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Guard cell-specific upregulation of *sucrose synthase 3* reveals that the role of sucrose in stomatal function is primarily energetic

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Summary

- Isoform 3 of *sucrose synthase* (*SUS3*) is highly expressed in guard cells; however, the precise function of *SUS*3 in this cell type remains to be elucidated.
- Here, we characterized transgenic *Nicotiana tabacum* plants overexpressing *SUS3* under the control of the stomatal-specific KST1 promoter, and investigated the changes in guard cell metabolism during the dark to light transition.
- Guard cell-specific *SUS3* overexpression led to increased SUS activity, stomatal aperture, stomatal conductance, transpiration rate, net photosynthetic rate and growth. Although only minor changes were observed in the metabolite profile in whole leaves, an increased fructose level and decreased organic acid levels and sucrose to fructose ratio were observed in guard cells of transgenic lines. Furthermore, guard cell sucrose content was lower during light-induced stomatal opening. In a complementary approach, we incubated guard cell-enriched epidermal fragments in ^{13}C-$NaHCO_3$ and followed the redistribution of label during dark to light transitions; this revealed increased labeling in metabolites of, or associated with, the tricarboxylic acid cycle.
- The results suggest that sucrose breakdown is a mechanism to provide substrate for the provision of organic acids for respiration, and imply that manipulation of guard cell metabolism may represent an effective strategy for plant growth improvement.

Introduction

Stomata are leaf epidermal structures that consist of two guard cells surrounding a pore whose aperture is actively regulated. The control of gas exchange by stomatal movements allows plants to occupy habitats with fluctuating environmental conditions, and it has been predicted that stomata are important contributors to speciation and evolutionary change (Hetherington & Woodward, 2003). In addition, stomatal closure is one of the most important responses to periods of drought, the abiotic stress that most affects global agricultural production (Rai & Takabe, 2006). Plants under water deficit may employ a range of different physiological strategies in order to optimize the influx of water from soil and/or to minimize water loss via transpiration (Jones, 1998; Acharya & Assmann, 2009). Considerable progress has been made towards obtaining drought-tolerant genotypes via the manipulation of abscisic acid (ABA) metabolism (Yu *et al.*, 2008; Nilson & Assmann, 2010; Yoo *et al.*, 2010). However, in most cases, the resultant drought-tolerant transgenic plants exhibit reduced stomatal opening, which, in turn, leads to a reduction in net photosynthetic rate and, consequently, reduced growth. Alternative strategies that can be used to obtain drought-tolerant plants with a relatively minor impact on net photosynthetic rate include the manipulation of ABA-independent pathways (Nelson *et al.*, 2007), the manipulation of guard cell sucrose metabolism (Antunes *et al.*, 2012), the manipulation of guard cell plasma membrane H^+-ATPase (Wang Y *et al.*, 2014b) and the manipulation of mesophyll organic acid metabolism (Nunes-Nesi *et al.*, 2007; Araújo *et al.*, 2011). A more complete understanding of stomatal regulation and how it is influenced by the surrounding mesophyll cells thus represents an important step towards obtaining plants with greater water use efficiency (Yang *et al.*, 2005; Gago *et al.*, 2014; Lawson *et al.*, 2014).

Changes in guard cell signaling and metabolism during stomatal movements involve a complex network of interactions between ions, hormones, secondary messengers and metabolites (Schroeder *et al.*, 2001; Desikan *et al.*, 2004; Vavasseur & Raghavendra, 2005; Kim *et al.*, 2010; Kollist *et al.*, 2014). In this context, potassium (K^+) and sucrose are proposed to be the major osmolytes responsible for the induction of reductions in the osmotic potential of guard cells, which, in turn, induce water influx and, consequently, stomatal opening (Talbott & Zeiger, 1996). The daily course of plant stomatal movements involves reversible changes in guard cell osmolyte concentration separated into two phases: the accumulation of K^+ in the morning and the accumulation of sucrose in the afternoon (Talbott & Zeiger, 1998). According to this theory, in the early period of the day, there is an influx of K^+ and Cl^- from the apoplast to the symplast of guard cells with concomitant

malate accumulation from starch breakdown (Talbott & Zeiger, 1996; Zeiger *et al.*, 2002; Outlaw, 2003; Shimazaki *et al.*, 2007). The highest guard cell K^+ concentration is found at around midday, and amounts then decline with an increase in sucrose concentration (Tallman & Zeiger, 1988; Talbott & Zeiger, 1993, 1996), with sucrose considered to be the major osmolyte during the afternoon period (Zeiger *et al.*, 2002). However, previous results from our research group have shown that, contrary to expectations, potato transgenic plants overexpressing a yeast invertase or an antisense construct targeted against isoform 3 of *sucrose synthase* (*SUS3*) display increased and decreased stomatal conductance, respectively (Antunes *et al.*, 2012). Furthermore, using kinetic isotope labeling experiments, we showed that sucrose can act as a substrate during light-induced stomatal opening (Daloso *et al.*, 2015). These results indicate that sucrose breakdown, not just sucrose accumulation, is important in the control of stomatal opening. Thus, although sucrose plays an important role in stomatal function, the exact mechanism by which it does so remains far from clear and needs to be further dissected.

Sucrose breakdown may occur in the cytosol by the operation of two sucrose-cleaving enzymes, named invertase and sucrose synthase (SUS) (Sturm & Tang, 1999). Tobacco guard cells present higher SUS activity than whole leaves, whereas the opposite is observed for invertase, suggesting that SUS may be important for guard cell sucrose metabolism (Daloso *et al.*, 2015). SUS is a key enzyme in sugar metabolism and catalyzes the reversible conversion of sucrose and (A)UDP to fructose and (A)UDP-glucose (Baroja-Fernández *et al.*, 2009). Six isoforms (*SUS1–6*) have been documented in the genomes of Arabidopsis, rice and rubber tree (Baud *et al.*, 2004; Hirose *et al.*, 2008; Xiao *et al.*, 2014), whereas seven (*SUS1–7*) are present in cotton (Chen *et al.*, 2012) and tobacco (Wang *et al.*, 2015). Although little information is available concerning *SUS3*, in Arabidopsis, this isoform is highly expressed in guard cells (Bieniawska *et al.*, 2007) in comparison with whole leaves (Bates *et al.*, 2012), whereas, in potato, *SUS3* expression is upregulated in guard cell-enriched epidermal fragments under drought conditions (Kopka *et al.*, 1997). These facts notwithstanding, the role of *SUS3* during stomatal opening remains to be clearly defined. Here, we aimed to contribute to the current knowledge concerning the role of sucrose in guard cell regulation by examining tobacco plants overexpressing potato *SUS3* under the control of the guard cell-specific KST1 promoter and by carrying out kinetic isotope labeling experiments on isolated guard cell-enriched epidermal fragments of both wild-type (WT) and transgenic lines. The combined results are discussed in the context of the regulation of stomatal movement and the feasibility of improving crop yields via the genetic manipulation of guard cell metabolism.

Materials and Methods

Plant material and growth conditions

In this study, we used WT and transgenic *Nicotiana tabacum* L. cv Havana 425 plants overexpressing *SUS3* from *Solanum tuberosum* (AY205302) under the control of the potato KST1 promoter (X79779) (Müller-Röber *et al.*, 1995), a promoter that exclusively directs guard cell-specific expression (Plesch *et al.*, 2001). The plasmid construction, plant transformation and confirmation of transgenic plants were performed exactly as described in Antunes *et al.* (2012). Briefly, the binary plasmid pBinAr (Höfgen & Willmitzer, 1990) was used as a start point, with the CaMV-35S promoter being replaced by the KST1 promoter and renamed pBinK. The cDNA of *SUS3* from *S. tuberosum* was amplified and inserted into pBinK in the sense direction between the KST1 promoter and OCS terminator (Fig. 1a). Transgenic plants were generated by *Agrobacterium tumefaciens* transformation and confirmed by PCR with specific primers for the neomycin phosphotransferase (*NPTII*) selectable marker gene (FW, 5′-GCGGTCAGCCCATTCGCCGCC-3′; REV, 5′-TCAGCGCAGGGGCGCCCGGTT-3′).

Seeds of the T_2 generation were germinated in Petri dishes containing Murashige and Skoog (MS) medium (Murashige & Skoog, 1962) with the addition of 100 mg l^{-1} of kanamycin for transgenic lines, and cultivated *in vitro* for 15 d. The resultant seedlings were transplanted to 0.1-l pots containing Plantimax® substrate and cultivated under growth chamber conditions with artificial 16 h : 8 h, light : dark photoperiod, 150 μmol photons m^{-2} s^{-1}, 23 ± 2°C and relative humidity 70 ± 5%. After this, plants were transplanted to 6- or 20-l pots and cultivated under glasshouse conditions with or without control of light, humidity and temperature for 1–2 months, depending on the experiment. The conditions in which the plants were grown are highlighted in the legends of the figures.

Screening of transgenic lines

After *in vitro* screening, seven of the 15 transgenic lines were selected. Analysis of infrared thermography and whole-plant transpiration during cultivation in 0.1-l pots was performed on all lines (Fig. 1b,d). Subsequently two lines (L18 and L37) were selected for further experiments. Specific primers for SUS3 were designed (FW, 5′-GACCAGACTGATGAGCATGTCG-3′; REV, 5′-TCTTCACTTTGTCGAGCCTCG-3′). Total RNA was isolated using Trizol® (Gibco BRL, Life Technologies, New York, NY, USA) following the manufacturer's recommendations. Quantitative real-time PCR was performed using a 7300 Real Time System (Applied Biosystems, Foster City, CA, USA) and gene expression was analyzed, normalizing the results to the expression of the *actin* gene (FW, 5′-AGCAAGGAAATTACCG CATTAGC-3′; REV, 5′-ACCTGCTGGAATGTGCTGAGA-3′) as a control.

SUS activity

SUS activity was assayed in the sucrose breakdown direction (Antunes *et al.*, 2012; Baroja-Fernández *et al.*, 2012). Frozen samples were ground to a powder in liquid nitrogen and *c.* 5 ml of powder were transferred to Falcon tubes (50 ml). Protein extraction was performed by the addition of 16 ml of a freshly prepared three-fold-concentrated extraction buffer containing 150 mM Hepes/KOH (pH 7.4), 30 mM $MgCl_2$, 3 mM EDTA, 3 mM EGTA, 3 mM benzamidine, 3 mM ε-aminocaproic acid, 30%

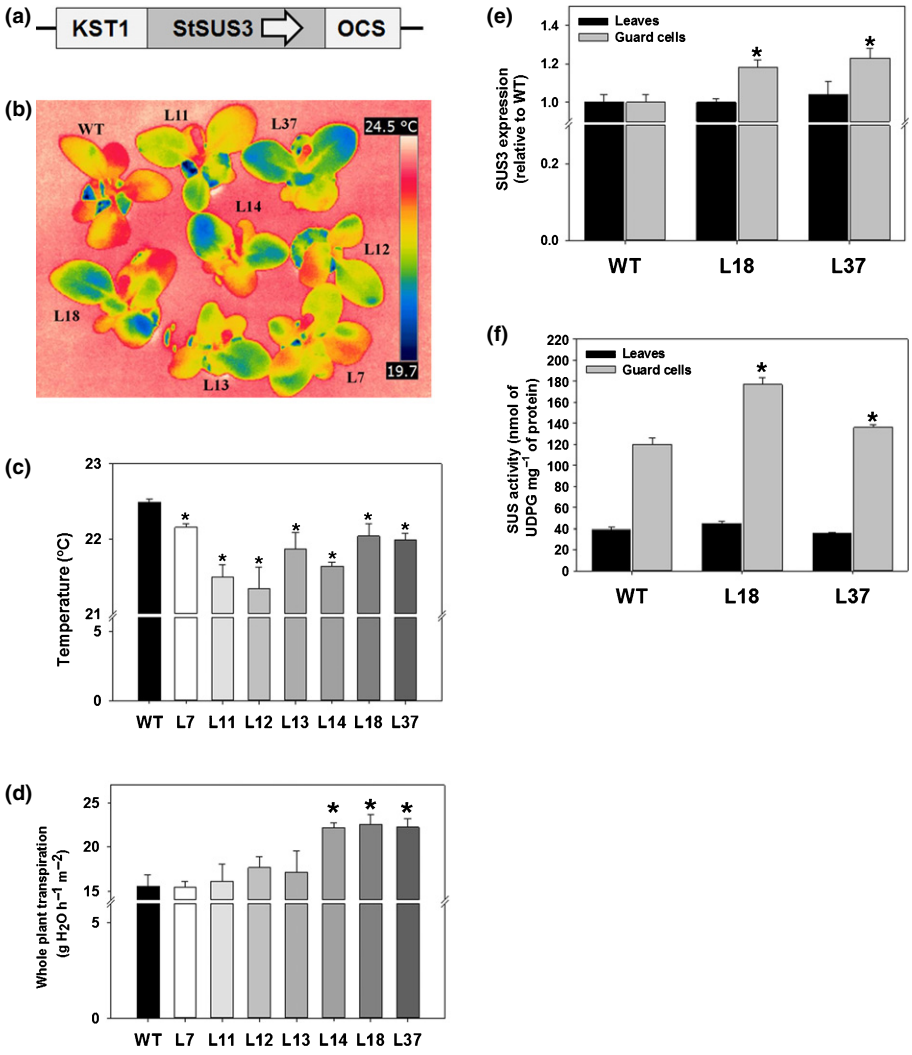

Fig. 1 Initial characterization of *Nicotiana tabacum* transgenic lines overexpressing *sucrose synthase 3* (*SUS3*) under the control of the KST1 promoter. (a) Construction used in this study consisting of the KST1 promoter, *SUS3* isoform (sense direction) and OCS terminator. (b, c) Infrared thermography of wild-type (WT) and several transgenic lines (b) and the corresponding leaf temperature (c). The leaf temperature was measured in the most expanded leaf (eight temperature points per leaf) from four independent plants ($n = 4 \pm$ SE). (d) Whole-plant transpiration (g H_2O h^{-1} m^{-2}) of WT and several transgenic lines between 07:00 and 13:00 h of the day ($n = 4 \pm$ SE). (e) Gene expression in leaves and guard cell-enriched epidermal fragments of WT and transgenic lines (L18, L37). *SUS3* gene expression was analyzed by quantitative real-time PCR normalized by *actin* (*ACT*) gene expression. Data are shown as relative expression normalized to WT ($n = 5 \pm$ SE). (f) SUS activity in the sucrose breakdown direction. Data are presented as nmol UDP-glucose (UDPG) produced min^{-1} mg^{-1} protein ($n = 6 \pm$ SE). Plants in (b–d) were grown under growth chamber conditions, whereas plants in (e, f) were grown under well-controlled glasshouse conditions (see the Materials and Methods section for details). Significantly different from WT by Student's *t*-test: *, $P < 0.05$.

(v/v) glycerol, 0.3% (v/v) Triton X-100 and 3 mM phenylmethylsulfonylfluoride (PMSF) to the Falcon tube. After homogenization and centrifugation (15 min, 4000 ***g***, 4°C), the supernatant was collected and transferred to another Falcon tube for an additional centrifugation. This step was repeated twice in order to collect a clean supernatant, after which 12 ml of the supernatant were collected, filtered using 0.2-µm filters and transferred to Falcon tubes (Ultracel 100K, Amicon® Ultra, EMD Millipore, Billerica, MA, USA; 15 ml) in order to concentrate the samples. These tubes were centrifuged at 4°C, 4000 ***g*** for 40–120 min. The samples were collected from the column when the volume reached 1 ml for the epidermal fragment samples and 3 ml for the leaf samples. The SUS activity assay was performed using 10 µl of the concentrated samples in two steps. First, the samples were incubated in a two-fold-concentrated assay buffer containing 40 mM Hepes/KOH, pH 7.4, 0.8 M sucrose and 4 mM UDP in a final volume of 100 µl at 37°C for 40 min. The reaction was stopped at 95°C for 5 min. After cooling, the samples were transferred to a microplate and the UDP-glucose produced was determined spectrophotometrically using a microplate reader (Biotek® EL 808, BioTek Instruments, Winooski, VT, USA) in a two-fold-concentrated reaction mixture containing 0.4 M glycine, pH 8.9, 10 mm $MgCl_2$, 4 mM NAD^+ and UDP-glucose dehydrogenase (0.005 U per reaction) (Sigma-Aldrich®). The amount of UDP-glucose produced by SUS in the reaction was calculated following a standard curve of UDP-glucose (0–30 nmol). The total protein content of the extract was determined according to Bradford (1976).

Gas exchange and chlorophyll *a* fluorescence analysis

Analyses of photosynthetic parameters were performed in completely expanded leaves of WT and transgenic lines using an infrared gas analyzer (6400 and 6400*xt*; LiCor, Lincoln, NE, USA) according to Flexas *et al.* (2006). Gas exchange was performed in saturating light (1000 µmol m^{-2} s^{-1}) containing 10% blue light to optimize stomatal opening and reference CO_2 concentration at 400 µmol mol^{-1}. The net photosynthetic rate (A, µmol CO_2 m^{-2} s^{-1}), stomatal conductance (g, mol H_2O m^{-2} s^{-1}), substomatal CO_2 concentration (C_i, µmol CO_2 mol^{-1}) and transpiration (E, mmol m^{-2} s^{-1}) were estimated. The effective quantum yield of photosystem II ($\Phi_{PSII} = (F'_m - F_s)/F'_m$) and

electron transport rates (ETR = $\Phi_{PSII} \times PAR \times 0.5 \times 0.84$) were calculated according to Genty *et al.* (1989), and F'_v/F'_m (maximum efficiency of PSII) was calculated according to Baker *et al.* (2007).

Whole-plant transpiration and growth analysis

Plants were cultivated in 0.1- or 20-l pots with a mixture containing soil, sand and manure (3 : 1 : 1), and maintained under growth chamber or non-controlled glasshouse conditions, respectively. Water loss by transpiration was determined using a gravimetric methodology (Cavatte *et al.*, 2012). For the experiment carried out under glasshouse conditions, the night before analysis the soil was completely irrigated. At predawn and the beginning of the night, the weights of the pots containing plants were recorded; the difference between these weights was estimated to be the evapotranspired water per plant (g H_2O per plant) which was recorded every day over 12 d. Under growth chamber conditions, we carried out whole-plant transpiration (g H_2O h^{-1} m^{-2}) of WT and transgenic lines between different time intervals of the day. In both experiments, pots filled with soil mixture without plants were used to estimate direct soil evaporation, and the difference between the total water loss (plant + soil) and evaporation (only soil) was considered as the plant transpiration. The leaf area was determined according to the model described in Antunes *et al.* (2008), which we adapted for tobacco leaves. The leaf area was then used to express transpiration on a per leaf area (g H_2O m^{-2}) basis. The growth parameters were determined following whole-plant transpiration analysis under glasshouse conditions. The final leaf area was determined, and leaf, stem and roots were harvested and dried at 70°C for 2 d and their dry weight (DW) was determined.

Drought stress experiments

A drought stress experiment was carried out in 6-wk-old plants growing in 6-l pots in well-controlled glasshouse conditions. The plants were subjected to a progressive water deficit by suspension of irrigation, and the relative water content (RWC) was determined daily. After 4 d of drought stress, the plants were watered again in order to analyze the capacity of recovery vegetative growth of the plants. Control plants were watered daily to maintain soil water close to field capacity, and the biomass accumulated in the leaves, stems and roots was determined at the end of the experiment and compared with that of plants experiencing drought stress.

Isolation of guard cell-enriched epidermal fragments

The isolation of guard cell-enriched epidermal fragments was performed according to Kruse *et al.* (1989) adopting the previously described adaptations for tobacco (Daloso *et al.*, 2015). This protocol resulted in a guard cell purity of 98% and low mesophyll contamination, as shown by the higher SUS activity, a marker enzyme of guard cells (Hite *et al.*, 1993; Willmer & Fricker, 1996; Bieniawska *et al.*, 2007; Bates *et al.*, 2012), in guard cell-enriched epidermal fragments compared with whole leaves (Daloso *et al.*, 2015).

Stomatal aperture measurements in isolated guard cell-enriched epidermal fragments

Stomatal aperture measurements were performed in previously isolated epidermal fragments as described previously (Wang Y *et al.*, 2014b), with modifications. The epidermal fragments were harvested predawn and submitted to light (180 μmol photons m^{-2} s^{-1}) or dark in a solution containing 5 mM Mes/NaOH (pH 6.5), 50 μM $CaCl_2$ and 5 mM KCl. The stomatal aperture was determined after the time (h) indicated in the figures. Time 0 in the figures means that samples were analysed immediately after collecting the epidermal fragments. In all experiments, images of the epidermal fragments were taken using an Olympus BX41 microscope and the stomatal aperture was determined in at least 30 intact stomata per time point using cellSens Dimension software (Olympus®, Tokyo, Japan).

Kinetic isotope labeling experiment following the metabolic fate of ^{13}C-$NaHCO_3$ by gas chromatography-time of flight-mass spectrometry (GC-TOF-MS)

Changes in guard cell metabolism during the dark to light transition were determined in predawn-harvested guard cell-enriched epidermal fragments submitted to white light. This approach was important to understand the photosynthetic flux distribution in guard cells during the dark to light transition (Daloso *et al.*, 2015). We aimed to investigate the photosynthetic flux distribution in plants with higher capacity to cleave sucrose. Briefly, following their extraction, guard cell-enriched epidermal fragments were pooled in a hyperosmotic solution (0.5 M mannitol) until sufficient material for metabolomic analysis was collected. After this, guard cell-enriched epidermal fragments were washed extensively and suspended in different solutions: either control (5 mM Mes/NaOH, pH 6.5 + 50 μM $CaCl_2$ + 5 mM ^{13}C-$NaHCO_3$ + 10 mM mannitol) or KCl (5 mM Mes/NaOH, pH 6.5 + 50 μM $CaCl_2$ + 5 mM ^{13}C-$NaHCO_3$ + 5 mM mannitol + 5 mM KCl). The guard cell-enriched epidermal fragments were incubated in these solutions and harvested after 30 and 60 min in the light. A sample was frozen before the start of the experiment which corresponds to time 0 in Figs 8 and 9 (see later). Lyophilized samples were extracted and derivatized as described later. The analysis of the relative abundance of mass isotopomers was carried out using Xcalibur 2.1 software (Thermo Fisher Scientific, Waltham, MA, USA) as described previously (Daloso *et al.*, 2015).

Extraction and analysis of metabolites

Leaves and guard cell-enriched epidermal fragments were harvested between 5 and 7 h after the beginning of the light period from plants growing under growth chamber conditions. Metabolite extraction for gas chromatography-mass spectrometry (GC-MS) and sugar/starch quantification by spectrophotometric

assays were performed according to Lisec *et al.* (2006). The GC-MS metabolite determinations were quantified as described by Roessner-Tunali *et al.* (2001). The levels of soluble sugars (sucrose, fructose and glucose) and starch were measured as detailed in Trethewey *et al.* (1999).

Stomatal density

Stomatal density was quantified on both adaxial and abaxial leaf surfaces according to Aharon *et al.* (2003).

Statistical analyses

The transgenic lines were statistically compared with WT using Student's *t*-test at 5% probability ($P < 0.05$). The level of sucrose over time (Fig. 9, see later) was compared by ANOVA and Tukey test ($P < 0.05$). All statistical analyses were performed using the algorithm embedded into Microsoft Excel (Microsoft, Redmond, WA, USA) or SigmaPlot 12 (Systat Software Inc., San Jose, CA, USA).

Results

The KST1 promoter induces *SUS3* overexpression specifically in guard cells

The decrease in sucrose that is observed during light-induced stomatal opening may occur mainly through SUS activity, given that guard cells present a high SUS activity compared with leaves (Daloso *et al.*, 2015). Furthermore, *SUS3* is mainly expressed in guard cells compared with mesophyll cells, and is upregulated in response to ABA in Arabidopsis guard cells (Yang *et al.*, 2008), as well as being upregulated in epidermal fragments of potato under drought conditions (Kopka *et al.*, 1997). Given these results, and in an attempt to avoid pleiotropic effects resulting from the use of a constitutive promoter, we created transgenic tobacco plants overexpressing potato *SUS3* under the control of the KST1 promoter (Fig. 1a), which has been well characterized and shown to drive expression preferentially in guard cells (Plesch *et al.*, 2001; Kelly *et al.*, 2013). Plants were transformed by an *Agrobacterium*-mediated protocol and, after initial screening, seven lines were selected. A careful screening was carried out in these lines which showed several transgenic lines with lower leaf temperature (Fig. 1b,c) and higher whole-plant transpiration (Fig. 1d), and two lines (L18 and L37) were selected for in-depth analysis (see the Materials and Methods section for details). Quantitative real-time PCR and SUS activity assays were then performed to analyze the level of *SUS3* expression and SUS activity in whole leaves and guard cell-enriched epidermal fragments. As expected, neither changes in *SUS3* expression nor in SUS activity were observed in the whole leaves of transgenic lines. However, significant increases in expression and SUS activity were observed in the guard cells of L18 and L37 (Fig. 1e,f). Given that the whole-leaf samples will be dominated by mesophyll cells, these data are precisely what would be anticipated if the transgene is expressed in a guard cell-specific manner.

Changes in guard cell *SUS3* expression lead to increased gas exchange and stomatal aperture

Given that gas exchange is highly dependent on the growth environment, we performed several experiments under different conditions in order to carefully verify the stomatal behavior of the transgenic plants. L37 plants showed higher stomatal conductance (g_s), transpiration rate (E) and ratio of substomatal and ambient CO_2 concentration ($C_i : C_a$) at 1 and 3 h after the beginning of the light period, whereas L18 plants exhibited these differences only at 3 h after the beginning of the light period (Fig. 2). Higher net photosynthetic rate (A) was observed in both lines at 3 h after the beginning of the light period (Fig. 2). In agreement with these data, transgenic lines showed higher stomatal aperture during light- and potassium-induced stomatal opening (Fig. 3a,b) and higher capacity to increase g_s during dark to light transitions (Fig. 4a,b). It is important to highlight that stomatal aperture opening may occur by a backpressure effect of the epidermal fragment isolation. Taking this into account, in the first experiment, we submitted the epidermal fragments to light and darkness for 2 h, followed by measurement of the stomatal aperture. No stomatal opening was observed in the dark (Fig. 3a), excluding the possibility of stomatal opening resulting from the release of backpressure.

Transgenic lines show increased whole-plant transpiration and plant growth with slightly increased drought tolerance

In order to determine whether leaf gas exchange measurements represent whole-plant transpiration, we carried out whole-plant transpiration measurements in WT and transgenic lines grown under both growth chamber and glasshouse conditions. Under growth chamber conditions, increased whole-plant transpiration ($g\ H_2O\ h^{-1}\ m^{-2}$) was observed across different time intervals of the day (Fig. 4c). Furthermore, transgenic lines showed higher accumulated water loss ($g\ H_2O$ per plant) than WT in the experiment performed under glasshouse conditions (Fig. 4d). The data from these experiments exclude the possibility that the increase in g_s and E revealed by infrared gas analysis is an effect limited to the small area used in the infrared gas analysis chamber. Moreover, the increase in g_s cannot be associated with changes in leaf stomatal density, as there was no difference detected in this parameter between WT and transgenic lines (Supporting Information Table S1).

Following the whole-plant transpiration experiment (Fig. 3d), the plants were harvested and DW was determined. The transgenic lines displayed an increased whole-plant, leaf and stem DW (Fig. 5a,c). However, no differences were detected in leaf, stem and root mass fraction (expressed as a percentage of total plant DW), in the number of leaves per plant or in the leaves/root and aerial part (leaves + stem)/root ratios (Table S1). The root DW (Fig. 5d) and stem length (Table S1) increased significantly only in L37 plants.

Given that the transgenic plants showed great differences in gas exchange (Fig. 2), stomatal aperture (Fig. 3) and whole-plant transpiration (Fig. 4c,d), we next carried out a drought stress

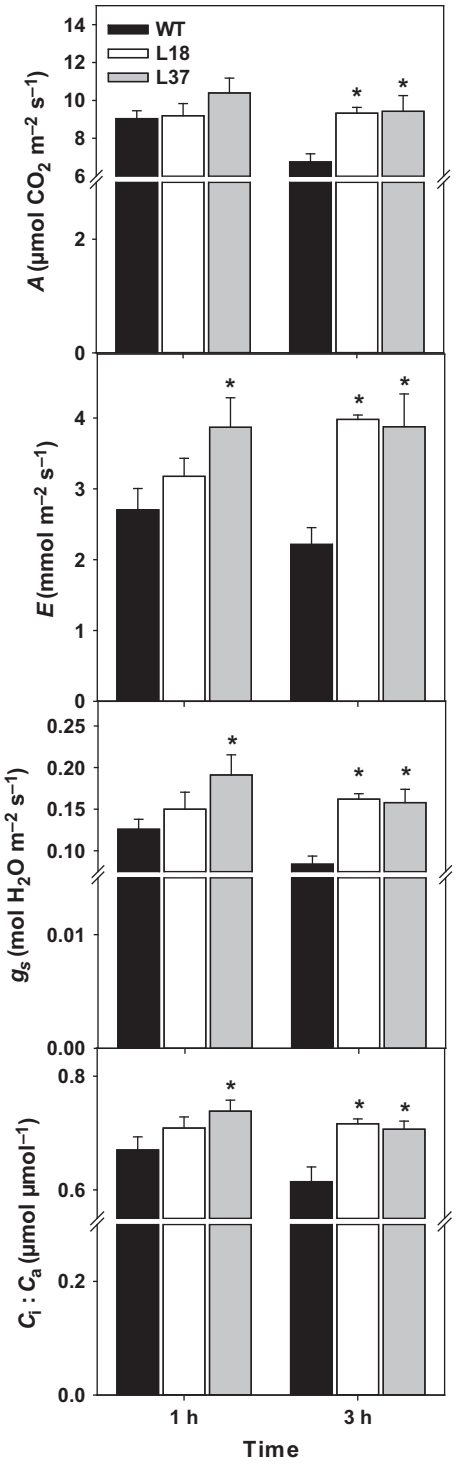

Fig. 2 Gas exchange measurements in completely expanded leaves of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L18, L37) at 1 and 3 h after the beginning of the light period. *A*, net photosynthetic rate; g_s, stomatal conductance; *E*, transpiration rate; $C_i : C_a$, ratio of substomatal and ambient CO_2 concentration. Plants were grown under growth chamber conditions (see the Materials and Methods section for details). Significantly different from WT by Student's *t*-test: *, $P < 0.05$ ($n = 4 \pm$ SE).

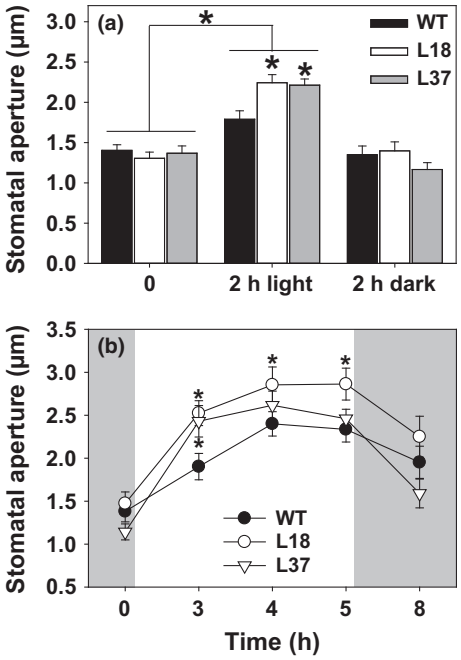

Fig. 3 Stomatal aperture measurements in epidermal fragments of *Nicotiana tabacum* wild-type (WT) and transgenic tobacco plants (L18, L37). Stomatal aperture was measured during dark to light and light to dark transitions. Epidermal fragments were harvested at predawn and submitted to light (180 μmol photons m^{-2} s^{-1}) or dark for the time (h) indicated in the figure in a solution containing 5 mM Mes/NaOH (pH 6.5) + 50 μM $CaCl_2$ + 5 mM KCl. Time 0 means that samples were analysed immediately after collecting the epidermal fragments. In all experiments, images of the epidermal fragments were taken using an Olympus BX41 microscope and the stomatal aperture was determined in at least 30 stomata per time point using cellSens Dimension software (Olympus®). Plants were grown under well-controlled glasshouse conditions (see the Materials and Methods section for details). Significantly different from WT by Student's *t*-test: *, $P < 0.05$ ($n = 30 \pm$ SE). In (a), asterisks above the lines indicate that values for 2 h of light were significantly different from those for time 0 for all genotypes.

experiment in order to investigate the behavior of these lines under water shortage conditions. Plants were subjected to a very rapid imposition of drought by the suspension of irrigation, and RWC and effects on photosynthesis were monitored. Plants were kept for 4 d under drought, followed by 3 d of rewatering. At the end of the experiment, drought-treated plants were harvested and the biomass was determined. A set of control plants not subjected to drought stress was harvested and the biomass was determined at the same time. Small reductions in RWC led to drastic reductions in g_s and all photosynthetic parameters after 1 d of drought stress (Fig. S1). Interestingly, transgenic lines showed higher RWC than WT at days 1, 2 and 4 of drought stress, as well as after 1 d of recovery with irrigation. Furthermore, line L37 recovered F'_v/F'_m values faster than WT after re-initiation of irrigation (Fig. S1), although the values did not recover to those exhibited by non-stressed plants (day 0 values). In agreement with the growth results shown above (Fig. 5), transgenic well-watered plants presented both higher plant and leaf DW compared with WT. However, these differences were eliminated during drought stress and recovery periods (Table S2). The root DW was lower in L37 after drought stress. Furthermore, L37 showed higher leaf and stem mass fraction and lower root mass fraction after drought stress when compared with WT (Table S2).

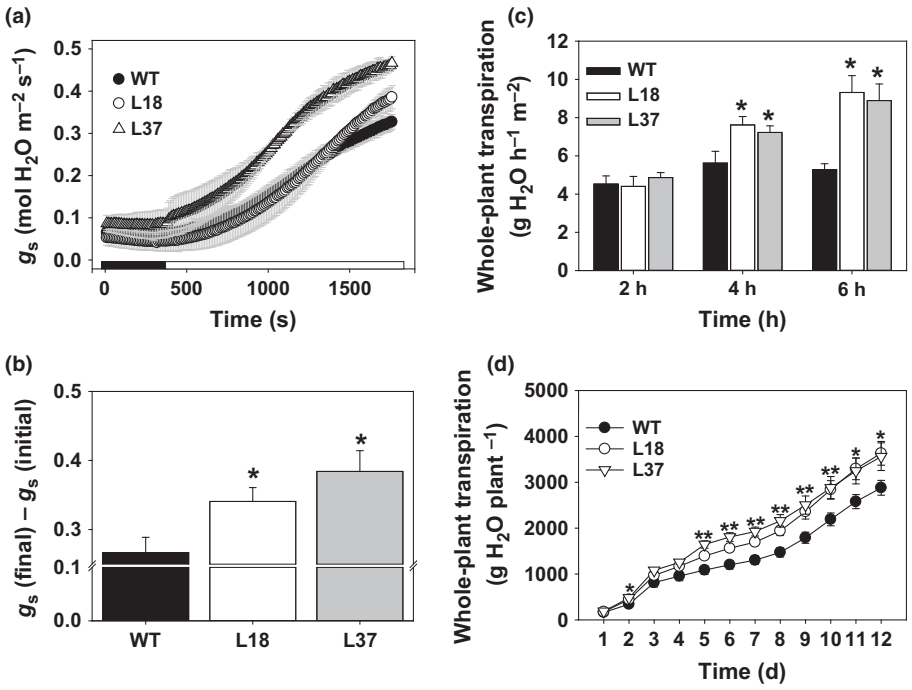

Fig. 4 Effect of guard cell *sucrose synthase 3* (*SUS3*) overexpression on stomatal conductance and whole-plant transpiration in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L18, L37). (a) Stomatal conductance (g_s) in dark to light transition in leaves ($n = 4 \pm$ SE). Black and white bars on the *x*-axis indicate dark and light periods, respectively. (b) Rate of increase in stomatal conductance (g_s) (g_s final − g_s initial) in dark to light transition in leaves ($n = 4 \pm$ SE). (c) Whole-plant transpiration (g H_2O h^{-1} m^{-2}). The whole-plant transpiration was determined in different time intervals of the day from 0 to 2 h, from 2 to 4 h and from 4 to 6 h after the beginning of the light period ($n = 4 \pm$ SE). (d) Whole-plant transpiration expressed as water loss accumulated (g H_2O $plant^{-1}$) during 12 d ($n = 6 \pm$ SE). Plants in (a, b, d) were grown under non-controlled glasshouse conditions, whereas plants in (c) were grown under growth chamber conditions (see the Materials and Methods section for details). Significantly different from WT by Student's *t*-test: *, $P < 0.05$. In (d), two asterisks (**) indicate that both transgenic lines are different from WT, whereas one asterisk (*) indicates that only one of the transgenic lines is different from WT.

Guard cell *SUS3* overexpression leads to changes in guard cell primary metabolism

Having demonstrated the elevated expression of *SUS3* in the transgenic lines and the consequences on gas exchange, stomatal aperture, whole-plant transpiration and, ultimately, plant growth, we next determined the metabolite contents in leaves and guard cell-enriched epidermal fragments in order to determine the consequence of *SUS3* overexpression on both guard cell and whole-leaf metabolism. Given that *SUS3* overexpression was delimited to guard cells of the transgenic lines, it is perhaps unsurprising that no changes were observed in the content of sucrose, fructose, malate and fumarate or the ratio of disaccharide to monosaccharide in whole-leaf extracts from transgenic plants (Fig. S2). By contrast, increased fructose content and decreased sucrose to fructose ratio and sucrose to fructose plus glucose ratio were observed in guard cell-enriched epidermal fragments of both L18 and L37. However, no differences in the levels of malate, fumarate or glucose were seen in guard cell-enriched epidermal fragments of either transgenic line (Fig. 6). The sucrose content and the ratio of sucrose to glucose decreased in guard cell-enriched epidermal fragments of L37 alone (Fig. 6).

GC-TOF-MS-based metabolite profiling was used in order to identify further changes in primary metabolism induced by *SUS3* overexpression. Minor changes were observed in leaves: for

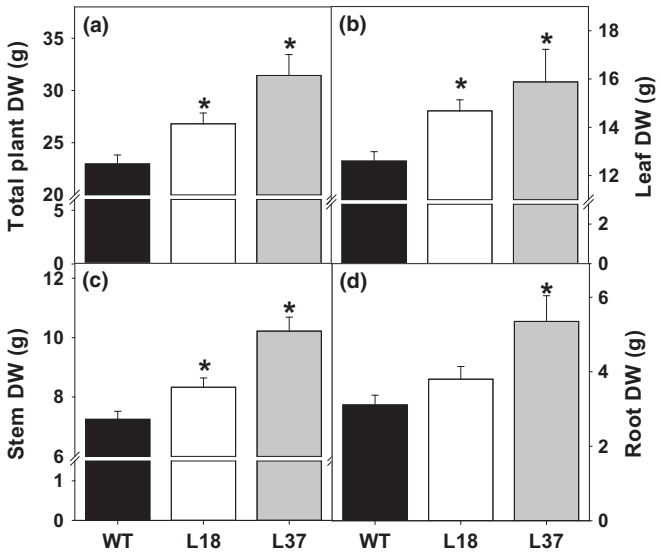

Fig. 5 Growth phenotype of *Nicotiana tabacum* wild-type (WT) and transgenic lines overexpressing *sucrose synthase 3* specifically in guard cells (L18, L37). After the end of the whole-plant transpiration experiment (Fig. 4d), plants grown under non-controlled glasshouse conditions (see the Materials and Methods section for details) were harvested and the biomass (dry weight, DW) of (a) the total plant, (b) leaf, (c) stem and (d) root was determined. Asterisks indicate values significantly different from WT by Student's *t*-test: *, $P < 0.05$ ($n = 6 \pm$ SE).

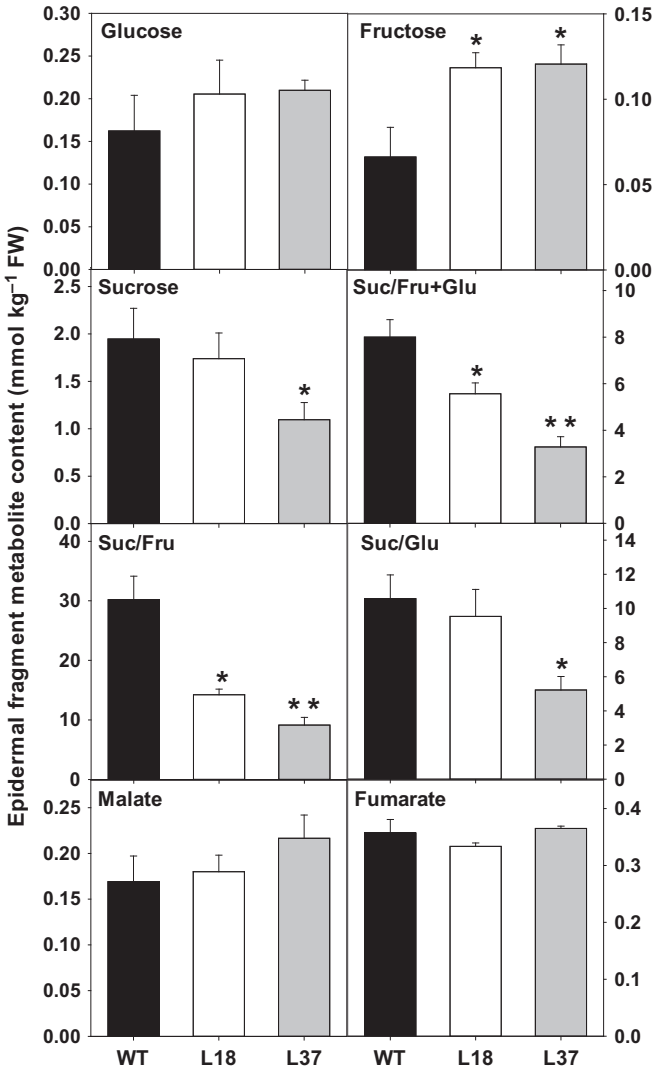

Fig. 6 Metabolite content in guard cell-enriched epidermal fragments of *Nicotiana tabacum* wild-type (WT) and transgenic lines overexpressing *sucrose synthase 3* specifically in guard cells (L18, L37). Glucose (Glu), fructose (Fru), sucrose (Suc), malate and fumarate were measured by a spectrophotometric method. Plants were grown under growth chamber conditions and harvested at midday (see the Materials and Methods section for details). Asterisks indicate values significantly different from WT by Student's *t*-test:
*, $P < 0.05$; **, $P < 0.01$ ($n = 5 \pm$ SE). FW, fresh weight.

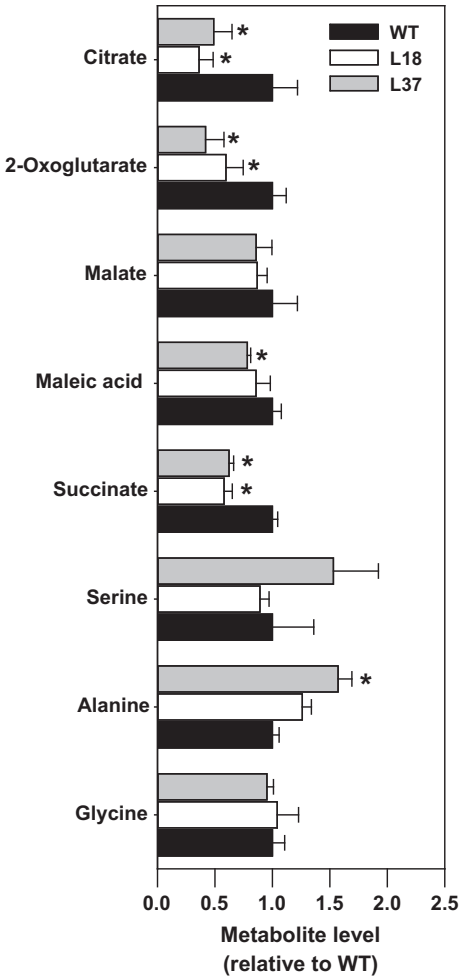

Fig. 7 Relative metabolite content in guard cell-enriched epidermal fragments of *Nicotiana tabacum* wild-type (WT) and transgenic lines overexpressing *sucrose synthase 3* specifically in guard cells (L18, L37). Data are normalized with respect to the mean response calculated for WT. Plants were grown under growth chamber conditions and harvested at midday (see the Materials and Methods section for details). Asterisks indicate values significantly different from WT by Student's *t*-test: *, $P < 0.05$ ($n = 5 \pm$ SE).

example, the amounts of serine increased in both lines, whereas aspartate and methionine increased in L37 alone. The amount of succinate decreased in both L18 and L37, whereas 2-oxoglutarate decreased only in L18 (Table S3). In guard cell-enriched epidermal fragments, the amount of the organic acids citrate, 2-oxoglutarate and succinate were reduced in both transgenic lines, whereas maleic acid decreased only in L37. By contrast, slight increases were observed in the amount of alanine in L37. No changes were observed in malate, serine and glycine (Fig. 7). These results suggest that the metabolites of the tricarboxylic acid (TCA) cycle may be involved in the altered phenotype of the transgenic plants, and that the effects seen are largely stomatal rather than mesophyll driven.

^{13}C kinetic isotope labeling experiments provide evidence connecting sucrose breakdown and organic acid metabolism during the dark to light transition

Given the relationship between sucrose cleavage and stomatal conductance observed in plants overexpressing SUS3, we next carried out a kinetic isotope labeling experiment using guard cell-enriched epidermal fragments from WT, L18 and L37, aiming to compare the redistribution of ^{13}C between these genotypes during the dark to light transition. Several differences were found between WT and transgenic lines before light treatment, with transgenic lines showing higher contents of pyruvate and glutamine and lower contents of citrate (Fig. 8) and fumarate (Fig. 9). Although no substantial changes in the levels of pyruvate and glutamine over time were observed, the levels of citrate tended to reduce over time (Fig. 8). Sucrose content was reduced over time during the dark to light transition (Fig. 9), and this

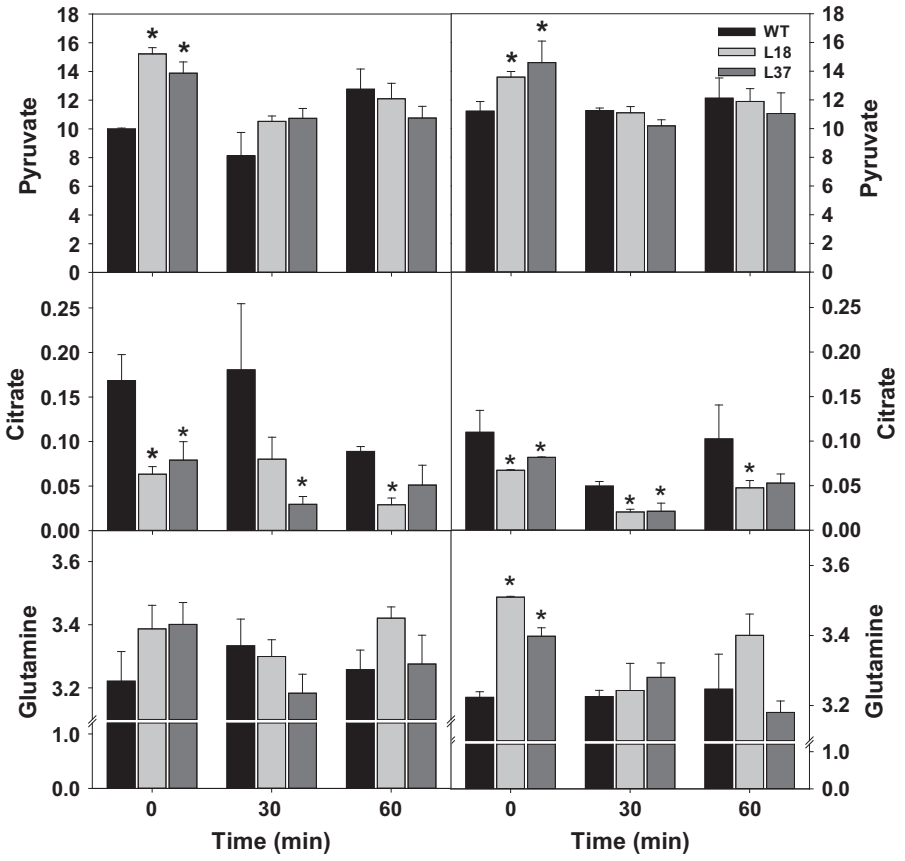

Fig. 8 Metabolite profiling in guard cell-enriched epidermal fragments of *Nicotiana tabacum* wild-type (WT) and transgenic lines overexpressing *sucrose synthase 3* specifically in guard cells (L18, L37) during the dark to light transition. Plants were grown under well-controlled glasshouse conditions. Guard cell-enriched epidermal fragments were harvested at predawn (0 min), transferred to solutions containing mannitol (left panel) or KCl (right panel), and collected after 30 and 60 min in the light (see the Materials and Methods section for details). The metabolite contents presented were normalized g^{-1} DW. Asterisks indicate values significantly different from WT by Student's *t*-test: *, $P < 0.05$ ($n = 3 \pm$ SE).

reduction was more pronounced in the transgenic lines under both control (mannitol) and K^+ treatments (Table S4). The abundance of the sucrose ion *m/z 366* (*m5*) relative to the parent ion *m/z 361* (*m0*), presented here as the ratio *m5*: *m0*, increased over time under both mannitol and K^+ treatments, and this increase was greater in WT compared with transgenic lines (Fig. 9). This is in close agreement with the lower level of sucrose observed in the transgenic lines, with higher rates of breakdown leading to lower ^{13}C enrichment in sucrose in transgenic lines. Together with sucrose breakdown, we observed an increase in the levels of fumarate and slight increases in succinate in all genotypes (Fig. 9). Although only small changes in the ^{13}C enrichment of fumarate (*m2*: *m0*) were observed over time, ^{13}C enrichment was observed at *m3* in succinate and a large increase in *m3/m0* (ratio between the abundances of *m/z 132* and the parent ion *m/z 129*) was observed over time. Interestingly, ^{13}C enrichment in succinate increased from 0 to 30 min and then decreased until 60 min, with this occurring to a greater extent in the transgenic lines (Fig. 9).

Discussion

Sucrose as substrate for tobacco guard cell regulation

Although guard cell metabolism has been studied for almost an entire century (Sayre, 1923), important questions regarding the metabolic changes that occur during stomatal opening remain to be answered, particularly with respect to the source and role of sucrose in guard cell metabolism. The role proposed for sucrose in guard cells is mainly linked to its function as an important osmolyte (Talbott & Zeiger, 1998). However, this theory was proposed by correlating the magnitude of stomatal aperture with the level of sucrose throughout the day. Alternative roles for sucrose in guard cell regulation, as a substrate for respiration or in organic acid biosynthesis have rarely been addressed in the literature (Ditrich & Raschke, 1977). On the basis of this information, and experiments indicating a link between sucrose cleavage and stomatal aperture (Antunes *et al.*, 2012; Daloso *et al.*, 2015), we generated tobacco plants overexpressing *SUS3* under the control of the KST1 promoter. Although SUS operates mainly in the direction of sucrose cleavage (Bieniawska *et al.*, 2007; Antunes *et al.*, 2012), this enzyme is capable of working in both the sucrose synthesis and sucrose breakdown direction. In this context, a clear reduction in the ratio of disaccharides to monosaccharides occurred in guard cell-enriched epidermal fragments of transgenic lines, most prominently reflected in the sucrose to fructose ratio, indicating that transgenic lines have increased capacity to cleave sucrose, as confirmed by the higher SUS activity in these lines. Furthermore, the isotope kinetic labeling experiment showed that transgenic plants have greater capacity to degrade sucrose (Fig. 9).

The higher capacity to cleave sucrose observed in the transgenic lines leads to increased stomatal conductance and stomatal aperture. These data are consistent with previous experiments in which

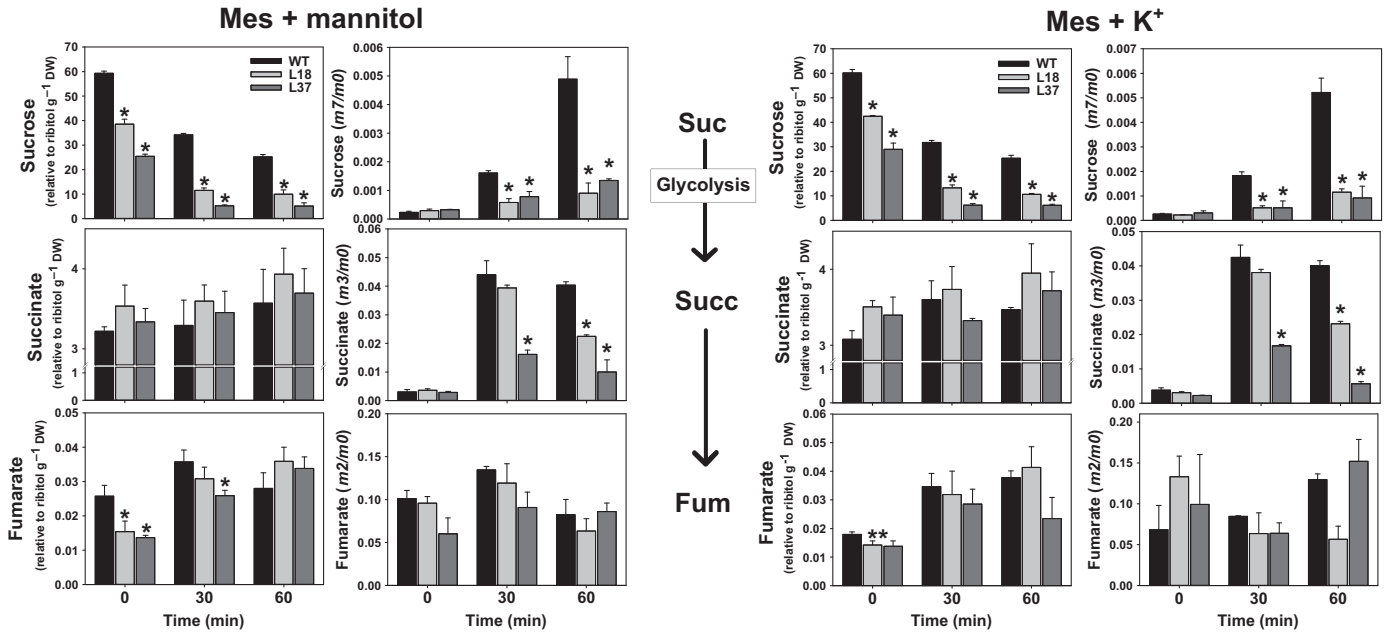

Fig. 9 Metabolite profiling (left graphs) and ^{13}C enrichment (right graphs) in guard cell-enriched epidermal fragments of *Nicotiana tabacum* wild-type (WT) and transgenic lines overexpressing the sucrose synthase 3 gene specifically in guard cells (L18, L37) during the dark to light transition. Plants were grown under well-controlled glasshouse conditions. Guard cell-enriched epidermal fragments were harvested at predawn (0 min), transferred to solutions containing mannitol (left panel) or KCl (right panel), and harvested after 30 and 60 min in the light (see the Materials and Methods section for details). The values of metabolite profiling are as described in Fig. 8. Asterisks indicate values significantly different from WT at the same time point by Student's *t*-test: *, $P < 0.05$ ($n = 3 \pm$ SE). The comparison of the rate of sucrose (Suc) breakdown across time between the genotypes is shown in Supporting Information Table S4. Succ, succinate; Fum, fumarate.

increasing guard cell acid invertase activity in potato plants via cell type-specific expression of the yeast *SUC2* gene resulted in increased g_s and net CO_2 assimilation rate (Antunes *et al.*, 2012). In the same study, decreasing levels of *SUS3* using an antisense strategy produced a decrease in g_s, net CO_2 assimilation rate and transpiration. Furthermore, experiments with WT guard cell-enriched epidermal fragments revealed reduced levels of sucrose, glucose and fructose during light- and potassium-induced stomatal opening (Daloso *et al.*, 2015), indicating that the breakdown of sucrose and other sugars is likely to be important during this process. Taken together, these results linking increased sucrose cleavage to greater stomatal aperture provide evidence for a role of sucrose in guard cell regulation beyond that of an osmolyte, and suggest that sucrose also acts as a substrate for guard cell regulation. It seems likely that this mechanism would occur under white light- and potassium-induced stomatal opening, as a higher stomatal aperture was observed in the transgenic lines under these conditions. This hypothesis is supported by the fact that line L18 presented significantly higher stomatal aperture than WT after 4 and 5 h of light- and potassium-induced stomatal opening (Fig. 3b), which is consistent with the higher SUS activity of this line (Fig. 1f). In this context, given that potassium-induced stomatal opening is ATP dependent (Raghavendra & Vani, 1989; Parvathi & Raghavendra, 1997; Tominaga *et al.*, 2001), sucrose breakdown would stimulate fluxes through glycolysis and mitochondrial metabolism in order to increase the amount of ATP produced during light- and potassium-induced stomatal opening (Daloso *et al.*, 2015). However, further experiments are needed to confirm whether this does indeed occur *in planta*.

Changes in organic acid levels suggest higher substrate supply for guard cell respiration

The contribution of organic acids and enzymes of the TCA cycle to the regulation of stomatal movements has been demonstrated recently, as transgenic tomato plants with constitutive reduced levels of fumarase (Nunes-Nesi *et al.*, 2007) and the iron–sulfur subunit of succinate dehydrogenase (SDH) (35S:SDH) (Araújo *et al.*, 2011) showed substantial changes in stomatal behavior. Here, the contents of malate, an organic acid previously shown to contribute to stomatal movements, were unaltered in both leaf and guard cell-enriched epidermal fragments of transgenic plants. However, the contents of citrate, succinate and 2-oxoglutarate were lower in guard cell-enriched epidermal fragments of transgenic plants harvested at midday (Fig. 7). In addition, the levels of fumarate and citrate were lower and the level of glutamine was higher in guard cell-enriched epidermal fragments of transgenic plants compared with WT harvested predawn (Figs 8, 9), suggesting that the TCA cycle is involved in the altered stomatal movements in the transgenic plants.

Succinate seems likely to be involved in stomatal movements, given that we observed ^{13}C enrichment in this metabolite during light-induced stomatal opening (Daloso *et al.*, 2015). As a substrate for complex II of the mitochondrial electron transport chain, the decrease in succinate observed in transgenic plants may be a result of a high rate of consumption by the respiratory process. This hypothesis is supported by results from the isotope kinetic labeling experiment, which showed a large increase in ^{13}C enrichment in the first 30 min under light, and a large decrease in

the next 30 min under light (Fig. 9). Furthermore, given that the sucrose breakdown and decrease in ^{13}C enrichment in succinate were more pronounced in the transgenic lines (Fig. 9), the results suggest that this mechanism may explain the greater stomatal aperture observed in transgenic plants. This idea is supported by the fact that guard cells contain numerous mitochondria (Pallas & Mollenhauer, 1972; Willmer & Fricker, 1996) and exhibit both high respiratory rates (Vani & Raghavendra, 1994; Araújo *et al.*, 2011) and high activity of ATP-dependent transporters (ATPases) (Lawson & Blatt, 2014; Wang Y *et al.*, 2014a). This high capacity for ATP production is necessary to support the activity of the ATPases localized within the guard cell plasma membrane that are responsible for membrane hyperpolarization during stomatal opening (Wang Y *et al.*, 2014a,b). ATP production in guard cells therefore represents a very important process during stomatal opening (Suetsugu *et al.*, 2014; Sun *et al.*, 2014; Wang SW *et al.*, 2014), highlighting the importance of mitochondrial metabolism and mitochondrial respiration for guard cell function. This hypothesis is further supported by the fact that mutation in phosphoglycerate mutase, a key glycolytic enzyme, leads to impaired blue light-induced stomatal opening (Zhao & Assmann, 2011).

It is worth highlighting, however, that transgenic plants with altered activity of TCA cycle enzymes show great differences in their stomatal behavior when the levels of malate are altered in the mesophyll cells (Nunes-Nesi *et al.*, 2007; Araújo *et al.*, 2011). It seems likely that the accumulation of malate in the mesophyll cells or in the apoplast space of guard cells may be a key point connecting photosynthesis and stomatal movements. However, antisense inhibition of SDH specifically in guard cells (MYB60:SDH) did not alter either leaf or apoplastic levels of malate, and no effect on stomatal conductance was observed in these plants (Araújo *et al.*, 2011). Given that we did not measure the levels of organic acids in guard cells of MYB60:SDH plants (Araújo *et al.*, 2011), it is difficult to compare with the results presented here. However, it is important to highlight that, although both 35S:SDH antisense lines and KST1:SUS3-overexpressing lines present higher g_s, it seems likely that the phenotypes of these transgenic plants are related to different mechanisms of stomatal regulation. Evidence for this hypothesis is mainly based on the fact that 35S:SDH antisense plants present great differences in the leaf and apoplast accumulation of malate, whereas, in SUS3 overexpression lines, the level of this metabolite did not change in leaves or in guard cells. It seems likely that the accumulation of the mesophyll-derived malate would have a high impact on g_s, but the importance of malate accumulation within guard cells is still not clear. Notwithstanding, we cannot rule out an important role for malate in the metabolic regulation of guard cells during stomatal opening, given that it has been shown that starch degradation in guard cell chloroplasts supplies the carbon necessary for malate accumulation under blue light (Talbott & Zeiger, 1993; Lascève *et al.*, 1997). Further experiments will be needed to better understand metabolic fluxes in guard cells during stomatal opening and, in particular, to understand in what flux mode (Tcherkez *et al.*, 2009; Gauthier *et al.*, 2010; Sweetlove *et al.*, 2010) the TCA cycle is operating.

Guard cell genetic manipulation as a strategy to increase photosynthesis and plant growth

Stomatal density and leaf area are directly related to transpiration rate (Lake & Woodward, 2008; Nilson & Assmann, 2010). Leaves with higher stomatal density and higher leaf area show higher transpiration rate (Yu *et al.*, 2008). Here, no changes were observed in stomatal density, and thus the higher stomatal aperture, g_s and E cannot be caused by changes in the number of stomata per leaf. Furthermore, higher whole-plant transpiration per area ($g\,H_2O\,h^{-1}\,m^{-2}$) and per plant ($g\,H_2O\,plant^{-1}$) were observed in the transgenic lines independent of growth conditions. These data exclude the possibility that the higher transpiration rate of L18 and L37 results from higher leaf area, higher stomatal density or growth conditions. It is interesting to highlight the large amount of water transpired by tobacco plants. We observed that tobacco plants lose *c.* $260\,g\,H_2O\,plant^{-1}\,d^{-1}$, on average, over all genotypes under non-controlled glasshouse conditions (Fig. 4d). The high transpiration rate of tobacco plants results in a very drought-sensitive genotype in which tissue dehydration was very often observed during the whole-plant transpiration experiments. Indeed, in the drought stress experiment, we observed that small reductions in RWC led to a strong reduction in the photosynthetic performance of the plants (Fig. S1). In this context, SUS3 lines presented higher water losses under irrigated conditions (Fig. 4c,d) and slight reductions in tissue dehydration compared with WT, as indicated by the higher RWC values under drought stress discussed later.

The stomatal pore is the first barrier to the influx of CO_2 for photosynthesis, and thus the magnitude of stomatal opening can limit the net photosynthetic rate (A) (Jones, 1998). Our previously published data suggest that the manipulation of guard cell sucrose metabolism can have a significant impact on plant growth and plant water use efficiency. Here, we showed that, when increasing stomatal conductance (g_s) through guard cell SUS3 overexpression, A increased. As a consequence, higher A led to increased growth and higher g_s led to higher transpiration in transgenic lines under favorable growth conditions. Furthermore, both transgenic lines produced the same amount of total plant DW and leaf DW compared with WT after a drought period. Interestingly, the transgenic line L37 showed a higher percentage of biomass allocated to leaves and stems after drought stress (Table S2). We suggest that the higher biomass accumulated before the imposition of stress may help these plants to adapt to subsequent drought conditions. Alternatively, given that SUS3 is upregulated in guard cells under drought conditions (Kopka *et al.*, 1997) or in response to ABA treatment (Yang *et al.*, 2008), plants overexpressing *SUS3* in their guard cells may have an increased drought tolerance by a mechanism still not understood. Further studies may help to identify the mechanism which underlies the phenotype observed in the transgenic lines under drought and other stress conditions.

In summary, transgenic tobacco plants overexpressing *SUS3* under the control of the KST1 promoter were characterized by increased stomatal conductance, stomatal aperture, photosynthesis and biomass production. The results suggest that guard cell

genetic manipulation may be a promising strategy to increase the photosynthetic activity and growth. Furthermore, the results suggest that sucrose breakdown may be a response in order to increase the supply of substrates for respiration, providing new links between sucrose and TCA cycle metabolism during the process of stomatal opening.

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Author contributions

D.M.D., T.C.R.W., W.C.A., M.E.L. and A.R.F. planned and designed the research; D.M.D., T.C.R.W., W.C.A., D.P.P. and C.M. performed experiments and analyzed the data; D.M.D., T.C.R.W., W.C.A. and A.R.F. wrote the manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Effects of drought stress on relative water content and photosynthetic activity.

Fig. S2 Metabolite content in leaves.

Table S1 Growth parameters and leaf stomatal density

Table S2 Growth parameter under well watered and drought stress conditions

Table S3 Leaf metabolite profile

Table S4 Sucrose level in guard cell-enriched epidermal fragments during dark to light transitions

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