DANILO DE MENEZES DALOSO

ROLE OF SUCROSE FOR TOBACCO GUARD CELL OSMOREGULATION:

OSMOLYTE OR SUBSTRATE?

Thesis submitted to Federal University of Viçosa, as part of the requirements for obtaining the *Doctor Scientiae* degree in Plant Physiology

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"This work is dedicate...

To my parents and all my family"

"Dedico esse trabalho...

Aos meus pais e à toda minha família"

"......years ago I knew everything; now I know nothing.

Education is a progressive discovery of our own ignorance."

William James Durant

Acknowledgements

To God, by the support during all my life.

To my parents and all my family, nothing is possible without you all.

To Department of Plant Biolgy of Federal University of Viçosa and to the Max Planck Institute for Molecular Plant Physiology, for the opportunity to develop this work.

To the supervisor's, Marcelo Loureiro, Thomas Williams, Alisdair Fernie, and Wagner Araújo, for the suggestions and contributions in this work.

To all professors of my life, everyone was very important for me. I really have respect and admiration of you all....many thanks.

To all my friends from different cities, states and countries, by the help in the experiments and analysis of these work's as well as the happy moments.

To all techinician of UFV and MPIMP, by the help during the experiments and analysis.

To FAPEMIG, for the fellowship.

Biography

DANILO DE MENEZES DALOSO, son of Roberto Belino Daloso and Maria Juciene de Menezes, was born in Rancharia, SP, Brazil in September 18th of 1982. He was under-graduated in Biological Sciences by University of State of Mato Grosso do Sul, MS. On 2009 he obtained his *Master Scientiae* degree in Plant Physiology at Federal University of Viçosa. On 2009 he started his doctoral studies in Plant Physiology program also at Federal University of Viçosa. Between February of 2012 and December of 2012 he developed the sandwich doctoral in Max Planck Institute for Molecular Plant Physiology in Potsdam-Golm, Germany. In February of 2013 was finished his works being here shown.

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RESUMO

A papel da sacarose em CG foi investigado através da caracterização de plantas de Tabaco transgênicas superexpressando a isoforma 3 do gene sacarose sintase (NtSuSy3) sob controle do promotor KST1 bem como por experimentos de análise de fluxo metabólico utilizando fragmentos epidérmicos enriquecidos com CG (EF) durante abertura estomática induzida pela luz. As plantas NtSuSy3 mostraram aumentos na condutância estomática, fotossíntese e nas taxas de transpiração a nível foliar e de planta inteira. As alterações observadas no metabolismo de CG das plantas transgênicas são discutidas no texto. Observou-se uma redução nos níveis de sacarose em diferentes experimentos de abertura estomática induzida pela luz, enquanto que os níveis de sacarose no meio não foram alterados, sugerindo que a sacarose foi degradada no simplasto de CG. A análise via LC-qTOF-MS de experimentos com EF submetidos a $NaH^{13}CO_3$ durante abertura estomática induzida pela luz mostraram um enriquecimento de ^{13}C em sacarose, malato, fumarate e glutamins. As possíveis funções exercidas por esses metabólitos são discutidas no texto. Em conjunto, os dados desse trabalho sugerem que a degradação da sacarose no simplasto de CG pode ser um mecanismo importante para a abertura estomática de Tabaco induzida pela luz.

ABSTRACT

A characterization of transgenic tobacco plants overexpressing potato sucrose synthase 3 gene (NtSuSy3) under control of KST1 promoter was performed in order to analyze the role of sucrose metabolism on GC osmoregulation. Also, we performed a metabolic flux analysis in guard cell enriched epidermal fragment (EF) of *Nicotiana tabacum* in order to investigate changes in GC metabolism during stomatal aperture light-induced. NtSuSy3 plants showed higher stomatal conductance, transpiration rate, whole plant transpiration, and net photosynthetic rate than wild type (WT). Several changes in GC metabolism were observed in transgenic plants are discussed in the text. In different stomatal aperture light-induced experiments, it was observed a decrease in sucrose content, while no changes were detected in the sugar content in the medium; suggesting that the sugars decreased observed is due to breakdown and not efflux of GC. Using a feeding strategy in EF submitted to $NaH^{13}CO_3$ followed by LC-qTOF-MS analysis, a ^{13}C-enrichment in sucrose, malate, fumarate and glutamine were observed. The possible function of these metabolites for GC osmoregulation are discussed in the text. Taken together, the data showed here provide evidence for another role of sucrose for GC osmoregulation. Our data suggest that sucrose breakdown, not just sucrose accumulation, can be performed to induce stomatal opening in tobacco.

General Introduction

The control of stomatal aperture involves reversible changes in the GC osmolytes concentration, mainly potassium and sucrose. The theory proposed by Talbott and Zeiger (1998) suggests that sucrose is an osmolyte in the GC symplast during the period of greater stomatal aperture. However, recent results of our research group have shown that this theory cannot be value for all conditions. Our results have demonstrated that the daily course of stomatal aperture of the plants grown in tropical climates is not consistent with the classical theory. Also, Antunes et al. (2012) showed that potato transgenic plants overexpressing invertase or antisense constructions of sucrose synthase have stomatal conductance increased and decreased, respectively. These results are opposite to the theory previously proposed by Zeiger and co-workers, and suggest that the sucrose breakdown is also important for stomatal aperture. However, it remains far from clear what metabolic pathways are activated after sucrose breakdown. In this context, the first chapter involves characterization of Tobacco transgenic plants sense to sucrose synthase 3 gene under control of KST1 promoter. The aim of this work is identify changes in guard cell metabolism with altered sucrose synthase 3 expression, checking the effect of this manipulation on whole plant transpiration and drought tolerance. The second chapter also involves the investigation of the role of sucrose for tobacco GC osmoregulation. However, different strategies of flux experiments in guard cell enriched epidermal fragment (EF) from wild type tobacco were used in order to determine the changes in GC metabolism during stomatal aperture induced by light and sucrose and/or K^+.

References

Antunes WC, Provart NJ, Williams TCR, Loureiro ME (2012) Changes in stomatal function and water use efficiency in potato plants with altered sucrolytic activity. Plant Cell and Environment, 35(4): 747-59.

Talbott LD, Zeiger E (1998) The role of sucrose in guard cell osmoregulation. J Exp Bot 49: 329–337

Chapter I

Mild increase in guard cell sucrose synthase 3 (SUS3) expression leads to increase in stomatal conductance, whole plant transpiration and growth associated with changes in guard cell metabolism.

Summary. Sucrose is proposed to be an important osmolyte for guard cell (GC) osmoregulation. Sucrose synthase (SuSy) is an enzyme related to both sucrose breakdown and sucrose synthesis. It has been previously demonstrated that isoform 3 (SUS3) has a high level of expression in GC, however, the function of SUS3 in GC metabolism and stomatal opening remains to be elucidated. A characterization of transgenic tobacco plants overexpressing potato SUS3 under control of KST1 promoter was performed in order to analyze the role of sucrose metabolism in guard cell osmoregulation and the effect of this genetic manipulation on whole plant drought tolerance. The GC SUS3 overexpression led to increased stomatal conductance (g_s), transpiration rate (E), whole plant transpiration, and net photosynthetic rate (A). Furthermore, an increase in plant, leaf, and stem dry weight as well as leaf carbon content was observed. Due to higher g_s and E, transgenic plants were more sensitive to drought stress and showed decreased instantaneous and intrinsic water use efficiency. No changes was found in electron transport rate, maximum rates of rubisco carboxylation, maximum capacity for photosynthetic electron transport, triose phosphate use limitation, light and CO_2 saturation point, light and CO_2 compensation point, mesophyll conductance to CO_2, dark respiration, photorespiration, stomatal density, and leaf concentration of photosynthetic pigments. Thus, the increase in g_s and A was not due to increase in photochemical performance, mesophyll capacity to transfer or to fix CO_2 as well as number of stomatal per area. Minor changes were observed in leaf sugar metabolism in transgenic plants. However, the sucrose/fructose ratio was reduced in epidermal fragments (EF) of transgenic lines. Several changes were observed in primary metabolites of leaves and EF of transgenic plants, with a clear reduction in the content of the organic acids succinate, citrate and 2-oxoglutarate observed in EF. Altogether, our data suggest that sucrose breakdown, not just sucrose accumulation, is an important mechanism that regulates stomatal opening in tobacco. The results are discussed in the context of the role of sucrose as a substrate molecule for GC osmoregulation.

Key Words: Sucrose synthase, guard cell metabolism, stomatal conductance, water use efficiency.

Introduction

Stomata are leaf epidermal structures which consist of two guard cells (GC) surrounding a pore. Stomatal closure is one of the most important responses to periods of drought, the abiotic stress that most affects global agricultural production (Rai and 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 and Assmann 2009). Although many progress has been made towards obtaining drought tolerant genotypes through manipulation of ABA metabolism (Yu et al. 2008; Nilson and Assmann 2010; Yoo et al. 2010), in most cases these drought-tolerant transgenic plants exhibit reduced stomatal opening, which in turn lead a reduction in net photosynthetic rate and consequently in reduction of growth, specially under moderate drought conditions (Galmés et al. 2007). An alternative strategy to obtain drought tolerant plants can be by manipulation of ABA-independent pathways (Nelson et al. 2007) or through manipulation of GC sucrose metabolism (Antunes et al. 2012). Therefore, an understanding of the processes that regulate stomatal movements would have potential biotechnological applications, since it offers a route to greater water use efficiency (WUE) (Yang et al. 2005).

The control of stomatal opening involves reversible changes in GC osmolyte concentration as sucrose as well as potassium (K^+) and the counter ions malate ($malate^{2-}$), chloride (Cl^-), and nitrate (NO_3^-) (Hedrich and Marten 1993; Talbott and Zeiger 1996; Guo et al. 2003). The relative importance of malate as counter ion depends on the Cl^- concentration (Raschke and Schnabl 1978; Van Kirk and Raschke 1978), whilst the relative importance of Cl^- or $malate^{-2}$ as counterions of K^+ depend on the growth conditions (Talbott and Zeiger 1996). The daily course of stomatal movements of plants grown in temperate climates involves reversible changes in GC osmolyte concentration separated in two phases, accumulation of potassium (K^+) in the morning and accumulation of sucrose in the afternoon (Talbott and Zeiger 1998). According to this currently accepted theory, in the early period of the day there is an influx of K^+ and Cl^- from apoplast to symplast of GC with concomitant malate accumulation from starch breakdown (Talbott and Zeiger 1996; Zeiger et al. 2002; Outlaw Jr, 2003; Shimazaki et al. 2007). The highest K^+ concentration is found at around midday, and levels then decline with an increase in sucrose concentration. Thus, sucrose is supposed to be the major osmolyte during the afternoon period (Tallman and Zeiger 1988; Talbott and Zeiger, 1993, 1996, 1998; Zeiger et al. 2002).

Sucrose synthase (SuSy) is a key enzyme for sugar metabolism which catalyzes the reversible conversion of sucrose and UDP or ADP to fructose and UDP-glucose or ADP-

glucose (Baroja-Fernández et al. 2009). Six isoforms (SUS1-6) were founded in the genome of Arabidopsis (Baud et al. 2004), of which isoform 3 (SUS3) is specifically expressed in guard cells (Bieniawska et al. 2007) during drought conditions (Kopka et al. 1997). However, the role of this gene in GC function remains poorly understood. Previous results from our group showed that mild reductions of SUT transporter expression in GC leads to reductions in GC sucrose content and stomatal conductance (g_s), including in the morning phase of stomatal opening, suggesting that sucrose is also important in this phase (Antunes et al. *unpublished*). Furthermore, contrary to expected, Antunes et al. (2012) showed that potato transgenic plants expressing a yeast invertase or an antisense construct targeted against sucrose synthase have increased and decreased stomatal conductance respectively. These results from Antunes et al. (2012) suggest that sucrose breakdown, not just sucrose accumulation, is important for the control of stomatal opening. However, the fate of carbon released by sucrose breakdown, and its importance in stomatal movements remains far from clear.

The role of sucrose during stomatal opening therefore remains to be clearly defined. This work aimed to contribute to our knowledge about the role of sucrose in GC osmoregulation using a reverse genetic approach. We created tobacco transgenic plants overexpressing isoform 3 of potato sucrose synthase (SUS3) under control of the KST1 promoter, which induced the overexpression only in GC. Based in the phenotype of plants expressing yeast invertase (Antunes et al. 2012), we hypothesised that SUS3 overexpression specifically on GC should lead to an increase in g_s. As expected, the results showed an increase in g_s, transpiration rate and whole plant transpiration in transgenic lines. The phenotype is not associated with photochemical performance, mesophyll capacity to transfer or to fixe CO_2 as well as the number of stomatal per area. Our data suggest that sucrose breakdown can be performed to induce stomatal opening in Tobacco.

Results

In silico analysis suggests an important role of SUS3 for guard cell function and stress tolerance

The amino acid sequence of sucrose synthase 3 isoform (SUS3) is highly conserved between the species analysed, showing many regions with more than 80% homology (Figure S1). As example, the identity and similarity of closed species *Solanum lycorpsicum* and *Solanum Tuberosum* (Figure S2 g) are 80 % and 98 %, respectively. A careful *in silico* analysis of the SUS3 gene (AT4G02280) of *Arabidopsis thaliana* was performed from available microarray data in order to investigate the function and the contribution of this gene

to guard cell metabolism and the stomatal opening process. Three different on-line platforms were used, Arabidopsis eFP Browser (Figure S2 a-d), Genevestigator (Figure S2 e) and AtGenexpress (Figure S2 f). At the whole plant level, SUS3 is mainly expressed in mature seeds of Arabidopsis (Figure S2 a). However, at cellular level SUS3 has a high expression in guard cells compared to mesophyll cells (Figure S2 b,e). This high expression can be related to processes regulated by abscisic acid (ABA), and a high expression of SUS3 has been found after ABA treatments (Figure S2 c,f). AtSUS3 does not appear to respond to other plant hormones (Figure S2 f), nevertheless is responsive to severe osmotic stress (Figure S2 d) and is coexpressed in a network composed by genes related to salt and stress tolerance (Figure S3).

KST1 promoter induce SUS3 overexpression specifically in guard cells

Aiming to avoid pleiotropic effects that could result from the use of a constitutive promoter, we created transgenic tobacco plants overexpressing SuSy isoform 3 of potato under the control of KST1 promoter (Figure S1 i), which is known to drive expression mainly in GC (Plesch et al. 2001). The plants were transformed by Agrobacterium and 20 lines were first selected by *polymerase chain reaction* (PCR) target to NPTII gene (Figure S1 h). After analysis of germination and root growth under kanamicin$^{®}$ treatment (data not shown), a quantitative *reverse transcript poli chain reaction* (qRT-PCR) was performed to analyze the level of SUS3 gene expression, normalized by actin (ACT) gene expression, in whole leaves and guard cell enriched epidermal fragments (EF). No changes in SUS3 expression were observed in leaves of transgenic lines, while mild increases were observed in EF of L11, L18 and L37, however only L18 and L37 were statistically different from WT ($p<0.05$) (Table 1).

Guard cell SUS3 overexpression has no effect on enzyme activity

Quantification of enzyme activity was performed in order to verify if the increase in SUS3 transcript levels leads to an increase in sucrose synthase (SuSy) activity and also if this genetic modification had some effect in the activity of Invertase (INV), another enzyme involved in sucrose breakdown. No differences were detected in SuSy activities in leaves and EF between WT and transgenic lines. SuSy activity was higher in the sucrose breakdown (SuSy$_{bre}$) direction than sucrose synthesis (SuSy$_{syn}$) direction. Interestingly, SuSy activity is higher in EF compared to whole leaves, while both INV$_{alk}$ and INV$_{aci}$ have lower activity in EF. No differences were detected in the activities of INV$_{alk}$, INV$_{aci}$, and total INV (INV$_{Total}$) as well as in the total capacity for sucrose breakdown, showed by the sum of SuSy$_{Bre}$ and INV$_{Total}$. An additional analysis of SuSy activity was carried out using desalted extracts and

expressing the activity by protein. The results showed no differences between WT and transgenic lines again. SuSy activity was almost 30 times higher in EF than whole leaves when expressed on a protein basis (Table 1).

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

Metabolite levels in leaves and guard cell enriched epidermal fragments (EF) were determined in order to test the effect of SUS3 overexpression on leaf and GC metabolism. Minor changes were observed in leaf sugar metabolism in transgenic plants. However, substantial differences were observed in the level of sugars and in the relationship between disaccharide/monodisaccharides in EF (Figure 1). No changes were observed in sucrose, fructose, malate, and fumarate content as well as in the disaccharide/monosaccharide relationship in leaves. A decrease in leaf glucose content was observed in L18 plants (Figure 1). No differences were observed in the level of malate, fumarate, glucose and sucrose in EF of transgenic lines, while an increase in fructose content was observed in L18. A clear tendency of reduction of sucrose and increase in fructose was observed in L37. Sucrose synthase is an enzyme involved in both sucrose synthesis and breakdown. Thus, the relationship between the level of sucrose and your products UDP-glucose and fructose are important to verify the balance of sucrose metabolism in the transgenic lines. In this context, a clear reduction was observed in the relationship between disaccharides/monosaccharide in transgenic lines, mainly due to changes in the sucrose/fructose relationship (Figure 1). Although the *in vitro* SuSy activity was not altered in transgenic lines compared to WT (Table 1), the overexpression of SUS3 induced several changes in GC sugar metabolism.

A metabolomic analysis by GC-TOF-MS was performed in order to identify changes in the primary metabolism induced by SUS3 overexpression (Tables S2-S3). Although no changes were observed in leaf SUS3 expression and leaf SuSy activity, several changes were observed in primary leaf metabolites. In general, the level of phosphoric acid, serine, aspartic acid and methionine increased in leaves of transgenic lines while ascorbic acid, succinic acid, glucose and trehalose decreased in the leaves of lines L18 and L37 (Figure 2). Despite the lower number of metabolites detected in EF samples, the level of organic acids citric acid, oxoglutarate, succinic acid and malic acid as well as in the amino acid aspartate were reduced in EF of transgenic lines. Slight increases were observed in the level of the sugars fructose, glucose and sorbitol as well as in the amino acids serine and alanine in EF of transgenic lines (Figure 3).

Table 1. Gene expression and enzyme activities in guard cell enriched epidermal fragments (EF) and leaves of wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37). Sucrose synthase 3 gene expression was analyzed by quantitative *reverse transcript poli chain reaction* (qRT-PCR) normalized by actin (ACT) gene expression. Data are shown as relative expression normalized to WT. Sucrose synthase activity (nmol UDPG min^{-1} mg^{-1} fresh weight or nmol UDPG min^{-1} μg^{-1} protein) in sucrose breakdown direction (SuSy$_{bre}$), sucrose synthase activity (μmol min^{-1} g^{-1} fresh weight) in sucrose synthesis direction (SuSy$_{syn}$), acid invertase activity (INV$_{aci}$) (nmol glucose min^{-1} mg^{-1} fresh weight), alkaline invertase activity (INV$_{alk}$) (nmol glucose min^{-1} mg^{-1} fresh weight) were performed in EF and leaf of wild type and transgenic lines. SuSy$_{bre}$ was measured following two different protocols (see M&M). Gene expression values shown in bold and underlined indicate values significantly different from wild type by Student's *t* test ($P < 0.05$) (n = 5 ± SE). No significant differences in SuSy activity were detected between transgenic lines and wild type plants by Student's *t* test (p<0,05) (n = 6 ± SE).

		WT	SuSy3 L11	SuSy3 L18	SuSy3 L37
	EF SUS3 expression	1.00 ± 0.04	1.13 ± 0.04	**$\underline{1.18 \pm 0.04}$**	**$\underline{1.23 \pm 0.05}$**
	Leaf SUS3 expression	1.00 ± 0.04	0.99 ± 0.09	1.00 ± 0.02	1.04 ± 0.07
		nmol UDPG min^{-1} mg^{-1} FW			
EF	**SuSy$_{Bre}$**	0.123 ± 0.02	0.110 ± 0.02	0.146 ± 0.01	0.090 ± 0.01
	SuSy$_{Syn}$	0.007 ± 0.001	0.007 ± 0.001	0.008 ± 0.001	0.007 ± 0.001
	INV$_{Aci}$	1.66 ± 0.14	1.62 ± 0.11	1.80 ± 0.04	1.84 ± 0.03
	INV$_{Alk}$	0.11 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.13 ± 0.01
	INV$_{Total}$	1.77 ± 0.15	1.75 ± 0.11	1.94 ± 0.04	1.97 ± 0.04
	SuSy$_{Bre}$+INV$_{Total}$	1.90 ± 0.16	1.86 ± 0.10	2.09 ± 0.04	2.06 ± 0.04
Leaf	**SuSy$_{bre}$**	0.031 ± 0.01	0.039 ± 0.01	0.042 ± 0.01	0.045 ± 0.02
	INV$_{aci}$	16.70 ± 1.83	18.05 ± 1.27	19.89 ± 1.37	20.04 ± 1.44
	INV$_{alk}$	1.65 ± 0.42	1.61 ± 0.22	2.40 ± 0.15	1.71 ± 0.27
	INV$_{Total}$	18.35 ± 2.23	19.66 ± 1.49	22.30 ± 1.47	21.75 ± 1.58
	SuSy$_{Bre}$+INV$_{Total}$	18.38 ± 2.23	19.70 ± 1.49	22.34 ± 1.49	21.79 ± 1.59
		nmol UDPG min^{-1} μg^{-1} protein			
EF	**SuSy$_{bre}$**	59.80 ± 2.80	54.03 ± 2.61	61.66 ± 1.00	58.35 ± 2.64
Leaf	**SuSy$_{bre}$**	2.17 ± 0.33	4.65 ± 0.76	2.35 ± 0.21	2.56 ± 0.63
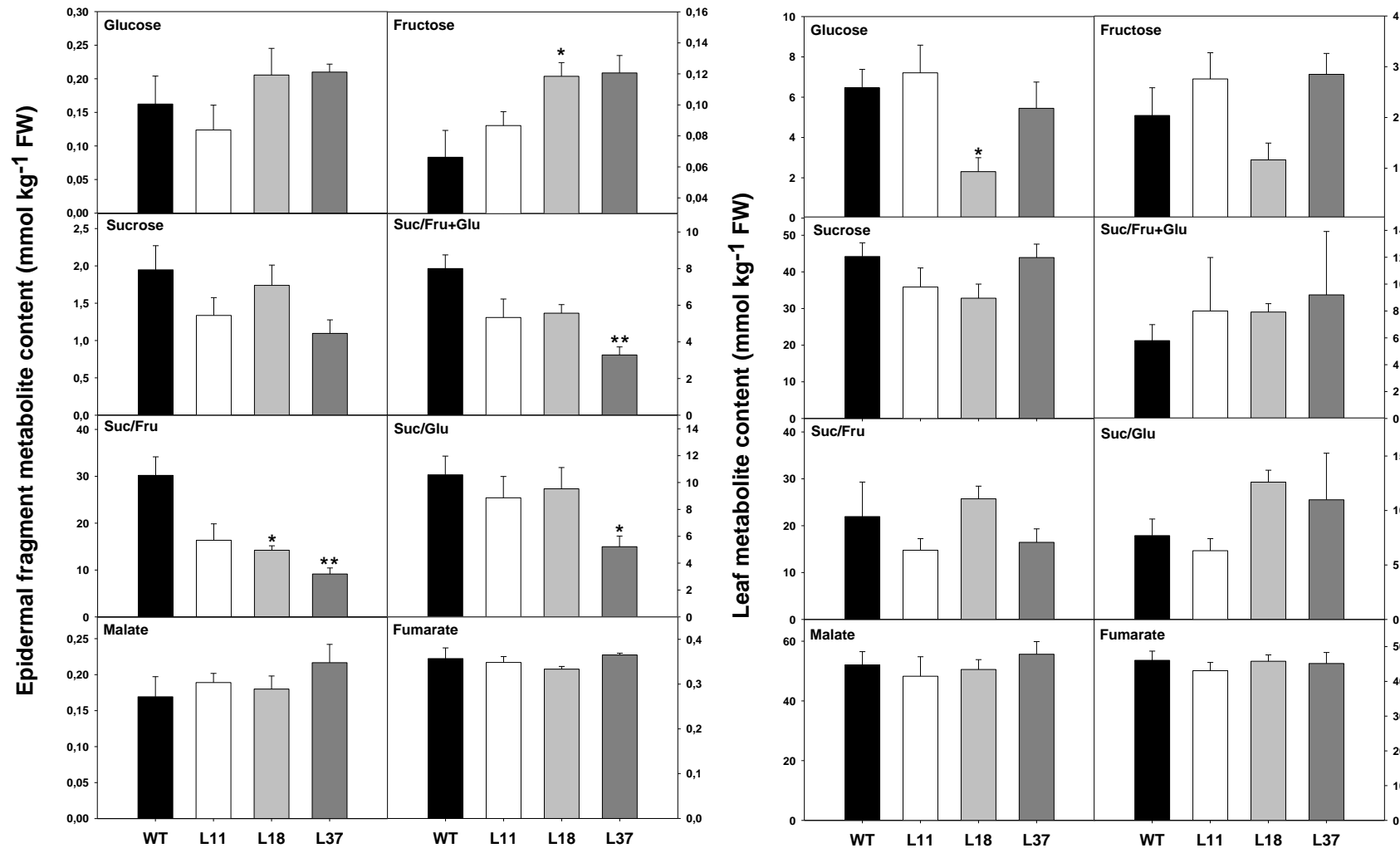
Figure 1. Metabolite content in epidermal fragment and leaves of wild type (WT) and transgenic lines (L11, L18, L37) sense to sucrose synthase 3 gene. Error bars indicate the standard error. * and ** asterisks indicate values significantly different from WT by Student's *t* test at 5% (p< 0.05) and at 1% (p< 0.01), respectively (n = 5 ± SE).
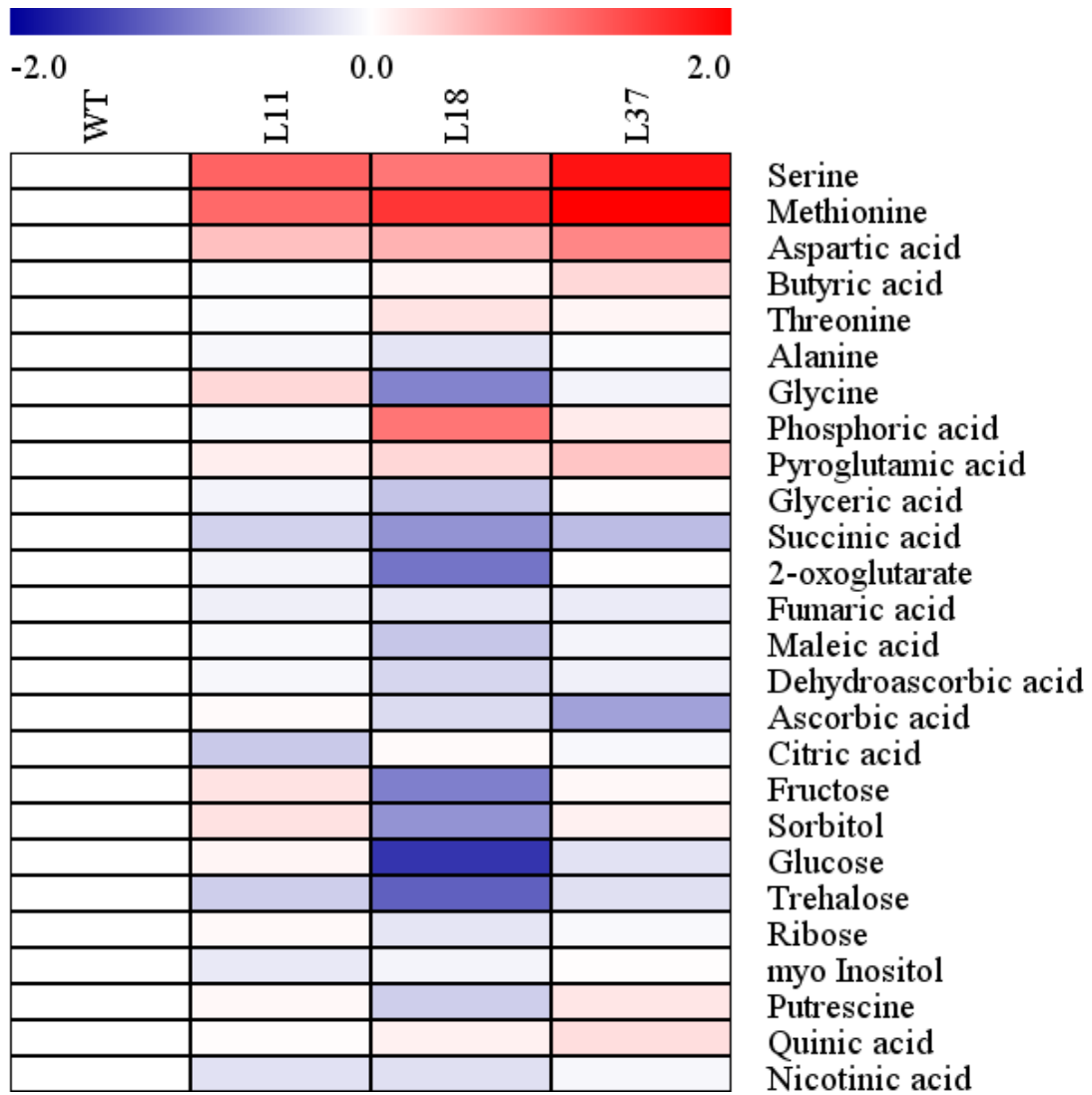
Figure 2. Heat map of leaf metabolic profile. The relative values of the table S2 were Log(2) transformed. The heat map was produced using MeV software (V.4.2.1) (n = 5 ± SE). The statics of the relative values are found in the Table S2.
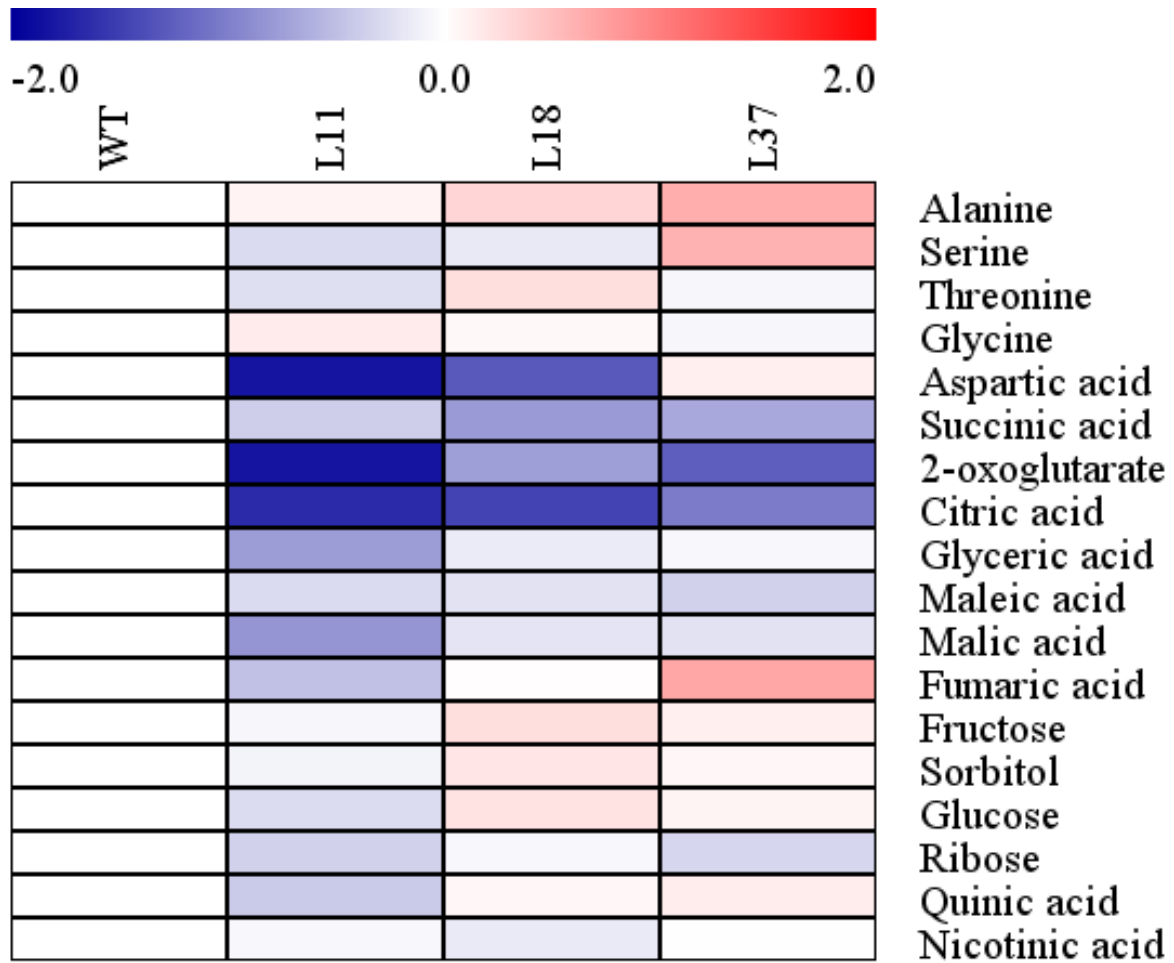
Figure 3. Heat map of guard cell enriched epidermal fragment metabolic profile. The relative values of the table S3 were Log(2) transformed. The heat map was produced using MeV software (V.4.2.1) (n = 5 ± SE). The statics of the relative values are found in the Table S3.

Changes in guard cell metabolism lead to increase in gas exchange

As gas exchange is highly dependent on growth conditions, we performed several experiments in different seasons, different years and under different conditions in order to carefully verify the stomatal behavior of transgenic plants. Analysis of thermal imaging by infra red thermography in young plants grown under artificial light conditions revealed certain transgenic lines with lower leaf temperature than wild type (WT) plants (Figure 4 a). It was observed whole plant transpiration increased in selected lines, principally in L37 (Figure 4 b). SuSy3 L37 plants showed higher g_s, E, and C_i/C_a at 08:00 and 10:00, while L18 only at 10:00 am. Higher rates of A were observed in both lines L18 and L37 at 10:00 am (Figure 5). The relationship between A and g_s suggest that the magnitude of A in tobacco is partially limited by g_s, since a direct linear relationship was observed between A and g_s (Figure 6). The

phenotype of lines with higher g_s and E were confirmed to be more sensitive to stress by analysis of fresh weight loss over time (Figure 4 c). In general L18 and L37 presented differences from WT in almost all analysis performed, while L11 was more similar, including at the transcript level (Table 1).
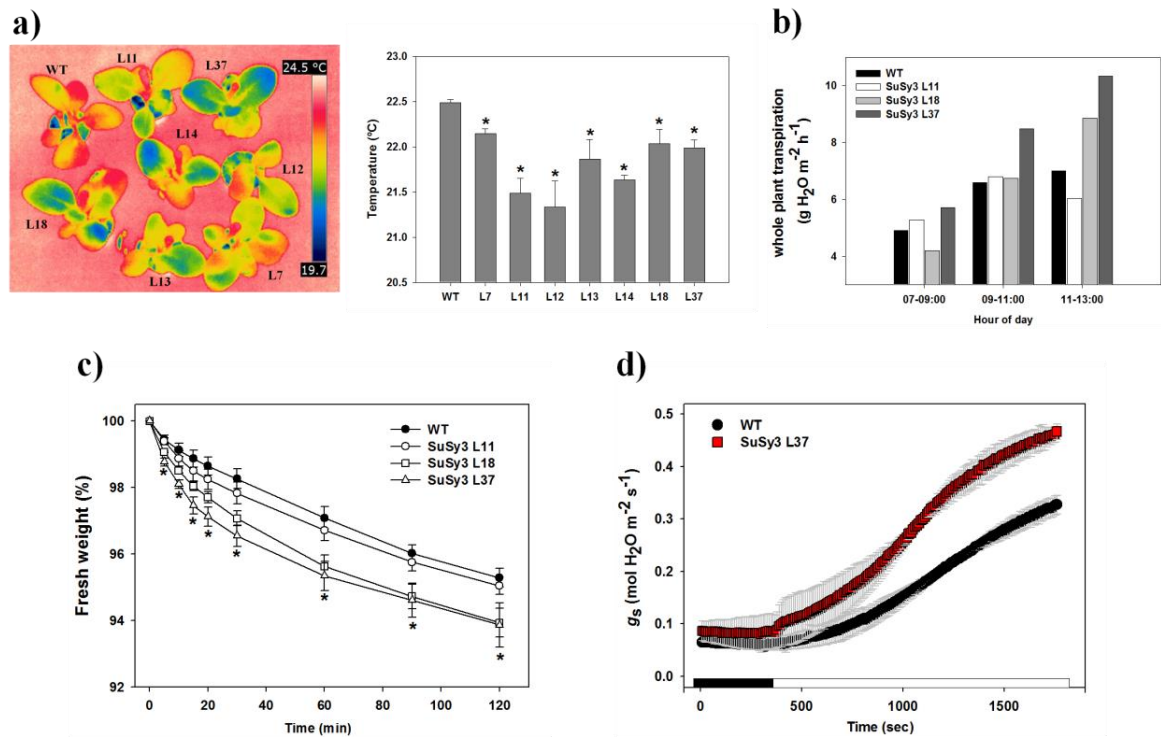
Figure 4. Physiological analysis of wild type (WT) and transgenic lines. The plants of all figures, except 4d, were growth in a chamber under light artificial conditions (150 μmol photons m^{-2} s^{-1}) with light/dark (16:8) period. Plants of the figure 4d were growth under green house conditions.

a) Infra red thermography of plants (left) and leaf temperature (right). The leaf temperature were measured in the most expanded leaf (n=8 temperature points per leaf).

b) Whole young plant transpiration (g H_2O h^{-1} m^{-2}) between different times of the day (n=4 ± SE).

c) Percentage of fresh weight loss along the time in detached young plants (n=4 ± SE).

d) Stomatal conductance (g_s) in dark-to-light transition in wild type (WT) and transgenic line (SuSy3 L37) (n=5 ± SE).

*Asterisks indicate values significantly different from wild type by Student's *t* test (P < 0.05)**.**

Figure 5. Gas exchange measures in completely expanded leaves of wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37). A − net photosynthetic rate, g_s − stomatal conductance, E − transpiration rate, C_i/C_a − ratio of sub-stomatal and ambient CO_2 concentration. Error bars indicate the standard error. *Asterisks indicate values significantly different from WT by Student's t test ($P < 0.05$) ($n=4 \pm SE$).

Figure 6. Relationship between photosynthetic rate (A) and stomatal conductance (g_s). Data from figure 2 ($n=8$). The linear regression was performed according equation: $f = yo + a*x$.

The daily course of gas exchange was analyzed in completely expanded leaves of plants grown in greenhouse conditions. In the first experiment, it was observed higher g_s (Figure 7 b) and E (Figure 7 c) in L18 at midday. No differences were detected in A along the day in the first (Figure 7 a) or the second experiment (Figure 7 d). The E was higher in L18 and L37 at 10:00 am in the second experiment, while no difference in g_s was detected in this experiment (Figure 7 e). Altogether, these two experiments developed in greenhouse showed the dynamic of gas exchange under tropical conditions. Low rates of g_s were detected during the pre-dawn period (05:00 and 06:00). However, is interesting to note that there is a very fast increase in g_s at early morning (Figure 7 b,e), the period of maximal g_s of the day. In this context, L37 plants showed higher capacity to increase g_s during dark-to-light transitions (Figure 4 d). The increase in g_s was followed by an increase in A (Figure 7 a,d) and E (Figure 7 c,f), since both process are dependent on stomatal opening.

Transgenic lines showed increased growth, whole plant transpiration and total leaf carbon content

Although mild increases were detected in g_s and E in transgenic lines analyzed by IRGA, a very sensitive analysis but one limited to a small area of the leaf, substantial differences was observed in transpiration at whole plant level through gravimetric methodology to quantify whole water loss by transpiration. An increase in the average daily water loss was observed in all lines analyzed (Figure 8 c). The lines L18 and L37 showed higher transpiration rates (%) than WT every day of the experiment (Figure 8 b), leading to higher values of water loss accumulated along of the 12 days (Figure 8 a) and lower leaf temperature (Figure 9 b,c). The leaf area was higher in L37 plants at the days 1 and 5, and higher in L18 plants at the day 12 of the experiment. When whole plant transpiration was expressed by leaf area (g H_2O m^{-2}), the results range according with the day of the experiment. Although on days 1 and 12 the rate of transpiration per area was similar between WT and transgenic lines, on day 5 of the experiment L18 and L37 plants showed higher values of transpiration per area (Figure 8 e). The data of this experiment exclude the possibility of the increase in g_s and E showed by IRGA analysis (Figures 5, 7) to be an effect limited to a small area used in the chamber of the IRGA. The increase in g_s cannot be associated with changes in stomatal density as there was no difference detected in this parameter between WT and transgenic lines (Table 2).
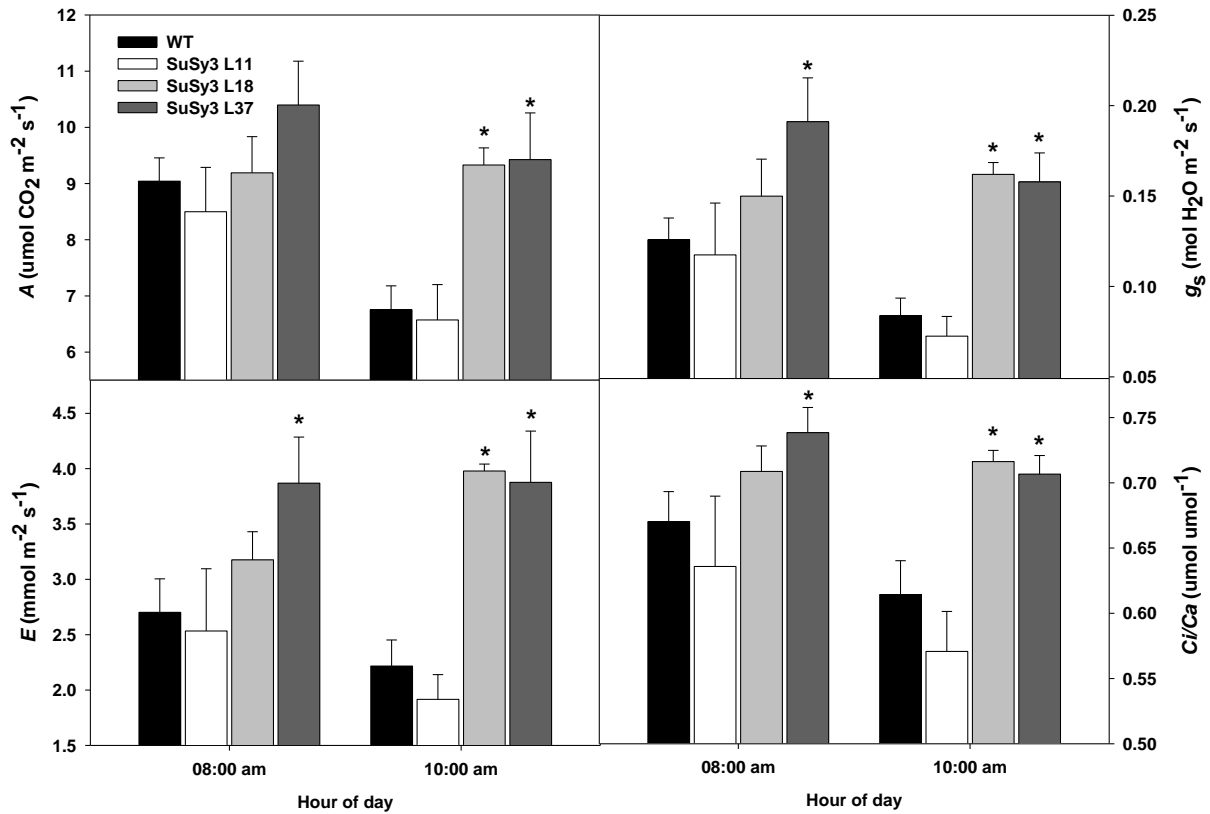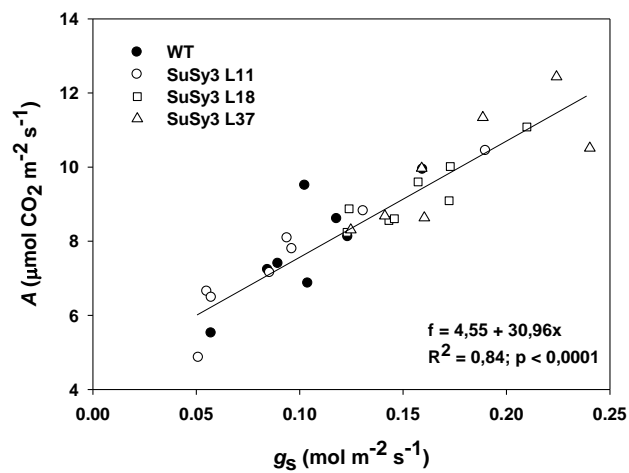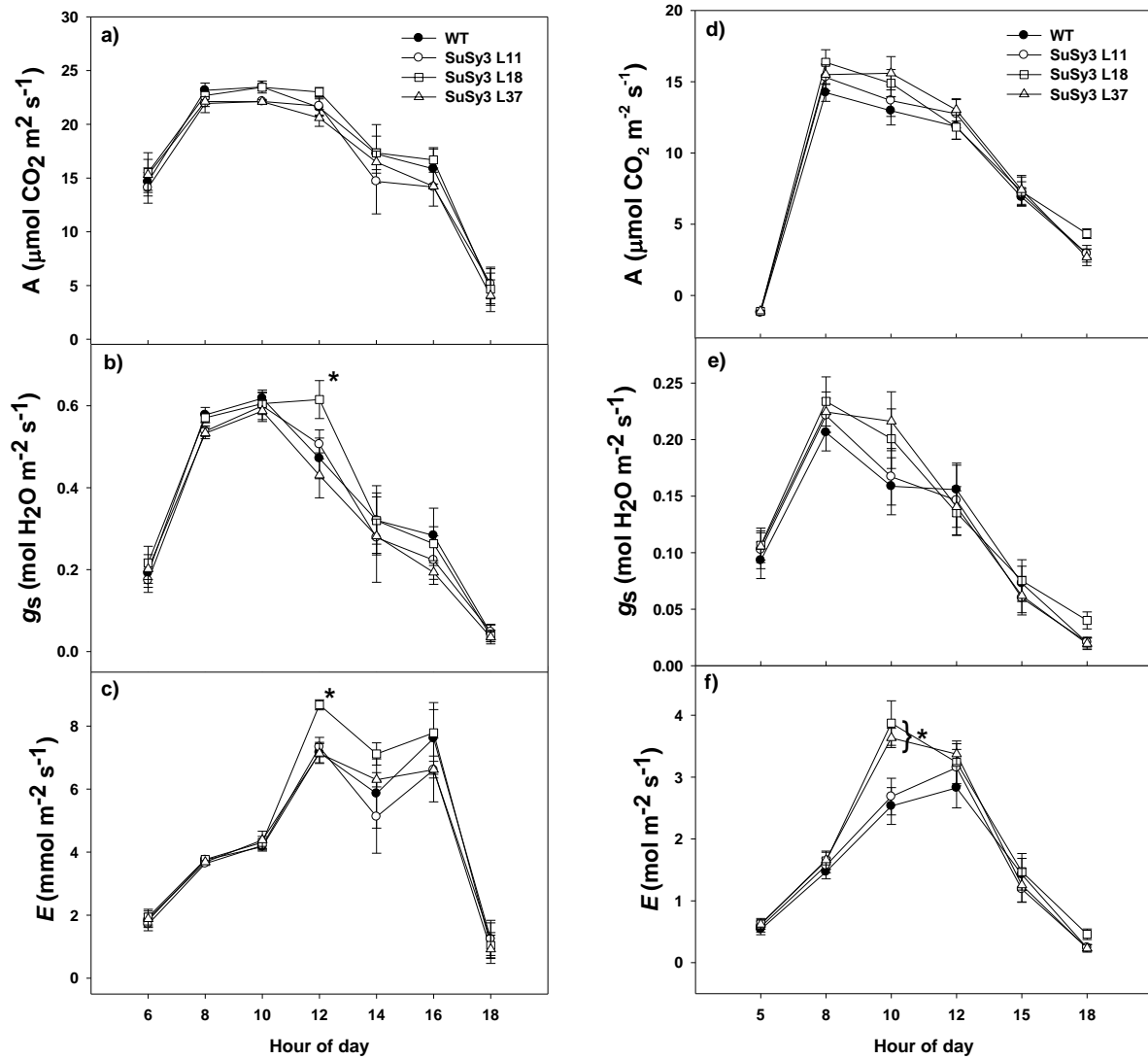
Figure 7. Daily course of gas exchange in completely expanded leaves of wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37) grown in tropical conditions. Data from first experiment (a,b,c) and second experiment (d,e,f). a,d - net photosynthetic rate (A); b,e - stomatal conductance (g_s); c,f – transpiration rate (E). Error bars indicate the standard error. *Asterisks indicate values significantly different from wild type by Student's t test ($P < 0.05$) ($n = 6 \pm SE$).
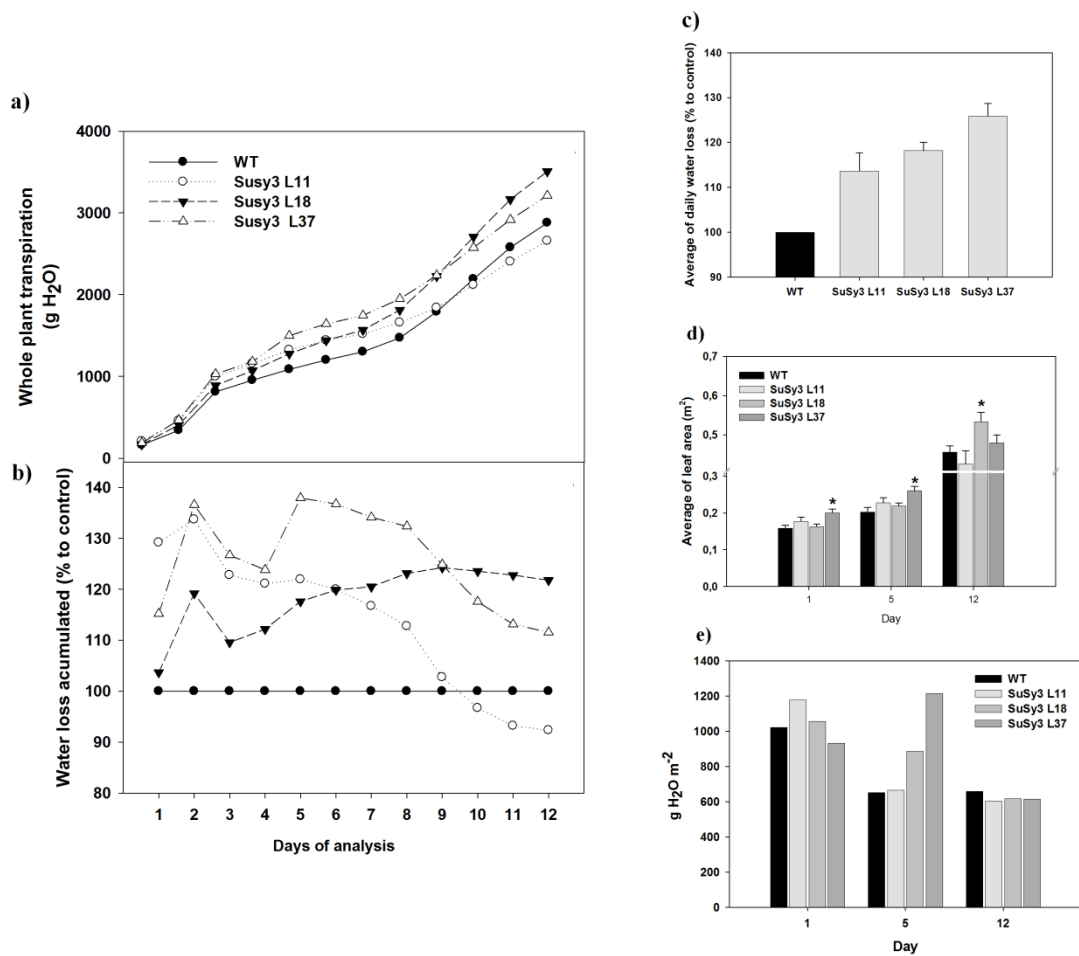
Figure 8. Whole plant transpiration of wild type and transgenic tobacco plants (n=6 ± SE).

a) Water loss accumulated (g H_2O) during all experiment (12 days)

b) Percentage of water loss accumulated relative to the control over the entire experiment (12 days)

c) Percentage of average daily water loss relative to the control in 12 days of the experiment.

d) Average of leaf area (m^{-2}) at days 1, 5, and 12 of the experiment. *Asterisks indicate values significantly different from wild type by Student's t test ($P < 0.05$).

e) Whole plant transpiration normalized by leaf area (g H_2O m^{-2}) at days 1, 5, and 12 of the experiment. Sum of the six whole plant transpiration (g H_2O) per total leaf area (m^{-2}) of these six plants.
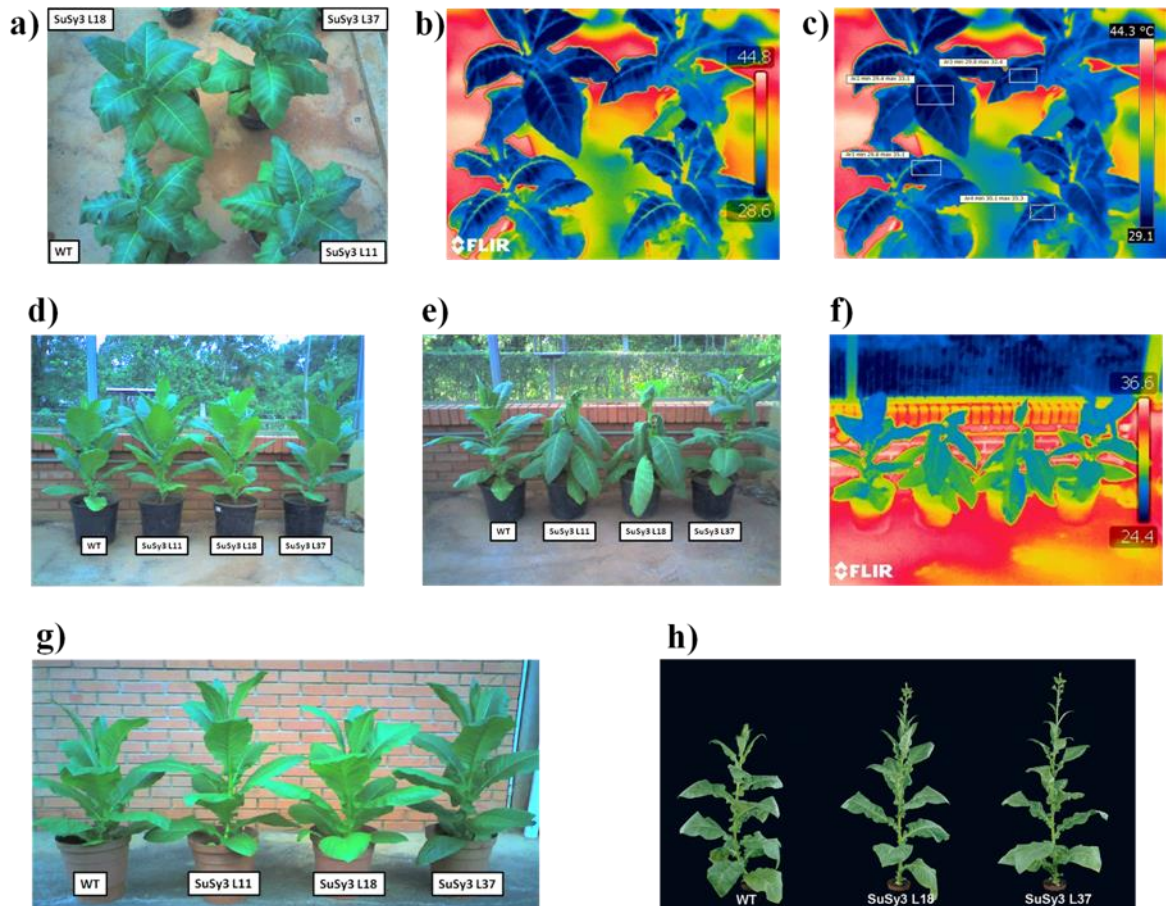
Figure 9. Phenotype of wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37).

a,b) On the top overview of wilt type and transgenic plants (a) and infra red thermography of the canopy of these plants (b) under irrigated conditions. The disposition of plants in thermal imaging is identical to figure a.

c) Thermal imaging showing the minimal and maximal temperature in the mid region of the leaf

d,e,f) Phenotype of plants used in the whole plant transpiration and growth analysis experiments under irrigated conditions (d) and two days after suspension of irrigation (e,f).

g) Phenotype of plants growth at green house used in the second experiment

h) Phenotype of plants grown at growth chamber with control of light, humidity and temperature.

At the end of the whole plant transpiration experiment, the plants were harvested and the dry weight was determined. Young leaves were also harvested to permit determination of leaf isotopic composition ($\delta^{13}C$) (DaMatta et al. 2008) for which no differences were detected (Figure 10 a). The lines L18 and L37 showed an increase in plant, leaves and stem dry weight

(Table 2) as well as in the total leaf carbon content (Figure 10 b), while no differences were detected in percentage of leaves, stem and root dry weight, in the number of leaves per plant and in the ratios leaves/root and aerial part (leaves + stem) to root (Table 2). The root dry weight and stem length (Figure 9 d,g,h) increased only in L37 plants (Table 2), although the increase in stem length was also observed in L18 in different experiments (Figure 9 d,h).
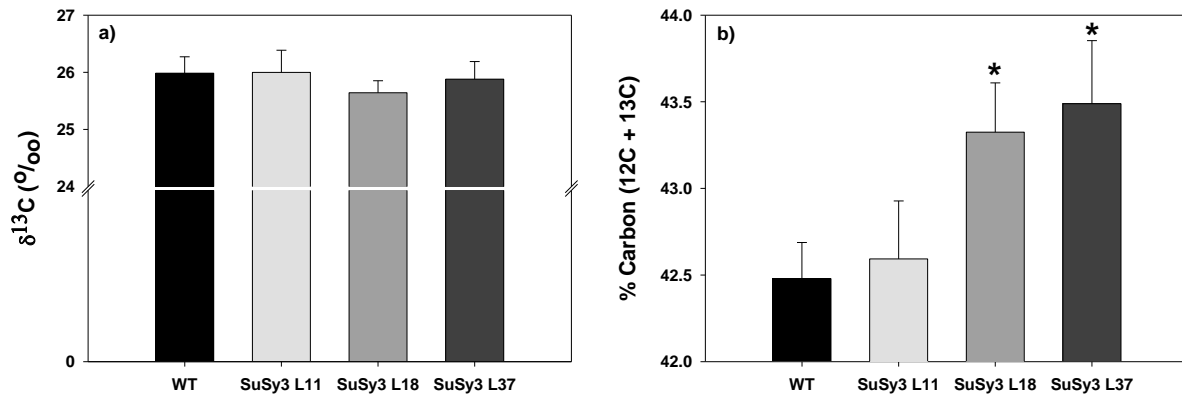
Figure 10. Leaf carbon isotopic composition (δ^{13}C) (a) and total leaf carbon content (% of the dry weight) (b) in wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, and SuSy3 L37). (n = 6 ± SE). Error bars indicate the standard error. *asterisks indicate values significantly different from wild type by Student's *t* test at 5% (p< 0.05).

Photosynthetic curves analysis confirms a stomatal-dependent phenotype in transgenic lines

   The level of photosynthetic pigment, light curve (*A*-PAR) and photosynthetic curves range CO_2 concentration (*A*/*C*$_i$) was carried out in WT and transgenic lines in order to determine if the transgenic lines has some increases in the photochemical performance or in the mesophyll capacity to transfer or to fix CO_2. Several photosynthetic parameters were estimated from photosynthetic curves (*A*-PAR, *A*/*C*$_i$, *A*/*C*$_c$) (Figures 11-12) and no changes were observed in dark respiration (R_d), electron transport rate (ETR), mesophyll conductance to CO_2 (g_m), photorespiration (R_I), maximum rates of rubisco carboxylation (V_{cmax}), maximum capacity for photosynthetic electron transport (J_{max}), in the ratio J_{max}/V_{cmax}, triose phosphate use limitation (TPU), CO_2 saturation and compensation point, and light saturation (LSP) and compensation (LCP) point (Table 4). No changes were observed in photosynthetic pigment content (Table 3). Altogether, these results showed that the overexpression of SUS3 in GC do not change mesophyll conductance to CO_2 and mesophyll capacity for CO_2 fixation.
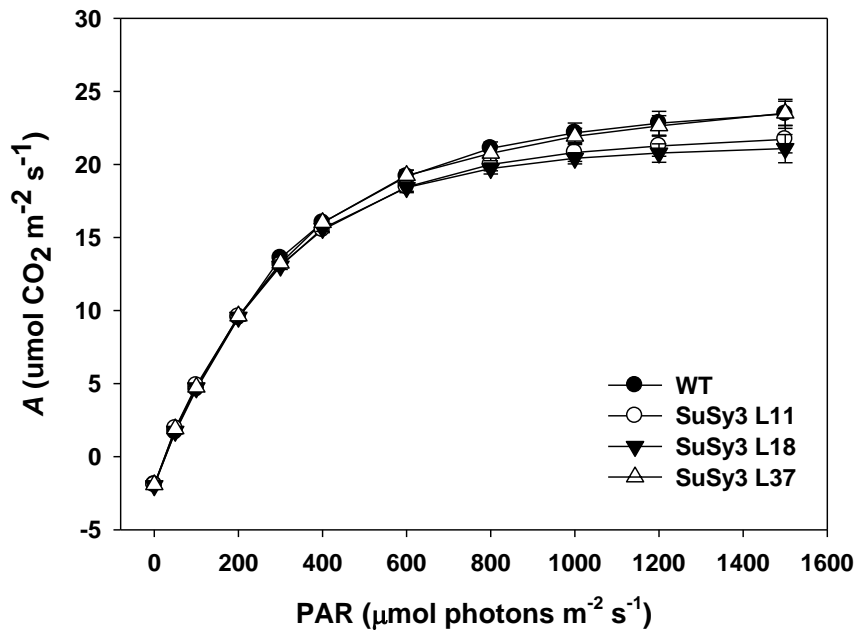
Figure 11. Net photosynthetic rate (*A*) under different photosynthetically active radiation (PAR) in wild type (WT) and transgenic lines overexpressing sucrose synthase 3 gene (L11, L18, and L37). Error bars indicate the standard error.
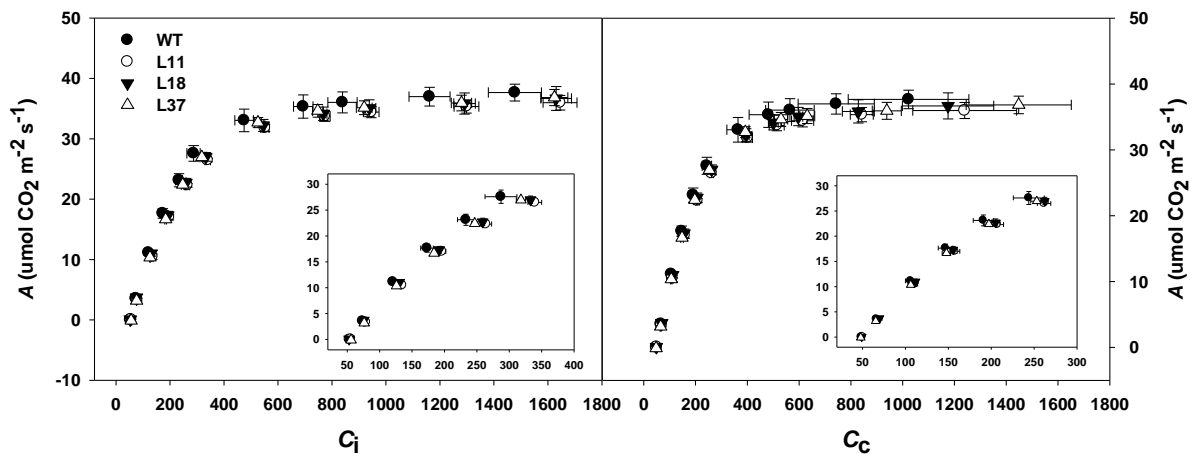
Figure 12. Net photosynthetic rate (*A*) under different CO_2 sub-stomatal concentration (C_i) (left) or CO_2 chloroplast concentration (C_c) (right) in Wild Type and transgenic lines (L11, L18, and L37). Error bars indicate the standard error.

Table 2. Growth parameters and stomatal density (stomatal/mm^2) in wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37) overexpressing sucrose synthase 3 gene specifically in guard cells. Means of growth parameters set in bold and underlined indicate values significantly different from wild type by Student's *t* test (P < 0.05) (n = 6 ± SE).

	WT	Sus3 L11	Sus3 L18	Sus3 L37
Plant DW (g)	22.97 ± 0.85	23.09 ± 2.04	**<u>26.81 ± 1.03</u>**	**<u>31.44 ± 2.00</u>**
Leaves DW (g)	12.60 ± 0.39	12.54 ± 1.01	**<u>14.68 ± 0.46</u>**	**<u>15.88 ± 1.35</u>**
Stem DW (g)	7.25 ± 0.27	7.55 ± 0.67	**<u>8.33 ± 0.31</u>**	**<u>10.22 ± 0.47</u>**
Root DW (g)	3.11 ± 0.26	3.00 ± 0.45	3.80 ± 0.34	**<u>5.35 ± 0.69</u>**
% leaves DW	54.95 ± 0.63	54.61 ± 1.45	54.83 ± 0.74	50.44 ± 2.02
% stem DW	31.59 ± 0.35	32.70 ± 0.76	31.11 ± 0.50	32.69 ± 0.89
% root DW	13.47 ± 0.75	12.69 ± 0.96	14.06 ± 0.79	16.88 ± 1.56
Leaves DW / root DW	4.17 ± 0.32	4.45 ± 0.42	3.97 ± 0.29	3.16 ± 0.44
Leaves DW + stem DW / root DW	6.56 ± 0.48	7.09 ± 0.57	6.23 ± 0.43	5.17 ± 0.64
Leaves number plant^{-1}	13.50 ± 0.22	13.00 ± 0.52	14.17 ± 0.31	14.60 ± 0.24
Specific leaf area (m^2 Kg^{-1} DW)	36.49 ± 2.01	34.57 ± 2.74	37.62 ± 2.43	32.13 ± 2.20
Stem length (cm)	56.83 ± 1.62	59.95 ± 3.85	56.83 ± 2.15	**<u>75.80 ± 2.37</u>**
Adaxial stomatal density	72.06 ± 5.10	82.94 ± 7.62	72.76 ± 6.06	65.27 ± 4.48
Abaxial stomatal density	147.14 ± 8.10	159.98 ± 15.33	141.08 ± 5.29	148.15 ± 0.13

Table 3. Photosynthetic characteristics of the wild type (WT) and tobacco transgenic lines overexpressing sucrose synthase 3 gene (SuSy3 L11, SuSy3 L18, SuSy3 L37). Rd – dark respiration, ETR – electron transport rate, g_m – mesophyll conductance to CO_2, R_I – photorespiration, V_{cmax} - maximum velocity of carboxylation, J_{max} - maximum capacity for electron transport, TPU - triose phosphate use limitation, LSP – light saturation point, LCP – light compensation point. Rd, LSP and LCP are derived from light curve (n=5 ± SE). The others parameters are derived from A-Ci curve (n=6 ± SE). No significant differences were detected between transgenic lines and wild type plants by Student's t test (p<0,05).

	WT	SuSy3 L11	SuSy3 L18	SuSy3 L37
R_d	1.98 ± 0.11	1.88 ± 0.13	2.04 ± 0.12	1.93 ± 0.06
ETR (μmol e$^-$ m^{-2} s^{-1})	182.83 ± 7.81	167.57 ± 3.27	170.70 ± 5.22	172.49 ± 2.83
g_m (mol CO_2 m^{-2} s^{-1})	0.394 ± 0.06	0.330 ± 0.04	0.339 ± 0.02	0.359 ± 0.02
C_c (μmol CO_2 m^{-2} s^{-1})	180.17 ± 11.63	192.36 ± 6.63	190.69 ± 4.51	183.19 ± 5.21
R_I (μmol m^{-2} s^{-1})	7.17 ± 0.56	6.15 ± 0.25	6.29 ± 0.26	6.54 ± 0.21
V_{cmax}	149.83 ± 6.45	145.84 ± 12.87	147.88 ± 13.16	133.06 ± 6.91
J_{max}	176.72 ± 7.10	166.60 ± 4.66	167.63 ± 4.54	171.24 ± 5.44
J_{max}/V_{cmax}	1.19 ± 0.08	1.19 ± 0.10	1.18 ± 0.11	1.30 ± 0.04
TPU	13.05 ± 0.62	12.65 ± 0.50	12.59 ± 0.75	12.86 ± 0.48
CO_2 saturation point	536.17 ± 33.30	606.88 ± 46.42	611.22 ± 62.95	557.22 ± 42.88
CO_2 compensation point	55.64 ± 1.96	57.86 ± 2.46	54.11 ± 2.18	57.46 ± 1.07
LSP	771.30 ± 56.44	720.20 ± 52.82	690.02 ± 59.79	796.94 ± 58.57
LCP	26.13 ± 1.38	24.31 ± 1.90	26.26 ± 2.16	24.94 ± 1.08

Table 4. Leaf concentrations of pigments (g kg^{-1} DW) in wild type (WT) and tobacco transgenic lines overexpressing sucrose synthase 3 gene (SuSy3 L11, SuSy3 L18, SuSy3 L37). Chl-a, chlorophyll *a*; Chl-b, chlorophyll *b*; Carot, carotenoids; Chl-a+b, total chlorophyll; Chl-a/b, ratio of chlorophyll *a/b*. No significant differences were detected between transgenic lines and wild type plants by Student's *t* test (p<0,05) (n = 6 ± SE).

	WT	SuSy3 L11	SuSy3 L18	SuSy3 L37
Chl-a	1.39 ± 0.06	1.38 ± 0.08	1.34 ± 0.01	1.45 ± 0.05
Chl-b	0.38 ± 0.04	0.38 ± 0.03	0.32 ± 0.01	0.36 ± 0.02
Carot	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.33 ± 0.01
Chl-a+b	1.78 ± 0.09	1.77 ± 0.11	1.68 ± 0.02	1.83 ± 0.07
Chl-a/b	3.82 ± 0.22	3.67 ± 0.10	4.18 ± 0.05	4.11 ± 0.11

Discussion

Guard cell genetic manipulation as strategy for photosynthesis improvement

The stomatal pore is the first barrier for the influx of CO_2 for the photosynthesis, thus the magnitude of stomatal opening can limit net photosynthetic rate (*A*) (Jones 1998). We showed that increasing stomatal conductance (g_s), by GC SUS3 overexpression, *A* increased linearly (Figure 6), suggesting that *A* is limited by g_s in wild type tobacco plants. As a consequence, higher *A* (Figure 5) led to increased growth (Table 2) and higher g_s led to higher transpiration in transgenic lines (L18 and L37) (Figures 4 b, 5, 7 f, 8), resulting in a more drought sensitive phenotype (Figures 4 c, 9 e).

Stomatal density and leaf area are directly related to transpiration rate (Lake and Woodward 2008, Nilson and Assmann 2010), given that leaves with higher stomatal density and higher leaf area show higher transpiration rate (Yu et al. 2008). In this study no changes were observed in stomatal density, thus the higher g_s and *E* cannot be associated with the number of stomata per leaf. Furthermore, although leaf area was higher in L37 line on day 5 of the experiment (Figure 8 d), whole plant transpiration per area (g H_2O m^{-2}) was also higher in this line on this day (Figure 8 e), showing that the increase in transpiration cannot be associated with a higher leaf area. These data exclude the possibility of the higher transpiration in L18 and L37 resulting from higher leaf area or higher stomatal density. Therefore, the increase observed in g_s and *E* in transgenic lines is attributed to changes in GC metabolism, as discussed below.

Guard cell metabolism as target to increase plant growth

Our previous published data suggest that the manipulation of GC sucrose metabolism may have a significant impact on plant growth and whole plant transpiration, what could be a promising strategy to improve plant growth and to obtain plants with altered water use efficiency (WUE) (Antunes et al. 2012). In this case, the GC SUS3 overexpression leads to a drought sensitive phenotype (Figures 4 c, 9 e-f), with both intrinsic and instantaneous WUE decreased (Figure S4). In the other hand, the drought tolerance showed by tobacco transgenic plants antisense to SUS3 under control of KST1 promoter confirms the goal of the genetic manipulation of GC sucrose metabolism as target to obtain drought tolerant plants (Daloso et al. *in progress*).

WUE is defined as CO_2 assimilation per unit water transpired (Bacon 2004). WUE can be used to relate the plant carbon gain from photosynthesis to plant water loss by transpiration, being a key parameter for the selection of genotypes to survive and maintain productivity in areas subject to water deficit (Condon et al. 2004). At the leaf level, WUE can be estimated from the A/g_s ratio, termed intrinsic WUE (WUEi), or the A/E ratio, termed instantaneous WUE (WUE), both obtained from gas exchange analysis. These two ratios decreased in transgenic lines (Figure S4), which means that the increase in transpiration is higher compared to increase in photosynthesis. In this context, the transgenic lines are characterized as a genotype with decreased WUE but increased effective use of water (EUW). EUW involves the maximal soil water capture for transpiration and minimal water loss by soil evaporation (Blum 2005). As biomass production is strongly related to transpiration rates, biotechnological strategies that aim to obtain plants with increased EUW are may prove an important strategy for increasing agricultural productivity in dry conditions (Blum 2009).

Albeit *A/E* and *A/gs* can estimates plant WUE, these values do not always correlate with measures of WUE obtained over time (Seibt et al. 2008). Thus, the selection of genotypes based on their WUE should be carried out with using reference to analyses performed at whole plant level, which provide more accurate data about the plant photosynthetic capacity at the expense of water loss by transpiration (Gilbert et al. 2011). In this study WUE was estimated at whole plant level by leaf carbon isotopic composition ($\delta^{13}C$) and no changes was found in transgenic plants (Figure 10). As $\delta^{13}C$ is also related to mesophyll conductance (g_m) (Flexas et al. 2010), we constructed A-C_i curves in order to estimate g_m and the chloroplastic CO_2 concentration (C_c). No changes were observed in g_m or C_c (Table 3), showing that although the level of sub-stomatal CO_2 concentration (C_i) is higher in transgenic leaves (Figure 5), g_m and C_c do not change, resulting in the same leaf $\delta^{13}C$.

Therefore, although the analysis of WUE by gas exchange showed that WUE decreased in transgenic lines, it's important to note that at whole plant level the transgenic plants showed increased dry weight and total leaf carbon content (Figure 10 b), without changes in $\delta^{13}C$. Altogether, the data suggests that the transgenic plants have higher CO_2 fixation and whole plant transpiration but the carbon isotopic discrimination by Rubisco was unaltered, thus there is no difference between WT and transgenic lines in WUE along the plant life time.

Sucrose as a substrate for tobacco guard cell osmoregulation

Tobacco transgenic plants overexpressing SUS3 from potato under our experimental conditions did not contain increased SuSy activity (Table 1). In a similar way, previously reported *sus* mutants have an uncommon but interesting phenotype as SuSy activity in the double mutant *sus5:sus6* and in the quadruple mutant *sus1:sus2:sus3:sus4* also do not change (Baroja-Fernández et al. 2012a). Furthermore, carbohydrate and starch levels are unaltered in the *sus3* mutant, *sus2:sus3* double mutant, and *sus1:sus2:sus3:sus4* quadruple mutant (Bieniawska et al. 2007; Barrat et al. 2009). These results indicate that certain modifications in the transcript levels of SUS genes do not necessarily lead to changes in SuSy activity or in the levels of the substrates and products of the reaction catalyzed by SuSy, demonstrating the level of complexity in sugar metabolism. It remains possible that samples collected at a different time of day might have shown differences, since SuSy activity range according with the time of the day (Antunes et al. 2012). Furthermore, there is several controversy about the conditions of the SuSy activity assay such pH, $MgCl_2$ concentration and temperature (Barratt et al. 2009; Baroja-Fernández et al. 2012a,b; Smith et al. 2012), thus suboptimal assay conditions might also tend to reduce any differences that exist in our samples.

Although SuSy$_{bre}$ and SuSy$_{syn}$ activities did not change in EF (Table 1), the ratio of sucrose/fructose was decreased (Figure 1), showing that SUS3 overexpression leads to changes in the balance of sugar metabolism, which in turn leads to a phenotype with changes in the stomatal behaviour. SuSy activity was almost thirty times higher in EF compared to whole leaves, suggesting the importance of this enzyme for GC metabolism. The role proposed for sucrose in GC is mainly linked as an important osmolyte (Talbot and Zeiger 1998). Alternatives roles, as substrate for respiration or organic acids biosynthesis are rarely addressed in literature (Dittrich and Raschke 1977). In this context, we demonstrate here that sucrose breakdown, indicated by the decrease in the ratio of sucrose/fructose, possibly in the symplast of GC since the EF's were extensively washed before to frozen, also appears to be an important mechanism in inducing stomatal opening. Furthermore, we carried out several

flux experiments in the second chapter in order to analyze the role of sucrose during light induced stomatal opening. The results showed that the level of sucrose is reduced under this condition, suggesting that sucrose is a possible substrate for GC osmoregulation.

Changes in organic acids levels suggest a higher substrates supply for respiration in GC

The contribution of organic acids and enzymes of TCA cycle for the regulation of stomatal opening has been recently demonstrated. Antisense tomato plants for fumarase (Nunes-Nesi et al. 2007) and the iron-sulphur subunit of succinate dehydrogenase (Araújo et al. 2011a) showed substantial changes in stomatal behavior, highlighting the importance of these enzymes as well as organic acids for normal stomatal function. Malate is the principle organic acid involved in stomatal movements, acting as counter ion and substrate for respiration in GC symplast as well as signalling molecule in the GC apoplast, communicating mesophyll cells and GC during stomatal closure induced by CO_2 (Lee et al. 2008; Negi et al. 2008; Kim et al. 2010). Fumarate, due to its chemical structure, the pattern of accumulation and degradation throughout the day, and its ability to induce stomatal closure in a dose-dependent manner (Nunes-Nesi et al. 2007; Araújo et al. 2011a), may have a similar role as malate in GC, however given that malate is present in the GC apoplast at higher concentrations, it would seem likely that it exerts greater influence on stomatal opening than fumarate does *in vivo* (Araújo et al. 2011b,c). In the second chapter of this thesis we showed that the fluxes of malate and fumarate are increased during light induced stomatal opening. However, the level of malate and fumarate was unaltered in transgenic plants overexpressing SUS3 (Figure 1). This may well be due to the time of harvest of EF (around the middle of the day), a period which K^+ is not the mainly osmolyte in GC, thus the level of malate is lower (Araújo et al. 2011a).

We carried out metabolic profiling of leaves and EF of WT and transgenic plants in order to identify changes in their metabolism induced by SUS3 overexpression. Although minor changes were observed in the metabolic profile, the results showed a reduction in the levels of some organic acids such citrate, succinate and 2-oxoglutarate in EF. These reductions in organic acids may be a response in order to increase the supply of substrates for respiration. In turn it would lead to an increase in ATP production, necessary for the ATPases on GC membrane (Vavasseur and Raghavendra 2005). Although speculative, possibly the sucrose breakdown could occur in order to keep a high production of ATP, necessary for the high activity of ATPases during stomatal opening.

Can SUS3 integrate ABA signaling and sucrose metabolism through a formation of a futile cycle in guard cells?

The *in silico* analysis from microarray available data provide some important information that support speculation about the role of SUS3 in stress tolerance and guard cell function. The data suggest that SUS3 can act in an osmotic stress network and as part of ABA signalling pathway. The ABA signalling pathway in GC is well investigated, however, the role of sucrose during GC ABA accumulation is poorly understood (Outlaw Jr 2003; Tallmam 2004; Vavasseur and Raghavendra 2005). GC ABA accumulation, by ABA treatment or drought, reduced the expression of genes related to starch metabolism (PGM, AGPase), sucrose metabolism (SUT1, Invertase), calvin cycle genes (Rbcs, G-3P-DH) as well as other genes of primary metabolism (UGPase, PDH, PEPCK, ADH, G-6P-DH) (Leonhardt et al. 2004; Zhao et al. 2008; Zhu et al. 2009; Böhmer e Schroeder 2011; Wang et al. 2011). In the other hand, the expression of sucrose phosphate synthase (SPS) and sucrose synthase 3 (SUS3) are increased under ABA or drought treatment (Kopka et al. 1997; Wang et al. 2011). This simultaneous increases suggest a futile cycle formation around sucrose in GC (Figure S6), as observed in other plants (Trethewey et al. 1999; Lattanzi et al. 2012). Futile cycles are metabolic cycles in which the net energy balance or the carbon flux around is zero or near to it (Schwender et al. 2004). The formation of this cycle is favoured once Invertase is *downregulated* in GC under drought (Kopka et al. 1997) and the synthesis of sucrose-6-phosphate and hexose-6-phosphates (F6P, G6P) from sucrose need less energy by SuSy (1ATP) when compared to Invertase (2ATP) (Figure S6). Although still speculative, this hypothesis shed light in some missing information that could be mean full to better understanding of the function of SUS3 in GC metabolism as well as the role of GC sucrose metabolism in whole plant stress tolerance.

Conclusion

Transgenic tobacco plants overexpressing potato SUS3 under control of KST1 promoter are characterized as a phenotype with increased stomata conductance, photosynthesis, and biomass production. This results suggest that GC genetic manipulation can be a promising strategy to increase the photosynthetic activity and growth in plants where net photosynthetic rate is limited mainly by stomatal conductance. Altogether, our data suggest that sucrose breakdown, not just sucrose accumulation, is an important mechanism that regulates the stomatal opening, and justify the importance of SuSy in GC behavior. Although speculative, possibly the sucrose breakdown could occur in order to keep a high

production of ATP, necessary for the high activity of ATPases during stomatal opening induced by K^+, or, alternatively, to produce malate, a counter-ion of K^+ during stomatal opening (Hedrich and Marten 1993).

Supplemental data
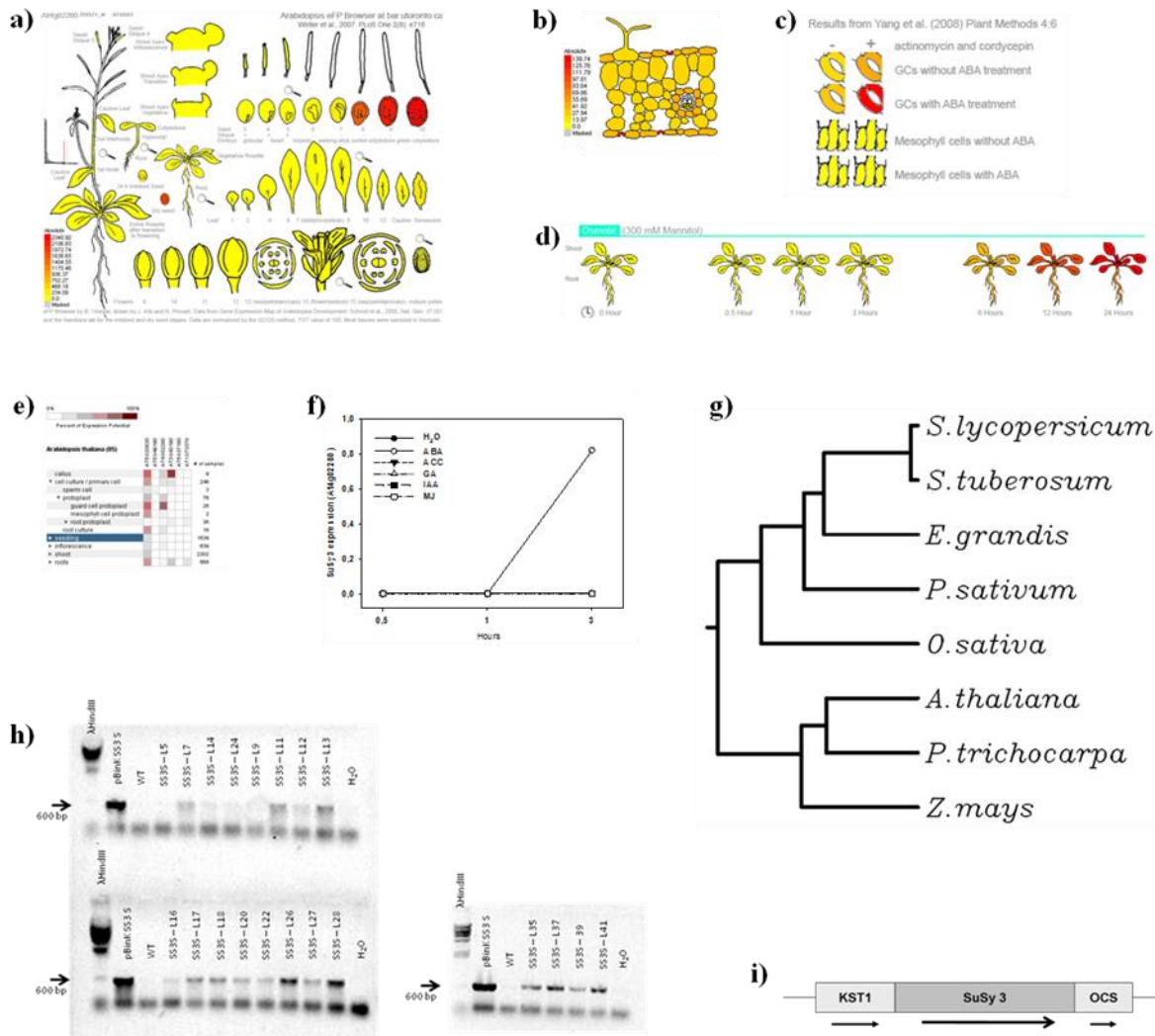
Figure S1. *In silico* analysis of Arabidopsis SUS3 (AT4G02280) (a-f), phylogenetic relationship between different species according SUS3 amino acid sequences (g), confirmation of transgenic transformation of tobacco plants by PCR (h), and construct used in this study (i).

a) Organ-specific expression of Arabidopsis SUS3 gene (AT4G02280) according eFP browser platform. The level of expression range according with the colour from yellow (low expression) to red (high expression) according with the legend in the left corner.

b) Cell-type-specific expression of SUS3 gene (AT4G02280) in Arabidopsis leaves. The level of expression range according with the colour from yellow (low expression) to red (high expression) according with the legend in the left side.

c) Expression of SUS3 gene in guard cells and mesophyll cells after ABA treatment. The level of expression range according the legend of the figure b.

d) Expression of SUS3 gene in Arabidopsis seedlings exposed to osmotic stress (300 mM mannitol) along 24 hours. The level of expression range according the legend of the figure b.

e) Organ-specific and cell type-specific expression of Arabidopsis SUS3 gene (AT4G02280) according Genevestigator platform. The level of expression range according with the colour from white (low expression) to red (high expression) according with the legend in the top of the figure.

f) Effect of hormones treatments on Arabidopsis SUS3 expression. ABA - abscisic acid, ACC - 1-aminocyclopropane-1-carboxylic-acid (precursor of ethylene synthesis), GA - gibberellin, IAA - auxin (indole-3-acetic acid), MJ – methyl jasmonate.

g) Phylogenetic relationship between the species listed using SUS3 aminoacid sequences.

h) *Poly chain reaction* (PCR) developed in genomic DNA extracted from whole leaves of *Nicotiana tabacum* wild type (WT) and different transgenic lines sense to SUS3. The PCR products (600 bp) were loaded in agarose gel to show the amplification of exogenous gene (*NPTII*), confirming exogenous DNA insertion.

i) Construction used to create transgenic tobacco plants overexpressing isoform 3 of sucrose synthase of potato under the control of KST1 promoter, which is known to drive expression mainly in GC (Plesch et al. 2001).

Table S1. Primers used in qRT-PCR analyses.

Gene	Orientation	Sequence (5' → 3')
ACT	Forward	AGCAAGGAAATTACCGCATTAGC
	Reverse	ACCTGCTGGAATGTGCTGAGA
SUS3	Forward	GACCAGACTGATGAGCATGTCG
	Reverse	TCTTCACTTTGTCGAGCCTCG

Figure S2. Identity of sucrose synthase 3 amino acids sequences from *Solanum tuberosum* (U24088.1), *Solanum lycopersicum* (HM180942.1), *Arabidopsis thaliana* (NM_116461.3), *Pissum sativum* (AJ311496.1), *Eucalyptus grandis* (DQ227994.1), *Populus trichocarpa* (GU592811.1), *Zea mays* (AY124703.1), *Oryza sativa* (L03366.1). Amino acid sequences were aligned with ClustalW (Higgins and Sharp 1988). in BioEdit package (Hall, 1999). Black region = 100% of homology, green region > 80% of homology, yellow region = > 60% of homology.

Figure S3. Coexpressed network around Arabidopsis SUS3 (AT4G02280) showing elements in the network that are stress responsive. The analysis of the coexpression network was performed using microarray data from on line platform ATTED-II (http://atted.jp/) (Obayashi et al. 2011).
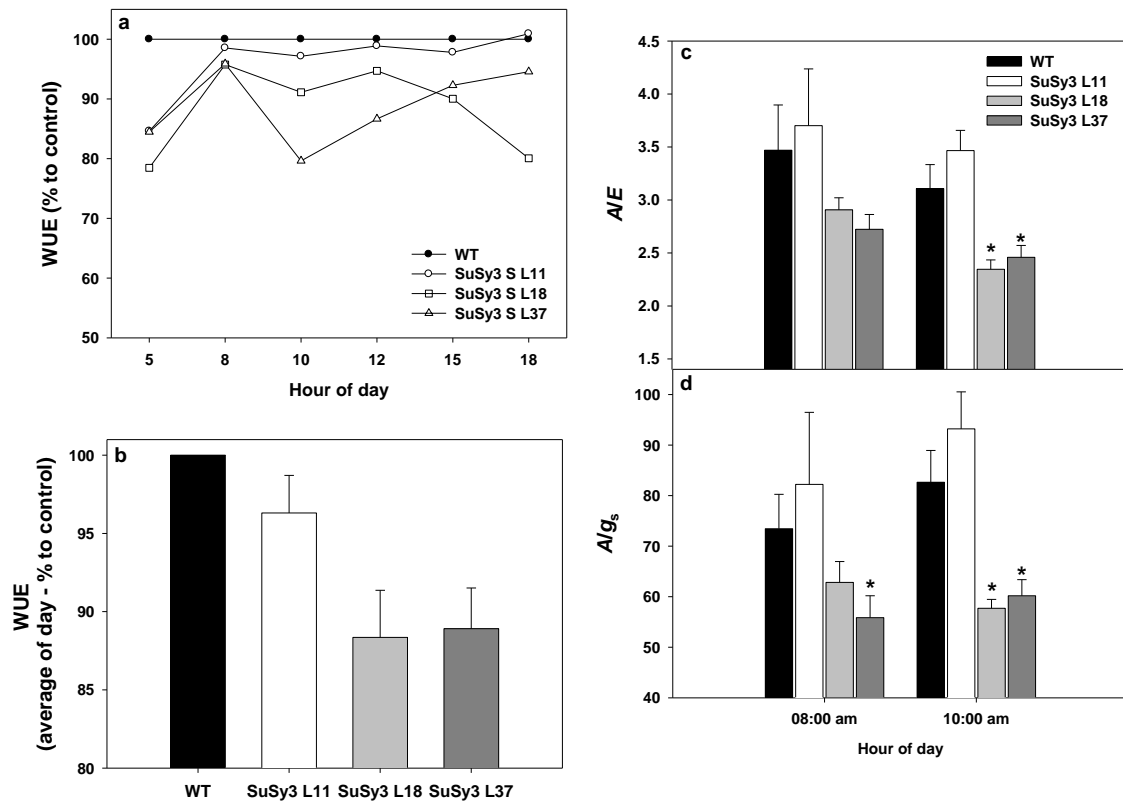
Figure S4. **Water use efficiency in wild type (WT) and transgenic lines (SuSy3 L11, SuSy3 L18, SuSy3 L37).**

a) Time course of water use efficiency (WUE) (A/E)

b) Average of WUE along the day. The data are percentage in relationship to WT (100%).

c) Intrinsic WUE - relationship between net photosynthetic rate (A) and stomatal conductance g_s.

d) Instantaneous WUE - relationship between net photosynthetic rate (A) and transpiration rate (E).

Error bars indicate the standard error. *Asterisks indicate values significantly different from wild type by Student's t test ($P < 0.05$).

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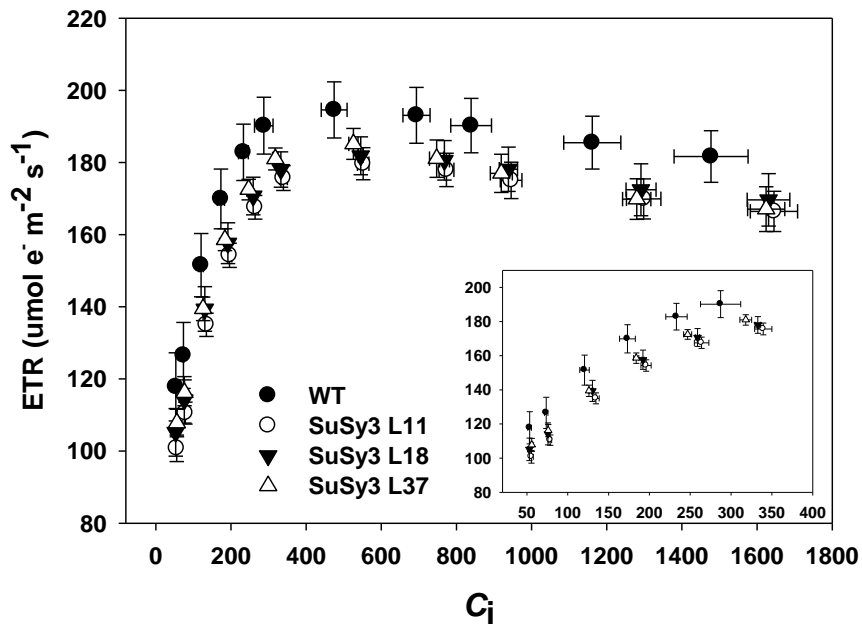
Figure S5. Electron transport rate (ETR) under different CO_2 internal concentration (C_i) in transgenic lines (L11, L18, and L37) and WT tobacco plants. Error bars indicate the standard error.
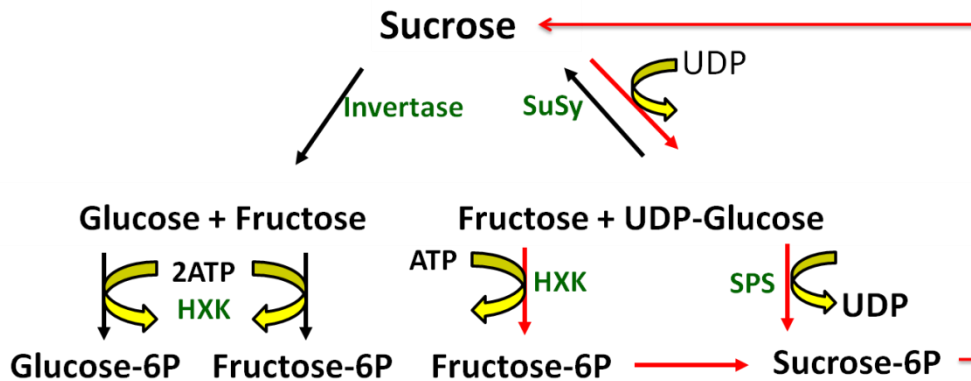
Figure S6. Proposed futile cycle formed during ABA accumulation in guard cells. Enzymes are in green, metabolites in black and the futile cycle proposed in red. SuSy – sucrose synthase; HXK – hexokinase; SPS – sucrose phosphate synthase.

Table S2. Metabolite levels in leaves of wilt type (WT) and transgenic lines (L11, L18, L37). The absolute values (nmol g^{-1} FW) were normalized by the WT values. Values in bold and underlined indicate values significantly different from WT by Student's *t* test (P < 0.05) (n = 5 ± SE).

	Leaves											
	WT			**L11**			**L18**			**L37**		
Serine	1.00	±	0.35	**2.35**	±	**0.17**	**2.12**	±	**0.13**	**3.64**	±	**0.07**
Methionine	1.00	±	0.74	2.26	±	0.42	3.01	±	0.18	**4.22**	±	**0.16**
Aspartic acid	1.00	±	0.21	1.42	±	0.15	1.53	±	0.08	**1.93**	±	**0.14**
Butyric acid	1.00	±	0.16	0.98	±	0.09	1.06	±	0.15	1.24	±	0.18
Threonine	1.00	±	0.05	0.98	±	0.11	1.16	±	0.05	1.06	±	0.10
Alanine	1.00	±	0.10	0.96	±	0.14	0.86	±	0.10	0.98	±	0.07
Glycine	1.00	±	0.22	1.24	±	0.28	0.51	±	0.24	0.94	±	0.23
Phosphoric acid	1.00	±	0.27	0.98	±	0.29	**2.12**	±	**0.09**	1.12	±	0.31
Glyceric acid	1.00	±	0.09	0.94	±	0.19	**0.73**	±	**0.09**	1.02	±	0.10
Succinic acid	1.00	±	0.06	0.79	±	0.12	**0.56**	±	**0.08**	**0.70**	±	**0.07**
Citric acid	1.00	±	0.14	0.75	±	0.13	1.03	±	0.07	0.96	±	0.12
Fumaric acid	1.00	±	0.14	0.92	±	0.06	0.88	±	0.08	0.90	±	0.06
Maleic acid	1.00	±	0.08	0.98	±	0.16	0.74	±	0.14	0.95	±	0.08
Pyroglutamic acid	1.00	±	0.09	1.10	±	0.14	1.25	±	0.07	**1.38**	±	**0.08**
2-oxoglutarate	1.00	±	0.17	0.94	±	0.11	**0.47**	±	**0.09**	1.01	±	0.28
Dehydroascorbic acid	1.00	±	0.14	0.96	±	0.12	0.80	±	0.07	0.93	±	0.08
Ascorbic acid	1.00	±	0.38	1.03	±	0.20	0.83	±	0.21	0.60	±	0.22
Fructose	1.00	±	0.17	1.16	±	0.18	0.50	±	0.28	1.04	±	0.22
Sorbitol	1.00	±	0.20	1.18	±	0.19	0.56	±	0.25	1.08	±	0.24
Glucose	1.00	±	0.15	1.06	±	0.24	**0.33**	±	**0.35**	0.86	±	0.23
Trehalose	1.00	±	0.51	0.77	±	0.18	0.42	±	0.41	0.84	±	0.17
Ribose	1.00	±	0.11	1.04	±	0.11	0.87	±	0.07	0.97	±	0.05
myo Inositol	1.00	±	0.05	0.89	±	0.06	0.95	±	0.05	1.01	±	0.06
Putrescine	1.00	±	0.14	1.04	±	0.16	0.77	±	0.08	1.15	±	0.05
Quinic acid	1.00	±	0.08	1.02	±	0.17	1.08	±	0.12	1.20	±	0.16
Nicotinic acid	1.00	±	0.09	0.85	±	0.04	0.85	±	0.08	0.96	±	0.05

Table S3. Metabolite levels in guard cell enriched epidermal fragment of wilt type (WT) and transgenic lines (L11, L18, L37). The absolute values (nmol g^{-1} FW) were normalized by the WT values. Values in bold and underlined indicate values significantly different from WT by Student's *t* test ($P < 0.05$) (n = 5 ± SE).

| | Epidermal fragment | | | |
	WT	L11	L18	L37
Serine	1.00 ± 0.36	0.82 ± 0.36	0.89 ± 0.08	1.53 ± 0.39
Aspartic acid	1.00 ± 0.43	0.28 ± 0.39	0.41 ± 0.21	1.09 ± 0.50
Threonine	1.00 ± 0.20	0.84 ± 0.27	1.19 ± 0.07	0.96 ± 0.15
Alanine	1.00 ± 0.06	1.07 ± 0.11	1.26 ± 0.08	**1.57 ± 0.12**
Glycine	1.00 ± 0.11	1.12 ± 0.24	1.04 ± 0.19	0.95 ± 0.06
Glyceric acid	1.00 ± 0.26	0.59 ± 0.16	0.90 ± 0.08	0.97 ± 0.08
Succinic acid	1.00 ± 0.05	**0.77 ± 0.09**	**0.58 ± 0.07**	**0.62 ± 0.04**
Citric acid	1.00 ± 0.22	**0.32 ± 0.20**	<u>**0.36**</u> ± <u>**0.12**</u>	0.49 ± 0.16
Fumaric acid	1.00 ± 0.18	0.72 ± 0.08	1.01 ± 0.08	1.62 ± 0.11
Maleic acid	1.00 ± 0.08	0.82 ± 0.11	0.86 ± 0.12	0.78 ± 0.03
Malic acid	1.00 ± 0.22	0.56 ± 0.21	0.87 ± 0.09	0.86 ± 0.14
2-oxoglutarate	1.00 ± 0.20	**0.28 ± 0.37**	0.60 ± 0.26	0.42 ± 0.34
Fructose	1.00 ± 0.19	0.96 ± 0.26	1.20 ± 0.11	1.09 ± 0.06
Sorbitol	1.00 ± 0.20	0.94 ± 0.25	1.16 ± 0.11	1.05 ± 0.07
Glucose	1.00 ± 0.22	0.83 ± 0.16	1.17 ± 0.12	1.06 ± 0.09
Ribose	1.00 ± 0.30	0.78 ± 0.30	0.96 ± 0.12	0.80 ± 0.19
Quinic acid	1.00 ± 0.16	0.75 ± 0.12	1.05 ± 0.07	1.11 ± 0.07
Nicotinic acid	1.00 ± 0.07	0.97 ± 0.06	0.90 ± 0.02	1.01 ± 0.04

Material and methods

Plant material and growth conditions

In this study were used wild type (WT) and transgenic plants of *Nicotiana tabacum* L. overexpressing sucrose synthase 3 gene from *Solanum tuberosum* (SuSy3) (AY205302) (SuSy3 plants) under control of potato KST1 promoter (X79779) (Müller-Röber et al. 1995), an promoter that direct the expression exclusively on guard cells (Plesch et al. 2001). The plasmid construction, plant transformation and confirmation of transgenic plants were performed exactly according with Antunes et al. (2012). In brief, a binary plasmid pBinAr (Hofgen and Willmitzer 1990) was used. The CaMV-35S promoter was replaced by KST1 promoter by specific restriction enzymes, now denominated pBinK. The cDNA of sucrose synthase 3 gene from *S. tuberosum* was aplified and inserted into pBinK in sense direction between KST1 promoter and OCS terminator. The transgenic plants were generated by *Agrobacterium tumefasciens* transformation and confirmed for PCR with specific primers to neomycine phosphotransferase gene (FW 5' GCGGTCAGCCCATTCGCCGCC 3'; Rev. 5' TCAGCGCAGGGGCGCCCGGTT 3'), producing a fragment of approximately 600 bp (Figure S1).

The seeds of T2 generation were surface-decontaminated by shaking 70% ethanol for 1 min, rinsed with sterile distilled H_2O. In sequence treated with 2% sodium hypochlorite for 5 min, rinsed three times with sterile distilled H_2O. The seeds were then allowed to germinate in Petri dishes containing MS medium (25 cm3) (Murashige and Skoog, 1962) cultivated *in vitro* for 15 days. The seedlings were transplanted to 0,1 L pots containing Plantimax® substrate and cultivated under photoperiod of 14 h illumination and light intensity of 120 μmol m-2 s-1 for 15 days. After this, the plants was transplanted for 20 L pots and cultivated in green house for 3-4 months.

Screening of transgenic lines

After screening *in vitro* by germination of T2 transgenic seeds in medium containing Kanamicin®, seven of the fifteen transgenic lines were selected. Analysis of infra red termography, gas exchange, water loss of detached plants along of the time and whole plant transpiration during the cultivation in 0,1 L pots were performed to choice three lines (L11, L18, and L37) for green house cultivation and further experiments. Specific primers for isoform 3 of sucrose synthase gene (SUS3) were designed (Table S1). Total RNA was isolate using Trizol® following recommendation of the company. The quantitative real time PCR was performed using 7300 *Real Time System* (*Applied Biosystems*, Foster, CA, USA) and the gene

expression was analyzed according Araújo et al. (2011a) using the expression of Actin gene (ACT) as control for the normalization.

Gas exchange and chlorophyll-a fluorescence analysis

These analysis were done in completely expanded leaves of wild type and transgenic lines using infra red gas analyzer (IRGA, Licor 6400 and Licor-6400*xt*, Lincoln, USA) according Flexas et al. (2006). For time course analysis, gas exchange were performed in saturating light (1000 umol m^{-2} s^{-1}) with 10% of blue light for optimize stomatal opening , estimating net photosynthetic rate (*A,* μmol CO_2 m^{-2} s^{-1}), stomatal conductance (*g*$_s$, 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}).

A-Ci curve with simultaneous chlorophyll-*a* fluorescence determination were performed using IRGA Licor 6400*xt* (leaf chamber 2 cm^2) with saturating light (1000 umol m-2 sec-1) and leaf temperature maintained at 25ºC. The CO2 was injected into the open circulating gas-stream of the photosynthesis system using its proper auto controlled CO2 injector. An A-Ci curve was performed in leakage leaf for estimate the error in CO2 fixation according Bernacchi et al (2002). Ci was calculated according Long and Bernacchi (2003). The A-Ci corrected curve was utilized for estimate maximum velocity of carboxylation (*V*$_{cmax}$) (Sharkey et al 2007), maximum capacity for electron transport (*J*$_{max}$) (Sharkey et al 2007), mesophyll conductance for CO_2 (*g*$_m$) (Harley et al 1992; Epron et al 1995), triose phosphate use (TPU) limitation (Sharkey et al 2007), photorespiration (*R*$_I$) (Epron et al 1995), chloroplast CO_2 concentration (*C*$_c$) (Epron et al 1995, Bernacchi et al 2002). Carboxylation efficiency was obtained by regression trough of the linear model (f=y0+a*x) using Sigma Plot Software (Farquhar and Sharkey 1982). Effective quantum yield of photosystem II (Φ_{FSII} = (Fm' – Fs)/Fm') and electron transport rate (ETR = Φ_{FSII} x RFA x 0,5 x 0,84) were calculated according Genty et al. (1989).

Light curve (*A*-PAR) was realized using IRGA Licor 6400 (leaf chamber 6 cm^2) with leaf temperature maintained at 25ºC and CO2 concentration in the chamber at 400 μmol mol^{-1}. CO2 saturation point and CO2 compensation point of A-Ci curve and Light saturation point (LSP), light compensation point (LCP), and dark respiration of A-PAR curve were obtained by regression analysis using the Hyperbola model (f= a-b/(1+c*x)^(1/d)) by Sigma Plot Software.

Infrared thermography

Thermal images of wild type (WT) and transgenic leaves were obtained using an infrared camera (Flir®) following Merlot et al. (2002). Images were analyzed using FLIR/QuickReport1.2 Software.

Whole plant transpiration and growth analysis

Plants were cultivated in 20 L pots with a mixture containing soil, sand and manure (3:1:1) maintained in green house. Water loss by transpiration was determined by gravimetric methodology (Cavatte et al. 2012). At previous night of analysis, the soil was completely irrigated. At 6h and 19h of next day, the weights of pots containing plants were recorded; the difference between these weights was estimated as evapo-transpired water. Pots without plants were used to estimate direct soil evaporation, and the difference between total water loss (plant + soil) and evaporation (only soil) was considered plant transpiration (n= 5). The leaf area was determined according with model developed for Antunes et al. (2008) adapted for tobacco leaves in the days 1, 5 and 12 of the experiment. The leaf area was used to correct the transpiration per leaf area (g H_2O m^{-2}). The growth parameters were determined after whole plant transpiration analysis. The final leaf area was determined and leaves, stem and roots were harvest, dried at 70ºC for two days, and the dry weight was determined.

Guard cell epidermal fragment enriched (EF) isolation

The isolation of guard cell epidermal fragment enriched (EF) was performed according with method proposed by Kruse et al (1989) and adapted for tobacco. The leaves were blended for three minutes (three pulses of 1 minute) using a warring blender (Phillips, RI 2044) with internal filter to isolate EF of the mesophyll and fibers. After this, the EF were collected in nylon membrane and washed before the frozen for avoid apoplast contamination. This protocol result in guard cell purity of 98% (Antunes et al. *data not published*), and all extraction procedures timed approximately 5 min until the epidermal fragments frozen in N_2 liquid.

Extraction and analysis of metabolites

The metabolites extraction for gas chromatography coupled mass spectrometry (GC-MS) and sugar/starch quantification by Elisa assays was performed according Lisec et al (2006). 10 mg of epidermal fragments lyophilized were submitted at 1000 µL of methanol (100%), 70 ºC for 1 h under 950 rpm. In GC-TOF-MS analysis, 60 µl of Ribitol (0,2 mg/ml stock in dH_2O) was added as an internal quantitative standard. The extract was centrifuged at 11000g for 10', and 600 µL of the supernatant was collected to another tube, where was added 500 µL

of chloroform (100%) (LC grade, Sigma) and 800 µL of deionized water. After vortex for 10 s, other centrifuge was done for 15' at 2200g. 1000 µL of polar (upper) phase was collected and transfer for tube of 1,5 mL, dried in speed vac and ressuspend in 100 µL of deionized water. For GC-TOF-MS analysis, the sample derivatisation was done according Lisec et al (2006) through N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS 24589-78-4, Macherey & Nagel, Düren, Germany) and methoxyamine hydrochloride (CAS 593-56-6, Sigma, Munich, Germany) dissolved at 20 mg/ mL in pure pyridine (CAS 110-86-1, Merck, Darmstadt, Germany). The levels of soluble sugars (sucrose, fructose and glucose) as well as starch were done following Threthwey et al. (1998). The GC-MS metabolite determination was quantified as described Roessner et al. (2001).

Enzyme activity

Extracts of whole leaves and GC enriched epidermal fragments will prepared according to Gibon et al. (2004). The enzyme activities of sucrose synthase (SuSy) and acid invertase were performed according Zrener et al. (1995). SuSy in the sucrose breakdown direction also was measured in desalted extract by NAP-5 column, which the activity was expressed in a protein base (nmol UDPG min^{-1} µg $protein^{-1}$).

Leaf carbon isotopic composition ($\delta^{13}C$)

The isotopic composition of carbon 13 (δ^{13}C) was determined in two young leaves per plant collected and dried for 72 h at 70 ºC. The δ^{13}C determination was performed with six replicates using Pee Dee Belemnite as standard according to DaMatta et al. (2008).

Stomatal density

Stomatal density was performed in adaxial leaf epidermal and abaxial leaf epidermal accordingly Aharon et al. (2003).

Pigments determination

It was retired two discs (6 mm^2) per leaf, putt in glass tube containing 7 mL of dimethyl sulfoxide (DMSO) and maintaining at 65 ºC for 3 h. After extraction, 600 µL was utilized for spectrophotometric determination (Shimadzu, model UV-2550) of chlorophylls and carotenoids at 490, 646 and 663 nm. The discs were dried at 70 ºC for 72 h and dry weight (DW) was determined. The concentration of pigments (g pigments kg^{-1} DW) was calculated according Wellburn (1994).

Coexpression analysis

The analysis of the coexpression network was performed using microarray data from on line platform ATTED-II (http://atted.jp/) (Obayashi et al. 2011). The network of SUS3 (At4g02280) coexpressed genes was obtained using CoexViewer tool of ATTED-II 5.2 (Obayashi et al. 2009). The data are available in (http://atted.jp/top_draw.shtml#CoexViewer) Further information's about the significance of the mutual rank values for the gene coexpression can be found in Obayashi and Kinoshita (2009).

Statistical analyses

The transgenic lines were statistically compared to WT using t-test at 5% of probability (p<0.05). All statistical analyses were performed using the SAEG System version 9.0 (SAEG, 2007).

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Chapter II

Changes in guard cell metabolism during light induced stomatal opening

Summary. The pathway of light signaling during stomatal opening is a process well characterized. However, guard cell (GC) metabolic changes during this process remains poorly understood. Here, we carried out different experiments using guard cell enriched epidermal fragments (EF) of *Nicotiana tabacum* wild type in order to investigate changes in GC metabolism during light-induced stomatal opening. A pool of EF was extracted pre-dawn and stored in the dark in a hypertonic solution to avoid stomatal opening. Experiments were started by transferring the EF to solutions enriched with potassium (KCl) and/or sucrose in the light. During stomatal opening induced by light and KCl, a decrease in sucrose, fructose and glucose was observed, while no changes in starch content was observed. A linear relationship was observed between the decrease of sugars and the increase of stomatal aperture. Furthermore, no differences were detected in the sugar content in the medium; showing that the decrease in sugars observed is due to breakdown and not efflux from GC. Under sucrose solution, the increase in sucrose content, probably from both sucrose influx and GC photosynthesis, was accompanied by a simultaneous increase in fructose and glucose. No changes in starch content were observed, suggesting that the increase in fructose and glucose resulted from sucrose breakdown. Using a feeding strategy with $NaH^{13}CO_3$ followed by LC-qTOF-MS analysis, ^{13}C-enrichment in fumarate and glutamine was detected, mainly under KCl treatment, suggesting that these two metabolites are involved in stomatal opening processes induced by light. ^{13}C-enrichment was also observed in sucrose, evidence for CO_2 fixation by Rubisco. However, a simultaneous $^{13}CO_2$ fixation by PEPcase cannot be excluded given that ^{13}C-enrichment was also observed in malate and aspartate. As expected, the ^{13}C-enrichment in malate confirms its role as a counter-ion of K^+ in GC symplast. Beyond the role of sucrose as an osmolyte molecule for GC osmoregulation, the data showed here provide evidence for another role of this metabolite for GC osmoregulation, suggesting that sucrose is broken down in the symplast of GC in order to provide carbon skeletons to malate production, which in turn can be stored in vacuole acting as counter-ion of K^+ or metabolized to induce respiration.

Key Words: Stomatal opening, guard cell metabolism, K^+, sucrose.

Introduction

Guard cells (GC) are leaf epidermal cells that contain all the structures necessary for photosynthesis and respiration, with numerous mitochondria (Pallas Jr and Mollenhauer 1972), the presence of both photosystems (Outlaw Jr et al. 1981; 1982), photosynthetic pigments (Zhu et al. 1998), light harvest complex proteins (Leonhardt et al. 2004), enzymes for CO_2 fixation (Rubisco and Phosphoenol Pyruvate carboxilase - PEPcase) (Outlaw Jr et al. 1979; Tarczynski et al. 1989; Parvathi and Raghavendra 1997), as well as enzymes for the Calvin cycle (Shimazaki et al. 1989), glycolysis and the TCA cycle (Vani and Raghavendra 1994). However, although GC have both a high photosynthetic and respiratory rates (Vani and Raghavendra 1994; Lawson et al. 2003), they also depend upon metabolites obtained from mesophyll cells and/or the apoplast for normal function. Furthermore, due to the high energy demand of GC ATP-dependent ion pumps as well as a high demand for ions and osmolytes, GC cannot be considered an autotrophic cell (Raschke and Dittrich 1977; Zeiger et al. 2002; Lawson 2009).

GC metabolism has been investigated for almost an entire century. Initial studies predicted that the interplay between starch and sugars would be the mechanism that regulates the dynamic of stomatal movements, which stomatal opening would depend on starch breakdown and an increase in sugar levels (Sayre 1923, 1926). However, a new theory was created after the finding that cations can also act as osmolytes in GC (Fisher and Hsiao 1968; Humble and Hsiao 1969). The use of labeled ions (^{86}Rb, ^{42}K e ^{82}Br) and different salts such as KCl, KNO_3, K_2SO_4, and KBr helped to highlight the importance of cations/anions for charge balance in the GC membrane and, consequently, for stomatal movements (Humble and Hsiao 1969; Fisher 1971; MacRobbie 1981a,b; Vavasseur and Raghavendra 2005; MacRobbie 2006). These experiments were essential in discovering the importance of K^+ for GC osmoregulation. It is now apparent that Cl^- and NO_3^- are the two main inorganic counter-ions to balance the positive electrical charge of K^+ (Zeiger 1983; Guo et al. 2003; Kim et al. 2010). However, these inorganic ions are unable to balance the positive electrical charge of K^+, leading to a synthesis of organic counterions in GC, mainly malate (Hedrich and Marten 1993; Outlaw Jr 2003).

The relative importance of malate as a counterion depends of the Cl^- concentration (Raschke and Schnabl 1978; Van Kirk and Raschke 1978), and the relative importance of Cl^- or malate^{-2} as counter-ions of K^+ depend of the growth conditions (Talbott and Zeiger 1996). The daily course of the stomatal movements involves reversible changes in GC osmolytes concentration separated in two phases, accumulation of potassium (K^+) in the morning and

sucrose in the afternoon. In the early period of the day there is an influx of K^+ and Cl^- from apoplast to symplast of GC with the concomitant malate accumulation from starch breakdown (Talbott and Zeiger 1996; Zeiger et al. 2002; Outlaw Jr, 2003; Shimazaki et al. 2007). Sucrose is supposed to be the major osmolyte during afternoon (Tallman and Zeiger 1988; Talbott and Zeiger, 1993, 1996, 1998; Lu et al. 1995; Kang et al. 2007); however the role of sucrose during the stomatal opening induced by K^+ remains.

The high activity of PEPcase and the high rates of CO_2 fixation in the dark compared to mesophyll cells (MC) are evidence that support the idea that GC has a metabolism similar to cells with C_4 metabolism (Outalaw Jr and Kennedy 1979; Outlaw Jr 1989; Brown and Outlaw Jr 1982; Gautier et al. 1991; Vavasseur and Raghavendra 2005). In this context, comparing the metabolism of mesophyll cell (MC) against epidermal tissue, Willmer and Dittrich (1974) showed that the first products of $^{14}CO_2$ fixation by epidermal tissue of *Tulipa gesneriana* and *Commelina diffusa* in the light and dark are malate and aspartate. In contrast, MC of these species had a CO_2 fixation pattern typical of cells with C_3 metabolism. These authors conclude that GC are able to exhibit Crassulacean acid metabolism. In contrast, Gotow et al. (1988) using the same feeding strategy, reported evidence supporting the presence of C_3 metabolism in GC, since the products synthesized after $^{14}CO_2$ fixation were 3-PGA, triose phosphates and sugars, suggesting CO_2 fixation by Rubisco. Simultaneous CO_2 fixation by Rubisco and PEPcase cannot be excluded, since although GC exhibited high rates of CO_2 fixation in the dark, these rates increased around 50% in the light (Gautier et al. 1991), indicating fixing process stimulated by light, possibly via Rubisco (Outlaw Jr et al. 1979; Tarczynski et al. 1989). Parvathi and Raghavendra (1997) suggest that the activity of not only PEPc but also Calvin cycle plays a positive role during stomatal opening, particularly when the other pathway is restricted.

GC are a very dynamic and complex cell type, with a high capacity to transport ions and metabolites between the apoplast and symplast. For these reasons, it is very difficult to quantify metabolic fluxes in GC, mainly because the presence of a metabolic steady state is almost impossible to verify in these cells. Metabolic steady state is a state in which all the fluxes in a network are invariant, so that the concentrations of the metabolic intermediates are constant over time (Ratcliffe and Shachar-Hill 2006). The steady state metabolic flux analysis cannot be readily applied to analysis of fluxes in tissues that are undergoing marked diurnal variations in metabolic activities (Colón et al. 2009), an intrinsic characteristic of GC. For these reasons, it's important to include analysis over the time in the stomatal opening experiments in order to investigate the dynamic of the changes in GC metabolism. In this

context, several experiments of stomatal opening over the time were developed here altering the presence of K^+ and sucrose in the medium. Two hypotheses about the role of sucrose in the GC symplast were tested here: (i) - the sucrose is not degraded, acting as an osmolyte molecule; (ii) - that sucrose is degraded to produce other metabolites. The results showed that the level of sucrose, fructose and glucose are decreased during stomatal opening induced by light and KCl, while no changes in starch content was observed. The data presented here provide evidence supporting the hypothesis (ii) that sucrose is broken in the symplast of GC and this is suggested to provide carbon skeletons for malate production.

Results

Creating the methodology: from guard cell enriched epidermal fragment extraction to flux experiments

We initially developed a framework to adjust the methodology for metabolic flux analysis in guard cell enriched epidermal fragments (EF). The concentration of all solutions was selected based in the literature regarding light induced stomatal opening experiments as well as using kinetic data for the Sucrose transporter (Outlaw 1995). To perform experiments investigating metabolic changes in EF with sufficient material to carry out metabolite profiling, we created a methodology to extract and to store a pool of EF before of the beginning of the experiment. The EF extraction was carried out during the pre-dawn period in order to harvest fragments with the stomatal pore closed and avoid light-induced changes in GC metabolism. EF extraction was carried out for 1 to 2 hours using approximately 100 fully expanded leaves of tobacco per experiment. The EF extracted were transferred to a glass beaker (1 L) with water (Figures 4-6; 12-14) or a hypertonic solution 0,5 M of mannitol (Figures 7-11) maintained in the dark. After the last EF extraction, an aliquot was analyzed under a microscope to measure the stomatal aperture and another aliquot was washed extensively and frozen as a control. To start the experiment, similar aliquots were put in petri dishes (Figure 1) with manual shaking or in Beakers (50 ml) with magnetic stirring. Immediately after placing the EF in the solutions an aliquot was collected and stomatal aperture was measured. Another aliquot was then collected on a membrane (200 μm), washed extensively and frozen as a second control (time 0). The EF were exposed to white light (~300 μmol photons m^{-2} s^{-1}) separated by a water layer to avoid heating of the samples. Thermal imaging confirms a lack of transfer of heat to the samples (Figure 1). The time which the EF were maintained in the solutions and light was varied in different experiments. Forty minutes

was sufficient to induce stomatal opening (Figure 2-3), which was accompanied with several changes in carbohydrate metabolism (Figures 4-6).
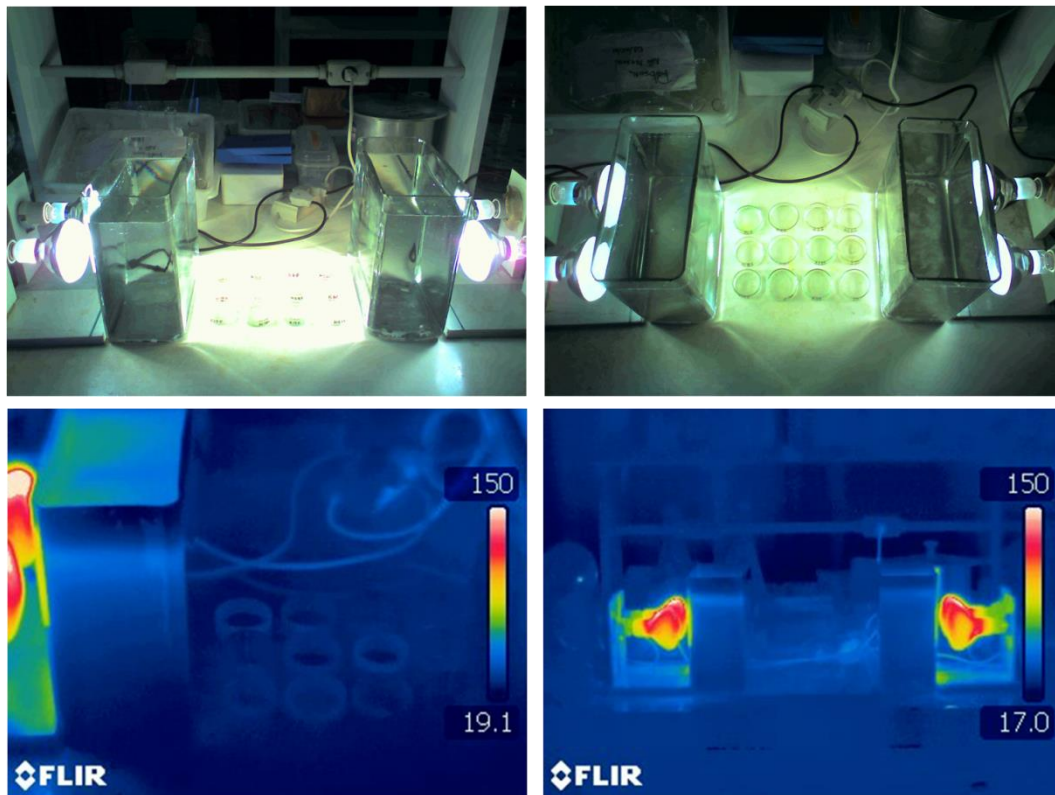

Figure 1. The material used in light induced stomatal opening experiments. Pictures (on the top) showing the distribution of the petri dishes with epidermal fragments between two water layer to avoid heat excess. Thermal imaging confirm lack of heating of the samples.
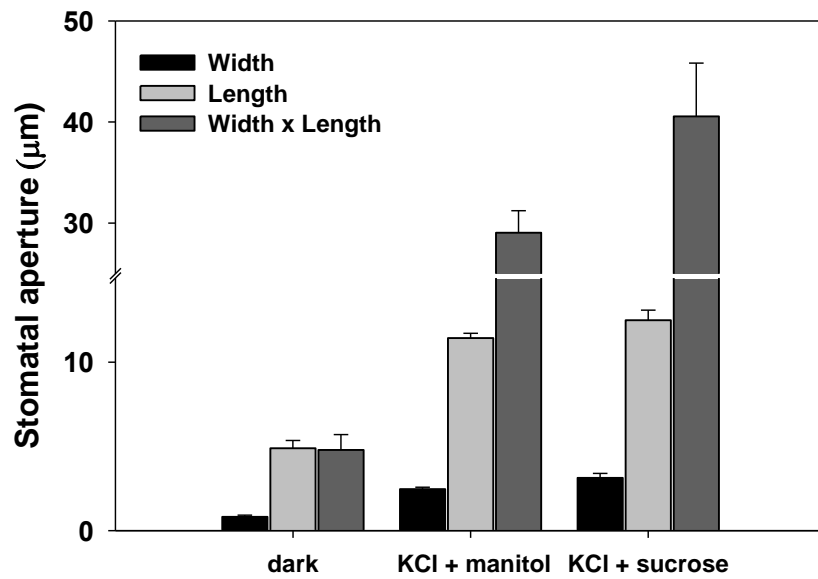
Figure 2. Light induced stomatal opening in wild type guard cell enriched epidermal fragment under different solutions. KCl + Mannitol – (MES/NaOH 5 mM pH 6,5 + CaCl$_2$ 50 µM + Mannitol 20 mM + KCl 5 mM); KCl + Sucrose (MES/NaOH 5 mM pH 6,5 + CaCl$_2$ 50 µM + KCl 5 mM + Sucrose 20 mM) (n = 4 ± SE).
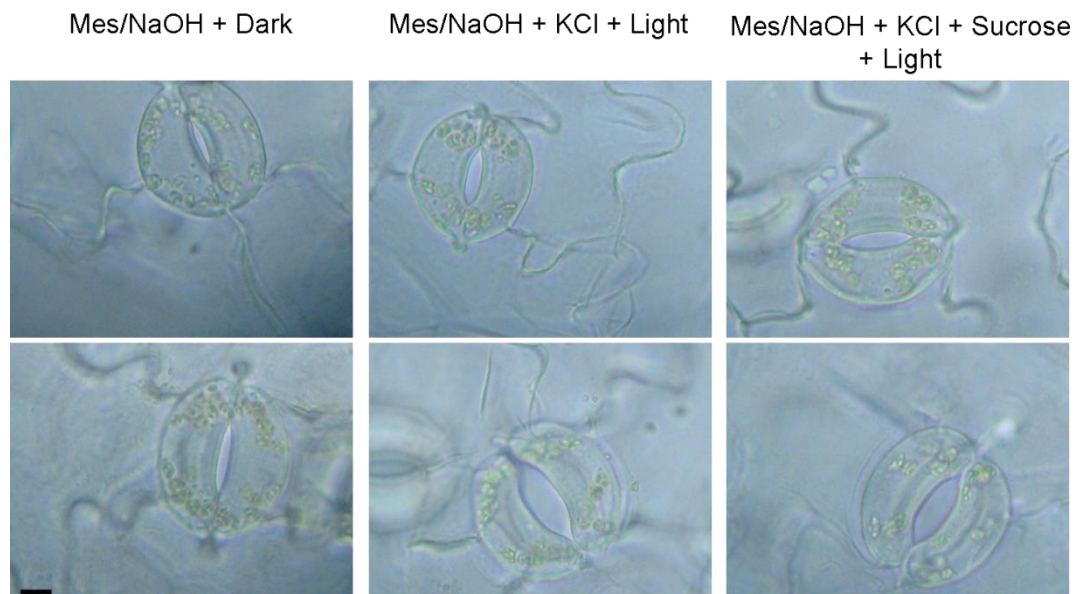

Figure 3. Pictures of stomata's of light induced stomatal opening experiment using wild type guard cell enriched epidermal fragment (EF) and different solutions under 90 minutes of dark or light. The EF were submitted to solutions containing MES/NaOH (5 mM) pH 6,5 + CaCl$_2$ 50 µM + Mannitol 20 mM; + KCl 5 mM); KCl + Sucrose (MES/NaOH 5 mM pH 6,5 + CaCl$_2$ 50 µM + KCl 5 mM + Sucrose 20 mM). Black bar = 5 µm

Changes in soluble sugars and starch during light induced stomatal opening

Potassium (K^+) is an osmolyte which induces stomatal opening (Fisher 1971). However, the role of sucrose during stomatal opening induced by K^+ remains poorly understood. Thus, in order to analyze the role of sucrose, and its breakdown products fructose and glucose, as well as the starch content during light and potassium induced stomatal opening, we incubated EF in different solutions containing potassium, sucrose or both in the light for 40 minutes. Also, in the treatment with sucrose and without potassium, we aimed to check the role of sucrose after influx into GC. No changes in starch content were observed in all treatments in the light or in the dark (Figures 4-6 inset). Under treatment with KCl solution, an increase in the K^+ content was observed accompanied with a decrease in sugars (sucrose, fructose, and glucose) (Figure 4). Under sucrose solution treatment, there were increases in sucrose and fructose without changes in glucose (Figure 5). In the presence of both osmolytes K^+ and sucrose, there were increases in the K^+ and sucrose content and a decrease in glucose content (Figure 6).
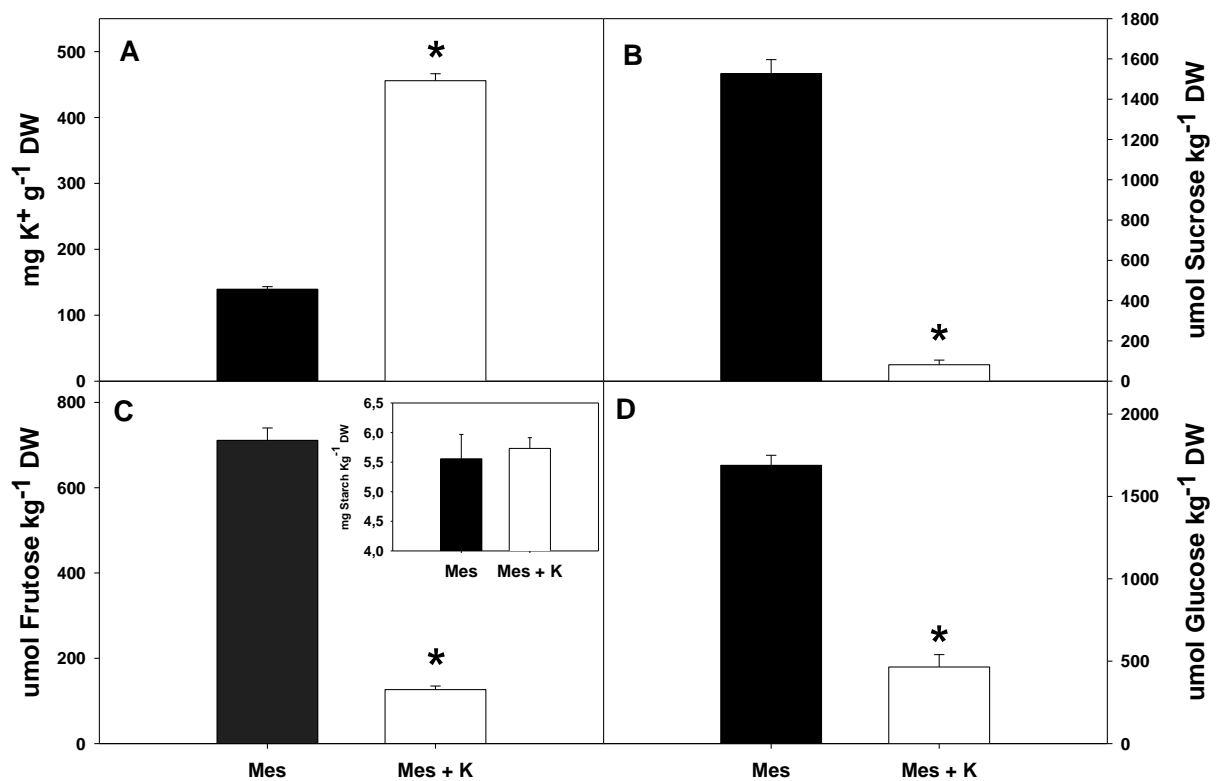

Figure 4. Changes in sucrose, glucose, fructose, and starch (inset) levels after influx of potassium (K^+) in guard cell enriched epidermal fragments. The epidermal fragments were submitted to treatment for 30 minutes with MES buffer solution (MES NaOH + $CaCl_2$) or MES + K solution (MES NaOH + $CaCl_2$ + KCl). * showed statistic difference by Student's *t* test (p<0,05) (n = 4 ± SE).
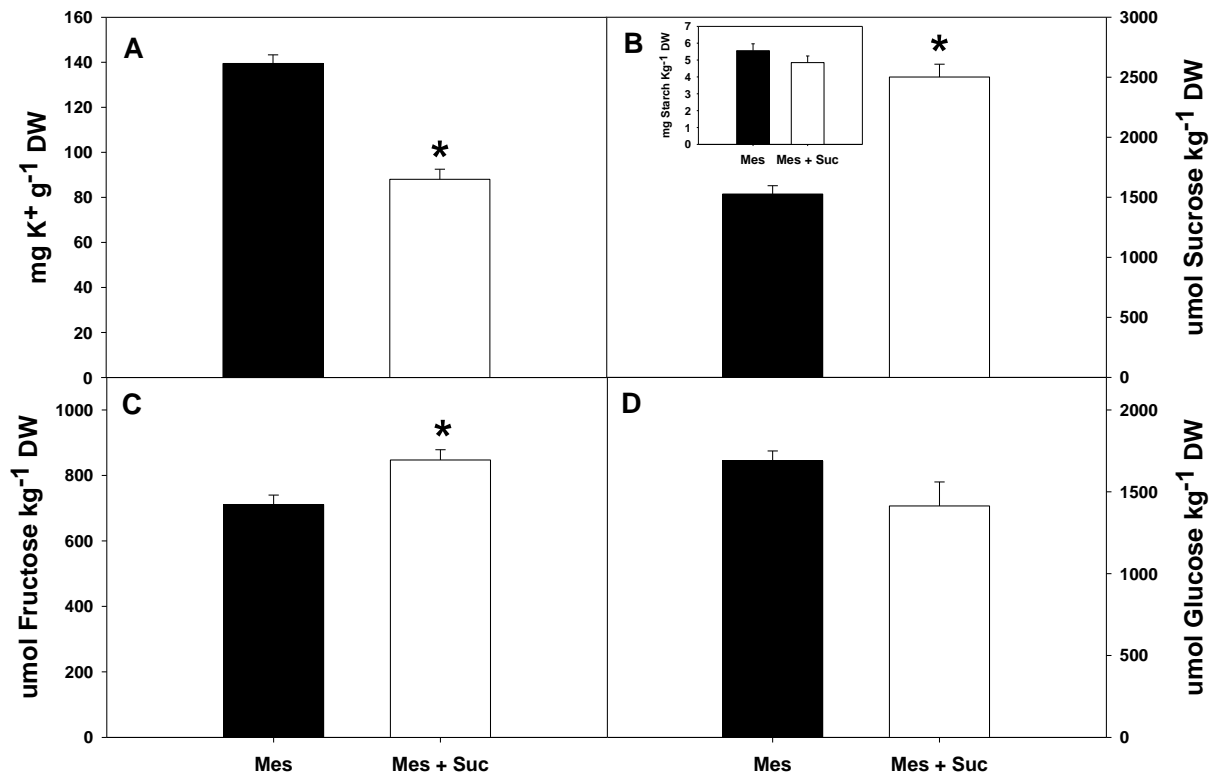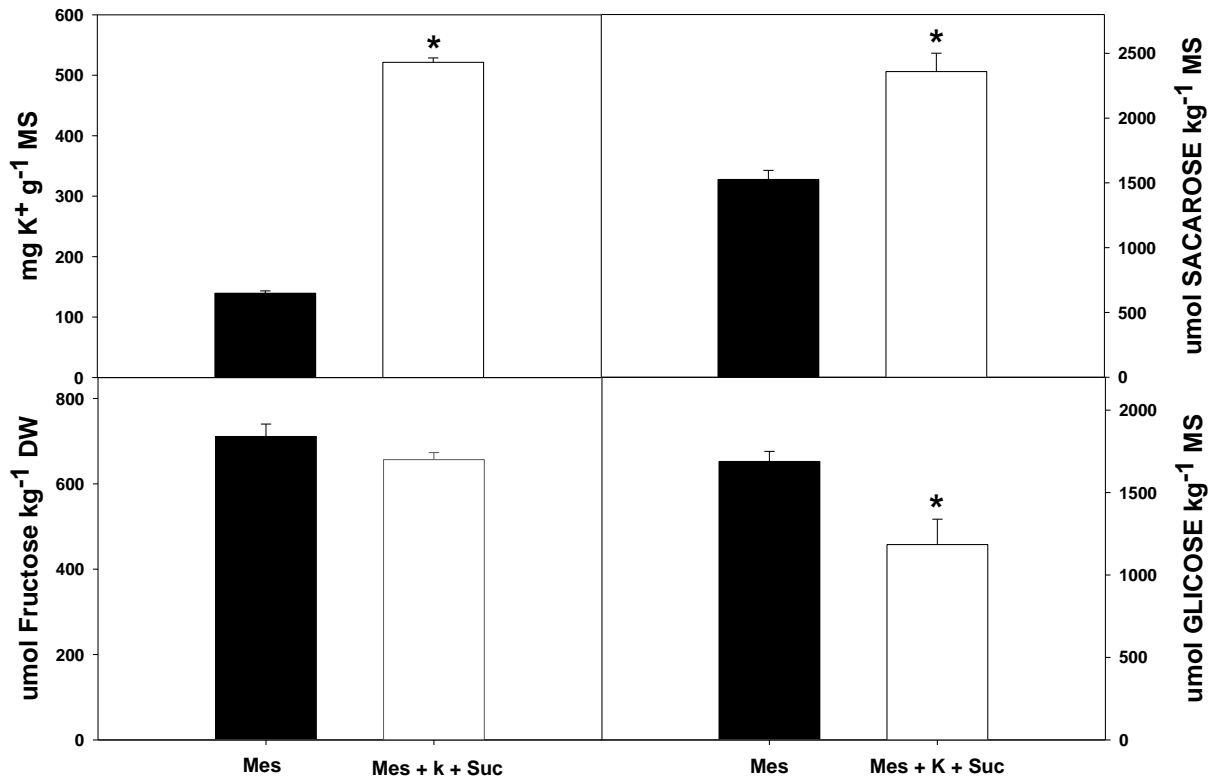
Figure 5. Changes in sucrose, glucose, fructose, and starch (inset) levels after influx of sucrose (Suc) in guard cell enriched epidermal fragments. The epidermal fragments were submitted to treatment for 30 minutes with MES buffer solution (MES NaOH + $CaCl_2$) or MES + sucrose solution (MES NaOH + $CaCl_2$ + Sucrose). * showed statistic difference by Student's t test ($p<0,05$) ($n = 4 \pm SE$).

Figure 6. Changes in sucrose, glucose, fructose, and starch (inset) levels after influx of K^+ and sucrose (Suc) in guard cell enriched epidermal fragments. The epidermal fragments were submitted to treatment for 30 minutes with MES buffer solution (MES NaOH + $CaCl_2$) or MES + K^+ + sucrose solution (MES NaOH + $CaCl_2$ + KCl + Sucrose). * showed statistic difference by Student's *t* test (p<0,05) (n = 4 ± SE).

Why is soluble sugar content reduced during light and potassium induced stomatal opening; breakdown or efflux from guard cells?

The levels of sucrose, fructose and glucose were reduced in response to K^+ treatment (Figure 5). Were these sugars metabolized in the GC symplast or was there an efflux of these sugars from the symplast to apoplast of GC? In order to verify this case we performed another experiment over a longer period analyzing the sugar content in the EF and in the medium. Stomatal aperture increased from 0 to 40 minutes but did not increase during further incubation. No difference was detected in stomatal aperture between the control and K^+ treatment at 40 and 80 minutes. However, after 120 minutes the stomatal aperture was greater in K^+ treatment (Figure 7). Sucrose and glucose were significantly reduced in only K^+ treatment over the time, while fructose showed the same tendency but this was not statistically different (Figure 8 a-c). A careful analysis of sugar content of the medium was

performed. The values were very low; closed to limit of detection of the spectrophotometric assays, which lead to high standard error in some time points. However, no differences were detected over time in both treatments (Figure 8 d-f), suggesting that the decrease in sucrose observed is due to breakdown and not efflux from GC. Interestingly, there is a linear relationship between the sugar levels and stomatal aperture, where the increase in stomatal aperture is accompanied by a decrease in sucrose, fructose and glucose and in the sum of these sugars (Suc + Fru + Glu). However, the stomatal aperture was statistically related only to sucrose and total sugar levels of the control treatment (Figure 9).
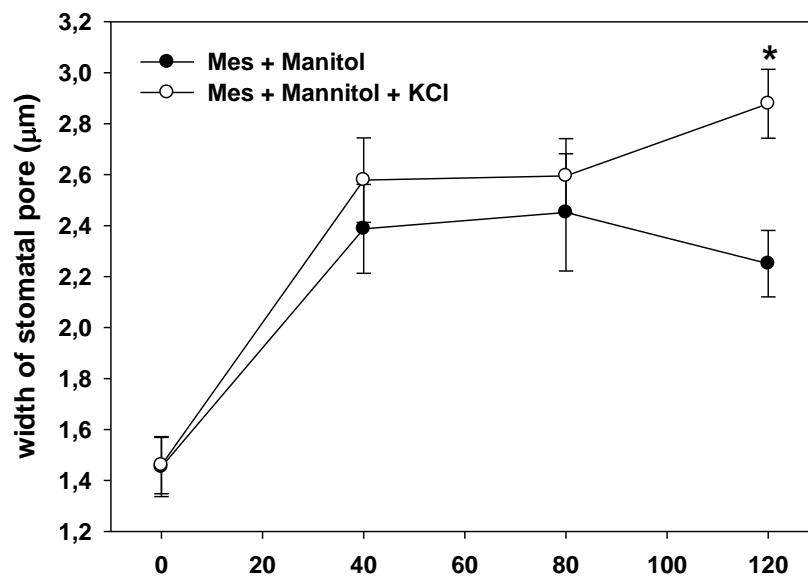

Figure 7. Stomatal aperture light-induced in wilt type guard cell enriched epidermal fragments during treatment with different solutions. Control (MES+ $CaCl_2$ + Mannitol), K^+ treatment (MES+ $CaCl_2$ + Mannitol + K^+) (n = 4 ± SE).
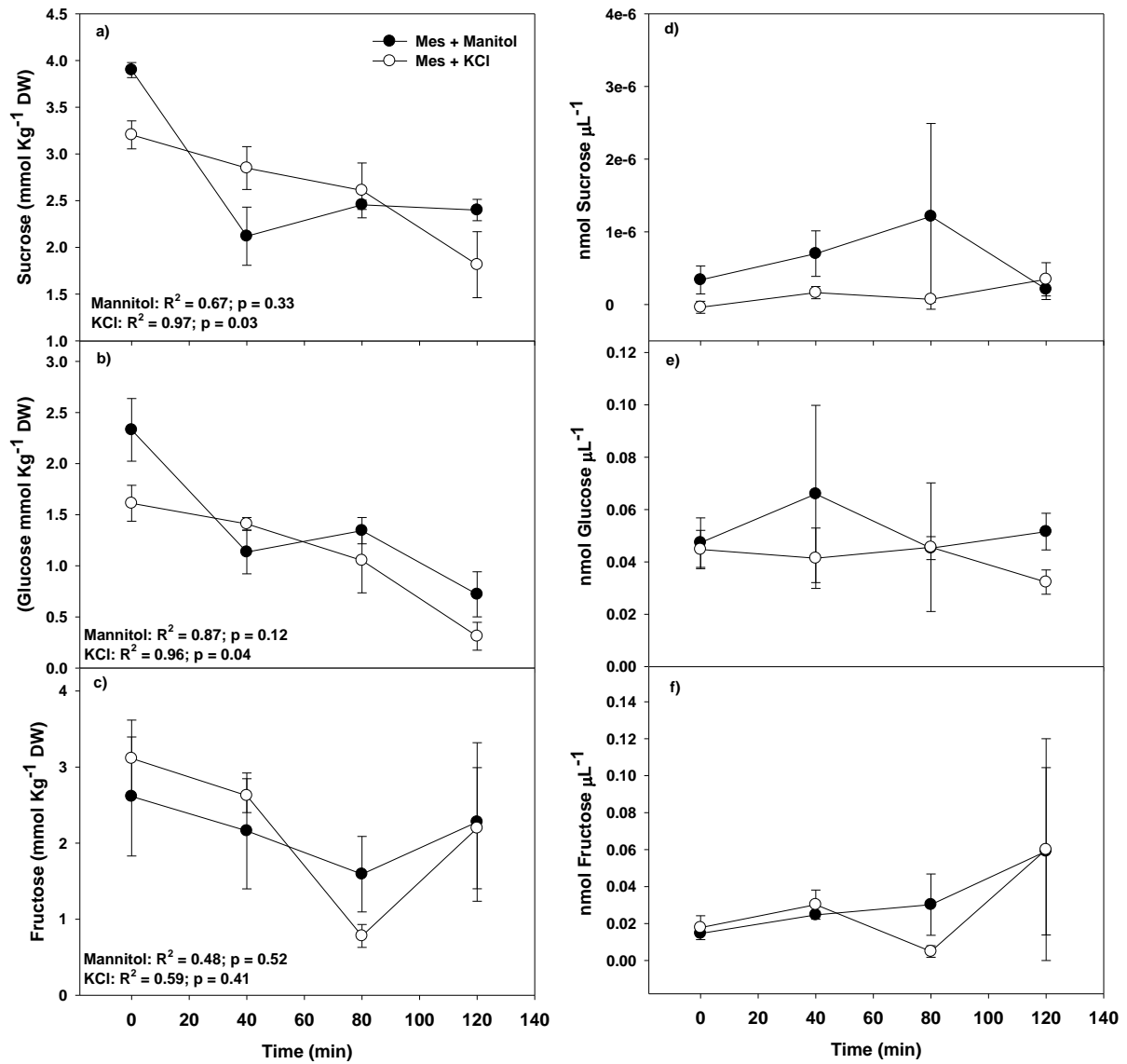
Figure 8. Changes in sucrose, glucose, and fructose levels after influx of K^+ in guard cell enriched epidermal fragments. The epidermal fragments were submitted to treatment for 0, 40, 80 and 120 minutes with MES buffer solution (MES + $CaCl_2$ + Mannitol) or K^+ solution (MES + $CaCl_2$ + KCl). The linear regression was performed according equation: $f = yo + a*x$. ($n = 4 \pm SE$).
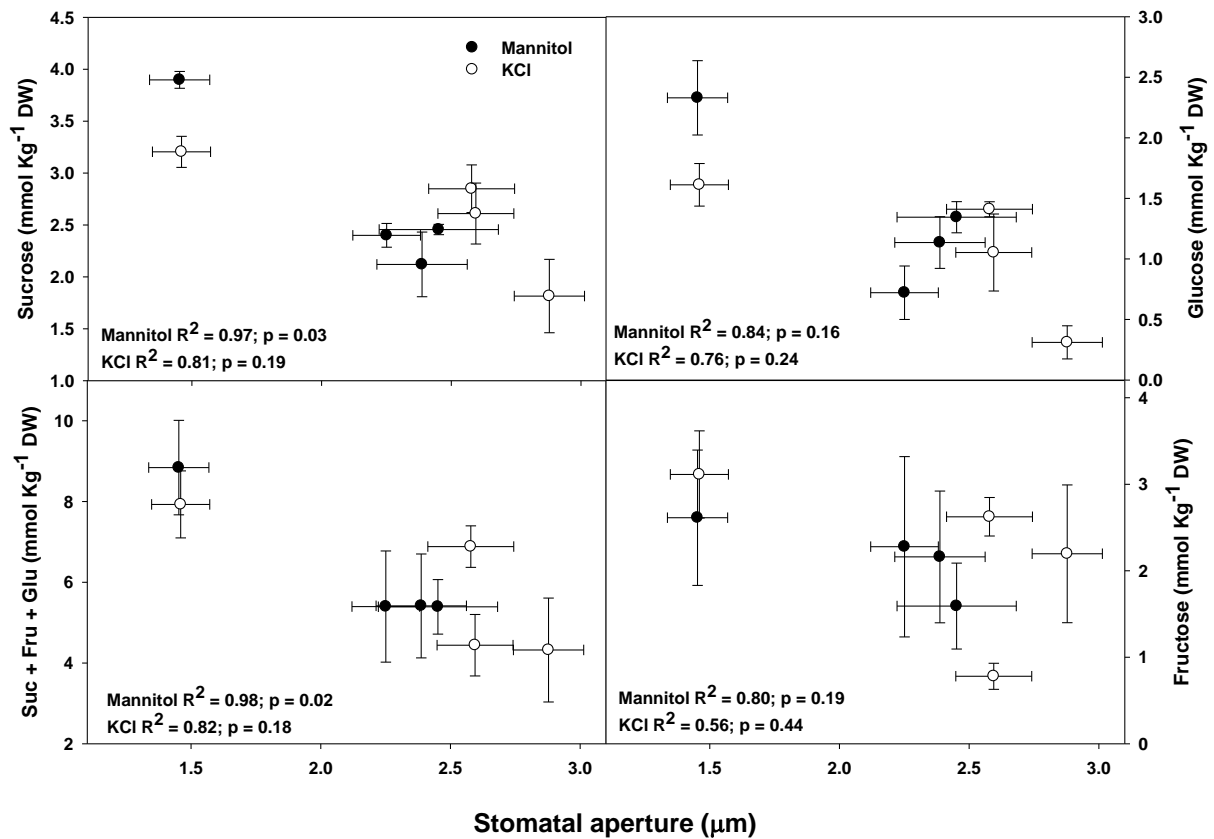
Figure 9. Relationship between sugar levels and stomatal aperture (data from figures 8 and 9). The linear regression was performed according equation: f = yo + a*x.

Role of sucrose after the influx on guard cell symplast

It was previously shown that forty minutes was sufficient to induce stomatal opening and several changes in sucrose metabolism. In the next step, it was developed a flux experiment spanning the time between 0 and 40 minutes, evaluating stomatal aperture, sugars and starch by enzymatic and colorimetric methods. This experiment, contrary to the others showed until now, was performed in glass beakers, not in petri dishes. Stomatal aperture increased linearly over time in both treatments control (mannitol 30 mM) and under sucrose solution (mannitol 10 mM + sucrose 20 mM) (Figure 10). However, this increase was accompanied by changes in sugars content only in the sucrose treatment. Starch content did not change over time, independent of the treatment. We also analyzed starch content using a colorimetric method and no changes were observed again (data not shown). No difference was detected between control (EF frozen before the start of the experiments) and the time 0 (EF frozen immediately after the submission to the solutions), showing a good adjustment of the methodology. Under sucrose solution, the increase in sucrose content, probably from

sucrose influx and from GC photosynthesis, was followed by a simultaneous increase in fructose and glucose. Compared to the control values, sucrose, fructose and glucose levels were significantly increased (Anova and Tukey test - p<005) after 8, 32 and 24 minutes, respectively. On the other hand, no significant differences were observed in sucrose, fructose and glucose levels over time in the control (Mannitol) treatment (Figure 11).
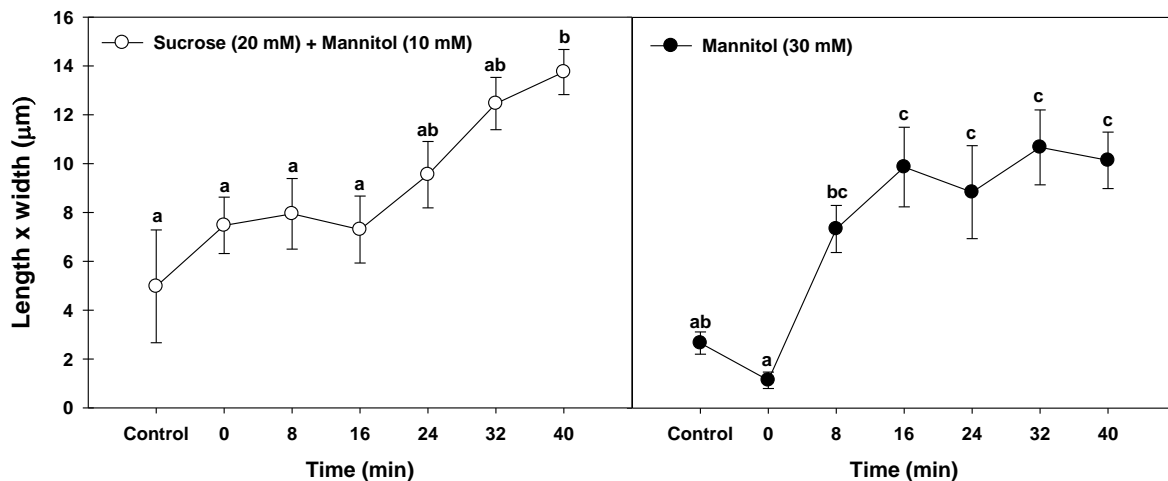

Figure 10. Stomatal aperture (length x width of stomatal pore) along the time under control solution (MES NaOH 5 mM pH 6.5 + CaCl$_2$ 50 µM + mannitol 30 mM) and sucrose solution (MES NaOH 5 mM pH 6.5 + CaCl$_2$ 50 µM + mannitol 10 mM + sucrose 20 mM). Means followed by different letter indicate values significantly different from control by ANOVA and Tukey test (P < 0.05) (n = 4 ± SE).

Guard cell carbon fixation during light induced stomatal opening

EF were collected, pooled in water and stored in the dark. Following this, EF were treated with NaH^{13}CO$_3$ under four conditions; control (MES + CaCl$_2$), KCl, sucrose and KCl + sucrose for 1 hour in the light (see M&M for details). After this period, EF were collected on a membrane, washed extensively and frozen. Samples were extracted and analyzed by LC-qTOF-MS according Bajad et al (2006). An increase in ^{13}C-enrichment in sucrose was observed under control and K$^+$ treatments (Figure 12), probably from ^{13}C incorporation in sucrose via GC photosynthesis. Furthermore, an increase in ^{13}C-enrichment was observed in malate and glutamine, principally under K$^+$ treatment (Figures 13-14). Under sucrose treatment, the ^{13}C-enrichment of sucrose decreased (ratio M+1/M+0, M+2/M+0, M+3/M+0, M+4/M+0) probably due the influx of ^{12}C-sucrose. The ^{13}C-enrichment in malate, fumarate

and glutamine were also lower under presence of sucrose compared to K^+ treatment. Glutamine was labeled at M+1 and M+2 in all treatments but to a greater extent in the K^+ and control treatments. In addition, in another experiment performed with $NaH^{13}CO_3^-$ analyzed by GC-TOF-MS, preliminary data showed a ^{13}C-enrichment in sucrose, glutamine and aspartate (Figure S1).
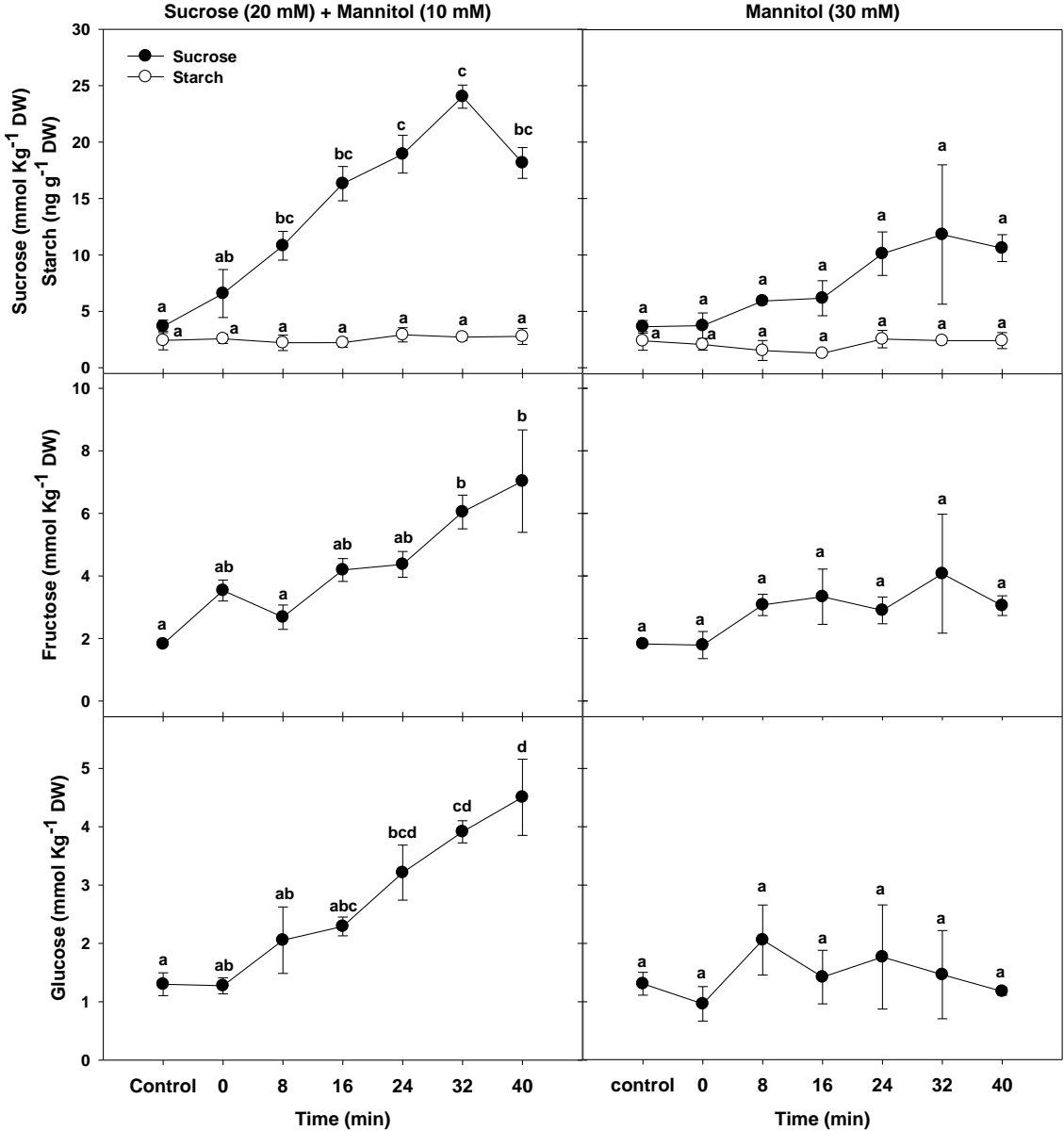

Figure 11. Changes in sucrose, glucose, fructose, and starch levels after influx of sucrose in guard cell enriched epidermal fragments. The treatments and statistics are described in figure 10.
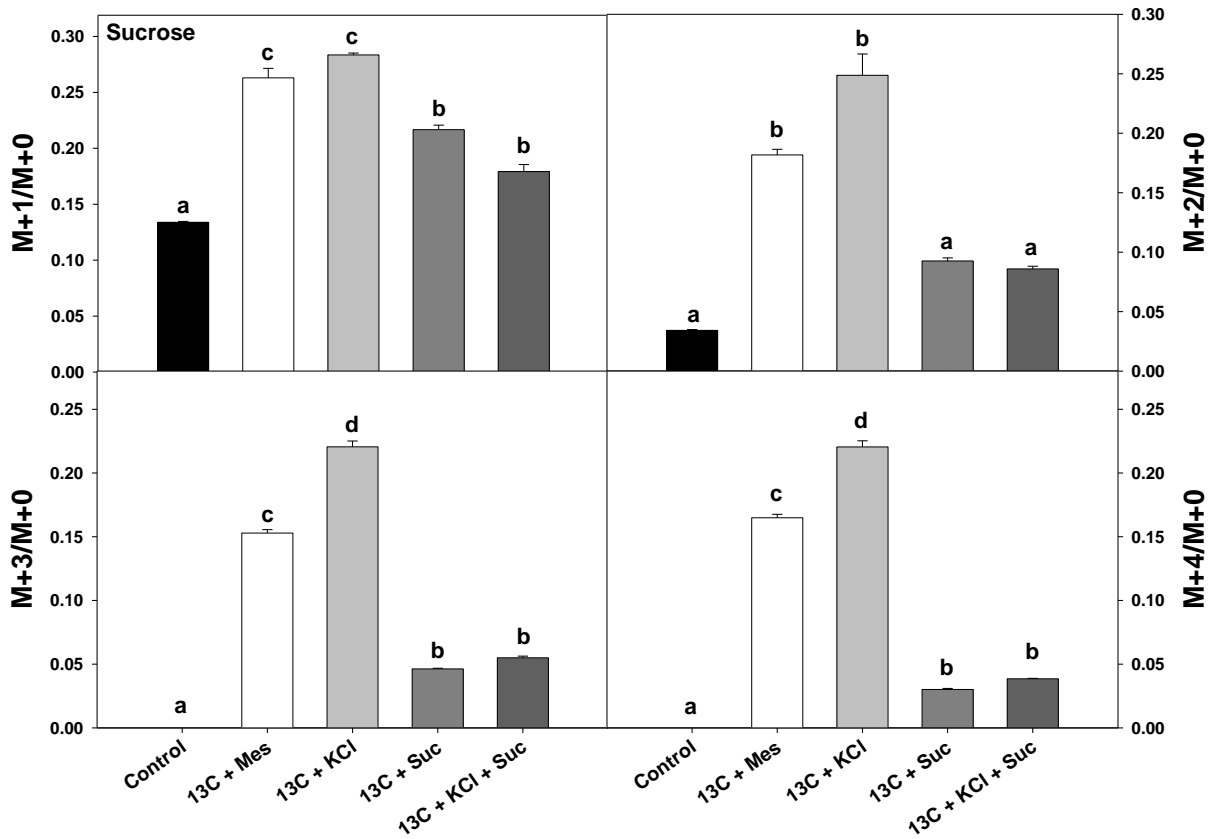
Figure 12. ^{13}C-enrichment of sucrose in epidermal fragments after 1 hour of labelling with ^{13}C-NaHCO$_3^-$. The treatments are described in figure 13. The guard cell enriched epidermal fragment were incubated in solutions Mes (MES/NaOH 5 mM + CaCl$_2$ 50 μM), KCl (MES/NaOH 5 mM + CaCl$_2$ 50 μM + KCl 5 mM), Suc (MES/NaOH 5 mM + CaCl$_2$ 50 μM + sucrose 20 mM), and KCl + Suc (MES/NaOH 5 mM + CaCl$_2$ 50 μM + KCl 5 mM + sucrose 20 mM) for 1 hour under light (~300 μmol photons m^{-2} s^{-1}). Control means samples frozen immediately before the experiment. Means followed by different letter indicate values significantly different from control by ANOVA and Tukey test ($P < 0.05$) (n = 4 ± SE).

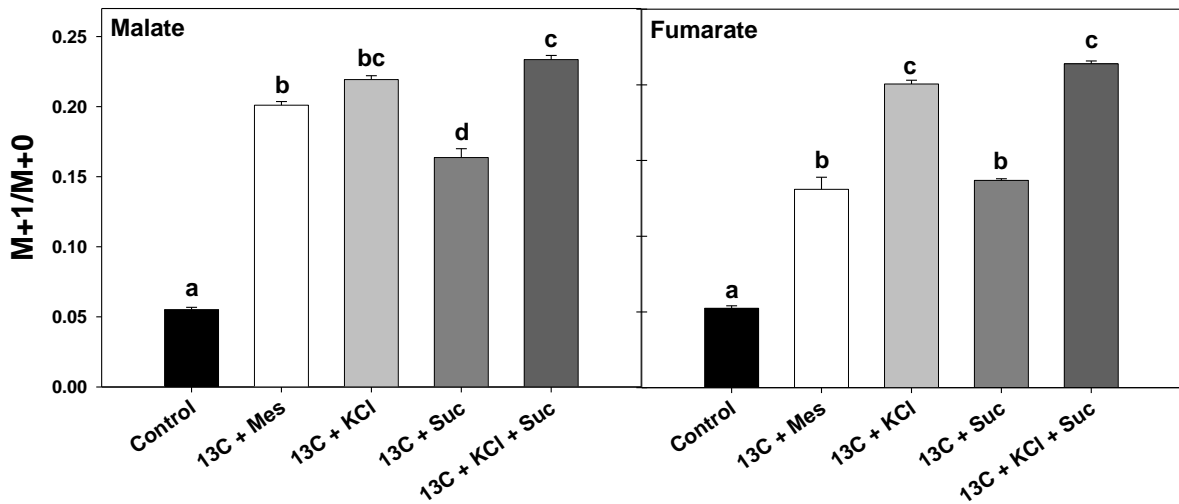**Figure 13.** ^{13}C-enrichment of malate (left) and fumarate (right) in epidermal fragments after 1 hour of labelling with ^{13}C-NaHCO$_3^-$. The treatments and statistics are described in figure 12.
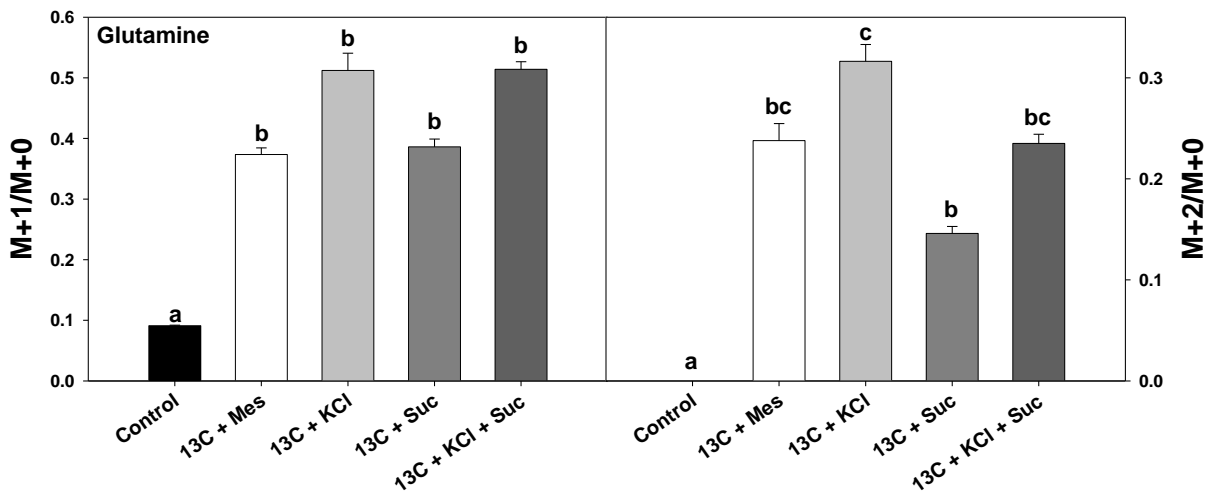
kn

Figure 14. ^{13}C-enrichment of glutamine M+1/M+0 (left) M+2/M+0 (right) in epidermal fragments after 1 hour of labelling with ^{13}C-NaHCO$_3^-$. The treatments and statistics are described in figure 12.

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Discussion

Sucrose as a substrate for guard cell osmoregulation

Guard cells (GC) are a very dynamic cell type with a complex signalling network that integrates endogenous and environmental signals (Fan et al. 2004). The control of gas exchange by stomatal movements allows the plants to occupy habitats with fluctuating environmental conditions and so it can be predicted that stomata must be important contributors to speciation and evolutionary change (Hetherington and Woodward 2003). The changes in GC signalling and metabolism during stomatal aperture process involves a complex network of interactions between hormones, secondary messengers and metabolism (Schoroeder et al. 2001; Desikan et al. 2004; Vavasseur and Raghavendra 2005; Kim et al. 2010). In this context, potassium (K^+) and sucrose are proposed to be the main osmolytes responsible for inducing changes in the osmotic potential of GC, which in turn induces water influx and consequently stomatal opening (Tallbot and Zeiger 1998).

Although the role of sucrose in GC osmoregulation is well documented (see reviews Zeiger et al. 2002; Outlaw Jr, 2003), new evidence for the function of this metabolite come from studies developed in our laboratory. Using transgenic tobacco plants expressing an antisense construct targeted against the plasma membrane sucrose transporter (SUT) under control of KST1 promoter, it was showed that the reduction of sucrose content in tobacco GC leads to stomatal conductance (g_s) reduced (Antunes et al. *in preparation*). Furthermore, transgenic potato plants expressing an antisense construct targeted against sucrose synthase 3 (SuSy3) exhibited decreased g_s while transgenic potato plants with increased GC acid invertase activity had increased g_s (Antunes et al. 2012). In the same way, an increase in GC SUS3 expression in transgenic tobacco plants leads to increase in g_s (data from chapter I of this thesis). In agreement with these studies, the decrease in sucrose levels during stomatal opening induced by K^+ (Figures 5, 9, 13), in the absence of changes in starch content and sucrose content in the medium, provide more evidence that the sucrose breakdown in GC is an important mechanism during stomatal opening process.

The increase in sucrose content observed under sucrose treatment (Figure 11) was accompanied by an increase in fructose and glucose, without changes in starch content (Figure 11). It suggests that the increase in fructose and glucose is from sucrose breakdown, not starch breakdown. Furthermore, the lower ^{13}C-enrichment in malate, fumarate and glutamine observed under sucrose treatment compared to K^+ treatment (Figures 13-14), suggest that sucrose is broken under K^+ treatment, not stored in vacuole. Altogether, these

results highlight the importance of sucrose as a substrate for GC osmoregulation, in addition to the role as osmoticum. Probably the sucrose breakdown can provide carbon skeletons for malate production (Dittrich and Raschke 1977), which in turn can be stored in vacuole acting as counter-ion of K^+ (Hedrich and Marten 1993) or metabolized to induce respiration, since GC have a high respiration rate (Vani and Raghavendra 1994).

Contribution of TCA cycle related metabolites to light induced stomatal opening

The increase in ^{13}C-enrichment in malate during light and K^+ induced stomatal opening is expected based on the literature, and suggests that the methodology used in this work is appropriate. Interestingly, the ^{13}C-enrichment observed in fumarate under these conditions is a great evidence for the contribution of this metabolite and/or the operation of the TCA cycle in the stomatal opening process. In a similar way, a small ^{14}C-enrichment in fumarate already was observed in *Vicia faba* GC protoplasts (Brown and Outlaw Jr 1982). The importance of fumarate for the normal stomatal functioning has been shown. Fumarase activity of *Vicia faba* was 4-fold higher in GC than mesophyll cells in proteins basis (Hampp et al. 1982). Further, tomato transgenic plants with a reduced fumarase activity, which have elevated fumarate content, showed a impaired stomatal function (Nunes-Nesi et al. 2007), suggesting that fumarase is important for the generation of malate for stomatal opening (Fernie and Martinoia 2009). Probably, fumarate by some similarities with malate such their chemical structure, the pattern of accumulation and degradation throughout the day, and to be able to induce stomatal closure in a dose-dependent manner (Nunes-Nesi et al. 2007; Araújo et al. 2011 a,b), may have the same role as malate in GC. Nevertheless, given that malate is present in the GC apoplast at higher concentrations (Araújo et al. 2011 a), it would seem likely that it exerts greater influence on stomatal opening than fumarate does *in vivo* (Araújo et al. 2011 c).

The ^{13}C-enrichment in glutamine during light induced stomatal opening (Figure 14) suggests that this metabolite also participate of the metabolic network of the stomatal opening process and/or GC photosynthesis. Furthermore, the lower ^{13}C-enrichment in glutamine under sucrose treatment compared to K^+ treatment (Figure 14) possibly is due ^{12}C-sucrose breakdown. Alternatively, the synthesis of glutamine as well fumarate and malate (Figure 13) in illuminated GC can be stimulated by the presence of K^+. Though the roles of sugars and organic acids during the stomatal opening are well studied, little is known about the contribution of amino acid metabolism to GC functioning. The labelling of glutamine (Figure 14, S1) and aspartate (Figure S1 - data preliminary) after GC CO_2 fixation has previously

been observed in feeding experiments using $^{14}CO_2$ (Willmer and Dittrich 1974; Brown and Outlaw Jr 1982). However, the role of these metabolites during stomatal opening remains unclear. In illuminated leaves, the carbon source for glutamine production comes from 2-oxoglutarate directly from TCA cycle or *via* the breakdown of citrate stored during dark period (Figure 15). Under this condition, the TCA cycle does not appear to operate like a proper cycle (Tcherkez et al. 2009; Gauthier et al. 2010). While the conventional TCA cyclic flux remains important, especially in heterotrophic tissues, a variety of non-cyclic flux modes occur in specific instances (Sweetlove et al. 2010). Thus, although the role of the glutamine *per si* for GC functioning is unknow, the ^{13}C-enrichment in glutamine is a great evidence that TCA cycle is operating during light induced stomatal opening. A non-cyclic flux modes of TCA cycle cannot be exclude to operate in iluminated GC; however further feeding experiments are need to demonstrate how glutamine contribute for GC functioning and how TCA cycle operate under light induced stomatal opening.

Can tobacco guard cells produce all energy and osmolytes required for stomatal opening?

No changes in starch content during light induced stomatal opening were detected (Figures 4, 5, 11). The changes in starch content in GC during stomatal opening depend on light quality, and starch breakdown appears to occur only under blue light (Talbott and Zeiger 1993), since the blue light-induced stomatal opening was severely impaired in the Arabidopsis *pgm* mutant, which did not accumulate starch in GC chloroplasts (Lascève et al. 1997). It is important to note that we did not control light quality in our experiments, which make it impossible to discuss these results based on light quality. Starch breakdown in GC chloroplasts has been shown to be an important mechanism to increase the level of osmoregulatory molecules, counter-ions (mainly malate) and/or substrates for respiration (Shimazaki et al. 2007). GC starch content has also been quantitatively related to stomatal aperture in *Vicia faba* (Outlaw Jr and Manchester 1979); however the relative rates of sugar import and consumption can determine whether a net change in the starch content of GC occurs (Dittrich and Raschke 1977). Therefore, it is not surprising that under certain conditions the starch content of GC does not change during stomatal movement (Lawson 2009), as was shown in this study.

Interestingly, in the control treatment (Mannitol + $CaCl_2$) the stomatal opened significantly ($p<0.05$) in 8 minutes keeping opened until 40 minutes without changes in starch content, suggesting that the GC are photosynthesizing all the osmoticum and all energy that they need to open. Several authors suppose that the energetic demand for stomatal opening

can be sufficiently produced by GC (Humble e Hsiao, 1970; Inque et al. 1985; Tominaga et al. 2001; Zeiger et al. 2002). Further, although there are evidences demonstrating that stomatal responses to light depend on photosynthetic rates of mesophyll cells (Shimazaki et al. 2007; Doi e Shimazaki 2008; Mott 2009), antisense plants for cytochrome b_6f complex (Baroli et al. 2008), SBPase (Lawson et al. 2008), and Rubisco (von Caemmerer et al. 2004; Baroli et al. 2008) does not showed changes in stomatal conductance, suggesting that the photosynthetic rates of mesophyll cells do not contribute for the stomatal movements. In agreement with this studies, GC of tobacco apparently produce all energy and osmolytes necessary for stomatal opening, which explain no changes observed in starch content (Figure 11). However, whether this is a characteristic of tobacco GC or whether this is a characteristic of plants growing under tropical conditions remains unclear.

Do tobacco guard cells present a CO_2 fixation by both Rubisco and PEPcase?

The very clear ^{13}C-enrichment in sucrose (M+4) observed suggest a CO_2 fixation by Rubisco. However, a simultaneous [13]CO_2 fixation by PEPcase cannot be excluded given that ^{13}C-enrichment was also observed in malate and aspartate (Willmer and Dittrich 1974; Brown and Outlaw Jr 1982; Gotow et al. 1988). Alternatively, the ^{13}C-enrichment in malate and aspartate can be from sucrose breakdown (Dittrich and Raschke 1977). Several controversies exist regarding GC metabolism, mainly concerning the contribution of the photosynthetic carbon reduction pathway (PCRP) for GC. Vavasseur and Raghavendra (2005) in a classical review pointed that although a matter of some controversy, it is generally accepted that the Calvin cycle plays a minor role in stomatal movements. These authors base their discussion on the fact that GC have few and smaller chloroplasts, low chlorophyll content as well as low level of Rubisco. In the same way, Outlaw Jr (1989) concluded that GC do not photosynthetically reduce significant quantities of CO_2, suggesting that PCRP would not contribute significantly to the overall carbon balance of these cells. In contrast Zeiger and co-workers have shown that photosynthetic carbon assimilation within GC can produce osmotically active sugars (Tallman and Zeiger, 1988; Talbott and Zeiger, 1993, 1998; Zeiger et al., 2002). In this context, Gotow et al. (1988) showed that the radioactivity of [14]CO_2 fixation in the light was found in 3-phosphoglyceric acid (PGA), sugar monophosphates, sugar diphosphates, and triose phosphates, suggesting that PGA is the primary carboxylation product in illuminated GC and that the detected labeled RuBP was produced from the regeneration of the CO_2 acceptor via PCRP activity.

It is now widely accepted that the Calvin cycle enzymes are present in GC chloroplasts; however, the debate over their activity, function and role in stomatal behaviour remains (Lawson 2009). Zeiger et al. (2002) pointed that, given that some controversies regarding GC metabolism have been surprisingly long-lived, a primary reason behind the controversies may not be scarcity or lack of quality of data, but the unusual complexity and functional plasticity of the GC. Thus, it is clear that GC metabolism remains a complex field to study. Due the connectivity between GC and MC as well as the transport of ions and metabolites between these two cells and within GC it is very difficult to analyze the distribution of photosynthetic fluxes in GC metabolism *in vivo*. Nevertheless, this study contributed to knowledge of GC metabolism highlighting the role of sucrose as a substrate for stomatal opening. Although more evidence is needed, these results suggest that the sucrose breakdown can provide carbon skeletons for malate production, a mechanism already shown to be possible (Dittrich and Raschke 1977). Also, this study provides evidences that glutamine and fumarate also participate of the stomatal opening process (Figure 15). Further metabolic flux analysis using labeled substrates, including sugars, organic acids and amino acids, accomplished with mass spectrometry and/or NMR analysis can provide important information regarding the fate of carbon from sucrose breakdown, helping to understand the interesting, but complex, GC metabolism.
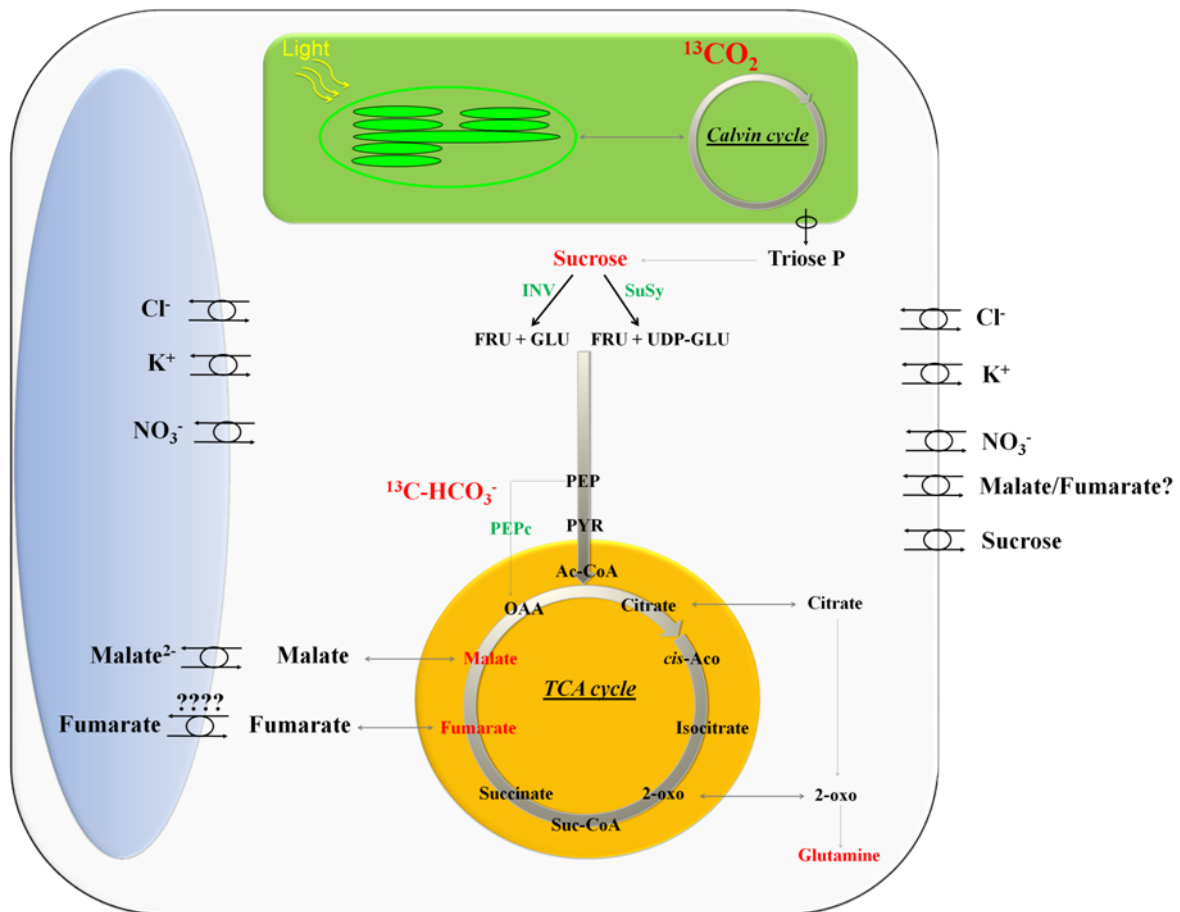
Figure 15. Schematic representation of the changes of guard cell (GC) symplast metabolism during light induced stomatal opening observed in this study. Subcellular compartments are differenced by color: Blue – vacuole, Green – chloroplast, Orange – mitochondria, White – cytosol. Molecules in red means labeled metabolites in ^{13}C-NaHCO$_3$-feeding experiment. The results showed that the level of sucrose, fructose and glucose are decreased during light and K$^+$ induced stomatal opening, while starch content does not changes. Interestingly, stomatal opened in the control treatment also without changes in starch content, suggesting that GC are photosynthesizing all the osmoticum that they need to open. A ^{13}C-enrichment in sucrose is evidence to CO$_2$ fixation from Rubisco; however a simultaneous CO$_2$ fixation by PEPcase cannot be excluded given that ^{13}C-enrichment was also observed in malate and aspartate. As expected, the ^{13}C-enrichment in malate confirms its importance for GC functioning. Probably malates produced under these conditions are transported from mitochondria to vacuole, where is counter-ion of K$^+$. The ^{13}C-enrichment observed in fumarate and glutamine is a great evidence that TCA cycle is operating during light induced stomatal opening. Although this model do not show the connection between GC and mesophyll cells, it's important to highlight that other cell types are important to provide ions (Cl$^-$, NO$_3^-$, K$^+$) and metabolites such sugars and organic acids for GC functioning.

Conclusion

Since that stomatal closure is an important mechanism to plant drought tolerance, the GC signalling pathway ABA-dependent which leads to stomatal closure is well documented. However, the metabolic changes during stomatal closure and/or stomatal opening remain poorly understood. The majority of the information's about GC metabolism are from studies developed in 70's and 80's, which provide the initial knowledge about the flux distribution in GC through the use of radiolabeled metabolites (Willmer and Dittrich 1974; MacRobbie 1981a,b; Brown and Outlaw Jr 1982). Actually, with the development of mass spectrometry technologies and the availability of several ^{13}C-labeled metabolites make possible to perform metabolic flux analysis under several conditions and different substrates. This combination contributed significantly for the understanding of the different cell types and organisms. However, to our knowledge, there are no studies about GC metabolism using ^{13}C-labeled metabolites coupled with mass spectrometry analysis. Thus, using a combination of different methods to analyze the changes in GC metabolism during light induced stomatal opening, this study contribute to knowledge of GC metabolism highlighting the role of sucrose as a substrate for the stomatal opening. Sucrose is related to be an important osmolyte for GC when the level of K^+ decreased (Talbot and Zeiger 1998). However, the results showed here suggests that during K^+ and light induced stomatal opening sucrose breakdown can be an important mechanism to provide carbon skeletons to malate production, which in turn can be stored in vacuole or degraded to induce respiration.
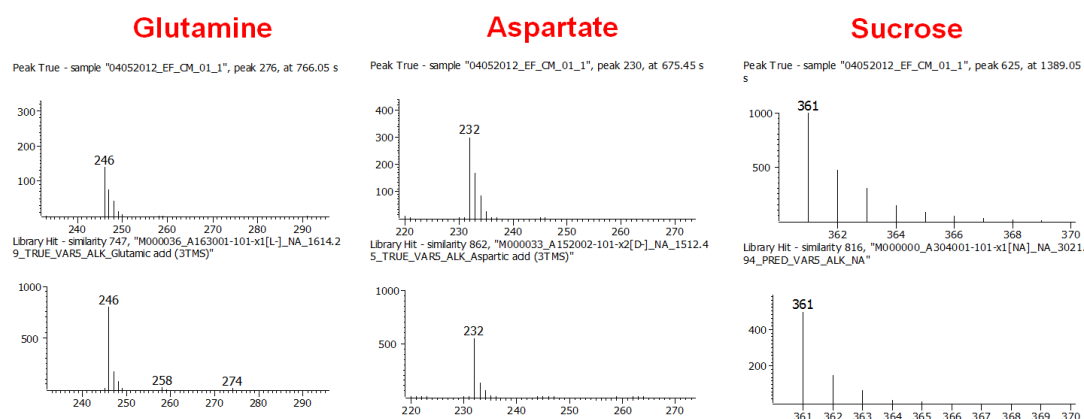
Supplemental data

Figure S1. Preliminary data from flux experiment in epidermal fragment under ^{13}C-NaHCO$_3^-$. Mass spec from GC-TOF-MS showing the ^{13}C-enrichment in glutamine, aspartate and sucrose.

Material and methods

Plant material and growth conditions

In this study, we used wild type (WT) of *Nicotiana tabacum* L. The seeds of tobacco were surface-decontaminated by shaking 70% ethanol for 1 min, rinsed with sterile distilled H$_2$O. In sequence treated with 2% sodium hypochlorite for 5 min, rinsed three times with sterile distilled H$_2$O. The seeds were then allowed to germinate in Petri dishes containing MS medium (25 cm3) (Murashige & Skoog, 1962) cultivated *in vitro* for 15 days. The seedlings were transplanted to 0,1 L pots containing Plantimax® substrate and cultivated under photoperiod of 14 h illumination and light intensity of 120 µmol m-2 s-1 for 15 days. After this, the plants was transplanted for 20 L pots and cultivated in green house for 3-4 months.

Guard cell enriched epidermal fragment (EF) isolation

The isolation of guard cell enriched epidermal fragment (EF) was performed according with method proposed by Kruse et al (1989) and adapted for tobacco. The leaves were blended for three minutes (three pulses of 1 minute) in warring blender (Phillips, RI 2044) with internal filter for isolate epidermal fragment of mesophyll and fibers. After this, the EF were collected in nylon membrane, washed and submit to different solutions. This extraction protocol result in guard cell purity of 98% (Antunes et al 2013), and all extraction procedures timed approximately 5 min until the epidermal fragments frozen in N$_2$ liquid.

After the extraction, the EF were treated different solutions; control (MES/NaOH 5 mM + CaCl₂ 50 µM + Mannitol 30 mM), KCl (MES/NaOH 5 mM + CaCl₂ 50 µM + Mannitol 20 mM + KCl 5 mM), sucrose (MES/NaOH 5 mM + CaCl₂ 50 µM + Mannitol 10 mM + sucrose 20 mM), KCl and sucrose (MES/NaOH 5 mM + CaCl₂ 50 µM + KCl 5 mM + sucrose 20 mM). The EF were maintained in the light range the time according with the experiment. After this, the EF were collected on membrane, washed extensively to avoid apoplast contamination and frozen.

Potassium quantification

Lyophilised EF (10 mg) were transfered to tubes (2 ml) contaninig nitric-percloric acid solution (4:1) maintained at 100ºC for 1 hour. After, the volume was completed to 2 mL with deionised water. The K^+ concentration was determinate by flame specphotometer.

Extraction and analysis of metabolites

The metabolites extraction for gas chromatography coupled mass spectrometry (GC-MS) and soluble sugar quantification by Elisa assays was performed according Lisec et al (2006). Briefly, 10 mg of epidermal fragments lyophilized were submitted at 1000 µL of methanol (100%), 70 ºC for 1 h under 950 rpm. In GC-TOF-MS analysis, 60 µl of Ribitol (0,2 mg/ml stock in dH₂O) was added as an internal quantitative standard. The extract was centrifuged at 11000g for 10', and 600 µL of the supernatant was collected to another tube, where was added 500 µL of chloroform (100%) (LC grade, Sigma) and 800 µL of deionized water. After vortex for 10 s, other centrifuge was done for 15' at 2200g. 1000 µL of polar (upper) phase was collected and transfer for tube of 1,5 mL, dried in speed vac and ressuspend in 100 µL of deionized water. For GC-TOF-MS analysis, the sample derivatisation was done according Lisec et al (2006) through N-Methyl-N-(trimethylsilyl) trifluoroacetamide (MSTFA, CAS 24589-78-4, Macherey & Nagel, Düren, Germany) and methoxyamine hydrochloride (CAS 593-56-6, Sigma, Munich, Germany) dissolved at 20 mg/ mL in pure pyridine (CAS 110-86-1, Merck, Darmstadt, Germany). The levels of soluble sugars (sucrose, fructose and glucose) were done following Threthwey et al. (1998).

Feeding experiment using NaH¹³CO₃ analyzed by LC-qTOF-MS

The EF's were treated with NaH¹³CO₃ 5 mM under 4 solutions described above for 1 hr in the light. After this, 1/3rd of the total material was immediately frozen and the remaining 2/3rds placed back into the same treatment solution, though with ¹²C bicarbonate in place of ¹³C, and incubated in the light or dark for 1 hr. After this period fragments were harvested,

washed and frozen. Lyophilized samples were extracted at -20°C with methanol/chloroform (7:3), phase partitioned with water, and the aqueous phase analyzed by LC-MS, or by direct infusion of samples into the MS. The detection was performed in the positive ion mode using electron spray as source of ionization (ESI) and *time-of-flight* (TOF) as analyzer (Bruker). The spectral were analyzed through AMDIS software according Bajad et al (2006).

Statistical analyses

The treatments were statistically compared to control using Student's *t* test (p<0.05) when there was only one time point and ANOVA and Tukey test (p<0.05) when there were more than one time point. All statistical analyses were performed using the SAEG System version 9.0 (SAEG, 2007).

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General Conclusion

The characterization of transgenic tobacco plants overexpressing potato SUS3 under control of KST1 promoter showed that the genetic manipulation of GC metabolism leads to a phenotype with changes in the stomatal behavior, which in turn results in plants with water use efficiency altered. Furthermore, the results from second chapter showed that the levels of sucrose are decreased during light induced stomatal opening. Although is well documented that sucrose is proposed to be an osmolyte for GC osmoregulation, the data showed here suggest that sucrose breakdown, not just sucrose accumulation, is an important mechanism that regulates the stomatal opening. Although speculative, possibly the sucrose breakdown could occur in order to keep a high production of ATP, necessary for the high activity of ATPases during stomatal opening, or to production of malate and fumarate, acting as a counter-ion in vacuole of GC.