). The increase in leaf sugar and starch contents (Fig. 5) is perhaps surprising, given the slightly decreased rates of net CO_2 assimilation that were measured for these plants (Fig. 1c), and could reflect changes in source-to-sink transport of photosynthate in the leaves of these plants.

The relationship between sucrolytic activity and stomatal function

The gas exchange and WUE data presented here indicate that stomatal function is altered in plants with altered sucrolytic activity, and this in turn suggests the presence of a link between leaf sucrolytic activity and stomatal aperture. Moreover, data from the $^{14}CO_2$ labelling experiments suggest that this link is not due to altered mesophyll capacity for carbon fixation; this is a particularly important consideration for the SuSy 3 antisense plants, where the reduction in SuSy (and invertase) activity was not restricted to guard cells.

Research carried out over the last two decades has generally supported a role for sucrose in the generation of turgor pressure within guard cells (Talbott & Zeiger 1998; Outlaw 2003; Lawson 2009). However, in the experiments described here, decreased leaf sucrolytic activity (SuSy3 antisense) was correlated with data indicative of reduced stomatal aperture (Figs 1a,c & 3a), while increased guard cell sucrolytic activity (SUC2 expression) was correlated with data indicative of increased stomatal aperture (Figs 1b,d & 3b). This was despite the fact that whole leaf sucrose levels tended to be higher in the SuSy3 antisense plants (Fig. 5a) while sucrose levels in leaves and epidermal fragments from SUC2 lines were unaffected (Supporting Information Fig. S6).

One explanation may be that the breakdown products of sucrose (hexose sugars, for example) might also play a role in turgor generation (MacRobbie 1987; Talbott & Zeiger 1993). Thus, decreased leaf SuSy3 and invertase activity (Fig. 4) in the SuSy3 antisense lines might restrict production of these compounds and reduce stomatal opening, while increased guard cell invertase activity might have the opposite effect. No changes in whole leaf levels of glucose or fructose were detected in SuSy3 antisense lines, though this does not preclude there being an effect specifically within guard cells as a result of altered SuSy activity. Significant increases in whole leaf hexose phosphate levels were detected in the SUC2 lines, and a significant increase in glucose in epidermal fragments was also detected (Supporting Information Fig. S6), lending some support to this hypothesis as an explanation for altered stomatal function.

Sucrose breakdown may also be required for the accumulation of K^+/malate as osmoticum. Carbon skeletons for the synthesis of malate via PEP carboxylase may ultimately derive from sucrose (Raschke & Dittrich 1977; Dittrich & Mayer 1978). ATP is also necessary for K^+ accumulation (MacRobbie & Smyth 2010), and may be generated through operation of glycolysis and mitochondrial oxidative phosphorylation (Vavasseur & Raghavendra 2005) using sucrose as a substrate. Likewise, sucrose uptake by guard cells is likely to occur via proton symport (Boorer *et al*. 1996; Sauer 2007) and hence may also require ATP generated from breakdown of a proportion of cellular sucrose. Thus, decreasing the capacity of guard cells to cleave sucrose and generate ATP might decrease (SuSy3 antisense) solute accumulation and stomatal aperture while increasing this capacity could increase (SUC2 expression) solute accumulation and thereby increase stomatal aperture.

Several studies have also revealed the importance of the contents of the apoplast in the control of stomatal aperture. High concentrations of apoplastic sucrose, which can be achieved under transpiration rates, can lead to decreased stomatal aperture (Outlaw & Vlieghere-He, 2001), while the apoplastic concentration of the organic acids malate and citrate also exert an effect over stomatal aperture (Araújo *et al*., 2011). Thus, alterations in sucrolytic activity could affect stomatal aperture by altering the accumulation of apoplastic solutes. Given the small alterations in metabolite levels detected in the SUC2 lines, and the fact that transgene expression was limited to guard cells, this appears an unlikely explanation in this line, and measurements of apoplastic content will be required to determine if such an effect occurs in the SuSy3 antisense lines.

Current data do not allow us to determine which of these alternatives is correct, or whether a combination acts *in planta*. However, stable isotope labelling experiments (Ratcliffe & Shachar-Hill 2006), expanding on pioneering work carried out using radioisotopes (Dittrich & Raschke 1977) and GC-MS based metabolite profiling (Lisec *et al*. 2006) of epidermal fragments may help to distinguish between these hypotheses by revealing the composition of guard cell osmoticum throughout the day and the metabolic fluxes necessary to support the accumulation of these osmolytes. The results presented here do not necessarily diminish the importance of sucrose as guard cell osmoticum, but indicate that processes that are involved in its accumulation and degradation in guard cells are likely to play a role in controlling stomatal aperture.

Conclusions

Analysis of transgenic potato plants with either decreased SuSy activity or increased guard cell invertase activity suggests that sucrose, in addition to its role as an important guard cell osmoticum, also plays an important role as substrate in these cells. Decreased sucrolytic activity was linked to decreased stomatal conductance and increased short-term and long-term WUE, while the opposite was observed in transgenic plants with increased sucrolytic activity. These data support the hypothesis that manipulations in guard cell metabolism could be a way to fine-tune stomatal turgor regulation in order to increase plant tolerance to water deficit. Experiments under field conditions are needed to test the feasibility of these manipulations in order to improve plant productivity under drought stress.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Total protein and activity gels indicating invertase activity in epidermal fragments isolated from leaves of SUC2 overexpression and wild-type potato plants. Four hundred μg of protein extracted from epidermal fragments was subjected to semi-denaturing SDS-PAGE and stained for invertase activity. Arrow indicates the location of invertase activity on the gel.

Figure S2. (a) Invertase and (b) SuSy activity in leaves from wild-type plants and SUC2 overexpression lines. Values are the mean and standard error of the mean ($n = 5$).

Figure S3. Gas exchange parameters of SuSy3 antisense plants after 25 and 55 d growth. (a) Stomatal conductance in wild-type and SuSy3 antisense lines throughout the day after 25 d growth. (b) Rate of CO_2 assimilation in wild-type and SuSy3 antisense lines throughout the day after 25 d growth. (c) Stomatal conductance in wild-type and SuSy3 antisense lines throughout the day after 55 d growth. (d) Rate of CO_2 assimilation in wild-type and SuSy3 antisense lines throughout the day after 55 d growth.

Figure S4. Transpiration of wild-type and SuSy3 antisense plants throughout the day. (a) 25-day-old plants, (b) 35-day-old plants, (c) 55-day-old plants. Values are mean and standard error of the mean ($n = 5$). Asterisks indicate statistically significant differences between the SuSy3 antisense line and wild-type plants at the indicated time ($P < 0.05$, Duncan's test).

Figure S5. Rate of CO_2 assimilation by wild-type and SUC2 overexpression plants as a function of internal leaf CO_2 concentration. Horizontal and vertical bars represent the standard error of the mean ($n = 5$).

Figure S6. Measurements of glucose, fructose and sucrose content in epidermal fragments from SUC2 overexpression plants. (a) glucose, (b) fructose, (c) glucose + fructose (hexose), (d) sucrose. '4' and '72' and INV 4 and INV 72 SUC2 overexpression lines, respectively. Abundance per GC pair was calculated using measurements of the number of guard cell pairs per gram dry weight of epidermal fragments. Error bars represent standard deviations from six replica measurements from three separate epidermal fragment preparations per line. *t*-Test results: * denotes significant differences to wild type at $P = 0.05$; ** denotes significant difference to wild type at $P = 0.1$.

Table S1. Enzyme activity in leaf discs from wild-type and SuSy3 antisense plants. Values are mean ±standard error of the mean ($n = 5$). Activity is expressed in nmol min^{-1} mg^{-1} protein. Significant differences between wild type and transgenics are indicated in bold ($P < 0.05$, Duncan's test). ADP-glucose pyrophosphorylase (AGPase), fructose-1,6-bisphosphatase (FBPase), sucrose-phosphate synthase [SPS; maximum (Vmax) and selective (Vsel) activities], and activation state (activation).

Table S2. Levels of phosphorylated metabolites in leaves from wild-type and SuSy3 antisense plants. All values are given in nmol g^{-1} FW apart from Pi (μmol g^{-1} FW) and are given as the mean ± the standard error of the mean ($n = 5$). Bold and underlined data are significantly different ($P < 0.05$, Duncan's test) from WT plants.

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