WERNER CAMARGOS ANTUNES

ENGINEERING FOR DESICCATION POSTPONEMENT: ANTISENSE OF SUCROSE TRANSPORTER IN TOBACCO SPECIFICALLY ON GUARD CELLS RESULTS IN REDUCED STOMATAL CONDUCTANCE AND INCREASED WATER USE EFFICIENCY

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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ACCEPTED: July 31st, 2009.

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Nairam Félix de Barros	Antônio Teixeira Cordeiro

Marcelo Ehlers Loureiro
(Advisor)

To my parents, Orlei and Zelita

To my brothers Roberto and Viviane

To my girlfriend Natália

I dedicate this work

Acknowledgements

To God, for all…

To the Federal University of Viçosa for the opportunity to develop this work

To the Plant Physiology teachers for everything taught, not only plant physiology theories, but most important, for their friendship

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I'm so grateful for all

Biography

Werner Camargos Antunes, son of Orlei Antunes Vieira and Zelita Camargos Antunes was born in Esmeraldas, MG, Brazil in may 15th of 1981. He was under-graduated in Agronomy by Federal University of Viçosa, MG. On 2005 he obtained his *Master Scientiae* degree in Plant Physiology at UFV. On 2005 he started his doctoral studies in Plant Physiology program also at UFV. In July 2009 was finished his works being here shown.

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Resumo

ANTUNES, Werner Camargos, D.Sc., Universidade Federal de Viçosa, julho de 2009. **Transformação genética visando resistência à seca: plantas de tabaco transgênicas antisenso do transportador de sacarose apresentam menor condutância estomática e aumento na eficiência do uso da água.** Orientador: Marcelo Ehlers Loureiro. Co-orientadores: Dimas Mendes Ribeiro e Fábio Murilo DaMatta.

Nesse trabalho foi avaliada a importância do transportador de sacarose especificamente em células guarda (CG) e o papel da sacarose sobre os movimentos estomáticos. Utilizou-se plantas de tabaco transformadas com o antisenso do gene do transportador de sacarose sob controle do promotor *KST1*, específico de GC. As CG das plantas transgênicas apresentaram menores teores de sacarose, maiores nos de amido e um modesto incremento nos de K^+. O menor conteúdo de sacarose nas CG das plantas transgênicas esteve associado com menores valores de condutância estomática (g_s). Essa associação sugere a importância da sacarose no simplasto na manutenção de baixos potenciais osmóticos nas CG. Foi observada uma rápida redução nos teores de amido quando os estômatos estavam se abrindo, fato não observado nas plantas não-transformadas. Nas plantas transformadas, com menor g_s, foi possível demonstrar uma restrição difusional (estomática) à fotossíntese (*A*). As plantas transformadas também apresentaram menor taxa de transpiração (*E*) e menor concentração de CO_2 na câmara sub-estomática, além de maiores valores da razão de composição isotópica ($\delta^{13}C$). Entretanto, maiores valores da razão *A*/*E* esteve associado com menores valores de *A*, conseqüentemente, a uma menor taxa de crescimento, porém não a uma menor eficiência baseada nas taxas de crescimento relativas. Os dados de $\delta^{13}C$ confirmaram a menor g_s e reforçam que esse fenótipo se prolongou pelo desenvolvimento das plantas. Por meio de plantas de tabaco com menor g_s foi possível demonstrar que o fenótipo de retardamento à seca foi a principal característica desta transformação, proporcionando as plantas transgênicas um menor consumo de água. Os resultados sugerem que a manipulação do transporte de sacarose em CG foi um mecanismo prático e efetivo na aquisição de plantas mais resistentes à seca.

Abstract

ANTUNES, Werner Camargos, D.Sc., Universidade Federal de Viçosa, July, 2009. **Engineering for desiccation postponement: antisense of sucrose transporter in tobacco specifically on guard cells results in reduced stomatal conductance and increased water use efficiency.** Adviser: Marcelo Ehlers Loureiro. Co-Advisers: Dimas Mendes Ribeiro and Fábio Murilo DaMatta.

It was evaluated the importance of guard cell (GC) sucrose transporter and the role of sucrose as osmotic on GC. We transformed tobacco plants with antisense gene construct for sucrose transporter driven by KST1, GC specific promoter. Transgenic plants GC have less sucrose, more starch and modest increase in K^+ contents. Low sucrose contents in GC of transgenic lines were associated with low stomatal conductance (g_s), suggesting the importance of sucrose transporter and symplastic sucrose in maintaining low osmotic potential on GC. It was observed rapid starch disappearance when the guard cells are swelling, fact not observed in control plants. By means of low g_s tobacco plants demonstrated diffusional (stomatal) restriction of photosynthesis (A), low transpiration rate (E) and low sub-stomatal CO_2 concentration, high A/E and higher carbon rate composition ($\delta^{13}C$). However, higher A/E was associated with lower A, consequently, a slower crop growth rate, but not smaller "efficiency index" as showed by relative growth rate. The $\delta^{13}C$ data confirms the low conductance, showing that it represents a common stomata behavior over all plant development. By means of low g_s tobacco plants, we got desiccation postponement phenotype as principal feature of this transformation, being high water saving plants. These results suggest that manipulation of sucrose transport in GC may be developed as a practical mechanism for drought avoidance and water conservation during irrigation. These results illustrate the importance of fine tuning of sucrose metabolism transport and metabolism in the fitness of stomatal function in contributing to plant survival or growth under unfavorable water conditions.

Preface

General Introduction

Stomata are specialized epidermal structures consisting of two guard-cells (GC) involving a pore whose opening is actively regulated. Stoma possesses some degree of control of the water and carbon dioxide exchange between the plant and atmosphere, and thus plays a key role in survival of land plants. The regulation of the pore opening is mediated by several factors and it is closely linked to photosynthetic rate and water loss by transpiration (Schroeder et al., 2001ab, Ward et al., 2009). Stomata make a significant contribution to global carbon and water cycles. It was estimated that 440 x 10^{12} kg CO_2 (120 x 10^{12} kg C) and 32 x 10^{15} kg of water vapor pass through stomatal pores annually (Hetherington & Woodward, 2003). Different and more detailed carbon cycles estimates on earth are available (Lal, 2008). The relative influx to CO_2 and efflux of water vapor are used to estimate the *water use efficiency* (WUE) at single leaf until or at higher environmental complexity. Global (biosphere) estimative of WUE based on above statistics is equal to 13.75 g CO_2 kg^{-1} H_2O.

At plant level, in most cases WUE can be defined by increase in dry mass to water transpired (Bacon, 2004). However, there is not a single and simple definition for WUE. Its definitions depends on the particular context in which it is been discussed. At leaf level, by means of gas exchange analysis, the ratio of net photosynthetic CO_2 influx (A) to water loss by transpiration (E) defines *instantaneous water use efficiency* (WUE_L; A/E, µmol CO_2 $mmol^{-1}$ H_2O), or *intrinsic water use efficiency* by ratio of A to stomatal conductance (g_s) (WUE_i, A/g_s, µmol CO_2 $mmol^{-1}$ H_2O). At crop level, it can be defined by yield production (harvestable) to water (evapo)transpirated ($_yWUE$). Gas exchange studies generally give an estimative of immediate water use efficiency, while harvest analysis measured on accumulated plant tissue tends to give a time-integral WUE. At long term or *season-long water use efficiency* give us the best estimative of WUE (WUE_{sl}). The WUE_{sl} includes variation in climatic conditions along the day and among different days, including water availability both in soil and atmosphere. It also includes

plant carbon loss by respiration and exudates/emissions at all levels. As emphasized by Jones (2004), the integral WUE reflects mainly the value when stomata are most open because majority of carbon gain and water transpiration occurs on opened stomata.

Stomatal opening occurs when the osmolytes in the apoplast entry into the interior (mainly vacuole) of the CG, generating differences of water potential between symplast to apoplast. Stomatal aperture is regulated by both internal physiological and external environmental factors (Fan et al., 2004). The stomatal movements are rapid and driven by osmotic changes. An increased concentration of osmolytes reduces the osmotic potential and governs an increase of cell turgor, increasing the cell volume, thus promoting stomatal pore opening. The opposite is true for the closure (Ward et al., 2009).

The main hypothesis that explains the alteration of volume of CG involves an increase in concentration of ions K^+ and counter-ions $malate^{2-}$ and Cl^-, (Ward et al., 2009). The renewed interest on mechanisms regulating the changes in metabolism of CG have come from publications that points out that sucrose and carbohydrate metabolism have an important contribution to osmotic adjustment in CG (Lu et al., 1995; Talbott & Zeiger, 1996; Lu et al., 1997; Talbott & Zeiger, 1998; Outlaw, 2003; Vavasseur & Raghavendra, 2005; Lawson, 2009). In a landmark study, Talbott & Zeiger (1996) showed that K^+ increases faster in morning governing rapid stomatal opening, while sucrose increases slowly in the morning and faster in midday and afternoon when K^+ levels fell down. This change in osmolytes keeps stomatal pore opened until sucrose concentration is decreased, followed then by stomatal closure. This hypothesis presupposes the dual mechanism: K^+ (and counter-ions) as major osmolytes in the morning and sucrose in the afternoon. Nevertheless, the relative importance on solutes involved in stomatal aperture still are a controversial issue, once it is questionable value of the histochemical quantification of K^+, method of low sensibility and accuracy. Additionally, there is no any similar publication with other species and diverse experimental conditions confirming Talbott & Zeiger's (1996) results. In summary, there is still no consensus regarding the relative importance of sucrose and K^+ in the mechanism of osmotic regulation in GC, and it is still unknown the mechanism that explains the alteration of sucrose concentration along the day. If sucrose comes

from photosynthetic carbon reduction, it seems less probable once GC have low photosynthetic carbon assimilation rate (Lawson, 2009). Sucrose transported from apoplast is more acceptable (Lu et al., 1997; Outlaw & DeVlieghere-He, 2001). Furthermore, gluconeogenesis process (Dittrich & Raschke, 1977) and sucrose/glucose from starch breakdown are also possibilities (Talmann & Zeiger, 1988) for the role of sucrose on stomatal opening, notwithstanding those hypotheses do not exclude each other.

The reason why sucrose increases in GC is mainly due to sucrose transport from mesophyll cells (Lu et al., 1997), action performed by a sucrose-H^+ symporter (Boorer et al., 1996; Sauer, 2007). The high rates *in planta* are consistent with a high concentration of sucrose in GC apoplast (Kang et al., 2007). On a membrane-area basis, the lower-limit estimated (up to 10.6 pmol sucrose cm^2 GC surface area s^{-1}) is approximately the same as the rate of K^+ accumulation during stomatal opening, which suggest that CG has sufficient and high sucrose-transport capacity (Outlaw, 1983; Lu et al., 1997; Outlaw & DeVlieghere-He, 2001). Because of the mechanism for plant-cell sucrose uptake would be depolarizing and, thus, consuming H^+ from apoplast as well as K^+ uptake consumes. Sucrose inward through the transporter is dependent on the extracellular sucrose, H^+ concentrations and the membrane voltage (Boorer, 1996). At present, however, it is not possible to establish the quantitative significance of competition between K^+ uptake and sucrose uptake by GC (Tallmann, 2004).

An additional argument for the possible role of sucrose, or their by-products in the osmotic regulation of stomata, is based on experimental data showing that the enzymes of synthesis and degradation of sucrose such as sucrose-phosphate synthase (SPS) and sucrose synthase (SuSy) have high activities in GC compared with mesophyll cells on a protein basis (Hite et al., 1993). Moreover, SuSy expression increases under drought stress specifically in guard cells, playing a probable role in the regulation of stomata movements (Kopka et al., 1997; Loureiro, 1999). Other authors also argument in favor of sucrose showing that minor alterations of sucrose content in GC could have dramatic effects on photosynthesis (Riesmeier et al., 1994; Kühn et al., 1999). These results strongly suggest that sucrose transport and metabolism are important to

stomatal regulation of its regular functions (Lu et al, 1997; Outlaw & DeVlieghere-He, 2001).

Testing the hypothesis that sucrose uptake in GC is important to stomatal movements, we have engineered transgenic plants of *Nicotiana tabacum* L. antisense to sucrose transporter gene from potato (*stSUT1*; X69165; Riesmeier et al., 1993) with strong and specifically expression on GC driven by *KST1* promoter (Plesch et al., 2001). The purpose of using specifically GC promoter was to study sucrose transport restrict at GC level avoiding any pleiotrotic interference from the sucrose transport metabolism at a whole plant level. Studies using constitutive expression of an antisense sucrose transporter construct showed deleterious effects in exporting sucrose from source leaves. The reduced carbon export capacity led to chlorotic lesions in leaves, inhibition of sink development and consequently to reduction of plant growth as observed in tobacco [*StSUT1::CaMV 35S*; constitutive] and tomato [*LeSUT1::rolC* or *LeSUT2::rolC*; companion cell-specific] antisense plants (Bürkle et al., 1998; Hackel et al., 2006). In this work, additional investigations assessed the importance of an osmotic role of sucrose, K^+, reducing sugars, and role of "starch" in GC along the day and at different water availability. Its consequence on gas exchange analysis, growth and dry matter accumulation, and WUE_L, WUE_{sl} and $_yWUE$. The results showed us that reducing activity of this transporter could increase WUE_L and $_yWUE$ and, as a result, improve drought stress avoidance.

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Chapter 1

Sucrose transporter is essential for sugar uptake in guard-cells and its decreased expression leads to reduced stomatal conductance

Key words: Sucrose transporter, stomatal conductance, guard cell, osmorregulation

ABSTRACT

In this study, we aimed to evaluate the importance of guard cell (GC) sucrose transporter and the role of sucrose as osmotic on GC. We transformed tobacco plants with antisense gene construct for sucrose transporter driven by KST1, GC specific promoter. Transgenic plants GC showed less sucrose, more starch and modest increase in K^+ contents. Low sucrose contents in GC of transgenic lines was associated with low stomatal conductance (g_s), suggesting the importance of sucrose transporter and symplastic sucrose in maintaining low osmotic potential on GC. It was observed rapid starch disappearance during stomatal opening, fact not observed in control plants. By means of low g_s transgenic tobacco plants it was demonstrated diffusional (stomatal) restriction of photosynthesis (*A*), low transpiration rate (*E*), low sub-stomatal CO_2 concentration, higher *A*/*E*, and higher carbon rate composition ($\delta^{13}C$). However, these plants showed higher *A*/*E* and also lower *A*, consequently, a slower crop growth rate, but not smaller "efficiency index" as showed by relative growth rate. The $\delta^{13}C$ data confirms the low conductance, and show that it represents a common stomata behavior over all plant development. Despite small reduction in dry weight accumulation, the strategy used to generate these transgenic plants indicates the viability to obtain plants more efficient in water-use through changes in the rate of sucrose influx to guard cells.

INTRODUCTION

Sucrose is the major form of soluble carbon and energy used for higher plants for long-distance transport in the phloem. It works as energy storage for a diverse group of plants like sugarcane, sugar-beet, carrots, and many other crop species or wild plants. In source leaves, sucrose is a photosynthetic product distributed to others sinks organs, tissues or cells. There are two different forms of transport of sucrose: I) by symplast, *i.e*., cell to cell through plasmodemata connections or II) by apoplastic transport until phloem. For sucrose to achieve the apoplastic loading is indispensable the sucrose transporter activity (Riesmeier et al., 1993; Kühn et al., 1999, Sauer, 2007; Braun & Slewinski, 2009). Therefore, sucrose transport, including long distance transport from source to sink, must be highly regulated and sucrose transporters must have indispensable roles in regulation of photo-assimilate redistribution (Lalonde et al., 2004; Shiratake, 2008).

Sucrose transporter has a mechanism of transport that follows a Michaelis-Menten-type kinetics with 1:1 H^+:sucrose stoichiometry symport (Boorer et al., 1996). Detailed patch clamp analyses demonstrated that the direction of transport by a sucrose carrier may be reversed depending on the direction of sucrose gradient, pH or trans-membrane potential (Carpaneto et al., 2005). The sucrose:H^+ co-transport and its affinity for sucrose is surprisingly similar (0.1-2 mM) among different species (Lemoine, 2000; Williams et al., 2000; Shiratake, 2008). All their characteristics are in agreement with those demonstrated at physiological level of sucrose concentration, and membrane potential in plants (Lu et al., 1997, Outlaw & DeVlieghere-He, 2001, Kang et al., 2007a, Pandey et al., 2007, Ward et al., 2009).

Immunolocalization studies confirm the presence of sucrose transporter in sieve elements, companion cells and xylem parenchyma (Schmitt et al., 2008). Despite not identified by Schmitt et al. (2008) in other cell types, many evidences corroborate with the presence of sucrose transporters on pollen (Lemoine et al., 1999), seeds (Weber et al., 1997; Bick et al., 1998), trichomes, and guard cells (GC) (Stadler et al., 2003; Weise et al., 2008).

In agreement with the role of sucrose transport in phloem function, the reduction of its expression by antisense techniques using constitutive promoters in potato, tobacco, Arabidopsis, and tomato plants leads to chlorotic lesions in their source leaves, inhibition of sink development, and consequently reduces plant growth (Riesmeyer et al., 1994; Bürkle et al., 1998; Gottwald et al., 2000; Hackel et al., 2006, respectively). Those plants contained a great excess of starch, and sugar failed to be transported efficiently to roots and inflorescences. In Arabidopsis sucrose transporter mutant *Atsuc2*, in which sucrose transport is impaired, also accumulated great excess of sucrose and starch [±20X] at vegetative parts. Complementation studies using GALACTINOL SYNTHASE promoter of *Cucumis melo* (CmGAS1p), which confers expression only in the minor veins of mature leaves and not in the transport phloem of larger leaf veins and stems, reveals that mutant plants expressing AtSUC2 cDNA also accumulated sugars and starch [2-3X], but they have normal morphology (Srivastava et al., 2008). These results confirmed that AtSUC2 is compromised in phloem loading but not in long-distance transport (Srivastava et al., 2008). These contrasting results illustrate the importance of use of other promoters rather than constitutive promoters in the study of gene function using transgenic plants.

Several evidences pinpoint a role for the sucrose transporter for stomatal function (Lu et al., 1995; Kopka et al., 1997; Lu et al., 1997; Outlaw & DeVlieghere-He, 2001; Stadler et al., 2003). GC is singular among epidermal cells, because they do not have plamodemata connections (Wille & Lucas, 1984). However, it accumulates sucrose at regular functions (Lu et al., 1995; Talbott & Zeiger, 1996; Lu et al., 1997; Talbott & Zeiger, 1998). Therefore, sucrose transporters must have indispensable role on its functions.

The mechanism which explains stomatal aperture is based on osmotic changes: when osmotic potential (or more precisely, water potential) decreases as a result of osmolytes uptake in GC, water enters to interior of cells increasing the cellular volume. The special configuration of their cellulose microfibrils allows them to open when their volume increases. The physical mechanism of closing is the opposite; involving efflux of solutes and water of GC cells (Roelfsema & Hedrich, 2005). The first hypothesis proposed to explain increases in osmolytes contents at GC was based on Lloyd

observations (Lloyd, 1908; cited by Lawson, 2009). It showed that stomata contained more starch when closed in the dark than when open during the day. As a result, he concluded that starch was converted into osmoticum during the day, which results in osmotic changes leading to alteration in GC turgor. Many years later, Fischer (1968) highlighted the importance of K^+ on stomatal opening. The starch↔sugar hypothesis was replaced by K^+-malate^{2-} theory. The carbon source was theme of many studies, despite the photosynthesis of GC be the likely choice, but GC has insufficient carbon-reducing capacity to replenish carbohydrate storage (Outlaw, 1989; Reckmann et al., 1990, Outlaw, 2003; Lawson, 2009). But other studies based on $^{14}CO_2$ fixation in epidermal fragments pointed out phospho*enol*pyruvate carboxilase fixation into malic acid and trans-amination by-products, not into sugar or their phosphates (Raschke & Dittrich, 1977; Dittrich & Mayer, 1978). Also based on $^{14}CO_2$, Dittrich & Raschke (1977a) observed that epidermal tissue forms starch from malic acid, and appearance of labeled sugar indicates that gluconeogenesis can occur in epidermal tissue of *Commelina communis* L. and *Vicia faba* L., (Outlaw et al., 1981). Testing the hypothesis that GC can maintain their carbon balance or import assimilates from mesophyll, Dittrich & Raschke (1977b) exposed isolated epidermal fragments and whole leaf to $^{14}CO_2$, and obtained evidences of an intensive transfer of assimilates, particularly sugars, from mesophyll to epidermis. Twenty years later, Lu et al. (1997) repeated $^{14}CO_2$ incorporation experiment at various times after pulse-labeling on *Vicia faba* L. leaflets. In GC dissected from whole leaf retaining apoplastic contents and those from rinsed peels containing only symplastic contents confirmed the sucrose-specific radioactivity, and the source of sucrose is the photosynthesis of mesophyll cells. These experiments opened new windows on osmoregulation in GC, nor based on starch↔sugars nor K^+-malate^{2-}, but multi-osmoregulatory pathways in GC (Pandey et al., 2007; Lawson, 2009; Ward et al., 2009). Osmorregulation on GC seems highly complex and species specific as well as environmental variable. As a consequence, it is unlikely to get a simple explanation for all mechanisms of changes in GC turgor (Talbott & Zeiger, 1998; Outlaw, 2003, Kang et al., 2007a; Lawson, 2009).

In a landmark study, Talbott & Zeiger (1996) pointed out a dual mechanism of stomatal opening. They showed that GC had two distinct osmoregulatory phases. In

the first (morning) phase, opening was correlated with K^+ uptake and, to a lesser extent, sucrose accumulation. In the second (afternoon) phase, which apertures were maximal, K^+ content declined and sucrose became the dominant osmotica. $Malate^{2-}$ and Cl^- are the counter-ions of K^+, but its accumulation seems to be environmental dependent. Nevertheless, the effective contribution of each one of these osmolytes was not enough elucidated. If the histochemical semi-quantification of K^+ [as expressed as % of area] can be associated to minor changes of stomatal aperture, this can be questionable. The quantitative association between sucrose and stomatal aperture can be made in morning, notwithstanding it fits better in the afternoon. In addition, there is no any further extent similar publication with other species or diverse experimental conditions confirming Talbott & Zeiger's (1996) results. In resume, there is still no consensus regarding the importance of sucrose on the mechanism of osmotic regulation in GC. Several reviews about this issue completely forget to mention its existence, of if do so, relegate an secondary and not know mechanism (Schroeder et al., 2001; Fan et al., 2004; Pandey et al., 2007; Neill et al., 2008; Sirichandra et al., 2009) as well as individual contribution of each described osmotica to drive changes in GC turgor. Talbott & Zeiger (1996) hypothesis presupposes enough sucrose in GC apoplast, as confirmed by Lu et al. (1997) and Kang et al. (2007b), and GC takes up exogenous sucrose probably via sucrose transporter present in GC plasma membrane. On a membrane-area basis, the estimative rate of K^+ accumulation during stomatal opening is the same than sucrose import, indicating that CG have sufficient and high sucrose-transport capacity (Outlaw, 1983; Lu et al., 1997; Outlaw & DeVlieghere-He, 2001). It is important to emphasize that K^+ uptake by GC is necessary favorable trans-membrane potential driven by ATPases activities, and it shares, with sucrose, the same biophysical mechanism of transport into the symplast, co-transport with H^+. At present, however, it is not possible to establish the quantitative significance of competition between K^+ uptake and sucrose uptake by GC, (Lu et al., 1997, Schroeder et al., 2001; Outlaw, 2003; Tallmann, 2004; Ward et al., 2009).

In this study, we aimed to evaluate the importance of GC sucrose transporter and the role of sucrose as osmotic on GC, and its consequence on stomatal aperture.

We transformed transgenic tobacco plants with an antisense construct for sucrose transporter driven by *KST1*, GC specific promoter, in order to evaluate the effects of reduction of sucrose transporter expression on sucrose uptake in GC as well as on leaf gas exchange, plant growth and its effects on water use efficiency.

RESULTS

Plant transformation

After leaf co-culture with transformed *Agrobacterium tumefaciens*, different and potentially *Agrobacterium*-mediated *Nicotiana tabacum* L. transformation lines were regenerated by tissue culture as showed by representative line in Fig. 1. As a result of kanamicin resistance, the different lines have apparently normal growth, similar to wild type (WT) plants at non-selective culture medium.

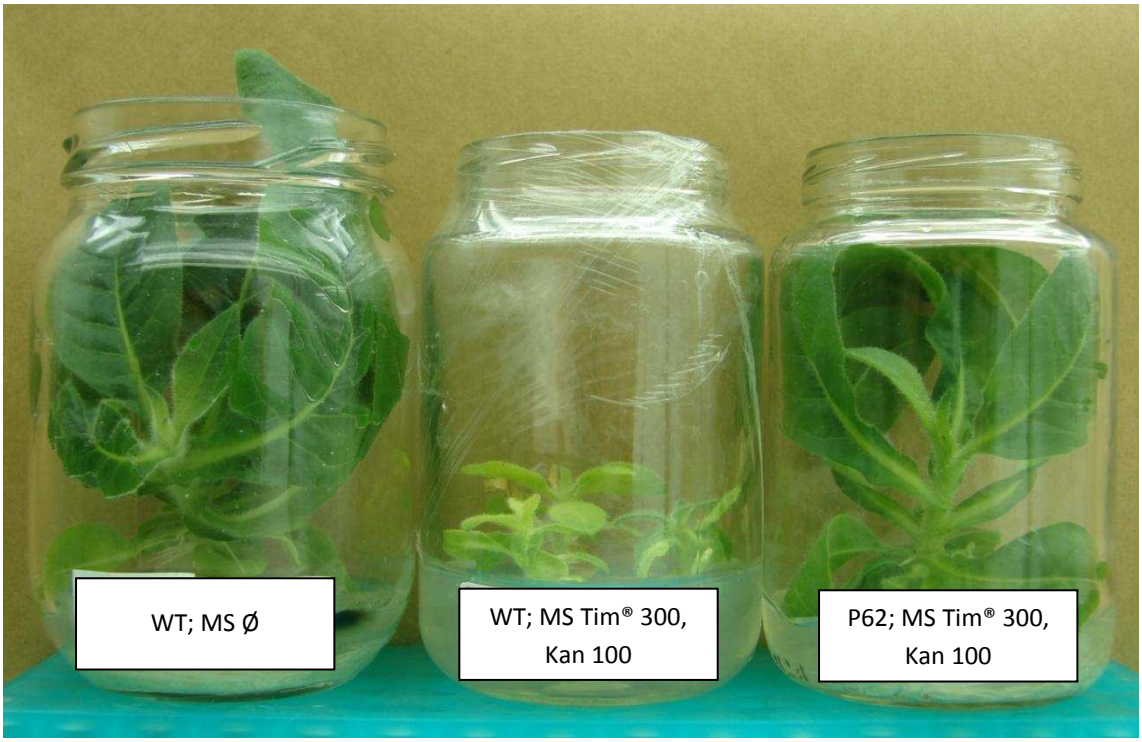

Figure 1: *Nicotiana tabacum* L. plants (Havana 425) cultivated *in vitro*. Left, non-transformed plant (wild type; WT) cultivated in MS media without antibiotics (MS Ø). Centre, WT plants cultivated in MS media with 100 mg L^{-1} kanamicin and 300 mg L^{-1} of Timentin® (MS Tim® 300, Kan 100). Right, representative antisense of sucrose transporter regenerated line (P62), probably transformed, and cultivated in MS media with antibiotics (MS Tim® 300, Kan 100).

The confirmation of tobacco transformation was completed by specific primers in PCR for *NPTII* (kanamicin resistance) gene. As showed in Fig. 2, fourteen different regenerated lines confirmed the presence of *NPTII* gene (600 bp), thus confirming exogenous DNA insertion. After that, pre-selection of transformed lines based on gas exchange analysis occurred (data not shown). Four T2 lines of sucrose transporter antisense plants with contrasting phenotypes were selected and denominated as P62 L6, P62 L9, P62 L24 and P62 L44, and confronted to wild type (WT, non-transformed) plants.

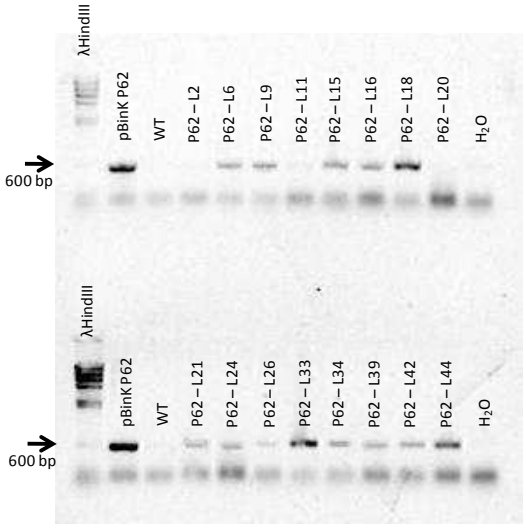

Figure 2: PCR product (600 bp) in agarose gel showing exogenous gene (*NPTII*, kanamicin resistence), confirming exogenous DNA insertion in the construction with sucrose transporter (P62) antisense driven by *KST1* promoter (guard cell specific driven-by expression).

Osmoregulation in Guard Cells

The reduction of sucrose transporter expression on GC led to a reduction of sucrose contents in epidermal fragments [*composed mainly by more than 98% of GC cells*; data not shown) along all daylight except for P62 L6 where changes were restricted to midday and afternoon (Fig. 3C). These results are expected if sucrose inside stomata is mainly a consequence of transporter mediated by a GC sucrose transporter.

There is a diurnal rhythm phase on sugars contents in GC. It was observed an increase of sugar at morning until approximately midday and its decrease at afternoon

(Fig. 3). Such behavior was little evident in GC of transformed plants, which the sucrose content was low and approximately constant along the time of analysis.

Glucose (Fig. 3A) or fructose (Fig. 3B) contents were smaller than sucrose (Fig. 3C) in GC, but did not differ statistically from control plants (P>0.05) , with exception in fructose content in P62 L44 line at 12:00h in which that sugar content has reduced (Fig. 3B).

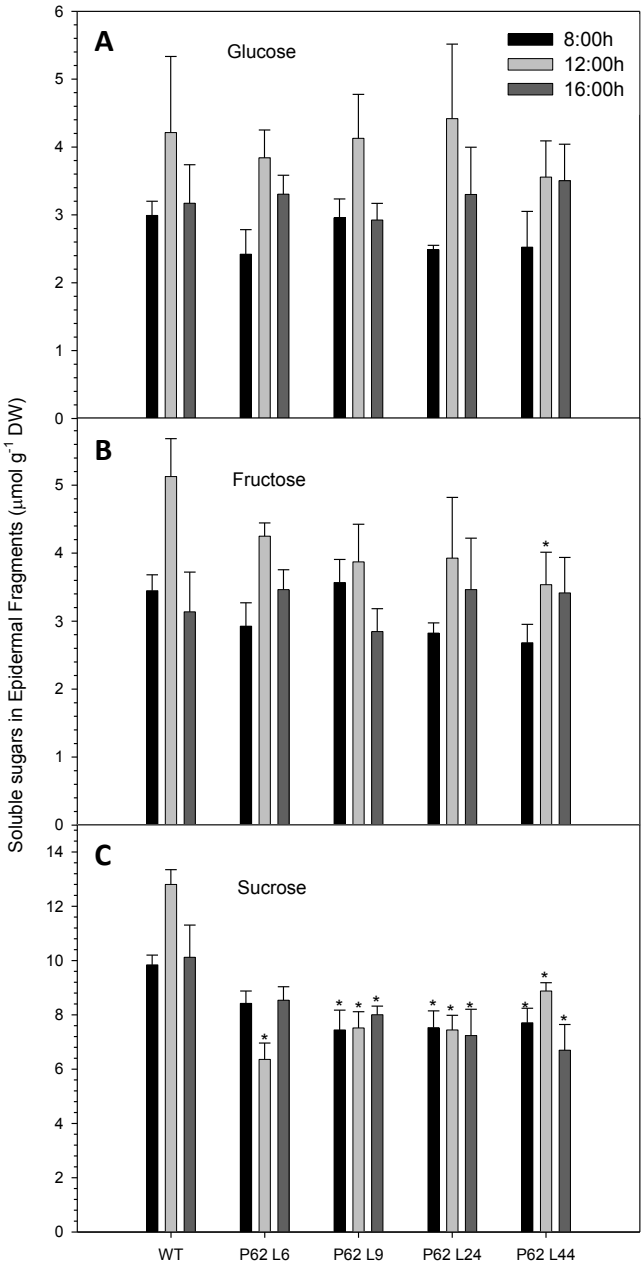

Figure 3: Glucose (A), fructose (B) and sucrose (C) contents in epidermal fragments (>98% of guard cells) at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

When analyzed as a group, the soluble carbohydrates (glucose + fructose + sucrose) (Fig. 4) clearly show reduction in GC transformed lines as compared to sugars contents in GC of control untransformed plants.

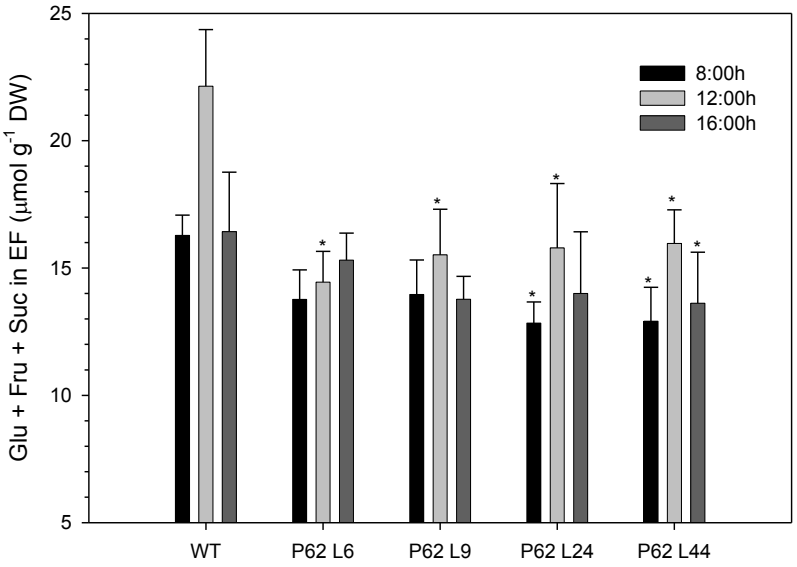

Figure 4: Glucose plus fructose plus sucrose content in epidermal fragments (>98% of guard cells) at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT ($P<0.05$; Dunnett's test). n=5 +/- SE

The GC of transgenic lines accumulated more starch than WT's GC, especially at 16:00h (except for line P62 L9, $P>0.05$). Two different lines (P62 L6 and P62 L9) also accumulated more starch than control plants at midday. As typical for mesophyl cells, starch levels also increases in epidermal fragments along the period analyzed (8:00 to 16:00h) (Fig. 5).

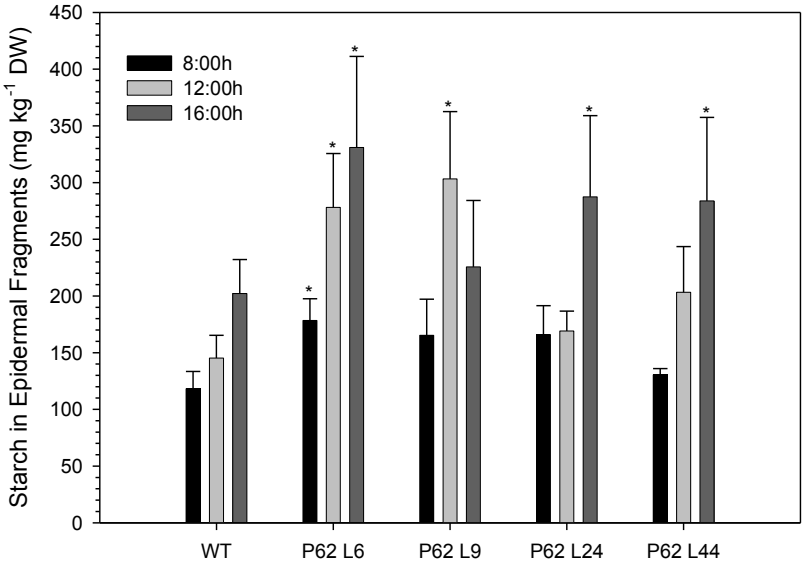

Figure 5: Starch content in epidermal fragments (>98% of guard cells) at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

K^+ contents in epidermal fragments have a sharp increase just for the transgenic line 6, both at 12:00 and 16:00h (Fig. 6). This increase corroborates with the hypothesis that sucrose and potassium could compete with each other regarding GC membrane depolarization.

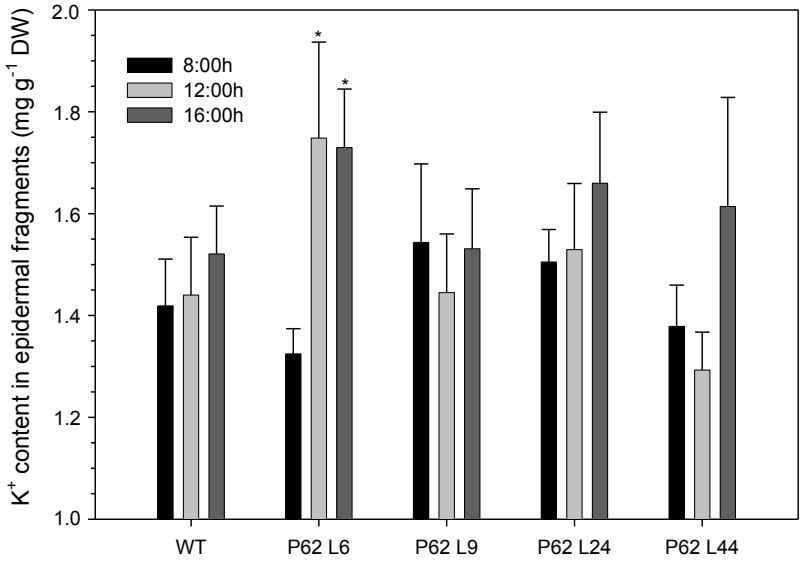

Figure 6: K^+ content in epidermal fragments (>98% of guard cells) at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

Gas exchange analysis

At different time points, several transgenic plants show decreased net photosynthesis (*A*, Fig. 7), with exception of P62 L44. The differences were more pronounced at and after the midday.

In agreement with the phenotype of transgenic lines for *A*, several transgenic lines had shown clear reduction in stomatal conductance (g_s) at different time periods (Fig. 8). The strongest reduction in g_s was also observed at and after the midday.

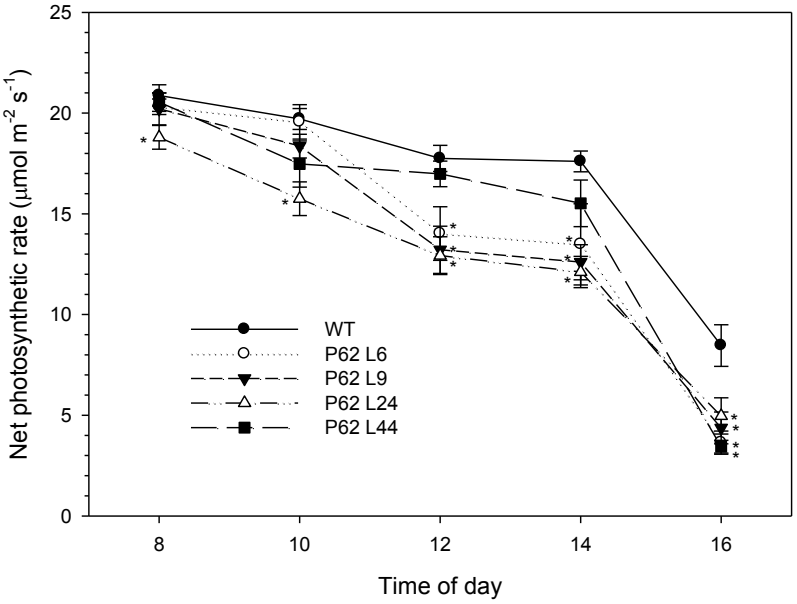

Figure 7: Time course of net photosynthetic rate at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

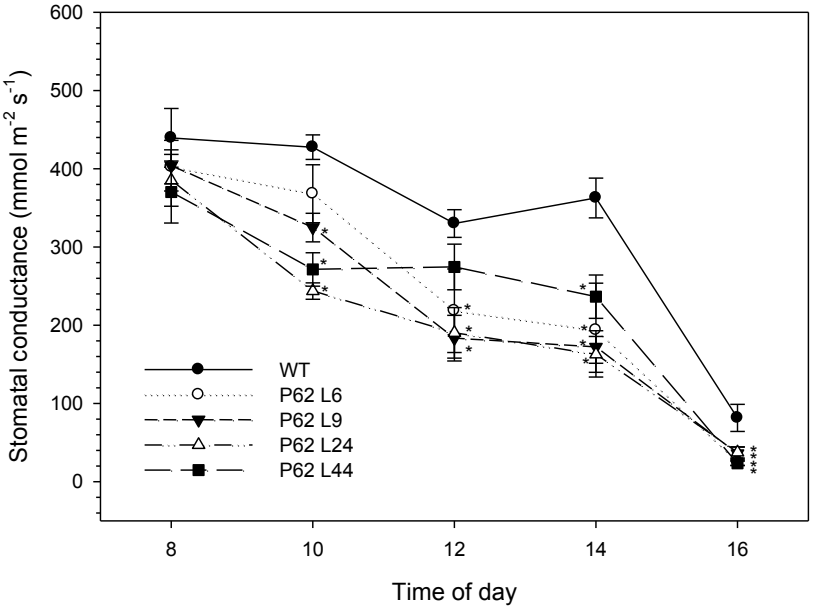

Figure 8: Time course of stomatal conductance at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

The sub-stomatal to environmental CO_2 concentrations ratio (C_i/C_a) decreased in transformed plants principally at 10, 12 and 14h (Fig. 9). These results also support the hypothesis that there are diffusive (stomatal) limitations of *A* in transformed lines.

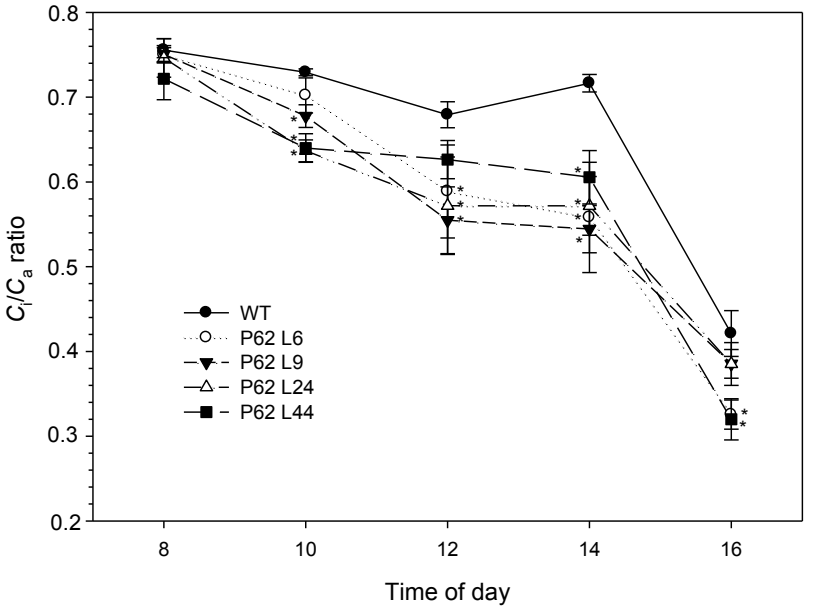

Figure 9: Time course of sub-stomatal (C_i) to environmental (C_a) ratio at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

Stomatal limitation of *A* was confirmed by equal *A* rates when diffusive barriers were negligible through the utilization of high CO_2 concentration (1000 µL L^{-1}) (Fig. 10). Regardless of the measurement have occurred in just one transgenic line (P62 L9), there is no any evidence that there is a significant effect in other lines based on growth analysis.

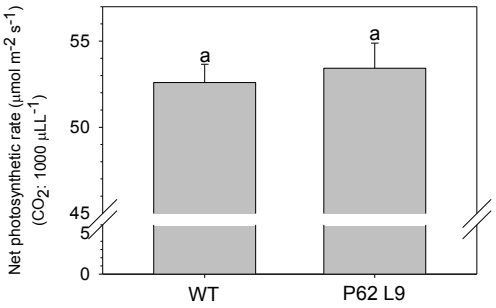

Figure 10: Net photosynthetic rate at transformed line (P62 L9) and wild type (WT) tobacco plants at high CO_2 (1000 µL L^{-1}). Average points followed by same letter do not differ statistically (P>0.05; *t* test). n=5 +/- SE

As consequence of lower g_s on P62 plants, the transpiration rates (*E*) were also reduced at all daylight for several transgenic lines at different time points (Fig. 11). Albeit *E* depends on g_s, it also varies along the day as result of changes in vapour pressure deficit (VPD) in the atmosphere. These results emphasized that reduction in *E* was not in the same extent as reduction in *A* along the day, fact that explains the increase in instantaneous water use efficiency (WUE_L), which was, in average, around 15%. (Fig. 12)

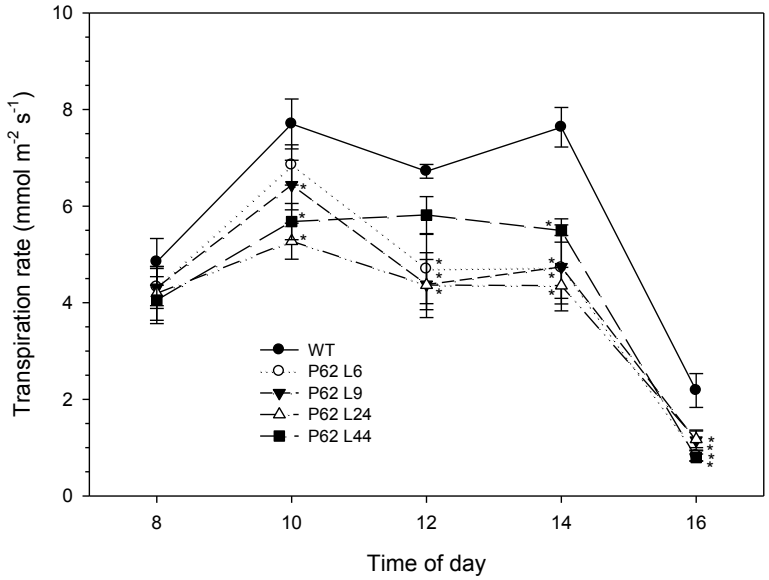

Figure 11: Time course of transpiration rate at transformed (P62) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

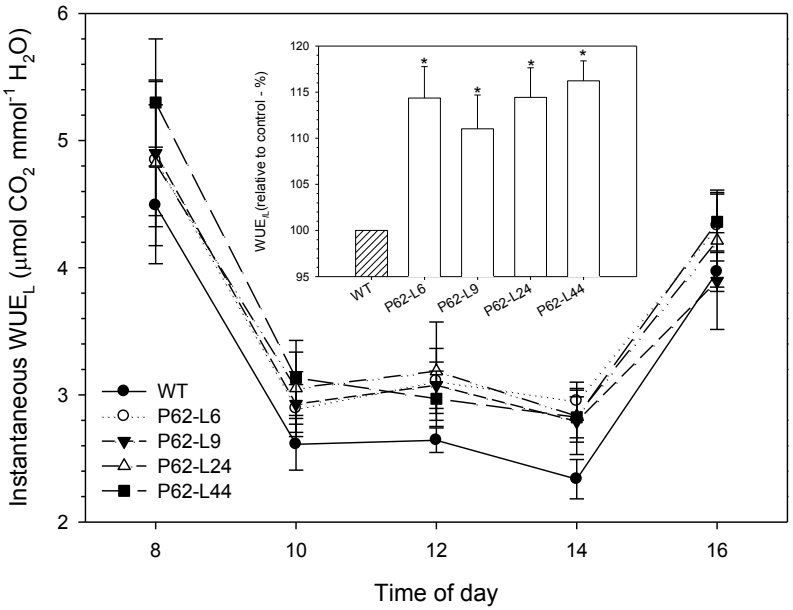

Figure 12: Time course of instantaneous water use efficiency at leaf level (WUE_L) at transformed (P62) and wild type (WT) tobacco plants. In set, the mean WUE_L of day, relative (%) to control (WT). For each time, average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

The increase in WUE_L at transformed plants was not just a temporal isolated event ("instantaneous") on those plants. The carbon isotope ratio composition ($\delta^{13}C$) (Fig. 13) confirms that transgenic plants had a constitutive reduction on g_s.

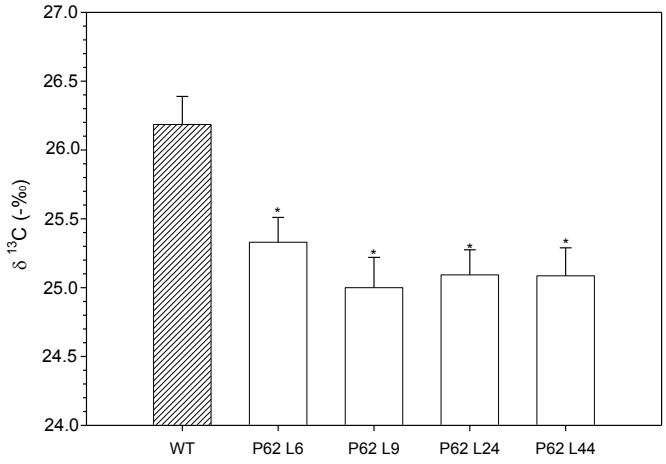

Figure 13: Carbon isotope composition ($\delta^{13}C$) at transformed (P62) and wild type (WT) tobacco plants. Average points followed by asterisks differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE

Growth analysis and water use efficiency

As showed before, reduced g_s in transformed plants had implication on reduction in *A*. Has this punctual detection of reduction on *A* any connotation on plant biomass accumulation? In general, the transgenic plants accumulated less biomass (Tables 1A, B, C). At the first day of analysis, corresponding to the initial phase of exponential growth, transgenic lines (P62 L6 and P62 L9) that had the smallest g_s and *A* showed smaller biomass gain for the most part due to less biomass allocation on stem (% DW stem; Table 1A). Other changes were observed for line 9 only, remaining uncertain the meaning of this differences. Surprisingly, those lines changed their biomass allocation giving higher proportion of biomass accumulated on leaves and less for stem. Transgenic lines do not change roots mass fraction, and do not change proportion of root to shoot biomass or root to leaves; both in area or dry mass basis. The root length was either unaffected. Even though P62 L6 and P62 L9 have changed leaf biomass accumulation, they had also smaller leaf area. They do not have a reduced number of leaves and, apparently, do not have changed specific leaf area.

Table 1A: Biometric analysis at transformed (P62) and wild type (WT) tobacco plants. Average points in bold and underlined differ statistically from WT (P<0.05; Dunnett's test). n=6 +/- SE.

First day of analysis.

Parameter	WT	S.E.	P62 L6	S.E.	P62 L9	S.E.
Plant DW (g)	3.4072	0.4794	2.9488	0.3395	2.3826	0.0815
Leaves DW (g)	2.5602	0.3563	2.2562	0.2572	1.8106	0.0729
% DW leaves	75.22	0.40	76.54	0.69	75.94	0.94
Stem DW (g)	0.3624	0.0589	0.2588	0.0357	**0.1808**	0.0061
% DW stem	10.50	0.41	**8.73**	0.52	**7.61**	0.28
Roots DW (g)	0.4846	0.0658	0.4338	0.0509	0.3912	0.0206
% DW roots	14.28	0.23	14.73	0.43	**16.45**	0.87
Root DW/shoot DW (g g^{-1})	0.1666	0.0032	0.1728	0.0058	**0.1974**	0.0126
Root DW/leaves DW (g g^{-1})	0.1898	0.0035	0.1926	0.0067	0.2173	0.0144
Plant leaf area/roots DW (m^2 kg^{-1})	235.83	10.79	225.76	8.39	204.35	13.42
Nº leaves (> 5 cm length)	7.60	0.24	7.20	0.37	7.00	0.00
Plant leaf area (m^2)	0.1119	0.0119	0.0975	0.0110	0.0789	0.0021
Specific leaf area (m^2 kg^{-1})	44.718	1.939	43.357	1.527	43.697	0.892
Stem length (cm)	6.68	0.55	4.92	0.63	**3.44**	0.29
Root length (cm)	24.32	0.49	26.38	1.54	26.94	1.04
Stem diameter (cm)	1.087	0.045	1.038	0.040	0.979	0.012
Traspiration* (g)	n.a.		n.a.		n.a.	
WUE_{sl} (g DW kg^{-1} H_2O)	n.a.		n.a.		n.a.	
$_yWUE$ (g DW leaves kg^{-1} H_2O)	n.a.		n.a.		n.a.	

*It were not computed the water transpired between 19:00h until 06:00h in next day. Accumulated in seven days.
n.a. Not applicable. DW: dry weight; WUE_{sl}: season-long water use efficiency; $_yWUE$: yield water use efficiency

The clear reduction in biomass accumulation in transgenic plants (average 22.7 %, in L6 and 39.6 % in L9), was followed by divergent reduction in total leaf area (average 15.9 %, in L6 and 27.2 % in L9). The transgenic lines showed less accumulated water loss in the period between 1st to 7th or 7th to 14th days (transpiration) (Table 1B and 1C, respectively). Since transgenic plants showed equal (or higher) root to shoot, or root to leaf, or plant leaf area to root biomass ratios, these results associated to smallest g_s on those plants, strongly suggest that reduction in transpiration is not only consequence of smaller leaf area but smaller *E* (in same area basis; Fig. 11).

Table 1B: Biometric analysis at transformed (P62) and wild type (WT) tobacco plants. Average points in bold and underlined differ statistically from WT (P<0.05; Dunnett's test). n=6 +/- SE.

Between 1st to 7th days

Parameter	WT	S.E.	P62 L6	S.E.	P62 L9	S.E.
Plant DW (g)	13.6573	1.2846	**9.9950**	0.9970	**7.2103**	0.3751
Leaves DW (g)	9.8095	0.8914	**7.3107**	0.6989	**5.2738**	0.2594
% DW leaves	72.21	1.84	73.28	0.39	73.21	0.66
Stem DW (g)	2.2327	0.3346	1.5398	0.2132	**0.9228**	0.0810
% DW stem	15.98	1.45	15.15	0.62	**12.72**	0.66
Roots DW (g)	1.6152	0.1819	**1.1445**	0.0894	**1.0137**	0.0505
% DW roots	11.81	0.66	11.57	0.29	**14.07**	0.07
Root DW/shoot DW (g g^{-1})	0.1342	0.0084	0.1310	0.0037	**0.1637**	0.0009
Root DW/leaves DW (g g^{-1})	0.1649	0.0124	0.1579	0.0036	0.1922	0.0021
Plant leaf area/roots DW (m^2 kg^{-1})	227.95	17.02	249.84	7.46	232.16	6.67
Nº leaves (> 5 cm length)	12.33	0.56	11.67	0.21	11.33	0.33
Plant leaf area (m^2)	0.3537	0.0244	**0.2845**	0.0191	**0.2345**	0.0108
Specific leaf area (m^2 kg^{-1})	36.755	1.833	39.405	1.162	**44.647**	1.499
Stem length (cm)	25.67	1.99	**20.95**	1.35	**16.33**	0.89
Root length (cm)	32.08	1.12	32.15	1.07	35.17	0.70
Stem diameter (cm)	1.602	0.040	**1.487**	0.027	**1.376**	0.027
Transpiration* (g)	1,379.7	102.5	**1,002.5**	83.6	**803.2**	61.5
WUE_{sl} (g DW kg^{-1} H_2O)	7.233	0.579	6.884	0.386	6.038	0.280
$_yWUE$ (g DW leaves kg^{-1} H_2O)	5.136	0.396	4.942	0.260	4.341	0.218

*It were not computed the water transpired between 19:00h until 06:00h in next day. Accumulated in seven days.
n.a. Not applicable. DW: dry weight; WUE_{sl}: season-long water use efficiency; $_yWUE$: yield water use efficiency

As consequence of intense reduction in water loss by transpiration and reduction of biomass accumulation, when analyzed as a ratio, such ratio does not allows transgenic plants to show increased *season-long water use efficiency* (WUE_{sl}) (Table 1B and 1C). As a consequence of differential allocation of biomass in leaves, the transgenic plants showed increase *yield water use efficiency* ($_yWUE$). Based on agronomical use of tobacco crop, the leaves are the harvestable part of plant used economically. The increase in $_yWUE$ is an important feature of these lines. These plants produces approximately the same quantity of biomass with less quantity of water supplied (Table 1C).

Table 1C: Biometric analysis at transformed (P62) and wild type (WT) tobacco plants. Average points in bold and underlined differ statistically from WT (P<0.05; Dunnett's test). n=6 +/- SE.

Between 7^{th} to 14^{th} days

Parameter	WT	S.E.	P62 L6	S.E.	P62 L9	S.E.
Plant DW (g)	35.1175	1.1580	**25.3042**	2.7849	**20.5675**	0.9715
Leaves DW (g)	17.6242	0.5166	15.1837	1.2675	13.0617	0.7166
% DW leaves	50.32	1.45	**60.82**	1.71	**63.50**	1.57
Stem DW (g)	13.1283	0.9708	**6.4382**	1.1513	**4.6145**	0.3211
% DW stem	37.23	1.96	**24.65**	1.61	**22.45**	1.07
Roots DW (g)	4.3650	0.2325	3.6823	0.4438	2.8913	0.2176
% DW roots	12.45	0.59	14.54	0.44	14.06	0.76
Root DW/shoot DW (g g^{-1})	0.1425	0.0078	0.1702	0.0060	0.1640	0.0103
Root DW/leaves DW (g g^{-1})	0.2470	0.0073	0.2404	0.0123	0.2233	0.0175
Plant leaf area/roots DW (m^2 kg^{-1})	152.14	5.27	162.32	11.89	188.91	10.15
Nº leaves (> 5 cm length)	15.67	0.21	14.67	0.56	15.33	0.49
Plant leaf area (m^2)	0.6597	0.0228	**0.5785**	0.0426	**0.5385**	0.0259
Specific leaf area (m^2 kg^{-1})	37.428	0.694	38.415	1.489	41.390	1.449
Stem length (cm)	65.98	2.54	**52.47**	3.03	**42.62**	1.30
Root length (cm)	37.13	2.03	39.43	1.09	38.45	2.50
Stem diameter (cm)	1.833	0.043	1.790	0.039	1.789	0.028
Traspiration* (g)	2,696.3	114.6	**2,230.3**	153.8	**1,930.8**	92.3
WUE_{sl} (g DW kg^{-1} H_2O)	7.977	0.363	6.649	0.832	6.928	0.406
$_yWUE$ (g DW leaves kg^{-1} H_2O)	2.887	0.096	**3.433**	0.363	**4.030**	0.306

*It were not computed the water transpired between 19:00h until 06:00h in next day. Accumulated in seven days.
n.a. Not applicable. DW: dry weight; WUE_{sl}: season-long water use efficiency; $_yWUE$: yield water use efficiency

As expected by low biomass accumulation in transgenic plants, in general they showed low absolute growth rate (AGR) (Table 2) for all parameter analyzed (plant dry weight, stem or root dry weights; leaf area or stem length) at the first week of analysis.

Relative growth rate (RGR), bearing in mind that it represents in broad-spectrum, the "efficiency index", it expresses growth in terms of a rate of increase in size per unit of size. This allows more equitable comparisons than absolute growth rate. As showed in Table 2, the RGR was smaller in transgenic lines for dry weight accumulation in the first week analyzed, but not in the second one. Different from AGR, the RGR for leaves dry weight in transgenic plants showed higher rates in the second week data, indicating preferential carbon allocation into the leaves. In general terms, it was not observed major changes in root dry weight or stem length RGR for both genotypes.

Table 2: Absolute and relative growth rates of transformed (P62) and wild type (WT) tobacco plants. The data represent the average calculate rate. n=6

Absolute growth rate - Hunt (1990)

	1st week			2nd week		
	WT	**P62-L6**	**P62-L9**	**WT**	**P62-L6**	**P62-L9**
Plant DW (g day^{-1})	1.464	1.007	0.690	3.066	2.187	1.908
Leaves DW (g day^{-1})	1.036	0.722	0.495	1.116	1.125	1.113
Stem DW (g day^{-1})	0.267	0.183	0.106	1.557	0.700	0.527
Root DW (g day^{-1})	0.162	0.102	0.089	0.393	0.363	0.268
Leaf area (cm^2 day^{-1})	345.47	267.18	222.33	437.18	419.95	434.23
Stem length (cm day^{-1})	2.712	2.290	1.842	5.760	4.502	3.755

Relative growth rate - Hoffmann & Poorter (2002)

	1st week			2nd week		
	WT	**P62-L6**	**P62-L9**	**WT**	**P62-L6**	**P62-L9**
Plant DW (g g^{-1} day^{-1})	0.2003	0.1748	0.1575	0.1389	0.1318	0.1499
Leaves DW (g g^{-1} day^{-1})	0.1943	0.1686	0.1523	0.0872	0.1049	0.1294
Stem DW (g g^{-1} day^{-1})	0.2578	0.2540	0.2304	0.2616	0.2005	0.2311
Root DW (g g^{-1} day^{-1})	0.1721	0.1404	0.1360	0.1468	0.1643	0.1488
Leaf area (m^2 m^{-2} day^{-1})	0.1659	0.1550	0.1551	0.0906	0.1010	0.1186
Stem length (cm cm^{-1} day^{-1})	0.1917	0.2100	0.2236	0.1369	0.1314	0.1377

Time course of sugar and K^+ contents in guard cells and stomatal regulation

A second experiment was performed with plants in linear phase of growth rate in order to better evaluate the temporal profile of sugars and K^+ in GC, including dark periods when stomata are closed, both at predawn and at early-night times. Glucose (Fig. 14A) and fructose (Fig. 14B) had a sharp increase in early-morning, when stomatal are opening. Sucrose (Fig. 14C) raises continually since stomatal are closed (05:00h) to midday, after that, the sucrose content decreases linearly until approximately 20h.

The starch levels (Fig. 14E) [as showed before in Figure 5] increased during daylight and transgenic plants accumulate more starch compared to WT at the end of afternoon. Its contents were maintained relatively constant in WT until 20h when stomata are closed. For the transgenic line, a modest decrease in its content may

explain an increase in soluble sugars (Fig. 14D) (the most part due to sucrose Fig. 14C) contents. In the face of high starch content observed at night, it can be rapidly metabolized at morning when stomata are opening.

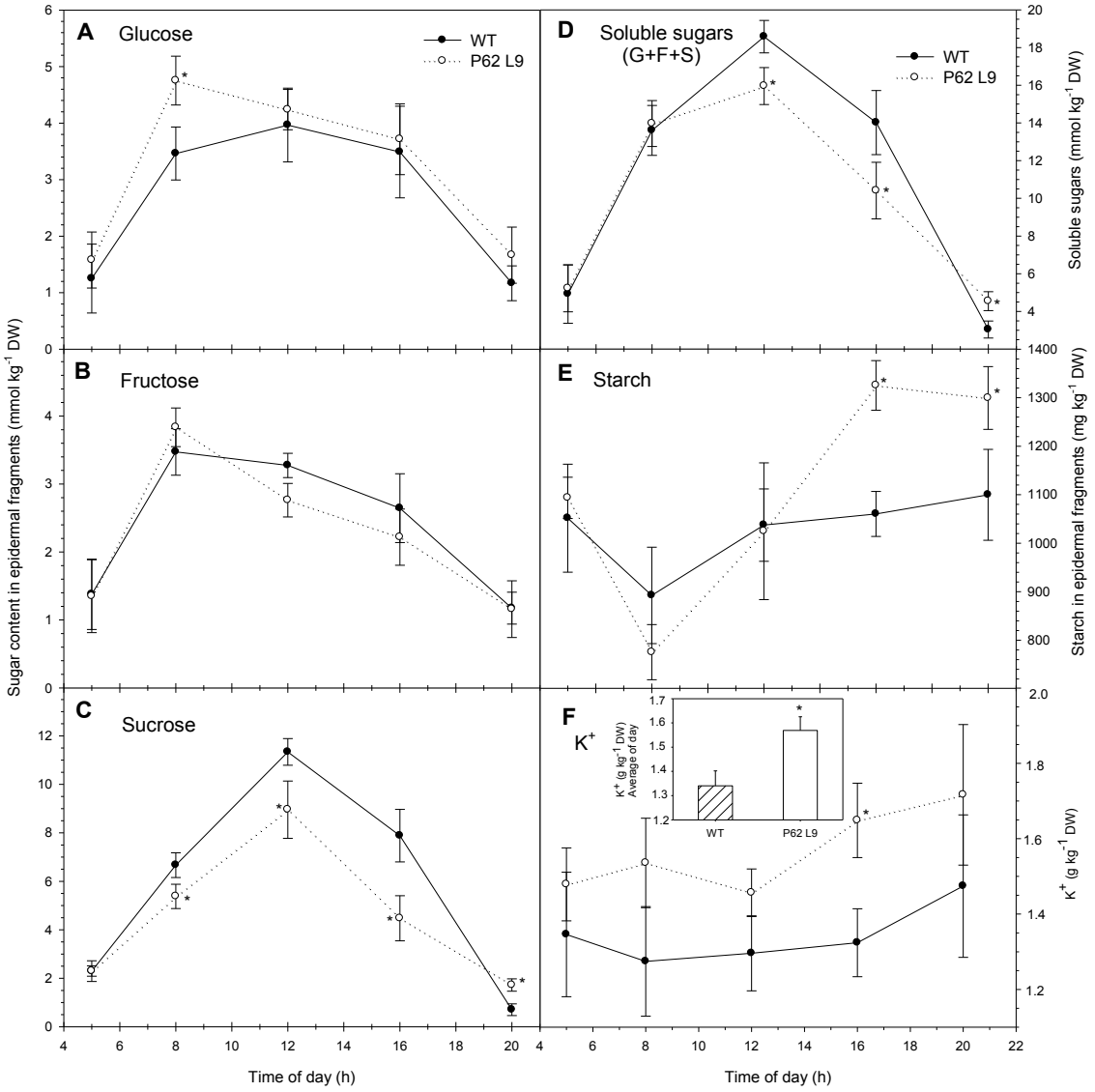

Figure 14: Time course of glucose (A), fructose (B), sucrose (C), total sugars (D), starch (E) and K^+ (F) contents in epidermal fragments (>98% of guard cells) at transformed line (P62 L9) and wild type (WT) tobacco plants. For each time, average points followed by asterisks differ statistically from WT ($P<0.05$; *t* test). n=5 +/- SE

K^+ contents in GC were approximately constant along the day, contrasting from Talbott & Zeiger (1996) results that found out high contents in morning and low at afternoon. The transgenic line has higher K^+ content than WT in GC. The increase varies between 15 to 20% depending on time analyzed, as average of day, it increases was around 16%.

DISCUSSION

Effects on osmoregulation in guard cells

Using epidermal fragments collected in transgenic lines along the day, it was observed low sucrose content in transgenic plants in higher extent at midday and afternoon in two independent experiments (Fig. 3C, 14C).These results supply convincing indirect evidences that occurs significant reduction in sucrose import in GC from transgenic plants, as expected.

Despite lower influx of sucrose, increase of starch was observed at and after afternoon whereas an increase in glucose in epidermal fragments was observed for line P62 L9 only in the second experiment at morning (Fig. 3 and 14A). The increases in starch and glucose could represent a compensatory mechanism to mitigate the negative effect on GC turgor due to reduction of sucrose import in GC, fact that can suggest the presence of homeostatic mechanism in the control of stomatal movements involving sugar metabolism in this cells. As a carbohydrate, glucose can follow glucolytic and tricarboxylic acid pathways in numerous mitochondria present in GC (Willmer & Fricker, 1996; Vavasseur & Raghavendra, 2005) supplying huge quantity of ATP necessary to function a motor-cell, with “energy” necessary for the role of a plasma membrane H^+-ATPase to drive both K^+ and sucrose influx and other osmolytes dependents on favorable trans-membrane potential (Schwartz & Zeiger, 1984; Parvathi & Raghavendra, 1995). In green tissues of plants, chloroplasts and mitochondria are the two potential sources of energy, providing ATP and reducing power. The prevailing view is that GC possesses a high respiratory rate together with limited photosynthetic capability (Vavasseur & Raghavendra, 2005). High respiration rates were observed in

GC by Raghavendra & Vani (1989), suggesting that ATP produced through oxidative phosphorylation was important for stomatal movements (Parvathi & Raghavendra, 1997). The reduction in mitochondrial fumarase activity impairs photosynthesis in tomato plants via an effect on stomatal function (Nunes-Nesi, et al., 2007). As in Antunes (2005), g_s decreases in transgenic potato plants with reduction in sucrose cleavage in GC (*i.e.*, sucrose synthase 3 antisense plants); an opposite phenotype (high g_s) was found in transgenic plants with increased sucrose degradation activity exclusively in GC (*i.e.*, sense invertase plants). Those results suggest the importance of carbohydrate metabolism on GC function. The osmotic component of total sugars (Fig. 4 and 14D) did not change significantly in the morning (8:00h); corroborating for support the hypothesis that sugar metabolism has fundamental importance for stomatal opening.

The reduction of sucrose contents on transgenic plants at midday and afternoon (Fig. 4 and 14C, 14D) agree with Talbott & Zeiger (1996) results. Smaller sucrose contents in GC of transgenic lines was associated with low stomatal conductance on that time, confirming the importance of sucrose transporter and symplastic sucrose in maintaining low osmotic potential on that time of day. GC sucrose uptake activity and expression of sucrose transporter genes at the RNA level in guard cells have been previously reported (Ritte et al. 1999; Reddy & Rama Das, 1986; Talbott & Zeiger, 1996; Kang et al. 2007a).

Reduced sucrose flux through plasma membrane probably was associated to higher starch content in GC of transgenic plants at afternoon. This metabolic phenotype was not due to sole reduction to sucrose influx to GC, but also reduction of its efflux; once the direction of transport by a sucrose carrier may be reversed depending on the direction of sucrose gradient, pH or trans-membrane potential (Carpaneto et al., 2005).

Starch accumulation effect in stomatal aperture is an old hypothesis, nowadays reduced as only a source of malate as counter-ion for the K^+ influx (Lawson, 2009). However, a time limited decrease in starch content in control and transgenic plants were paralleled by a significant increase in glucose, whereas no significant change in K^+

levels was observed. This parallelism suggests that increase in starch synthesis and its time limited hydrolysis plays not a role for increase in malate levels as counter-ion, but was mainly linked to supply the needed glucose for energetic supply of ATP synthesis and the strong ATPase activity in this cells, or alternatively, to allow a sufficient drop in GC water potential to allow enough turgor recovery and stomatal opening to support leaf photosynthesis. Also in *Vicia faba* plants, GC starch concentration was higher in the leaflets with closed stomata than in leaflets with open stomata (Outlaw & Manchester, 1979). Because of low photosynthetic CO_2 assimilation rate in GC (Outlaw, 1989; Lawson, 2009), the high level of starch and glucose could be a metabolic way of these cells to store carbon and energy in cells of transgenic plants with low sucrose import capacity. Nevertheless, some observations points out that starch stored in GC can only provide a limited amount of sucrose (Outlaw, 2003; Vavasseur & Raghavendra, 2005).

The external concentration of sucrose is sufficient to decrease stomatal aperture size in an isolated system (Kang et al., 2007a). In this way, not only sucrose in symplast acts as osmolytes, but also sucrose can accumulate more in the apoplast in low sucrose import capacity of transformed GC, decreasing the stomatal aperture by dropping the osmotical driven force. GC are isolated from the neighbor cells (Wille & Lucas, 1984) and their apoplast are continuous with other mesophyll cells, being the GC apoplast the terminal point in the evaporative pathway (Ewert et al., 2000). Thus, solutes can accumulate achieving high concentrations in the apoplast of these cells, reducing stomatal aperture size in intact leaves (Outlaw & DeVlieghere-He, 2001).

Despite not measured, we speculate that in transgenic GC a higher malate^{2-} and/or Cl^- or NO_3^- contents could exist (Cotelle et al., 1999; Guo et al., 2003; Roelfsema & Hedrich, 2005), in order to compensate the increased content of K^+ observed mainly at midday in both experiments. The data are consistent with the hypothesis that starch degradation provides the carbon skeletons for anion synthesis in GC during stomatal opening.

According to Kang et al. (2007b), the increase in stomatal aperture size follows the onset of illumination, corresponding to an increase in GC K^+ content (Fig. 6) and a

decrease in guard cell starch content (Fig. 14E). In two independent experiments, we were not able to observe this increase in K^+ at the morning period (Fig. 6 and 14E). What osmotic changes explain the stomatal opening at morning in tobacco plants? This is, so far, an unresolved question, a "Holy Graal of Stomatal Physiologists" (Vahisalu et al. 2008; Acharya & Assmann, 2009; Lawson, 2009; Sirichandra et al., 2009; Ward et al., 2009). Our data suggest that an increase in glucose and sucrose contents can explain part of stomata opening in the morning on tobacco plants. Nevertheless, the total sugar contribution (Fig 14D) does not allows us to drive solely sugar importance for stomatal aperture size, because total sugars in transgenic and WT plants are the same at 8:00h. $Malate^{2-}$, Cl^-, aspartate could be also other "commons" osmolytes in GC (Dittrich & Raschke, 1977b). Organic metabolites can be a significant source of osmotica. In *Vicia faba*, sucrose, glucose, and fructose account for 51% of measured osmoticum, whereas malate and citrate account for 37% after light treatments (Talbott & Zeiger, 1993). In addition to symplastic changes, it could be associated to apoplastic osmolytes accumulation driven by low import capacity of transgenic GC. It is important to remember that transgenic plants show low g_s also in the morning, thus the osmotic drive force for stomatal opening was lower than in WT GC. This suggests us that organic acids, others sugars (Kang et al., 2007a) and free amino acids can participate actively on GC osmorregulation inside GC (Raschke & Dittrich, 1977).

The increase in K^+ contents (Fig. 6) in transgenic GC reflects that uptake systems for K^+ and sucrose (considering that sucrose is imported from apoplast; Dittrich & Raschke 1977b, Lu et al., 1997, Kang et al., 2007a) appear to be competitive as showed competition between glucose and K^+, (Dittrich & Mayer, 1978). These osmolytes share the same mechanism of transport, co-transport with H^+, dependent on the extracellular osmolyte, and H^+ concentrations and the membrane voltage (Boorer, 1996). As showed before, it is still not possible to determine the degree of competition.

Osmoregulation in epidermal peels is likely to be essentially based on K^+ exchange with the bathing medium, which in the majority of experiments only contains KCl, explaining the strong phenotype of the mutants in these conditions. By

contrast, in whole plant experiments, the accumulation of sucrose in guard cells would lead to a less pronounced phenotype. It was the case of *Gork-1* mutant. It has K^+ extrusion defect and *gork-1* phenotype is much more pronounced in epidermal strips experiments than in whole plant experiments (Hosy et al., 2003). These results highlight the importance of sucrose in addition to other carbohydrate contents and its role on metabolism on stomatal movements. As a general rule, it should not be possible to extrapolate bathing medium experiments to whole plant physiology.

Effects on gas exchange

As consequence of alterations in sucrose transport specifically on GC, transgenic tobacco plants exhibit low constitutive g_s behavior (Fig. 8) and stomatal limitation to *A* (Fig. 7). The reduced g_s in transgenic plants were largely observed at midday and afternoon, but not exclusively so. Since C_i depends on g_s and, because g_s (and net *A*) were reduced in transgenic plants, but not potential *A*, measured under high $C0_2$ concentration (Fig. 10), between the transgenic and control plants. These results suggest that a stomatal restriction and not biochemical variables was responsible for the reduction in photosynthesis. Increases in stable carbon isotope ratio composition ($\delta^{13}C$) (Fig. 13) supply important evidence for that propositions (Farquhar et al., 1989; Condon et al., 2004, Roussel et al., 2009), which include a detection of an accumulated difference, explained here basically by the decrease in g_s.

Effects in water use efficiency

An important way to enhance the ratio A/E (WUE_L), and thereby to improve the transpiration efficiency of biomass production, is to decrease the value of C_i/C_a ratio (Farquhar et al., 1989; Condon et al., 2004). A small value of C_i/C_a in transgenic lines was associated to equal photosynthetic capacity (amount and activity of photosynthetic machinery per unit leaf area) compared to non-transformed control plants (Fig. 10). Seeing that it is a diffusive process, transpiration varies with vapour pressure deficit (VPD) in the environment. At higher VPD (data not shown) the

transgenic plants transpired 30-40% less than WT plants (Fig. 11) and they showed not proportional reduction in *A*, giving to them an approximately 15% of increase (average of day) in instantaneous water use efficiency (WUE_L) (Fig. 12). This process can be exploited in breeding for high water use efficiency, acquiring more carbon (biomass) in exchange for the water transpired by the crop (Table 1C), *i.e*. improving crop transpiration efficiency (Condon et al., 2004). An important consequence, not revealed in Figure 12, is that low g_s plants had higher leaf temperature (data not shown). Increases in leaf temperature causes increases in vapour pressure in stomatal cavity and therefore, an increase in the gradient-driving transpiration, resulting in an increase in *E* per unit change in conductance. As temperature increase, it is possible to increase respiration and photorespiration rates, effects that could perhaps explain part of the changes in biomass accumulation observed (Tables 1A-C).

Due to special property of RUBISCO to discriminate between $^{13}CO_2$ and $^{12}CO_2$ (McNevin et al., 2007), the $\delta^{13}C$ varies when the availability of CO_2 varies. The $\delta^{13}C$ varies with *A* and g_s, as *A* reduce, it tends to increase C_i if g_s is constant, *i.e*., increasing the availability of $^{12}CO_2$ and $^{13}CO_2$ for RUBISCO. In that way, it has a propensity to decrease $\delta^{13}C$, or fix less $^{13}CO_2$ in relation to $^{12}CO_2$. When g_s reduce, it tends to reduce C_i (if *A* constant) and increase $\delta^{13}C$, *i.e.* fixing proportionally more $^{13}CO_2$ when CO_2 availability is low, *i.e*., has a low $\Delta^{13}C$ (Farquhar et al., 1989; Condon et al., 2004). As $\delta^{13}C$ increase in P62 lines, we ascertain those plants present higher WUE_L (Condon et al, 2002; Chaves et al., 2004; Condon et al., 2004), consequence mainly due to smaller g_s. For C3 species, measuring $\delta^{13}C$ provides a powerful means of estimating WUE_L, but experience has shown that improvements in leaf-level water use efficiency may not always translate into higher season-long water-use efficiency (WUE_{sl}) or yield (Table 1B and 1C). In the absence of soil water deficit, low-$\Delta^{13}C$ wheat genotypes tend to grow slower than high-$\Delta^{13}C$ genotypes, resulting in lower total biomass production and grain yield (Condon & Richards, 1993; Lopez-Casteneda et al., 1995; Condon et al., 2002, 2004; Centritto et al., 2009); similar results were obtained with our tobacco lines (Table 1B and 1C). High $\delta^{13}C$ (low $\Delta^{13}C$), could be associated with “conservative” crop growth rate in cereals if differences in $\Delta^{13}C$ in the absence of soil water deficit are the result of differences in stomatal conductance (Condon & Richards, 1993). Antisense

sucrose transporter (P62) genotypes fitted on this described situation, with lower stomatal conductance, high *A*/*E* and higher $\delta^{13}C$. Our transformed lines had smaller accumulated transpiration and data shows that the main cause is reduction of g_s, less extent consequence of smaller leaf area.

Effects on growth parameters

In our transgenic plants, higher *A*/*E* was associated with lower photosynthetic rate per unit of leaf area (Fig. 7), as in the wheat described case (Condon & Richards, 1993; Lopez-Casteneda et al., 1995; Condon et al., 2002, 2004; Centritto et al., 2009). Consequently, a slower crop growth rate (Table 2), but not smaller "efficiency index", based on the effects on relative growth rate (RGR) was observed. RGR of plant biomass in the transgenic lines was virtually equal to WT plants in a second week of analysis. Even though, the RGR for leaf biomass accumulation were higher in both lines, while stem biomass accumulation rate were smaller, and root biomass accumulation were equal; indicating the preferential carbon allocation into leaves in detriment of stem carbon buildup.

Since transgenic plants showed equal (or higher) root to shoot, or root to leaf, or plant leaf area to root biomass ratios, it is improbable that the reduction of transpiration per plant are consequences of reduction of water absorbing capacity. It is either not probably consequence of smaller stem water conductance based on modest stem diameter reduction. It seems consequence of reduction plant growth rather than cause of it.

In irrigated environments the slow growth rate of high $\delta^{13}C$ genotypes (P62 lines) had translated directly into low final biomass production, as reported for wheat, with low grain yield (Condon et al., 1987; Sayre et al., 1995; Fischer et al., 1998; Condon et al., 2002). High $\delta^{13}C$ tobacco genotypes have achieved less biomass and lower yield (Tables 1A-C). However, in transgenic lines were partitioning more of the achieved biomass into the harvested product *i.e*. leaves. The P62 lines increased approximately 20-25% of carbon (DW) allocation into leaves. For farmers and

agronomists, the unit of production is much more likely to be the yield of harvested product achieved from the water made available to the crop through precipitation and/or irrigation, *i.e.* a farmer's definition is one of agronomic or yield water-use efficiency ($_{y}$WUE) (Bacon, 2004; Condon et al., 2004). Irrespective of its physiological basis, "conservative" crop growth by high $\delta^{13}C$ transgenic tobacco genotypes had important implications for $_{y}$WUE (Tables 1BC).

A common feature in plant physiology is the capacity of plants to adjust metabolic and in a broad spectrum "physiologically" to adverse environment. For example, there are preferential carbon allocations into and deeper roots when plants are submitted to drought (Huang & Gao, 2000; Pinheiro et al., 2005); or increased chlorophyll contents under shade environments (Gonçalves et al., 2001). Another common phenotype of the shade-avoidance syndrome is the relocation of "energy resources" from storage organs into the stems and petioles, so that, the plant outgrows its competitors (Collins & Wein, 2000; Tao et al., 2008). All those features drive to get better chances of plants to survive in an adverse environment. In resume, plants maximize carbon and energy resources investments in growth parameters that could contribute to increased survival under unfavorable conditions. In our view, the increase in carbon relocation into leaves follow this idea, trading-off resources, maximizing plant photosynthesis in a low photosynthetic rate per unit of area. In this way, transgenic plants acquired more leaf area in relation to total biomass, but not change consistently specific leaf area. As a final point, they could maximize plant carbon fixation capacity per unit of leaf dry matter.

The stomatal data reported here indicates that altering GC sucrose import capacity by genetic manipulation of sucrose transporter may be useful tool for the acquisition of higher water use efficiency, despite some reduction in dry matter accumulation, allowing an important economy in the water needed to harvest production ($_{y}$WUE) in tobacco.

CONCLUSION

Here, we described how a sucrose transporter can be capable of influence stomatal function. Using a tobacco plant antisense for sucrose transporter in GC, we showed that transgenic plants accumulate less sucrose, more starch and modest increase in K^+ contents. They presented constitutive low stomatal conductance behavior and demonstrated other gas exchange consequences like low *A*, *E* and C_i. By means of low g_s, we got plants with higher water use efficiency with some expense of dry matter accumulation, however, high water saving plants.

MATERIAL AND METHODS

Plant material

Seeds of *Nicotiana tabacum* L. CV. 'Havana 425' 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 cm^3) (Murashige & Skoog, 1962). The seedlings were transplanted to 500 cm^3 pots containing MS medium (80 cm^3) and cultivated under photoperiod of 14 h illumination and light intensity of 100 µmol m^{-2} s^{-1}, with day/night temperatures of 25 °C/20 °C.

Transformation via *Agrobacterium tumefasciens*

A binary plasmid pBinAr (Hofgen & Willmitzer, 1990) was used. The CaMV-35S promoter was replaced by KST1 promoter (Plesh et al., 2001) by specific restriction enzymes, now denominated pBinK. The antisense sucrose transporter cDNA (Riesmeier et al., 1993) was inserted into pBinK. Neomycine phosphotransferase (NPTII – kanamicin resistance) marker was used for tobacco transformation via *Agrobacterium tumefasciens* strain LBA 4404. Engineered plasmid was inserted into *E. coli* (DH5α) by coupled used of classical thermal and calcium treatment. The bacteria were kept to growth in LB media with 100 mg L^{-1} of kanamicin at 37 °C. The confirmation plasmid insertion was done by PCR after plasmid purification by midi-prep plasmid-DNA

isolation techniques, according to Sambrook & Russel (2001). Isolated plasmid was inserted in *A. tumefasciens* by eletroporation methodology (Brasileiro & Carneiro, 1998) and growth in Rhyzo medium with 100 mg L^{-1} of kanamicin at 29 °C. It was used leaf discs methodology for tobacco transformation exactly as described in Brasileiro & Carneiro (1998). Timentin® (300 mg L^{-1}) was used to kill *A. tumefasciens* in a selective (with 100 mg mL^{-1} of kanamicin) MS medium during all period of sterile cultivation of tobacco explants/regenerates. None regenerated explants were observed for untransformed HAV-425 (WT) in sterile selective regenerating medium. Some WT regenerates (cultivated in non-selective medium) and 50 transformation candidates regenerates (Brasileiro & Carneiro, 1998) were transferred to selective medium growth. The illustrative result was showed in Fig. 1.

Plant DNA extraction and PCR analysis

For molecular analysis of transgenic tobacco, plant genomic DNA was extracted from 1-1.5 g fresh leaf tissue according to (Murray et al., 1980). PCR analysis was performed using specific primers to detect NPTII gene (for. 5' GCGGTCAGCCCATTCGCCGCC 3'; Rev. 5' TCAGCGCAGGGGCGCCCGGTT 3') amplifying a fragment expected with size of about 600 bp. The PCR procedure was performed under the following conditions: DNA (400 ng), forward and reverse primers (0.7 µM), 3 mM $MgCl_2$, 0.25 mM dNTPs, 1X PCR buffer according to manufacture, 1 unit of Taq polymerase (GoTaq Flexi, Promega) at hot-start 94 °C for 5 min, followed by 6 cycles of *touchdown* consisting of 45s at 94 °C, 60s at 65°C, reducing 1 °C per cycle and, 60s at 72 °C. It was followed by 23 cycles of 45s at 94 °C, 60s at 60°C and 60s at 72 °C. Electrophoretic detection of PCR product was performed in 1% agarose gel stained with ethidium bromide.

Greenhouse tobacco cultivation

Regenerated lines were acclimated in 300 cm^3 pot filled with commercial seedlings substrate. The plant was covered with a plastic bag to reduce transpiration. It was kept to growth in 25 °C and photoperiod of 14 h in 250 µmol m^{-2} s^{-1} fluorescent lights

growth chamber. A week later, the plastic bag was removed and cultivated for some days on these conditions. The tobacco plants were transferred to 5 dm^3 pots with fertilized soil at greenhouse conditions. Irrigation was done to maintain soil moisture at field condition. T2 lines were obtained by self-pollination of regenerated lines protected by paper bag.

For all experiments plants, seed of T2 lines were surface-decontaminated as described before and cultivated in selective medium, and then transferred to 50 cm^3 pot, later for 300 cm^3 pots, and finally, to 5 dm^3 pots in a similar conditions described before. WT seedlings are cultivated in non-selective medium.

Gas exchange measurements

The gas exchange measurements were performed in approximately 20-30 cm tall plants in an expanded 5^{th} or 6^{th} leaf, counting-down for apical meristem. It was used Li-Cor Li-6400 gas exchange system (http://www.licor.com) with 1000 µmol m^{-2} s^{-1} photosynthetically active radiation. Temperature and CO_2 concentration were ambient. It was used 500 cm^3 min^{-1} flow air. For each repetition measurement, it was used average of 2 independent plants. n = 5.

Growth analysis

The growth analysis was performed with cultivated plants in 5 dm^3 pots with fertilized soil, beginning at the initial growth log phase (approximately 7 leaves, Table 1). Three independent biometric experiments were performed (destructive experiments). Tobacco leaf area was estimated using a model similar of proposed to coffee leaf area estimating model (Antunes et al., 2008) (appendix). Other parameters determined including stem diameter and length; root length. Dry weights of roots, stem and leaves were also measured (dried at 70 °C until constant weigh, with n = 6).

Epidermal fragments extraction

The epidermal fragments were extracted from 2 expanded leaves (from different plants), from which main and secondary nervures were excised out, following the method described by Kruse et al. (1989), Raschke et al. (1989), and Kopka et al. (1997). In resume, the leaf lamina was putted in warring blender (Phillips-Wallita, model RI 2044) with internal filter, and then blended with 200-250 cm^3 of cold deionized water for 5 pulses of 15 s at maximum power. It was filtered in 200 nm nylon membrane and rinsed exhaustively with 1.5 to 2 dm^3 of cold deionized water to completed removal apoplastic contents, debris of mesophyll and another cells, resting only intact guard cells and rare trichomes, in a matrix of skeletons of pavement epidermal cells (Fig. 15). The purity of guard cells was higher than 98% by optical microscopy inspection. None mesophyll cells were detected (data not shown). All extraction procedures timed approximately 5 min until the epidermal fragments freezed in N_2 liquid. The epidermal fragments were stained using fluorescein diacetate (FDA) and propidium iodide (PI) dyes, equally as described in Huang et al. (1986). By employing a double-exposure method to record the fluorescence from cells stained with both FDA (green – living cells) and PI (red – dead cells) could be easily distinguished on the basis of fluorochomasia (Fig. 15). It could be observed the high purity and viability of guard cells preparations, rare living trichomes and absence of mesophyll cells. It was not observed living pavement epidermal cells, just some sparse nucleus on preparation.

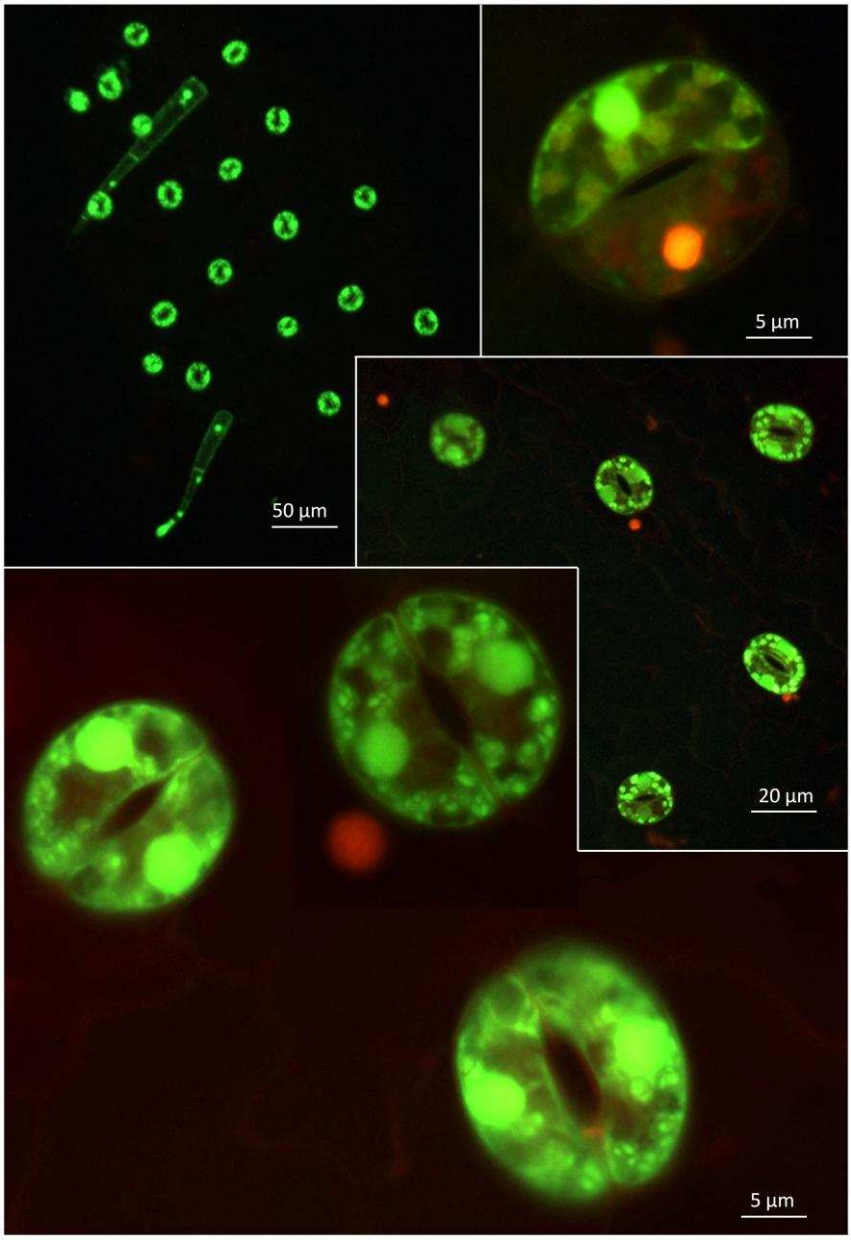

Figure 15: Fluorescence photomicrography of epidermal fragments highlighting the viability of guard cells, and absence of intact other pavement epidermal cells. They were stained with both fluorescein diacetate (FDA, green – living cells) and propidium iodide (PI, red – dead cells). It was showed at different magnification photomicrography.

Sugars and starch extraction and determination

It was used lyophilized epidermal fragments and methodology described by Lisec et al. (2006) for sugars extraction. In resume: 10 mg of dry weight (DW) was extracted in 1000 µL of pure methanol at 70 °C for 1h. It was centrifuged 17.000 xg for 10 min. 700 µL of supernatant was transferred to another tube, when 400 µL of deionized water and 500 µL of $CHCl_3$ were added and vortexed. 1000 µL of polar phase was collected and dried in speed vac. The pellet of methanol extraction was rinsed with 80% ethanol

3 times and used to starch extraction using hot KOH methodology described by Threthwey et al. (1998). For glucose, fructose, sucrose and starch determinations were used enzymatic methodology (coupled to NADH formation, accompanied at 340 nm) as described by Threthwey et al. (1998). All metabolites were quantified by standard curve of pure substance (0 to 30 nmol for soluble sugars and 0 to 50 nmol glucose equivalents for starch) in the same plate.

K^+ determination

It was used 10 mg of lyophilized epidermal fragments and digested with 1 cm^3 of mixture of pure nitro-percloric acids (4:1) at 100 °C. The volume was completed to 2000 µL with mili-q water. K^+ quantification was done in flame photometer in a specialized laboratory.

$\delta^{13}C$ composition

It was used two expanding leaves (each for independent plant) for $\delta^{13}C$ determination. The leaves were dried at 70 °C and powdered. The $\delta^{13}C$ determination was performed in specialized laboratory using Pee Dee Belemnite as standard according to DaMatta et al. (2003). n = 5.

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Chapter 2

Desiccation postponement in sucrose transporter antisense tobacco plants

Key words: Sucrose transporter, stomatal conductance, transpiration, drought avoidance.

ABSTRACT

In this work we described that a sucrose transporter in guard cell (GC) could affect water consume by plants. As a result of decreased expression of sucrose transporter specifically in GC, we show that they have constitutive low stomatal conductance (g_s) behavior as well as low transpiration rate, especially when there is high water availability. By means of low g_s tobacco plants, we got desiccation postponement phenotype as principal feature of this transformation, being high water saving plants. These results suggest that manipulation of sucrose transport in GC may be developed as a practical mechanism for drought avoidance and water conservation during irrigation. These results illustrate the importance of fine tuning of sucrose metabolism transport and metabolism in the fitness of stomatal function in contributing to plant survival or growth under unfavorable hydric conditions. This work allows including the GC sucrose transporter, absent in the several published list of possible target genes to improve drought resistance, and illustrate that a change in expression of a single gene can show visible desirable phenotypes regarding water stress responses.

INTRODUCTION

Drought is the major environmental threat to agricultural production worldwide. Fresh water scarcity has been identified as one of the principal global problems in near future, and plants account for around 65% of global fresh water use (Postel et al., 1996). For its survival, plants spent water through transpiration in order of cooling themselves and absorbing nutrients; water is also used as a substrate for photosynthetic and other metabolic reactions. As a rule, when stomatal pore opens to permit CO_2 entry in sub-stomatal cavity, they loss water vapour to environment. The rate of gas fluxes through stomatal aperture depends on specific plant characteristics, their physiology and environmental microclimate features such as irradiance, temperature, air moisture, and wind velocity (Kramer & Boyer, 1995). How do plants perceive these environmental conditions to respond and to adjust physiologically upon climate changes remain an unresolved question.

It is known that plants synthesize abscisic acid (ABA) when they perceive the soil drying. This new ABA or any pre-existing ABA can be transported inside the plant to induce the required adaptive response, the closure of stomata being the most studied of these. Some authors points out that the relative humidity is key factor controlling stomatal aperture size by ABA catabolism induced by CYP707A1 gene (CYP707A1 is proposed to be essential for ABA catabolism inside the guard cells). High humidity induces accumulation of its transcript levels, followed by the reduction of ABA contents under these environmental conditions (Okamoto et al., 2009). Besides that, relative humidity was identified as a key environmental factor controlling stomatal response to CO_2 (Talbott et al. 2003)

ABA is perceived by a proposed ABA receptor (Shen et al., 2006; Liu et al., 2007), and inside guard cells (GC), it induces increases in levels of some signaling intermediates like H_2O_2 and NO. ABA stimulates, among other secondary signals, elevation of free Ca^{2+} on cytosol of GC. The coupled effect of this secondary signals induces trans-membrane depolarization and inhibits inward K^+ channels and activates outward K^+ channels leading to stomatal closure as well as prevent closed stomata to open (MacRobbie, 1998; Blatt, 2000; Pei et al., 2000; Garcia-Mata & Lamattina, 2001;

Schroeder et al., 2001; Garcia-Mata & Lamattina, 2002; Fan et al., 2004; Pandey et al., 2007; Neill et al., 2008; Acharya & Assmann, 2009; Ward et al., 2009). Under dry conditions, effective concentrations of ABA might become high enough both to trigger ion efflux and to inhibit GC sucrose uptake. If so, stomata would open less and/or close earlier in the day (Tallman, 2004).

K^+ and sucrose are the major osmolytes that drive increases in GC volume (Talbott & Zeiger, 1996; 1998). K^+ and sucrose uptakes occur passively, through inwardly rectifying K^+ channels, in response to trans-membrane hyperpolarization that results from the activation of plasma membrane H^+-ATPases. Sucrose entries inside GC through specific sucrose transporter (Lu et al., 1997; Outlaw & DeVlieghere-He, 2001; Sauer, 2007). GC possess low photosynthetic rate to explain high sucrose contents in their symplast (Outlaw, 2003), and starch mobilization could not accomplish significant synthesis of sucrose (Talbott & Zeiger, 1993). In addition, there are no plasmodemata connections in mature GC (Wille & Lucas, 1984). This anatomical trait excludes GC symplastic sucrose loading hypothesis. Consequently, for leaf apoplastic loading and GC sucrose import is indispensable the role of sucrose transporter activity (Riesmeier et al., 1993; Kühn et al., 1999, Sauer, 2007; Braun & Slewinski, 2009). Corroborating to this affirmative, the GC sucrose transporter have high sucrose transport capacity (activity), similar to capacity of K^+ influx (Outlaw, 1983; Lu et al., 1997; Outlaw & DeVlieghere-He, 2001).

As predict response that sucrose acts as important osmolyte in GC (Dittrich & Raschke, 1977; Lu et al., 1995; Talbott & Zeiger, 1996; Lu et al., 1997; Talbott & Zeiger, 1998; Outlaw & DeVlieghere-He, 2001; Outlaw, 2003; Lawson, 2009), the reduction of its influx could lead to reduced stomatal conductances (g_s). It might allow plants to reduce water consumes and achieve drought resistance. Engineering of the stomatal GC optimizing openings could provide a major contribution to more sustainable water use during droughts. The reduced influxes of ions and sucrose associated to high efflux of osmolytes also could be achieved by increase in ABA contents (Zhang & Outlaw, 2001a,b,c). However, as proposed by Tallman (2004), ion efflux from GC are more sensible than sucrose efflux when ABA levels increase. These hypotheses could be

interpreted as indirect evidence for the hypothesis of Talbott & Zeiger, (1996) that postulates that K^+ is replaced by sucrose at midday and afternoon.

Genetic analysis has identified that inheritance of growth traits and water use efficiency are governed by QTLs (quantitative trait locus or loci) (Rönnberg-Wästljung et al., 2005), as well for carbon isotope discrimination ($\Delta^{13}C$) (Teulat et al., 2002; Laza et al., 2006), stomatal regulation (Price et al. 1997), and drought resistance (Yue et al. 2006). Achieving drought resistance through traditional breeding is a difficult task due to the gene complexity involved, and the number of physio-biochemical processes at both cellular and whole-plant levels that contribute to this phenotype at different stages of plant development (Wang et al., 2003; Valliyodan & Nguyen, 2006). The complexity of this whole-plant response to environmental volatility and interaction with the intensity and duration of water deficits and temperature make it a difficult challenge for achieving drought resistance (Witcombe et al., 2008). The limited knowledge of stress-associated metabolism is still a major gap in understanding stress tolerance in many plant species (Vinocur & Altman, 2005).

Drought resistance is the plant's capacity to avoid or mitigate the consequences of water deficits. Mechanisms include desiccation postponement (the ability to maintain tissue hydration) and desiccation tolerance (the ability to function while dehydrated) (Taiz & Zeiger, 2006). Drought resistance includes drought escape via a short life cycle or developmental plasticity, desiccation postponement (DP) via enhanced water uptake and reduced water loss, while drought tolerance (DT) via osmotic adjustment, antioxidant capacity and desiccation tolerance (Yue, et al., 2006). DP and DT involve different mechanisms and processes, and phenology is the single most important factor influencing whether a plant avoids drought (Witcombe et al., 2008).

Many efforts for achieving abiotic stress tolerance in plants had some agronomical penalties (Kasuga et al., 1999; Abe et al., 2003; Swindell et al., 2007). Drought resistant plants often accumulate less total biomass and exhibit low $\Delta^{13}C$. In other words, it frequently could be associated to low net photosynthetic rate (A) and low stomatal conductance (g_s), and consequently low transpiration rate (E). Probably

stomatal restriction (diffusive restriction) of *A* could result in lower total biomass production ("conservative" growth) and grain yield in absence of water deficit (Condon et al., 2004). In irrigated or rain-fed environments, faster growth of high-$\Delta^{13}C$ genotypes is usually translated directly into higher final biomass production and grain yield (Condon et al., 1987, Sayre et al., 1995; Fischer et al., 1998; Condon et al., 2002; Jones, 2004). Low-$\Delta^{13}C$ genotypes have achieved less biomass and lower yields. In low water availability environments, high-$\Delta^{13}C$ genotypes exhaust the available soil water too quickly and the agronomical penalty in terms of crop water use and yield, associated with faster growth is not always as great as might be expected (Condon et al., 2004). The "conservative" gas exchanges in low-$\Delta^{13}C$ associated to low g_s resulting in low water consumption (resulting in "stored-moisture" in soil) can ensure stable production at marginal lands (Condon et al., 2002). Such optimized stomatal behavior means that the plants should have higher water use efficiency (WUE) with less water supply (Zhang & Yang, 2004).

Contributions to more sustainable water use during droughts is one important research issue to be pursuit, in order to explore more possibilities to deal with the dramatic negative effects expected as result of environmental changes in water availability to plant growth in global scale. Testing the hypothesis that optimizing stomatal behavior can provide plants with high WUE and/or low water consumption, we engineered transgenic tobacco plants antisense for sucrose transporter driven by *KST1*, GC specific promoter, which shows constitutive low stomatal conductance, in order to achieve a biotechnological application for plants that consume less water. Detailed assessment of physiological responses and the comparative water consume under irrigated and drought conditions was performed to test if changes in stomatal behavior by the use of specific genetic modification of guard cells could contribute to obtain plants more efficient in water use.

RESULTS

Water consume in irrigated environment

The four transgenic lines used to compare water consume in irrigated environments (5 dm^3 pots, irrigated diary) have lower transpiration compared to wild type (WT) plants (Fig. 1A). The different lines showed diverse behavior, the P62 L9 had smaller total amount of water transpired (50% to 75% of WT transpiration) during all period analyzed. The P62 L6 and P62 L24 lines showed intermediary behavior, consuming more than P62 L9 but less than WT over all period analyzed. The P62 L44 shows a transient lower transpiration.

Some part of these differences in water consume are due to differences in plant's leaf area. Those plants had different growth rate and leaf area (see Chap. 1), as expected, small plants transpires less water, as the case of P62 L9. When transpiration rate was standardized to same leaf area, in general, all transgenic lines transpire less than WT, especially when the water availability is high, *i.e*. small plants growing in pots (Fig. 1B). In the same leaf area basis, this phenotype is explained by differences in g_s. The reduction in water consume demonstrates that smaller g_s and *E* measured in gas exchange system (see Chap. 1) could be extrapolated to whole plant and for long period of development, showing constitutive phenotype. Those features reinforce the importance of sucrose transport to stomatal function. As a result, when plants have smaller g_s, they may possibly save soil water, especially when there is plenty of water availability, and achieving DP phenotype.

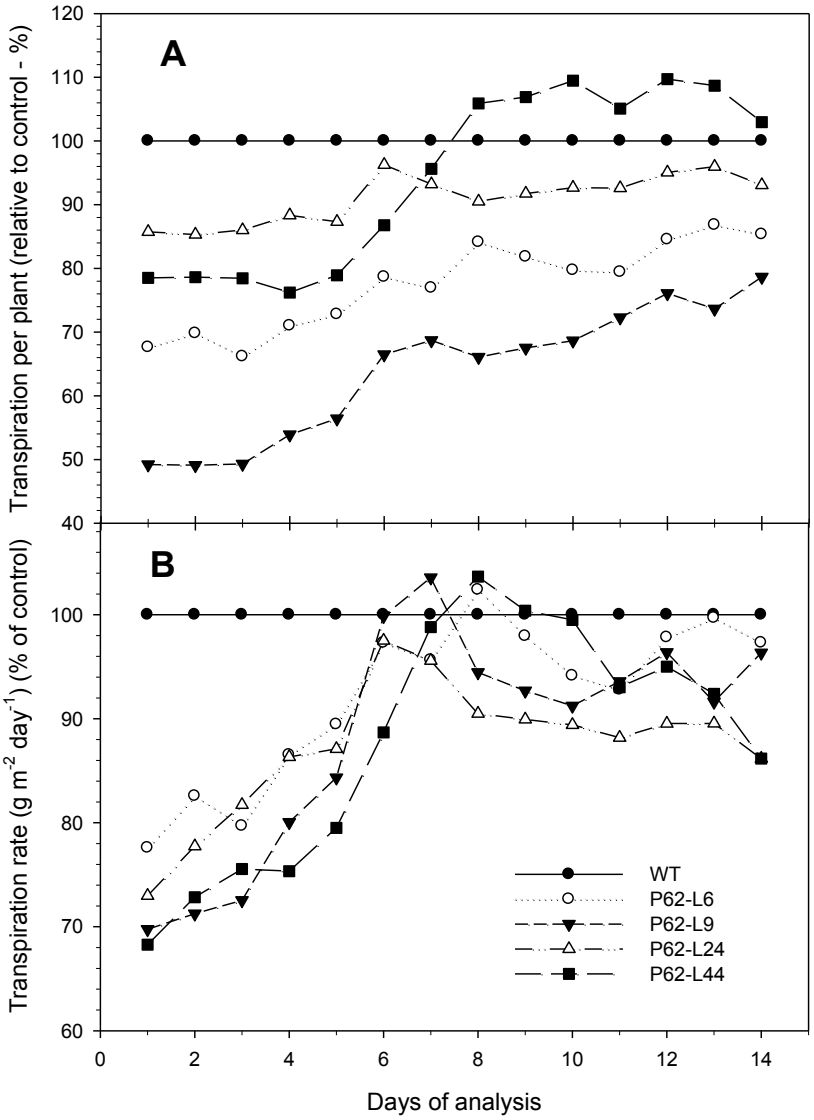

Figure 1: Relative transpiration rate of transgenic antisense (P62 L6, L9, L24 and L44) tobacco plants cultivated in 5 dm^3 pots. (**A**) Total plant transpiration, relative to control non-transformed plant (wild type; WT). (**B**) Transpiration rate standardized to leaf area, relative to WT . n = 6

Water consume in non-irrigated environment

Water consume of two transgenic lines (P62 L6 and P62 L9) were compared to WT in drought condition (non-irrigated experiment). As showed in Figure 2, the transpiration rate was smaller in transgenic lines than WT until approximately 28 hours of water stress, when this phenotype was "inverted", *i.e.* transpiration rate were higher in transgenic lines when compared to WT at same time (Fig. 2). The soil water evaporation was negligible during all period analyzed (data not shown).

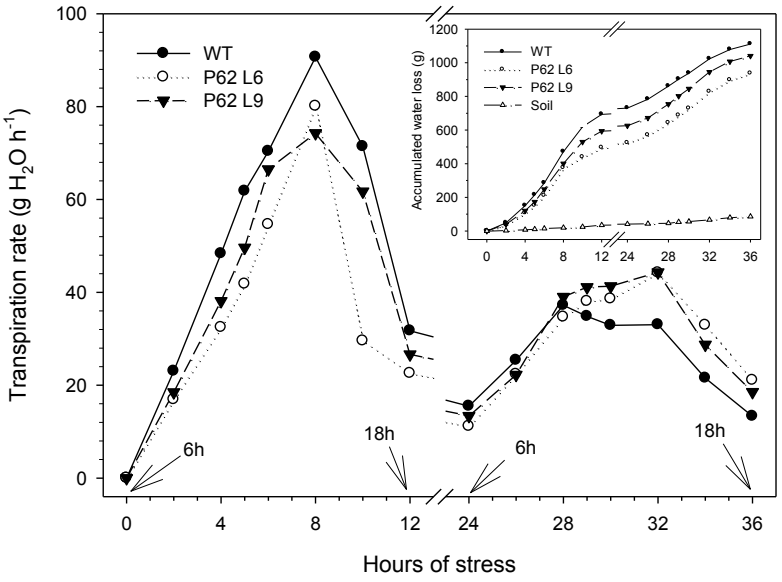

Figure 2: Transpiration rate of transgenic antisense (P62 L6 and P62 L9) and non-transformed (WT) tobacco plants cultivated in 5 dm^3 pots (5 kg dried soil) under imposed drought. Inset, the cumulative water loss data, with n = 5. Arrows indicate correspondent day time

As showed in Figure 2, transpiration rate was smaller in transgenic plants when soil water availability was not linked to a significant water deficit in plant. Progressive decrease in soil water, with probable increase in water deficit *in planta* was associated to an increase in transpiration in transgenic plants. Since a pot having transgenic plants presents lower accumulated water loss, depletion in soil moisture occurs more slowly than in WT pots (Fig. 2 inset, 3). Bearing in mind, in average, all plants do not have differences in leaf area (P>0.05) (due to non-random selection) (Tab. 1), but minor differences may exist among them. When transpiration was standardized to leaf area, clearly water consumption show a dual phase (Fig. 3): I) first phase; when there was plenty of water availability for transpiration until approximately 26h of stress. In this phase, repeated the results found from the irrigated plants*, i.e.* transgenic plants transpires less than WT. II) second phase; when transgenic plants transpires more than WT. The decrease in percentage soil moisture of plants grown in containers during this drought simulation, the sucrose transporter antisense transgenic plants presents lower accumulated water loss, depletion in soil moisture occurs more slowly than in WT pots (Fig. 2 inset). During this second phase of these experiments, wilting was observed in most plants during the light period; however a strongest phenotype was on WT plants (Fig. 4).

Table 1: Biometric analysis at transformed (P62) and wild type (WT) tobacco plants. Average points in bold and underlined differ statistically from WT (P<0.05; Dunnett's test). n=5 +/- SE.

Parameter	WT	S.E.	P62 L6	S.E.	P62 L9	S.E.
Plant DW (g)	28.0380	2.1837	22.5030	1.0268	23.3820	2.2193
Leaves DW (g)	15.8520	1.2548	13.4722	0.7105	13.8242	1.2072
% DW leaves	56.54	0.85	**59.82**	0.87	**59.30**	0.79
Stem DW (g)	8.2018	0.4415	**6.4442**	0.3055	**6.4248**	0.6243
% DW stem	29.48	0.88	28.69	0.98	27.55	0.97
Roots DW (g)	3.9842	0.5784	**2.5866**	0.1476	3.1330	0.4415
% DW roots	13.98	1.09	**11.48**	0.26	13.15	0.79
Root DW/shoot DW (g g^{-1})	0.1632	0.0148	**0.1297**	0.0033	0.1518	0.0103
Root DW/leaves DW (g g^{-1})	0.2481	0.0218	**0.1920**	0.0045	0.2222	0.0145
Plant leaf area/roots DW (m^2 kg^{-1})	148.34	14.08	**196.90**	6.28	169.61	15.59
Nº leaves (> 5 cm length)	13.80	0.37	14.20	0.49	14.00	0.32
Plant leaf area (m^2)	0.5627	0.0383	0.5068	0.0211	0.5051	0.0352
Specific leaf area (m^2 kg^{-1})	35.633	0.701	37.717	0.716	36.788	0.761
Stem length (cm)	59.14	1.12	**49.82**	2.05	**50.32**	3.75
Root length (cm)	36.04	2.58	36.98	1.86	36.26	1.97
Stem diameter (cm)	2.034	0.046	1.892	0.102	1.936	0.102

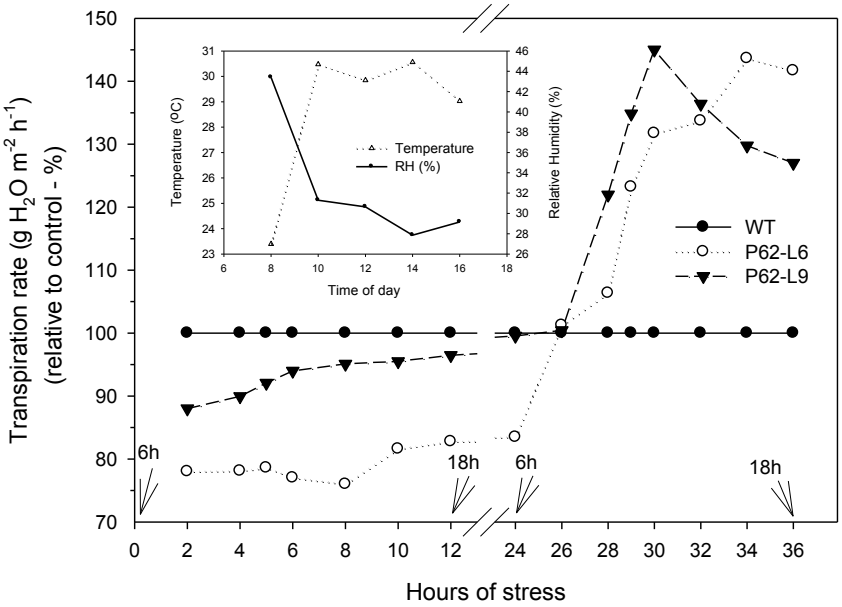

Figure 3: Transpiration rate of transgenic antisense (P62 L6 and P62 L9) tobacco plants. The data were transformed in relation to control non-transformed plant (wild type; WT) and corrected for soil evaporation and plant leaf area. (5 dm^3 pots; 5 kg dried soil). n = 5. Arrows indicate correspondent day time. Inset, the average of temperatures and relative humidity of time of analysis.

Figure 4: Photography of representative untransformed plant (control, WT) and transgenic lines (P62 L6 and P62 L9) at different times under drought simulation in two days of suspended irrigation. Left panel indicate first day and right panel the second day of evaluations

The wilt phenotype reflects perfectly the differences in leaf water content (Fig. 5). After few hours of stress in hot and low humidity conditions (Fig. 2), the relative water content (RWC) decreases slowly (91% to 82%) in transgenic plants whereas reduction to 76% occur in WT from the morning to at afternoon in the first day of drought induction. All plants could recuperate their RWC at night at expense soil water

consume. In the second day of analysis, higher transpiration rate of WT exhaust the available soil water too quickly and allow higher water transpiration as the transgenic plants, and even so reduces its RWC to 65%. At the same time as transgenic plants saved water and permit they restore water status to maintain higher RWC (77%; Fig. 4, 5). By the end of second day of water-stress the WT plants began to exhibit signs of irreversible damage. Once stressed the leaves of WT plants showed greater development of necrotic spots and yellowing relative to P62 plants (data not shown).

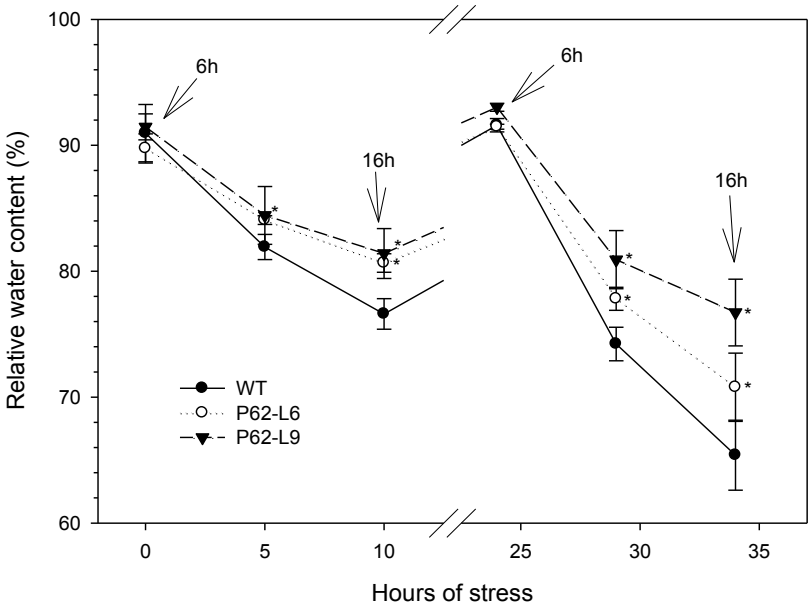

Figure 5: Relative water content of transgenic antisense (P62 L6 and P62 L9) tobacco plants and control non transformed plants (WT) along the time of drought stress. Arrows indicate correspondent day time

Gas exchange and fluorescence analysis under low water availability in hydroponics system

Gas exchange

We showed before those transgenic plants transpire less than WT at high water availability. The desiccation postponement phenotype is a principal feature of this transformation. In dry-down evaluations of potted plants, the genetic modified plants saved soil water and had higher RWC. To get better control of water potential and access the effect of manipulation of g_s by the effect of reduction in sucrose transporter expression on whole-plant water relations, plants were grown in hydroponics solution,

and a continuous drought stress treatment was produced using polyethylene glycol 6000 (PEG) to reduce water potential gradually at nutrient solution (acclimation for 3 days, 0.5% PEG for 4 days, 1% PEG for 7 days and 2% PEG for 7 days; Fig. 6) (Oliveira, 2001).

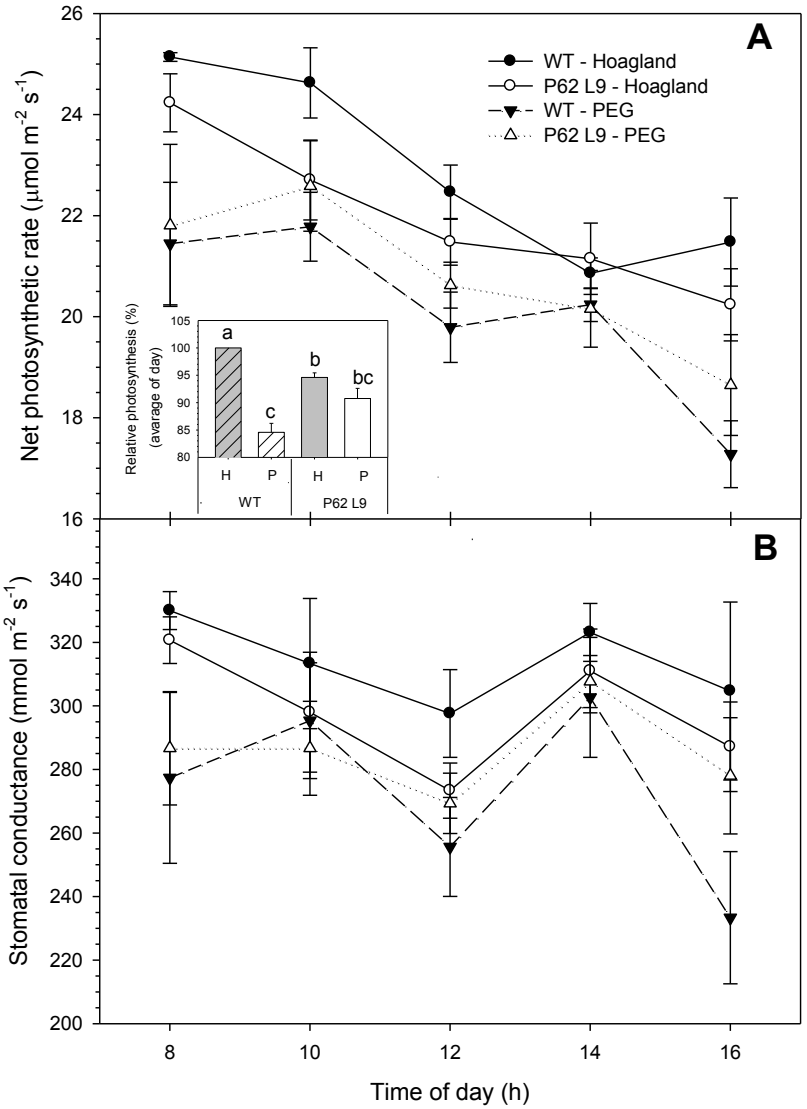

Figure 6: Time course of net photosynthetic rate (**A**) and stomatal conductance (**B**) at transformed (P62 L9) and wild type (WT) tobacco plants under Hoagland's nutrient solution or Hoagland's added to 2% PEG 6000 (Ψ_s = -0.14 MPa). n = 5 +/- SE. Inset, the average photosynthesis along the day (relative, % of control). Different letters over bars means statistical difference (P < 0.05) by Duncan's test. H = Hoagland; P = 2% PEG 6000

Without osmotic stress (no PEG), as expected based in the previous soil experiments, the transgenic plants show smaller *A* due reduced g_s (Fig. 6). In the presence of osmotic stress (2% PEG), the phenotype, as in soil experiment with drought stress, was opposed, *i.e.* higher *A* in P62 L9 than WT during all day (Fig. 6A,

inset). Changes in *A* were followed by changes in g_s along the day (Fig. 6B). This result was similar that was found out in drought induction in soil conditions. Under modest osmotic stress, transgenic plants achieved higher *A* than WT.

Fluorescence

In order to evaluate photochemical reaction in transgenic plants under stress, we evaluated fluorescence parameters under hydric stress (Table 2). Dark adapted fluorescence parameters (minimal fluorescence, F_o; maximal fluorescence, F_m; or variable to maximal fluorescence ratio, F_v/F_m) do not changed in all treatments (data not shown).

The light adapted fluorescence parameters (Tab. 2) showed negligible reduction of electron transport rate (ETR), and quantum yield of PSII (Y(II)) in the line P62 L9 without osmotic stress. Under osmotic stress, none parameters were altered in WT plants compared to WT in Hoagland's solution. In general, it was observed, only reduction in some parameters of P62 L9 at control's conditions (Hoagland) in relation to WT plants. Minor alteration were found in P62 L9 plants; a modest increase in Y(NO) and modest decrease in Y(NPQ). The ratio Y(II)/(Y(NO)+Y(NPQ)), which means of ratio of quantum yields do not differ in P62 L9 under stress, revealing that photochemical capacity do not changed in leaves growth under osmotic stress. These results highlight that changes in Y(NO) and Y(NPQ) were only circumstantial consequences between these competitive parameters.

Table 2: Light adapted fluorescence parameters at transformed (P62) and wild type (WT) tobacco plants. Average points in bold and underlined differ statistically from WT (P<0.05; *t* test) inside same solution treatment. Asterisks means differences (P<0.05; *t* test) between solutions inside same genotype. n=5 +/- SE. Noncyclic electron transport rate through PSII (ETR); Parameter estimating the fraction of open PS II centers based on a lake model (qL); non-photochemical quenching (qN); Quantum yield of photochemical energy conversion in PSII ((Y(II)); Quantum yield of non-regulated non-photochemical energy loss in PSII (Y(NO)); Quantum yield of regulated non-photochemical energy loss in PSII (Y(NPQ)); Photosystem II (PSII)

	WT-H	S.E.	WT-P	S.E.	P62 L9-H	S.E.	P62 L9-P	S.E.
ETR	21.16	0.080	21.06	0.180	**20.68**	0.110	20.60	0.100
qL	0.250	0.023	0.248	0.020	0.235	0.015	0.209	0.019
qN	0.386	0.025	0.344	0.011	0.426	0.003	0.377	0.010
Y(II)	0.625	0.003	0.623	0.005	**0.611**	0.004	0.609	0.003
Y(NO)	0.242	0.010	0.258	0.006	0.238	0.003	0.258*	0.006
Y(NPQ)	0.133	0.009	0.120	0.003	0.151	0.002	0.133*	0.004
Y(II)/(Y(NO)+Y(NPQ))	1.66	0.020	1.65	0.040	1.57	0.020	1.56	0.020

Growth under PEG

In nutrient Hoagland solution, it was observed minor reduction (P>0.05) of leaf area expansion of transgenic line. Under PEG solution, all genotypes reduce the leaf expansion, particularly on WT plants. On the other hand, as possibly consequences of higher *A* (Fig. 6) (Tab. 2), the P62 plants were capable of increase more rapidly their leaf area than WT plants at same conditions (Fig. 7). This result was observed in two different measurements on these plants.

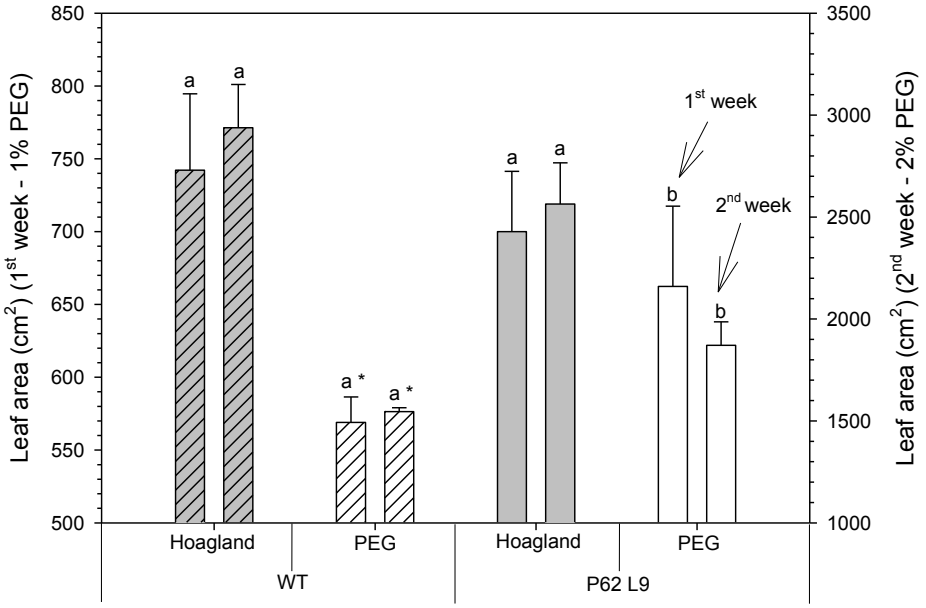

Figure 7: Leaf area of transformed (P62 L9) and wild type (WT) tobacco plants under Hoagland's hydroponics solutions or Hoagland's added to 1% PEG 6000 (Ψ_s = -0.06MPa) or 2% PEG 6000 (Ψ_s = -0.14 MPa) in two different weeks measurements, left and right bars, respectively, for each treatment. Different letters means statistical differences (P<0.05, *t* test) between genotypes in the same solution and week. Asterisks denote statistical differences (P<0.05, *t* test) between solutions inside same genotype and week. n = 5

DISCUSSION

Water consume

Low transpiration is mainly due to reduction in g_s, and any physiological change that affect stomatal aperture could affect water consume by plants. In our case, we propose that reduced sucrose contents in symplast of GC could be an important factor controlling stomatal behavior.

The transpiration rate (Fig. 1, 2) of transgenic lines was smaller than WT plants. High transpiration of WT plants depleted available soil water faster, and so, probably induces stomatal closures earlier than P62 lines along the day. Smaller transpiration rate of transgenic lines saved soil water (Fig. 2) giving them better water status as showed in Figure 4, and confirmed by higher RWC in Figure 5. This phenotype is a clear definition of DP pattern (Taiz & Zeiger, 2006).

When plants increase their leaf area (Fig. 1), the higher transpiration could exhaust the available soil water too quickly and probably had diminished the differences between WT to transgenic lines. It could explain part of the diminished

differences between water consume of small plants to bigger one. Transgenic plants had same (P>0.05) dry mass and number of leaves or specific leaf area. As expected (see Chap. 1), transgenic plants accumulate less biomass on stem and have higher proportion of dry matter of leaves in relation to total biomass accumulated. The transgenic plants had little alteration on root biomass, but showing higher leaf area to root biomass ratio. Even so, transgenic plants transpired less than WT both in irrigated (Fig. 1), as well as in modest drought stress (Fig. 2, 3). This result circumstantially suggests that are no differential hydraulics conductances or limitation on transgenic plants. It represents that modest slighter root dry mass could supply adequately shoot of available soil water.

In a dryer environment, P62 lines saved soil water (Fig. 2) and could replace transpired water in a way to maintain high RWC on leaves. On this time WT plants have exhausted soil water and showed lowest RWC (Fig. 5). The “high” water status could permit higher *A* even in drought. In field condition, we speculate that it can tolerate more, reducing the risk of depleting soil water faster reducing agronomical penalties and obtain higher biomass and higher yield.

Higher *A* was observed in P62 L9 under drought simulation in hydroponic-PEG solution. It could be consequence of water conservative stomatal behavior. With a higher RWC, transgenic plants have more stomatal opening allowing more photosynthesis rate than WT in same conditions. Higher *A* and intrinsic characteristics to relocation carbon for preferentially leaves (in detriment for stem carbon allocation; see Chap. 1) allow transgenic plants grow faster in low water availability. The minor differences in photochemical parameters are associated to higher demand for NADPH and ATP, both in PEG solution as in control conditions. Reduced ETR and Y(II) are associated to slower photochemical demand due to minor requirements for these compounds at low carboxylation rate, it is not suggested damage in electron transport system (Maxwell & Johnson, 2000; Baker, 2008).

Many examples of plants resistant to drought have been carried out in *Arabidopsis* using constitutive promoters. Frequent results found out in these transgenic plants or mutants resistant to drought are reduction in shoot growth

(Kasuga et al., 1999; Abe et al., 2003; Swindell et al., 2007). Many target proteins for engineering drought tolerance are listed by Schroeder et al. (2001); Umezawa et al. (2006); and Valliyodan & Nguyen (2006), and do not include sucrose metabolism and transport. Few examples are effective and tested under field conditions. The most used commercially wheat varieties, 'Drysdale', 'Ress', and 'Quarrion' (Farquhar & Richards, 1984, Richards et al., 2002; Condon et al., 2004), were selected by low $\Delta^{13}C$, having slower crop growth rate in absence of water stress, but in dryer conditions, the high water use efficiency can result in considerable yield gain (Condon et al., 2002). Comparable results were achieved by our transgenic plants with sucrose transport alterations in GC based on water consumption and growth parameters.

Here, it was described how can a sucrose transporter could affect water consume by plants. As a result of decreased expression of sucrose transporter specifically in GC, we show that they have constitutive low stomatal conductance behavior as well as low transpiration rate, especially when there is high water availability. By means of low g_s tobacco plants, we got desiccation postponement phenotype as principal feature of this transformation, being high water saving plants. These results suggest that manipulation of sucrose transport in GC may be developed as a practical mechanism for drought avoidance and water conservation during irrigation. Comparable phenotype as achieved in tobacco by GC NADP-malic enzyme manipulation, in which maximal g_s attained by the highest expression of malic enzyme (Laporte et al., 2002), then reduced malic enzyme also could achieved DP on those plants.

CONCLUSION

In conclusion, transformed tobacco plants with decrease in sucrose transporter expression specifically in GC reduced water consumption at irrigated conditions, and allow better growth and water use efficiency under drought or osmotic stress. The desiccation postponement phenotype of transformed plants could allow them save soil water and maintain higher relative water content on leaves under conditions. These results illustrate the importance of fine tuning of sucrose transport and metabolism in

the fitness of stomatal function, contributing for plant survival or growth under unfavorable hydric conditions. This work allows including the GC sucrose transporter, as of possible target genes to improve drought resistance, and illustrate that a single gene expression change could have visible desirable phenotypes regarding water stress responses.

MATERIAL AND METHODS

Plant material and cultivation conditions

Seeds of *Nicotiana tabacum* L. C.V. 'Havana 425' and transformed P62 lines were surface-decontaminated by shaking in 70% ethanol for 1 min and rinsed with sterile distilled H_2O. In sequence, seeds were treated with 2% sodium hypochlorite for 5 min and rinsed three times with sterile distilled H_2O. The seeds were then allowed to germinate in Petri dishes containing MS medium (25 cm^3) (Murashige & Skoog, 1962) and cultivated under photoperiod of 14h illumination and light intensity of 100 µmol m^{-2} s^{-1}, with day/night temperatures of 25 °C/20 °C. For all experiments plants, seeds of T2 lines and WT were surface-decontaminated as described before and cultivated in selective medium (MS medium added by Timentin® 300 mg L^{-1} and 100 mg mL^{-1} of kanamicin) and then transferred to acclimate in 50 cm^3 pot, later for 300 cm^3 pots, and finally, to 5 dm^3 pots at greenhouse. WT seedlings are cultivated in non-selective medium.

Water consumed

Plants were cultivated on 5 dm^3 pots and 5 kg (+/- 20 g) of a mixture containing soil, sand and manure. Water loss by transpiration was determined by gravimetric methodology. Briefly, at previous night, the soil was irrigated and left to drain all night. At 6h and 19h the weights (0.1 g precision) were recorded, the difference was estimated as evapo-transpired water. Filled pots (without plants) were used to estimate direct soil evaporation. The difference between total water loss and evaporation was considered plant transpiration using n= 6.

Drought induction was produced by interruption of irrigation of water saturated soil at prior night. The first weight (6:00h, day 1), of each pot weight was recorded and kept as reference. At regular intervals during the day, the weights loss was accompanied. Filled pots (without plants) were used to estimate direct soil evaporation using n = 5. The experiment had two days of measurements.

Relative water content

It was evaluated by leaf disks (13 mm diameter; 10 disks) putted over distilled water in anatomical position for 2h on a Petri dish at room conditions. It was recorded fresh weight (FW), turgid weight (TW) and dry weight (DW) (60 °C, 24h). Relative water content (%) was estimated by: RWC = 100(FW-DW)/(TW-DW).

Gas exchange measurements

The gas exchange was performed in approximately 20-30 cm tall plants in an expanded 5^{th} or 6^{th} leaf counting-down for apical meristem. It was used Li-Cor Li6400 gas exchange system (http://www.licor.com) with 1000 μmol m^{-2} s^{-1} photosynthetically active radiation. Temperature and CO_2 concentration were ambient. It was used 500 cm^3 min^{-1} flow air. n = 5.

Fluorescence measurements

The chlorophyll-*a* fluorescence parameters were determined using modulated light fluorometer system (MINI-PAM) (Walz, Effeltrich, Germany). It was measured minimal (F_o) and maximal fluorescence (F_m) in dark adapted leaves (30 min). It was estimated maximal quantum yield of PSII (F_v/F_m) according to Maxwell & Johnson (2000). It was evaluated minimal fluorescence of chlorophyll-*a* in leaves adapted to light ($F_o' = F_o/(F_v/F_m + F_o/F_m')$) (Klughammer & Schreiber, 2008), maximal fluorescence adapted to light (F_m'), electron transport rate (ETR = Y(II) x PAR x 0,5 x 0,84) (Genty et al., 1989). Effective quantum yield of PSII (Y(II)), the fraction of open PSII centers based on a lake

model (qL = (F_m'-F) x F_o'/(F_m'-F_o') x F), non-photochemical quenching (qN = F_m-F_m'/F_v), quantum yield of non-regulated non-photochemical energy loss in PSII (Y(NO) = F/F_m)), quantum yield of regulated non-photochemical energy loss in PSII ((YNPQ = (F/F_m') - (F/F_m)). The parameters Y(NPQ), Y(II) and Y(NO) were estimated as in Genty et al. (1989) and Hendrickson et al. (2004), and Klughammer & Schreiber (2008) in which Y(NPQ)+ Y(II)+Y(NO) = 1.

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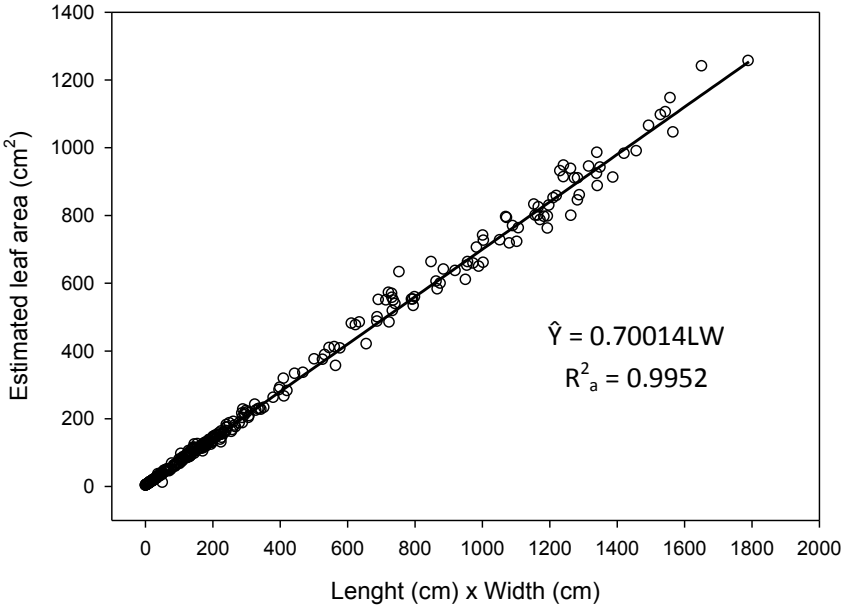
Appendix

Leaf area estimating model for *Nicotiana tabaccum* L. Hav. 425



The relationship between estimated leaf area and leaf dimensions (L, length and W, width) for tobacco using genotype "Havana 425"

Model Definition: $Y = \beta_1 x + e_i$

Variable	Value	Standard Error
β_1	0.700138211	0.002093082

Number of observations = 311
Average Residual = -0.548085868845541
Standard Error of the Estimate = 21.2766603114951
Adjusted coefficient of multiple determination (R^2_a) = 0.9952197262