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FRANCISCO BRUNO SILVA FREIRE

SUCROSE SYNTHASE 2 REGULATES WHOLE PLANT TRANSPIRATION IN *Nicotiana tabacum* L.

FORTALEZA
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Dissertação apresentada ao Curso de Mestrado em Bioquímica do Departamento de Bioquímica e Biologia Molecular da Universidade Federal do Ceará, como requisito parcial à obtenção do título de Mestre em Bioquímica. Área de Concentração: Bioquímica Vegetal.

Orientador: Prof. Dr. Danilo de Menezes Daloso.

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À minha família e amigos.

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RESUMO

Os estômatos são pequenas estruturas presentes principalmente na epiderme foliar das plantas, composto por duas células-guarda que circundam e regulam a abertura e o fechamento do poro estomático. Os movimentos estomáticos regulam a entrada de CO_2 atmosférico para a fotossíntese e a saída de água via transpiração, sendo importantes na regulação da eficiência do uso da água, sendo a razão entre a assimilação de CO_2 e a perda de água via transpiração. Resultados recentes indicam que a degradação da sacarose é um mecanismo responsável por induzir a abertura dos estômatos. Adicionalmente, foi demonstrado que a manipulação genética do metabolismo da sacarose de células-guarda é uma ferramenta potencial para a obtenção de plantas que consomem menos água e/ou maior eficiência do uso da água. Dentro desta perspectiva, este trabalho teve como objetivo avaliar o potencial biotecnológico da manipulação genética da atividade sacarolítica de células-guarda no desenvolvimento de plantas tolerantes a seca e/ou com maior eficiência do uso da água. Diferentes análises bioquímicas e moleculares somadas à uma extensa caracterização fisiológica foram desenvolvidas a fim de caracterizar o fenótipo de plantas transgênicas de *Nicotiana tabacum* antisenso para o gene *sacarose sintase 3* (*StSUS3*) de batata sob o controle do promotor KST1 específico de estômatos. Os resultados demonstraram que o transgene inserido (*StSUS3*) reduziu a expressão da isoforma *NtSUS2* de tabaco, que, por consequência, alterou substancialmente o metabolismo de células guarda. Além disso, as plantas diminuíram a condutância estomática e taxas de transpiração da planta inteira e elevou a eficiência do uso da água, além das plantas apresentarem um fenótipo de evitar a seca. Tomados em conjunto, nossos resultados indicam que o gene *NtSUS2* é um regulador chave da transpiração vegetal, sendo, assim, um importante alvo biotecnológico.

Palavras-chave: Atividade sacarolítica. Estresse de seca. Regulação do movimento estomático. Metabolômica. Eficiência do uso da água.

ABSTRACT

Stomata are small structures present primarily in the leaf epidermis of plants, composed of two guard cells that surround and regulate the opening and closing of the stomatal pore. Stomatal movements regulate atmospheric CO_2 input for photosynthesis and transpired water output, being thus important for plant water use efficiency, defined by the ratio of CO_2 assimilation to transpired water loss. Recent results indicate that the degradation of sucrose is a mechanism responsible for inducing stomatal opening. Additionally, it has been shown that genetic manipulation of guard cell sucrose metabolism is a potential tool for obtaining plants that consume less water and/or having greater water use efficiency. Within this perspective, this study aimed to evaluate the biotechnological potential of genetic manipulation of guard cells saccharolytic activity in the development of drought tolerant and/or more water use efficient plants. Different biochemical and molecular analyzes coupled with an extensive physiological characterization were developed in order to characterize the phenotype of transgenic *Nicotiana tabacum* plants antisense for the potato *sucrose synthase 3* gene (*StSUS3*) under the control of the stomatal-specific KST1 promoter. The results demonstrated that the inserted transgene (*StSUS3*) reduced the expression of tobacco *NtSUS2* isoform, which consequently altered substantially guard cell metabolism. Also, the plants decreased stomatal conductance and whole plant transpiration rates and increased the water use efficiency, besides the plants presented a drought avoidance phenotype. Taken together, our results indicate that the *NtSUS2* gene is a key regulator of plant transpiration and is therefore an important biotechnological target.

Keywords: Saccharolytic activity. Drought stress. Stomatal movement regulation. Metabolomics. Water use efficiency.

CONTENTS

1 GENERAL INTRODUCTION

Due to the current climate changes, it has been estimated that water demand for agricultural production will increase by 17% by 2025, mostly due the increase in the average global temperature and the fact that drought episodes will become more frequent according to the predicted climate change scenario. The growing demand for food for the constantly growing world population will be essentially dependent on the development of varieties with lower water demand and on the development of varieties with higher drought tolerance (PENNISI, 2008). Thus, it is urgently needed therefore to find strategies to improve plant water use efficiency (WUE), which is defined as the ratio between the amount of accumulated biomass per unit of water used at crop, whole plant or leaf level (BACON, 2004; BLUM, 2005; CONDON et al., 2004; FLEXAS et al., 2010; MEDRANO et al., 2015). At crop level ($_{c}$WUE) what matter is the field production, being the ratio between the plant production and the water used during the whole cultivation period (PASSIOURA, 2006). At whole plant level the ratio between total accumulated biomass to water used or transpired is defined as season-long water use efficiency ($_{sl}$WUE) and the ratio between individual yield production (harvestable) to water used or transpired over a certain period of time ($_{y}$WUE) (MEDRANO et al., 2010). At leaf level, gas exchange analysis is taken into account to determine instantaneous water use efficiency ($_{L}$WUE - ratio between net photosynthetic rate (A) and transpiration rate (E)) or intrinsic water use efficiency ($_{i}$WUE - ratio between A and stomatal conductance (g_s)) (CONDON et al., 2002; FLEXAS et al., 2013). Given that WUE depends fundamentally on the gain of carbon (C) and the water loss by the transpiration process, it is reasonable to assume that stomata is the master regulator of WUE (BRODRIBB; SUSSMILCH; MCADAM, 2019). However, the key regulators of plant transpiration remain elusive.

Plant scientists are looking to understand the dynamic of plant cells under increasing global environmental changes in order to improve plant growth and plant stress tolerance through genetic manipulation of plant metabolism. However, plants are controlled by modulation of complex regulatory networks acting at different spatial and temporal scales, which makes difficult to understand plant cell functioning, specially under adverse conditions, and to find important biotechnological targets (BERTOLLI; MAZZAFERA; SOUZA, 2014; SOUZA; DE OLIVEIRA; CARDOSO, 2004). The importance of knowing the WUE of a cultivar takes into consideration that when it is increased it means that the plant can produce the same biomass with less water consumption or can accumulate more biomass using the same amount of water. WUE measurements under water stress conditions are particularly useful in

the discovery of potential drought stress tolerance plants, as observed in low stomatal density poplar plants with high WUE, showing low loss of biomass and *A* in drought conditions (WANG et al., 2016). Also WUE measurements combined with drought cycles experiments are a great way to evaluate the plant recovery and differential acclimatization behavior between cycles (CYRIAC et al., 2018; FLEISHER et al., 2014; KELLY et al., 2019; SAINT PIERRE et al., 2012). In this context, although WUE measurements under water deficit conditions are particularly useful to unveil potential drought stress tolerant plants, it is important to highlight that WUE improvement is not always associated to increased drought tolerance, and *vice versa*. This is partially due the fact that with the increase of temperature and vapor pressure deficit (VPD) under drought, plants tend to transpire more and thus consume more water. Thus, although there are multiple ways to improve WUE (SINGH et al., 2012), one way to decrease plant water consumption is by genetic manipulation of key regulator(s) of plant transpiration, which is presumably located at the stomata (FLEXAS, 2016; GAGO et al., 2014).

Stomata are leaf epidermal structures consisting of two guard cells surrounding a pore that enable the gas exchange between plants and the environment. Those cells are highly specialized and the control of stomatal movements involves reversible changes in guard cell solute concentrations caused by environmental and endogenous signals such as plant water status, hormonal stimulus, mesophyll-derived metabolites, air humidity, light and CO_2 concentration (LAWSON et al., 2014; MATTHEWS; VIALET-CHABRAND; LAWSON, 2017). Maintaining the ideal balance between CO_2 assimilation and water loss through transpiration is totally dependent on stomatal movements, which is where gas exchange takes place. During the stomatal opening process, there is an increase in guard cell volume due to the influx and/or intracellular generation of solutes, favoring water entrance into the cell and consequently driving stomata to open (MEDEIROS et al., 2015).

The control of stomatal pore opening involves thus reversible changes in the concentrations of osmotically active solutes within guard cells, such as potassium (K^+), malate ($malate^{-2}$), chloride (Cl^-) and nitrate (NO_3^-) (EISENACH; DE ANGELI, 2017; SHIMAZAKI et al., 2007). By contrast, stomatal closure has been closely associated to hormonal stimulus, in which abscisic acid (ABA) plays a central role in guard cells, mediated by Ca^{+2}, which leads to increase pH, decrease of sugars by starch synthesis, K^+-intake channels closure and inhibition of H^+-ATPases leading to stomatal closure (ROELFSEMA; HEDRICH, 2005). Environmental cues such as dark and high CO_2 concentration close stomata while light and low CO_2 concentration stimulate its opening (ENGINEER et al., 2016). Light-induced stomatal opening demands large amount of ATP and occurs when GC phototropins sense light and initiate a

signal transduction pathway by activating H^+-ATPases and K^+-intake channels which ultimately leads to the vacuole accumulation of K^+, malate^{-2}, NO^{-3} and Cl^- (INOUE; KINOSHITA, 2017). The mechanisms by which CO_2 controls stomatal movements can be several, such as the interaction between phytohormones like ABA (WEBB; HETHERINGTON, 1997) and enzymes such as carbonic anhydrase (HU et al., 2010) and HT1 protein kinase (HASHIMOTO et al., 2006). Thus, stomatal movements follows a circadian rhythm according to external and endogenous stimulus (GORTON; WILLIAMS; ASSMANN, 1993; HUBBARD; WEBB, 2015), which is pivotal to sustain the demand of CO_2 for photosynthesis and to close the stomata in periods of high photosynthetic rate (MESSINGER; BUCKLEY; MOTT, 2006). Thus, stomatal movements is closely linked to the mesophyll photosynthetic activity, in which the transport of mesophyll-derived metabolites such as sucrose and malate and their import into guard cells seems to be key for stomatal movement regulation (DALOSO; DOS ANJOS; FERNIE, 2016; LAWSON et al., 2014; LIMA et al., 2018, 2019).

Numerous studies indicate the importance of carbohydrate and sucrose metabolism in the adjustment of stomatal movements (KOPKA; PROVART; MULLER-ROBER, 1997; OUTLAW; DE VLIEGHERE-HE, 2001; STADLER et al., 2003). Sucrose was proposed to act as a guard cell osmolyte and its accumulation would be responsible for maintaining the stomatal opening in substitution of K^+ in periods of the day when the concentration of this ion is low (TALBOTT; ZEIGER, 1998). However, recent studies suggest that it is not the accumulation but the degradation of sucrose in guard cells that is an important mechanism to provide light-induced stomatal opening, indicating that sucrose plays a major role in the regulation of guard cells (DALOSO et al., 2017; DALOSO; DOS ANJOS; FERNIE, 2016; ROBAINA-ESTÉVEZ et al., 2017). Recent studies demonstrated through kinetic [U^{13}C]-sucrose isotope labeling experiments that sucrose is degraded by entering the TCA cycle and providing carbon skeletons for Glutamine (Gln) biosynthesis.during light-induced stomatal opening (DALOSO et al., 2015; MEDEIROS et al., 2018). Furthermore, it has been shown that plants overexpressing the gene encoding hexokinase enzyme, one of the major enzymes of sucrose metabolism, lead to changes in stomatal conductance and WUE (KELLY et al., 2013, 2019; LUGASSI et al., 2015).

These results suggest that sugar breakdown within guard cells plays a major role in the regulation of stomatal movements. More importantly, these results highlight the energetic importance of sucrose metabolism to sustain stomatal opening and refute the previous idea that sucrose would act only as an osmolyte within guard cells (AMODEO; TALBOTT; ZEIGER, 1996; TALBOTT; ZEIGER, 1996, 1998). Furthermore, these studies indicate that genetic

manipulation of guard cell carbohydrate metabolism is an important approach to improve drought tolerance and/or WUE. For instance, transgenic plants with increased expression of hexokinase within guard cells have increased WUE and drought tolerance (KELLY et al., 2019), and antisense construction to sucrose transporter (*SUT1*) lead to plants with higher WUE, associated to reduced guard cell sucrose content and lower g_s compared with WT plants (ANTUNES et al., 2017). In addition, there are some evidence suggesting that high sucrose concentrations may lead to stomatal closure (KANG et al., 2007; LU et al., 1995). The existence of such contradictions in the literature and by the complexity of guard cell metabolism justifies the need for more studies to better understand the real role of sucrose in controlling stomatal movements.

Sucrose metabolism is important not only for the regulation of guard cells but mainly to the overall C distribution throughout the plant. In the cytosol of plant cells, sucrose is reversible degraded into hexoses by different invertase (INV) and sucrose synthase (SUS) isoforms (BIENIAWSKA et al., 2007; NGUYEN-QUOC; FOYER, 2001). The SUS-catalyzed reaction to sucrose breakdown is energetically more favorable than INV, because in this reaction it converts sucrose and UDP to fructose and UDP-glucose (GEIGENBERGER; STITT, 1993). The number and the expression of SUS isoforms vary among species and the organs, respectively. In tobacco (*Nicotiana tabacum* L.), there are seven SUS isoforms (*NtSUS1-7*), and isoforms 2 and 3 are the most abundant in mature leaves (WANG et al., 2015), while in potato (*Solanum tuberosum*) there are 5 isoforms (*StSUS1-5*), and isoform 3 is more abundant in GC (KOPKA; PROVART; MULLER-ROBER, 1997). In Arabidopsis, the expression of *SUS3* is nearly specifically to guard cells (DALOSO; DOS ANJOS; FERNIE, 2016), suggesting a specific role of this isoform in stomatal movements. Indeed, constructions of transgenic plants providing high capacity to degrade sucrose in GC exhibited higher g_s rates (ANTUNES et al., 2012; DALOSO et al., 2016; NI, 2012).

Given the need to develop plant crops with higher WUE and the existence of contradictions found in the literature about the role of sucrose in controlling stomatal movements, here we have characterized the phenotype of tobacco transgenic plants antisense for the isoform 3 of *SUS* gene of potato under control of the guard cell-specific KST1 promoter, evaluating the metabolic profile and WUE of transgenic plants.

2 OBJECTIVES

2.1 General objective

To observe the effects of genetic manipulation of guard cells saccharolytic activity on their primary metabolism, whole plant transpiration and water use efficiency.

2.2 Specific objectives

1. To analyze the gas exchange of tobacco transgenic plants antisense to potato *sucrose synthase 3* gene compared to wild type (WT).
2. To evaluate the growth and plant transpiration in transgenic lines and WT under irrigated and water deficit conditions.
3. To investigate changes in the metabolic profile of leaves and guard cells during dark-to-light transition in transgenic and WT plants.

3 EXPERIMENTAL STRATEGY

3.1 Plant material and growth conditions

Were utilized the commercial *Nicotiana tabacum* L. cv Havana 425 plants, due the large leaf area, wild type (WT) and transgenic genotypes antisense to *sucrose synthase 3* (*SUS3*) gene from *Solanum tuberosum* (STU24088) under control of the constitutive KST1 promoter (X79779), that is exclusive for expression specifically in guard-cell (MÜLLER-RÖBER et al., 1995; PLESCH; EHRHARDT; MUELLER-ROEBER, 2001). The transformation were realized by sub-cloning of the gene in the pBinK plasmid vector, which derivates from pBinAR-Kan (HÖFGEN; WILLMITZER, 1990), which had its CaMV-35S promoter switched to KST1 promoter. A 1567 pb fragment was obtained by the digestion of *SUS3* gene from potato (ANTUNES et al., 2012) using the KnpI restriction enzyme, which was cloned in the antisense direction in the pBinK vector, between KST1 promoter and OCS terminator. This construction was inserted in *Agrobacterium tumefasciens* (Strain GV 3101) by electroporation and cultivated in suspension with leaf discs from tobacco to achieve the plant transformation. The transformation was confirmed by PCR of the marker gene *NPTII* that confers kanamycin resistance.

Transgenic seeds of T_3 generation were obtained and germinated *in vitro*. All seeds were surface-decontaminated by shaking in 70% ethanol for 1 min and rinsed with sterile distilled H_2O. In sequence, seeds were treated with 0.2% sodium hypochlorite for 5 min and rinsed two times with sterile distilled H_2O. The seeds were then allowed to germinate in Petri dishes containing Murashige and Skoog (MS) medium (MURASHIGE; SKOOG, 1962) with addition of 50µM of Kanamycin® (KAN) for the transgenic lines and cultivated *in vitro* for 30 days under growth chamber conditions, 16 h:8 h, light : dark photoperiod, photosynthetic photon flux density (PPFD) of 100 µmol photons m^{-2} s^{-1}, 25 ± 1 °C and relative humidity 53 ± 5%. Seedlings showing KAN resistance were transferred to 0.1 liters seedbeds with substrate composed by a mixture of vermiculite, sand and soil (1:1:1) and kept well watered for 15 days under greenhouse conditions with natural 12 h light photoperiod, maximum PPFD of 500 µmol photons m^{-2} s^{-1}, 30 ± 4 °C and relative humidity 62 ± 10%. Subsequently, plants were dived in three groups. The first group was transferred to hydroponic system using Hoagland and Arnon nutritive solution in 2.5 L pots (HOAGLAND; ARNON, 1950), which was changed weekly and these plants used only for the isolation of guard cell-enriched epidermal fragments. The second group was transferred to 5.0 L pots containing the same substrate mentioned above. The first and second groups were cultivated under greenhouse conditions. The third group was cultivated into 2.5 L pots with the same substrate under growth chamber conditions. The plants grown on substrate were irrigated with Hoagland and Arnon nutritive solution three times per

week. All plants were cultivated during 45 or 60 days until the beginning of each experiment described below.

3.2 Phylogenetic analysis

To find out which tobacco SUS isoform most likely would have its expression decreased by the antisense sequence of SUS3 from potato, a phylogenetic tree was created in order to reveal which primers we need to use in the gene expression analysis. Amino acids sequences from previous work (WANG et al., 2015) were used as query sequence on tblastn from NCBI database to found CDS of *NtSUS1-7* (accession numbers: XM_016618980.1; XM_016595403.1; XM_016627888.1; XM_016610900.1; XM_016608012.1; XM_016585183.1; XM_016611295.1). Multiple sequence alignment was performed using ClustalW software (Kyoto University Bioinformatics Center, Kyoto, Japan) utilizing CDS of *NtSUS1-7* and *StSUS3* (accession number: AY205084.1) to build the phylogenetic tree by FastTree tool (PRICE; DEHAL; ARKIN, 2009).

3.3 Gene expression analysis

In order to quantify the expression level of the antisense inhibition of sucrose synthase in tobacco, an analysis of gene expression was performed. Total RNA from guard cells and leaves of plants grown in greenhouse were isolated and cDNA synthesized using SV Total RNA Isolation System and M-MLV Reverse Transcriptase (Promega Corporation, Madison, Wisconsin, EUA), accordingly to the manufacturer instructions. Total RNA was quantified (Epoch Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, Vermont, EUA) and its integrity checked in 50 $\mu g\ ml^{-1}$ ethidium bromide stained 1.5% agarose gel.

The primer for protein phosphatase 2A (*PP2A*) was designed by aligning the coding sequence (CDS) obtained from NCBI database using the muscle tool in Mega X software (FW,5'-CACTTCAGTCAATTGATAACGTC-3'; REV,5'-GCAAAATCCTACCAAAGAGGG-3'). The primers NtSUS2 were constructed as in previous work (WANG et al., 2015) (FW,5'- CACATTGATCCATACCACGGGGAT-3'; REV,5'-ACAGCAGCCAGTGTCAACAACCGA-3'). In order to estimate SUS silencing, qPCRs were performed by using GoTaq® qPCR Master Mix (Promega Corporation, Madison, Wisconsin, EUA) accordingly to the manufacturer instructions. The qPCRs were performed accordingly the following program: initial denaturation (95 ºC, 5 min), 40 x 3 steps cycle (denaturation 95

ºC 15 sec; annealing 56.3, 60.0 or 68.1 ºC 15 sec; elongation 60 ºC 20 sec) and final elongation (60 ºC, 2 min). Transcripts relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method, in which *PP2A* was used as internal control and WT was used as calibrator (LIVAK; SCHMITTGEN, 2001).

3.4 Gas exchange analysis

Photosynthetic response curves were performed using a portable infrared gas analyzer (LiCor 6400XT, Lincoln, NE, USA) in completely expanded leaves from 45 days-old plants of all genotypes, including WT, cultivated in hydroponic system and 5 liters pots. These curves were carried out aiming to observe the differences in the photosynthetic capacity between transgenic and WT plants in response to light and CO_2. Photosynthetic light responsive curve (A-PPFD) was measured using light intensities from high to low, in order: 2000, 1500, 1000, 800, 600, 400, 200, 100, 50 and 0 µmol photons m^{-2} s^{-1}, using 10% blue light (FLEXAS et al., 2008). For this measurement, *A* (µmol CO_2 m^{-2} s^{-1}) was recorded in each light intensity when steady state was reached at constant 400 ppm CO_2 and block temperature at 28 °C. Photosynthetic CO_2 responsive curve (A-C_i) was achieved using several CO_2 concentrations, in order: 400, 300, 200, 100, 50, 400, 500, 700, 1000, 1200 and 400 ppm CO_2. Substomatal CO_2 concentration (C_i, µmol CO_2 mol^{-1}) and *A* were recorded for each CO_2 concentration and constant 1000 µmol photons m^{-2} s^{-1} saturating light when steady state was reached. Also, were measured steady state of transpiration rate (*E*), *A*, g_s, and dark respiration (R_d) by measuring photosynthesis in dark and multiplying it by -1.

Both curves were constructed using SIGMAPLOT 14 (Systat Software Inc., San Jose, CA, USA). A three component exponential rise to maximum equation was used to fit the curves: $A = a\ (1 - e^{-bx}) + c$, where *A* = photosynthetic rate, x = PPFD, and a, b, c are parameters estimated by the non-linear regression (WATLING; PRESS; QUICK, 2000).

3.5 Whole plant transpiration

The determination of whole plant transpiration was achieved by using a gravimetric methodology (DALOSO et al., 2016). The whole plant transpiration was accomplished in order to help to observe phenotypic changes in stomatal movement behavior of transgenic plants. For 45 days-old plants cultivated in 2.5 and 5 liters pots with substrate and maintained in growth chamber and greenhouse conditions, respectively. Pots without plants were used to estimate

evapotranspiration of the soil. The pots were filled with water at beginning of the night almost in a daily basis, as shown in the legend of the figures. In the next day, the pots were weighted at predawn and at the end of the day. The WPT was then obtained by the difference between the two weights and by subtracting the evapotranspiration of the pots without plants (g H_2O d^{-1} $plant^{-1}$). WPT was recorded between different time intervals in a diel course as well as in a daily basis for eleven days and forty six days.

3.6 Growth analysis

The growth analysis was carried out in order to observe changes in development and growth, proportional biomass gain and leaf area that the transgene may have caused. For growth chamber and greenhouse conditions, the leaf area (LA) was estimated through a model described previously (ANTUNES et al., 2008). This model was adapted for tobacco by the equation: LA = 0.70014 * W * L. All leaves with more than 5 cm of length from all genotypes were measured. This model takes into account the length (L) and maximum width (W) of the leaves, which were obtained by measuring from the lamina tip to the point of the petiole intersection along the central nervure and by measuring at the widest point perpendicular to the central nervure, respectively. These measurements were recorded during the whole plant transpiration experiment on the first, middle and last day of experiment and on the days in between the data were estimated by linear regression ($r^2 > 0.98$). The leaf area (cm^2) was used to calculate the whole plant transpiration per leaf area (mg H_2O d^{-1} cm^{-2}) and specific leaf area (cm^2 g^{-1} leaf DW). For each plant, at the end of WPT experiments, was determined the total leaf number (> 5cm length), stem length, dry weigh (DW) of leaf, stem and roots by drying at 80 °C for 7 days. These parameters were used to determined total biomass DW (leaf DW + stem DW + roots DW), harvest index (leaf DW / total biomass DW), shoot DW (leaf DW + stem DW), shoot DW / root DW (g g^{-1}), leaves DW / root DW (g g^{-1}) and plant leaf area / roots DW (cm^2 g^{-1}). The relative growth rate (RGR, g g^{-1} day^{-1}) was obtained by the equation: RGR = ln (final weigh) – ln (initial weight) / final day – initial day.

3.7 Drought stress experiment

The drought stress experiment was performed in 45 days-old plants of all genotypes cultivated in 2.5 liters pots under growth chamber conditions. This experiment was composed by three drought cycles. These cycles were performed in order to analyze recovery effects in

hydric stress and to compare the possible differences in response to drought between transgenic and WT plants. On the first cycle, the irrigation was interrupted at the day 4 of the experiment for five days, and then irrigation was restored for seventeen days. On the second cycle, at day 26 of the experiment, the water supply was interrupted for seven days followed by seven days of recovery. On the third cycle, at day 40 of the experiment, the irrigation was ceased for more ten days and irrigated again. Approximately 10 leaf disks of 0.3 cm^2 were harvested per plant to calculate the relative water content (RWC). It was recorded fresh weight (FW) at the same instant the leaf disks were removed from each plant, turgid weight (TW) after the leaf disks were added to test tubes totally covered with distilled water for 3 hours at 25 ± 1 °C and DW after the leaf disks were kept in 80 °C for 72 hours. Each them were used to calculate RWC (RWC = 100 (FW - DW) / (TW - DW)).

3.8 Water use efficiency determination

The data collected for light curves and stomatal opening kinetics were used to calculate $_i$WUE (A/g_s, µmol CO_2 $mmol^{-1}$ H_2O). Only steady state, of A and g_s, under 1000 µmol photons m^{-2} s^{-1} and 400 ppm CO_2 from A-PAR curves and stomatal opening kinetics were used from plants grown in 5 liters pots under greenhouse conditions and in 2.5 liters pots under growth chamber conditions after drought experiments. For the determination of both $_y$WUE (g DW leaves kg^{-1} H_2O transpired) and $_{sl}$WUE (g DW kg^{-1} H_2O transpired), leaves DW and total biomass at the first day of the WPT experiment were estimated. The estimative of leaves DW at day 1 was achieved by linear regression ($R^2 > 0.82$) using the leaf area at the beginning of the experiment. In the estimation of total biomass at day 1, were used these previous leaves DW found in the first day to estimate by using exponential regression ($R^2 > 0.95$). The results of water use efficiency will indicate whether the transgene increased the plant productivity in terms of biomass per water saved.

3.9 Stomatal opening kinetics during dark-to-light transition

Stomatal opening kinetics was performed utilizing fully expanded leaves of 60 day-old plants after drought stress. Leaves were covered with aluminum foil for a few minutes keeping the plants adapted to the dark before measurements. Only four repetitions were performed per day at morning, thus avoiding effects caused by circadian rhythms on the measurements. Gas exchange was recorded every 10 sec for 300 sec in the dark and for 3200

sec under 1000 µmol photons m^{-2} s^{-1}, 400 ppm CO_2 and block temperature 28 °C using IRGA (LiCor 6400XT, Lincoln, NE, USA). Steady state of A and g_s were achieved by averaging the last 10 points at light condition. These measurements were used to calculate the maximum slope (Sl_{max}) of g_s, response to dark-light transition, and half-time ($t_{50\%}$) needed to reach g_s steady state (ELLIOTT-KINGSTON et al., 2016; HAWORTH et al., 2018; LIMA et al., 2019; MCAUSLAND et al., 2016). Basically, the stomatal opening kinetics help us to quantify the speed of stomatal movements.

3.10 Stomatal density and fresh weight loss in detached leaves

It was determined stomatal density of abaxial and adaxial leaf surfaces of 45 days-old plants cultivated in substrate under greenhouse conditions as previously described (DALOSO et al., 2016). The stomatal density was performed in order to observe whether changes in transpiration behavior would be caused by alterations in the number of stomata per leaf surface area. Colorless nail polishwas applied on both surfaces of full expanded leaves and leave it to dry. The nail polish was removed with clear adhesive tape and placed on a microscope slide. Epidermis photos were taken on optical microscopy and stomatal density (Number of stomata mm^{-2}) was determined utilizing ANATI QUANTI 2.0 software (UFV, Viçosa, MG, Brazil) (AGUIAR et al., 2007). Determination of fresh mass loss in detached leaves was performed by measuring leaf weight every 10 minutes for 2 hours, in order to evaluate the mutation interference on stomatal closured induced by leaf detachment. The result is demonstrated as % of leaf weight loss (water loss) over time relative to its initial weight.

3.11 Isolation of guard cell-enriched epidermal fragments

The isolation of guard cell-enriched epidermal fragments was realized adapting the protocol from previous work (KRUSE; TALLMAN; ZEIGER, 1989). This methodology results in epidermal fragments where at least 95% of the intact cells present are guard cells (ANTUNES et al., 2017). This was performed in 60 days-old plants of all genotypes cultivated in hydroponic system and 5 liters pots under greenhouse conditions. Samples from 5 liters pots were collected at morning and used for PCR in gene expression analysis. Samples from hydroponic system were harvested at dawn between 3-5 a.m. (dark) and at morning between 7-9 a.m. (light) and used to extract metabolites. The preparation was performed using expanded leaves of all genotypes from which main and secondary nervures were excised out and blended in warring

blender (Phillips-Wallita, model RI 2044) with internal filter in distilled water for 1 minute and 4 pulses of 5 seconds at half power. After, the solution containing guard cell-enriched epidermal fragments was filtered in 200 μm nylon membrane and the retained was rinsed vigorously with distilled water to remove the rest of ruptured mesophyll cells and apoplastic content. This retained was immediately frozen in liquid nitrogen and stored -80 °C freezer.

3.12 Extraction and analysis of leaf and epidermal fragments metabolites using GC-TOF-MS

Guard cell-enriched epidermal fragments and leaf samples from genotypes L3, L13 and WT cultivated in hydroponic system collected in dark and light period were used frozen to metabolite extraction. The line L11 was not used due to the fact that its results do not presented a strong phenotype caused by the transgene. The extractions were performed using ~50 mg of grinded leaf powder and ~200 mg of grinded epidermal fragments powder. The frozen powder was shaken for 15 minutes at 350 rpm and 70 °C with methanol containing 0.2 mg ml^{-1} of ribitol as internal quantitative standard. After 10 min centrifugation at 11000 g, the supernatant was collected and added to a new tube with water and chloroform. After 15 min centrifugation at 11000 g, the polar phase was separated and collected 150 μl and 1000 μl for leaf and epidermal fragments samples, respectively. Each extracted sample was dried in a vacuum concentrator to derivatization and analyzed by gas chromatography coupled to *time of flight* mass spectrometry (GC-TOF-MS) (LISEC et al., 2006). The resulting chromatogram and mass spectral analysis were evaluated using the TagFinder software (LUEDEMANN et al., 2008).

3.13 Statistical analyses

All data shown are the mean of four or five replicates ± standard error (SE), as indicated in the legend of each figure. The transgenic lines were statistically compared with WT using Student's *t*-test at 5% of probability ($P < 0.05$). These statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) or SIGMAPLOT 14 (Systat Software Inc., San Jose, CA, USA). The metabolomics data were analyzed using the MetaboAnalyst platform (CHONG et al., 2018) to run multivariate analysis such as partial least square-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (orthoPLS-DA). All data subjected to multivariate analysis were transformed to log and had its mean-centered and divided by the standard deviation of each variable using auto-

scaling mode of the MetaboAnalyst platform. These analyses were performed to reduce the scale variability of the dataset (XIA; WISHART, 2011).

4 *SUCROSE SYNTHASE* 2 REGULATES WHOLE PLANT TRANSPIRATION IN *Nicotiana tabacum* L.

(Unpublished manuscript)

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Summary

Guard cell carbohydrate metabolism is key for the regulation of stomatal movements. The sucrose synthase 3 gene (*SUS3*) is the most expressed SUS isoform in potato and Arabidopsis guard cells and encodes an enzyme that works on both sucrose synthesis and breakdown direction. Previous results showed that plants overexpressing *SUS3* specifically in guard cells had higher stomatal conductance, transpiration rate, and growth. We thus hypothesize that silencing guard cell *SUS3* results in reduction of stomatal conductance and possibly higher water use efficiency (WUE) and drought tolerance. Here, we characterized *Nicotiana tabacum* plants antisense for the potato *SUS3* gene under the control of guard cell-specific KST1 promoter. Phylogenetic analysis using amino acid sequences of all SUS isoforms demonstrated that potato

SUS3 (*StSUS3*) is equivalent to tobacco *SUS2* (*NtSUS2*). Slight reduction in guard cell *NtSUS2* expression decreased whole plant transpiration, stomatal conductance and biomass accumulation and increased yield WUE ($_{y}$WUE), defined as the ratio between yield production and water transpired over time. *NtSUS2* silencing altered the metabolite profiling of guard cells and leaves. Furthermore, the transgenic lines showed drought avoidance characteristics under successive drought stress cycles. Our results revealed that *NtSUS2* is a key regulator of whole plant transpiration and strengthen the idea that engineering guard cell metabolism is an effective strategy to decrease plant water consumption and to improve plant WUE.

Keywords: Saccharolytic activity; drought stress; stomatal movement regulation; water use efficiency.

Introduction

It has been estimated that water demand for agricultural production will increase by 17% by 2025, mostly due the increase in the average global temperature and the fact that drought episodes will become more frequent according to the predicted climate change scenario (PENNISI, 2008). It is urgently needed therefore to find strategies to improve plant water use efficiency (WUE), which is defined as the ratio between the amount of accumulated biomass per unit of water used at crop ($_{c}$WUE), whole plant or leaf level (BACON, 2004; BLUM, 2005; CONDON et al., 2004; FLEXAS et al., 2010; MEDRANO et al., 2015). $_{c}$WUE is related to the yield and the water used during the whole cultivation period (PASSIOURA, 2006). At whole plant level the ratio between total accumulated biomass to water used or transpired is defined as season-long water use efficiency ($_{sl}$WUE) and the ratio between individual yield production

(harvestable) to water used or transpired over a certain period of time ($_{y}$WUE) (MEDRANO et al., 2010). At leaf level, gas exchange analysis is taken into account to determine instantaneous water use efficiency ($_{L}$WUE - ratio between net photosynthetic rate (*A*) and transpiration rate (*E*)) or intrinsic water use efficiency ($_{i}$WUE - ratio between *A* and stomatal conductance (g_s)) (CONDON et al., 2002; FLEXAS et al., 2013). Given that WUE depends fundamentally on the gain of carbon (C) and the water loss by the transpiration process, it is reasonable to assume that stomata is the master regulator of WUE (BRODRIBB; SUSSMILCH; MCADAM, 2019). However, the key regulators of plant transpiration remain elusive.

Plant scientists are looking to understand the dynamic of plant cells under increasing global environmental changes in order to improve plant growth and plant stress tolerance through genetic manipulation of plant metabolism. However, plants are controlled by modulation of complex regulatory networks acting at different spatial and temporal scales, which makes difficult to understand plant cell functioning, specially under adverse conditions, and to find important biotechnological targets (BERTOLLI; MAZZAFERA; SOUZA, 2014; SOUZA; DE OLIVEIRA; CARDOSO, 2004). In this context, although WUE measurements under water deficit conditions are particularly useful to unveil potential drought stress tolerant plants, it is important to highlight that WUE improvement is not always associated to increased drought tolerance, and *vice versa*. This is partially due the fact that with the increase of temperature and vapor pressure deficit (VPD) under drought, plants tend to transpire more and thus consume more water. Thus, although there are multiple ways to improve WUE (SINGH et al., 2012), one way to decrease plant water consumption is by genetic manipulation of key regulator(s) of plant transpiration, which is presumably located at the stomata (FLEXAS, 2016; GAGO et al., 2014).

Stomata are leaf epidermal structures consisting of two guard cells that surround a pore. Guard cells are highly specialized and integrate endogenous and environmental signals such as plant water status, hormonal stimulus, light quantity and quality, CO_2 concentration, air humidity, among other conditions, to regulate stomatal opening (LAWSON et al., 2014; MATTHEWS; VIALET-CHABRAND; LAWSON, 2017). During the stomatal opening, there is an increase in guard cell volume due to the influx and/or intracellular generation of solutes, favoring water entrance into the cell and consequently driving stomata to open (MEDEIROS et al., 2015). The control of stomatal opening involves thus reversible changes in the concentrations of osmotically active solutes within guard cells, such as potassium (K^+), malate ($malate^{-2}$), chloride (Cl^-) and nitrate (NO_3^-) (EISENACH; DE ANGELI, 2017; SHIMAZAKI et al., 2007). By contrast, stomatal closure has been closely associated to hormonal stimulus, in

which abscisic acid (ABA) plays a central role (ROELFSEMA; HEDRICH, 2005). Environmental cues such as dark and high CO_2 concentration close stomata while light and low CO_2 concentration stimulate its opening (ENGINEER et al., 2016). Thus, stomatal movements follows a circadian rhythm according to external and endogenous stimulus (GORTON; WILLIAMS; ASSMANN, 1993; HUBBARD; WEBB, 2015), which is pivotal to sustain the demand of CO_2 for photosynthesis and to close the stomata in periods of high photosynthetic rate (MESSINGER; BUCKLEY; MOTT, 2006). Thus, stomatal movements is closely linked to the mesophyll photosynthetic activity, in which the transport of mesophyll-derived metabolites such as sucrose and malate and their import into guard cells seems to be key for stomatal movement regulation (DALOSO; DOS ANJOS; FERNIE, 2016; LAWSON et al., 2014; LIMA et al., 2018, 2019).

Several studies indicate the importance of carbohydrate metabolism in the regulation of stomatal movements (ANTUNES et al., 2012, 2017; DALOSO et al., 2016; KELLY et al., 2013, 2019; KOPKA; PROVART; MULLER-ROBER, 1997; LUGASSI et al., 2015; OUTLAW; DE VLIEGHERE-HE, 2001; STADLER et al., 2003; TALBOTT; ZEIGER, 1998; WANG et al., 2019). Recent studies suggest that the degradation of sucrose within guard cell is an important mechanism to provide substrate for Glutamine (Gln) biosynthesis during light-induced stomatal opening (DALOSO et al., 2015; MEDEIROS et al., 2018; ROBAINA-ESTÉVEZ et al., 2017). Furthermore, it has been shown that plants overexpressing either *hexokinase* or *sucrose synthase 3* (*SUS3*) specifically in guard cells altered stomatal movements (DALOSO et al., 2016; KELLY et al., 2013, 2019; LUGASSI et al., 2015). These results suggest that sugar breakdown within guard cells plays a major role in the regulation of stomatal movements. More importantly, these results highlight the energetic importance of sucrose metabolism to sustain stomatal opening and refute the previous idea that sucrose would act only as an osmolyte within guard cells (AMODEO; TALBOTT; ZEIGER, 1996; TALBOTT; ZEIGER, 1996, 1998). Furthermore, these studies indicate that genetic manipulation of guard cell carbohydrate metabolism is an important approach to improve drought tolerance and/or WUE. For instance, transgenic plants with increased expression of *hexokinase* within guard cells have increased WUE and drought tolerance (KELLY et al., 2019), and antisense construction to sucrose transporter (*SUT1*) lead to plants with higher WUE, associated to reduced guard cell sucrose content and lower g_s compared with WT plants (ANTUNES et al., 2017).

Sucrose metabolism is important not only for the regulation of guard cells but mainly to the overall C distribution throughout the plant. In the cytosol of plant cells, sucrose is

degraded into hexoses by different invertase (INV) and sucrose synthase (SUS) isoforms (BIENIAWSKA et al., 2007; NGUYEN-QUOC; FOYER, 2001). The SUS-catalyzed reaction to sucrose breakdown is energetically more favorable than INV, because in this reaction it converts sucrose and UDP to fructose and UDP-glucose (GEIGENBERGER; STITT, 1993). The number and the expression of SUS isoforms vary among species and the organs, respectively. In tobacco (*Nicotiana tabacum* L.), there are seven SUS isoforms (*NtSUS1-7*), and isoforms 2 and 3 are the most abundant in mature leaves (WANG et al., 2015). In Arabidopsis, the expression of *SUS3* is nearly specifically to guard cells (DALOSO; DOS ANJOS; FERNIE, 2016), similar to the observed in potato (*Solanum tuberosum* L.) (KOPKA; PROVART; MULLER-ROBER, 1997), suggesting a specific role of this isoform in stomatal movements. Indeed, genetic manipulation of potato *SUS3* substantially altered g_s (ANTUNES et al., 2012; DALOSO et al., 2016; NI, 2012). Here, we aim to contribute to a better understanding of the role of sucrose metabolism in guard cell by the characterization of tobacco transgenic plants antisense for the potato *SUS3* gene under control of KST1 promoter. We hypothesize that silencing the *SUS3* gene within tobacco guard cell results in reduction of g_s and transpiration with possibly higher WUE and drought tolerance. Our results are discussed in the context of stomatal movement regulation and manipulation of sucrose metabolism in guard cell as a feasible strategy on the development of less water consuming plants.

Material and methods

Plant material and growth conditions

In this study, we used the commercial *Nicotiana tabacum* L. cv Havana 425 wild type (WT) and transgenic plants antisense to *sucrose synthase 3* gene (*SUS3*) under control of the KST1 promoter (X79779), that drives the expression specifically to guard cells (KELLY et al., 2017; MÜLLER-RÖBER et al., 1995; PLESCH; EHRHARDT; MUELLER-ROEBER, 2001). The transformation was realized by sub-cloning the *SUS3* gene from *Solanum tuberosum* (*StSUS3*) (STU24088) into the pBinK plasmid vector, which derivates from pBinAR-Kan (HÖFGEN; WILLMITZER, 1990) and had the CaMV-35S promoter replaced by the KST1 promoter. A 1567 pb fragment was obtained by the digestion of *SUS3* gene using the KnpI restriction enzyme (ANTUNES et al., 2012), which was cloned in the antisense direction in the pBinK vector, between KST1 promoter and OCS terminator (Figure 1A). This construction was inserted into *Agrobacterium tumefasciens* (Strain GV 3101) by electroporation and cultivated

in suspension with leaf discs from tobacco to achieve the plant transformation. The transformation was confirmed by PCR of the marker gene *NPTII* that confers kanamycin resistance (Figure S1).

Transgenic seeds of lines L3, L11 and L13 of T_3 generation were obtained and germinated *in vitro*. These lines were chosen based on preliminary results of transpiration and leaf temperature (data not shown). All seeds were surface-decontaminated by shaking in 70% ethanol for 1 min and rinsed with sterile distilled H_2O. In sequence, seeds were treated with 0.2% sodium hypochlorite for 5 min and rinsed two times with sterile distilled H_2O. The seeds were then allowed to germinate in Petri dishes containing Murashige and Skoog (MS) medium (MURASHIGE; SKOOG, 1962) with addition of 50 µM of Kanamycin® (KAN) for the transgenic lines and cultivated *in vitro* for 30 days under growth chamber, 16 h:8 h, light:dark photoperiod, photosynthetic photon flux density (PPFD) of 100 µmol photons m^{-2} s^{-1}, 25 ± 1 °C and relative humidity 53 ± 5%. Seedlings showing KAN resistance (Figure S2) were transferred to 0.1 L pots with substrate composed by a mixture of vermiculite, sand and soil (1:1:1) and kept well-watered for 15 days under greenhouse conditions with natural 12 h light photoperiod, maximum PPFD of 500 µmol photons m^{-2} s^{-1}, 30 ± 4 °C and relative humidity 62 ± 10%. Subsequently, plants were dived in three groups. The first group was transferred to hydroponic system using Hoagland and Arnon nutritive solution in 2.5 L pots (HOAGLAND; ARNON, 1950) which was changed weekly and these plants used only for the isolation of guard cell-enriched epidermal fragments. The second group was transferred to 5.0 L pots containing the same substrate mentioned above. The first and second groups were cultivated under greenhouse conditions. The third group was cultivated into 2.5 L pots with the same substrate under growth chamber conditions. The plants grown on substrate were irrigated with Hoagland and Arnon nutritive solution three times per week. All plants were cultivated during 45 or 60 days until the beginning of each experiment described below.

Gene expression and phylogenetic similarity analyses

Total RNA from guard cells and leaves of 45 days-old plants grown in substrate under greenhouse conditions were isolated and the cDNA synthesized using SV Total RNA Isolation System and M-MLV Reverse Transcriptase (Promega Corporation, Madison, Wisconsin, EUA), accordingly to the manufacturer instructions. Total RNA was quantified (Epoch Microplate Spectrophotometer, BioTek Instruments Inc., Winooski, Vermont, EUA) and its integrity checked in 50 µg ml^{-1} ethidium bromide stained 1.5% agarose gel. The primer for

protein phosphatase 2A (*PP2A*) was designed by aligning the coding sequence (CDS) obtained from NCBI database using the muscle tool in Mega X software (FW,5'-CACTTCAGTCAATTGATAACGTC-3'; REV,5'-GCAAAATCCTACCAAAGAGGG-3'). The primers used to investigate NtSUS expression were obtained from previous work (WANG et al., 2015) (FW,5'- CACATTGATCCATACCACGGGGAT-3'; REV,5'-ACAGCAGCCAGTGTCAACAACCGA-3'). In order to estimate *SUS* silencing, qRT-PCR was performed by using GoTaq® qPCR Master Mix (Promega Corporation, Madison, Wisconsin, EUA) accordingly to the manufacturer instructions. The qRT-PCR was performed accordingly the following program: initial denaturation (95 ºC, 5 min), 40 x 3 steps cycle (denaturation 95 ºC 15 sec; annealing 56.3, 60.0 or 68.1 ºC 15 sec; elongation 60 ºC 20 sec) and final elongation (60 ºC, 2 min). Relative transcripts expression was calculated by the $2^{-\Delta\Delta Ct}$ method, in which *PP2A* was used as internal control and WT was used as calibrator (LIVAK; SCHMITTGEN, 2001).

Amino acids sequences from previous work (WANG et al., 2015) were used as query sequence on tblastn from NCBI database to found CDS of *NtSUS1-7* (accession numbers: XM_016618980.1; XM_016595403.1; XM_016627888.1; XM_016610900.1; XM_016608012.1; XM_016585183.1; XM_016611295.1). Multiple sequence alignment was performed using ClustalW software (Kyoto University Bioinformatics Center, Kyoto, Japan) utilizing CDS of *NtSUS1-7* and *StSUS3* (accession number: AY205084.1) to build a phylogenetic tree by FastTree tool (PRICE; DEHAL; ARKIN, 2009).

Photosynthetic light and CO_2 response curves

Photosynthetic response curves were performed in completely expanded leaves from 45 days-old plants of all genotypes cultivated in substrate under greenhouse conditions using a portable infrared gas analyzer (LiCor 6400XT, Lincoln, NE, USA). Photosynthetic light response curves (*A*-PPFD) was measured using light intensities from high to low, in order: 2000, 1500, 1000, 800, 600, 400, 200, 100, 50 and 0 μmol photons m^{-2} s^{-1}, using 10% of blue light to optimize stomatal opening (FLEXAS et al., 2008). For this measurement, *A* (μmol CO_2 m^{-2} s^{-1}) was recorded in each light intensity when steady state was reached at constant 400 ppm CO_2 and block temperature at 28 °C. Photosynthetic CO_2 response curves (*A*-C_i) was achieved using several CO_2 concentrations, in order: 400, 300, 200, 100, 50, 400, 500, 700, 1000, 1200 and 400 ppm of CO_2. Gas exchange was recorded for each CO_2 concentration and constant 1000 μmol photons m^{-2} s^{-1} when steady state was reached.

Both curves were constructed using SIGMAPLOT 14 (Systat Software Inc., San Jose, CA, USA). A three component exponential rise to maximum equation was used to fit the curves: $A = a (1 - e^{-bx}) + c$, where A = photosynthetic rate, x = PPFD, and a, b, c are parameters estimated by the non-linear regression (WATLING; PRESS; QUICK, 2000).

Whole plant transpiration and growth analysis

Whole plant transpiration (WPT) was determined by using a gravimetric methodology (DALOSO et al., 2016). WPT was determined in 45 days-old plants cultivated in substrate under greenhouse and growth chamber conditions. Pots without plants were used to estimate evapotranspiration of the soil. The pots were filled with water at beginning of the night almost in a daily basis, as shown in the legend of the figures. In the next day, the pots were weighted at predawn and at the end of the day. The WPT was then obtained by the difference between the two weights and by subtracting the evapotranspiration of the pots without plants (g H_2O d^{-1} $plant^{-1}$). WPT was recorded between different time intervals in a diel course as well as in a daily basis for eleven days and forty six days.

Leaves with more than 5 cm length were used to estimate leaf area (LA) through a model described previously (ANTUNES et al., 2008, 2017). This model takes into account the length (L) and maximum width (W) of the leaves, which were obtained by measuring from the lamina tip to the point of the petiole intersection along the central nervure and by measuring at the widest point perpendicular to the central nervure, respectively. These measurements were recorded during the WPT experiment in greenhouse in the first, middle and last day of experiment and in growth chamber at days 1, 3 and 5, the days in between the data were estimated by linear regression ($r^2 > 0.98$). The leaf area (cm^2) was used to calculate specific leaf area (cm^2 g^{-1} leaf DW) as well as to estimate WPT per leaf area (mg H_2O d^{-1} cm^{-2}). At the end of WPT experiments, it was determined the total leaf number (> 5cm length), stem length, dry weigh (DW) of leaf, stem and roots by drying at 80 °C for 7 days. These parameters were used to determined total biomass DW (leaf DW + stem DW + roots DW), harvest index (leaf DW / total biomass), shoot DW (leaf DW + stem DW), shoot DW / root DW (g g^{-1}), leaves DW / root DW (g g^{-1}) and plant leaf area / roots DW (cm^2 g^{-1}). The relative growth rate (RGR, g g^{-1} day^{-1}) was obtained by the equation: RGR = ln (final weight) – ln (initial weight) / final day – initial day.

Drought stress experiment

The drought stress experiment was performed in 45 days-old plants cultivated under growth chamber conditions. This experiment was composed by three drought cycles. On the first cycle, the irrigation was interrupted at the day 4 of the experiment for five days, and then irrigation was restored for seventeen days. On the second cycle, at day 26 of the experiment, the water supply was interrupted for seven days followed by seven days of recovery. On the third cycle, at day 40 of the experiment, the irrigation was ceased for more ten days and irrigated again. These cycles were performed in order to analyze recovery effects in hydric stress. The WPT and the relative water content (RWC) was estimated as indicated in the figures. Approximately 10 leaf disks of 0.3 cm^2 were harvested and used to determine fresh (FW), turgid (TW) and dry weight (DW), which were then used to calculate RWC (RWC = 100 (FW - DW) / (TW - DW)).

Determination of plant water use efficiency

Steady state values of A and g_s under 1000 µmol photons m^{-2} s^{-1} and 400 ppm CO_2 from A-PAR curves and stomatal opening kinetics (described below) were used to calculate $_i$WUE (A/g_s, µmol CO_2 $mmol^{-1}$ H_2O). For the determination of both $_y$WUE (g DW leaves kg^{-1} H_2O transpired) and $_{sl}$WUE (g DW kg^{-1} H_2O transpired), leaves DW and total biomass at the first day of the WPT experiment were estimated. The estimative of leaves DW at day 1 was achieved by linear regression ($R^2 > 0.82$) (Figure S3) using the leaf area at the beginning of the experiment. In the estimation of total biomass at day 1, were used these previous leaves DW found in the first day to estimate by using exponential regression ($R^2 > 0.95$).

Stomatal opening kinetics during dark-to-light transition

Stomatal opening kinetics was performed utilizing fully expanded dark-adapted leaves after ten days of the end of the drought stress experiment. In order to avoid circadian effects in the measurements, only four replicates were carried out during the morning period of the day. Gas exchange was recorded every 10 sec for 300 sec in the dark plus 3200 sec under 1000 µmol photons m^{-2} s^{-1}, 400 ppm CO_2 and block temperature 28 °C using IRGA (LiCor 6400XT, Lincoln, NE, USA). Steady state of A and g_s were achieved by averaging the last 10 points at light condition. These measurements were used to calculate the maximum slope (Sl_{max}) of g_s response and half-time ($t_{50\%}$) needed to g_s reach steady state during dark-to-light transition

(ELLIOTT-KINGSTON et al., 2016; HAWORTH et al., 2018; LIMA et al., 2019; MCAUSLAND et al., 2016).

Stomatal density and fresh weight loss in detached leaves

It was determined stomatal density of abaxial and adaxial leaf surfaces of 45 days-old plants cultivated in soil under greenhouse conditions as previously described (DALOSO et al., 2016). Colorless nail polish was applied on both surfaces of full expanded leaves and leave it to dry. The nail polish was removed with clear adhesive tape and placed on a microscope slide. Epidermis photos were taken on optical microscopy and stomatal density (Number of stomata mm^{-2}) was determined utilizing ANATI QUANTI 2.0 software (UFV, Viçosa, MG, Brazil) (AGUIAR et al., 2007). Determination of fresh weight loss in detached leaves was performed by measuring leaf weight every 10 minutes for 2 hours. The result is demonstrated as % of leaf weight loss (water loss) over time relative to its initial weight.

Isolation of guard cell-enriched epidermal fragments

The isolation of guard cell-enriched epidermal fragments was performed adapting the protocol from previous work (KRUSE; TALLMAN; ZEIGER, 1989). This methodology results in epidermal fragments where at least 95% of the intact cells present are guard cells (ANTUNES et al., 2017). This was performed in 60 days-old plants cultivated under hydroponic system under greenhouse conditions. Samples were harvested at pre-dawn between 3-5 a.m. (dark) and at morning between 7-9 a.m. (light) and used to extract metabolites. The guard cell-enriched epidermal fragments were obtained from expanded leaves in which main and secondary nervures were excised out and blended in warring blender (Phillips-Wallita, model RI 2044) with internal filter in distilled water for 1 minute and 4 pulses of 5 seconds at half power. After, the solution containing guard cell-enriched epidermal fragments was filtered in 200 μm nylon membrane and the retained was rinsed vigorously with distilled water to remove the rest of ruptured mesophyll cells and apoplastic content. This retained was immediately frozen in liquid nitrogen and stored -80 °C freezer.

Metabolite profiling analysis

Guard cell-enriched epidermal fragments and leaf samples collected in the dark and light during dark-to-light transition were used to metabolite profiling analysis. The extractions were performed using ~50 mg of grinded leaf powder and ~200 mg of grinded epidermal fragments powder. The frozen powder was shaken for 15 minutes at 350 rpm and 70 °C with methanol containing 0.2 mg ml^{-1} of ribitol as internal quantitative standard. After 10 min of centrifugation at 11000 *g*, the supernatant was collected and added to a new tube with water and chloroform. After 15 min of centrifugation at 11000 *g*, the polar phase was separated and collected 150 µl and 1000 µl for leaf and epidermal fragments samples, respectively. Sample were then dried in a vacuum concentrator to derivatization and subsequently analyzed by gas chromatography coupled to *time of flight* mass spectrometry (GC-TOF-MS) (LISEC et al., 2006). The resulting chromatogram and mass spectral analysis were evaluated using the TagFinder software (LUEDEMANN et al., 2008).

Statistical analysis

All data shown are the mean of three to five replicates ± standard error (SE), as indicated in the legend of each figure. Each replicate is an individual plant. The transgenic lines were statistically compared with WT using Student's *t*-test at 5% of probability ($P < 0.05$). These statistical analyses were performed using Microsoft Excel (Microsoft, Redmond, WA, USA) or SIGMAPLOT 14 (Systat Software Inc., San Jose, CA, USA). The metabolomics data were analyzed using the MetaboAnalyst platform (CHONG et al., 2018) to run multivariate analysis such as partial least square-discriminant analysis (PLS-DA) and orthogonal partial least squares-discriminant analysis (orthoPLS-DA). All data subjected to multivariate analysis were transformed to log and had its mean-centered and divided by the standard deviation of each variable using auto-scaling mode of the MetaboAnalyst platform. These analyses were performed to reduce the scale variability of the dataset (XIA; WISHART, 2011).

Results

Phylogenetic similarity among sucrose synthase isoforms from tobacco and potato and its expression in tobacco transgenic plants

Potato *sucrose synthase 3* gene (*StSUS3*) was cloned and inserted in the antisense orientation into tobacco leaves *via* agrobacterium-mediated transformation (Figure 1A). In

order to identify which tobacco SUS (*NtSUS*) isoform corresponds to the *StSUS3*, we first aligned all seven *SUS* CDS sequences from tobacco with *StSUS3*. The phylogenetic tree indicated that *StSUS3* is closest to *NtSUS2* (Figure 1B), as previously shown (WANG et al., 2015). Thus, the isoform that would have its expression decreased in transgenic guard cells is likely to be *NtSUS2*. In fact, qRT-PCR analysis showed that the expression of this isoform decreased by almost 40% in guard cells in lines L3 and L13, whilst no difference in L11 or in leaf gene expression was observed (Figure 1C). These results strengthen the idea that the KST1 promoter drives the expression specifically to guard cells, as previously demonstrated (ANTUNES et al., 2017; DALOSO et al., 2016; KELLY et al., 2017; LUGASSI et al., 2015).

Gas exchange analysis

Photosynthetic light and CO_2 response curves were initially performed to characterize the photosynthesis capacity of the transgenic lines in response to different light intensities and CO_2 concentrations. Among the three transgenic lines, only L3 showed lower net photosynthetic rate (A) compared to WT in both light and CO_2 response curves (Figures S4A,B). By contrast, no difference in A, transpiration rate (E) and dark respiration (R_d) in all transgenic lines and reduced stomatal conductance (g_s) in both L3 and L13 was observed under steady state condition (Figures 2A-D). Also, a positive relationship between the relative *NtSUS2* expression and g_s was observed among WT and the transgenic lines (Figure S5). These data indicate that antisense inhibition of *NtSUS2* did not have strong effect on the photosynthetic capacity, despite the reduction in g_s.

Antisense inhibition of NtSUS2 reduce whole plant transpiration under greenhouse conditions

Given that plant transpiration ranges according to the environmental condition surrounding the leaves (LUGASSI et al., 2015), gas exchange analysis may not provide a clear picture of the transgenic phenotype given that leaf transpiration was carried out under controlled atmosphere at the IRGA leaf chamber. Indeed, WPT have been demonstrated to be highly sensitive to identify plants with altered stomatal behavior (ANTUNES et al., 2017; DALOSO; DOS ANJOS; FERNIE, 2016). We thus have used a gravimetric methodology to investigate WPT in plants grown under non-controlled greenhouse conditions, which varies substantially in terms of light, temperature and humidity throughout the days (Figure S6). Under these

conditions, both L3 and L13 showed significant lower transpiration (up to 44%) compared to WT over eleven days of the experiment (Figures 3A,B). At the days 3, 6 and 11 of the experiment, the plants were not irrigated. Following the interruption of irrigation, WPT did not reduce at the day 3 and slightly reduced at the day 11. At the day 5 of the experiment, plants reached a peak of transpiration, which was associated to a sequence of sunny days (Figure S6). Following this day, a strong reduction in WPT in WT plants was observed (from 194 to 135 g H_2O d^{-1} $plant^{-1}$), whilst the transgenic lines maintain stable this parameter (Figure 3A). At this day, the percentage of WPT was not different among WT and transgenic lines (Figure 3B). Considering the accumulated WPT over the eleven days, L3 and L13 transpired approximately 32% less (893 g H_2O $plant^{-1}$) than the WT (1326 g H_2O $plant^{-1}$) (Figure 3C). Thus, L3 and L13 conserved 416 and 433 g H_2O $plant^{-1}$ at the end of 11 days, respectively (Figure 3C). Given that transpiration also ranges throughout the diel course, we next investigated whether the reduction in WPT is constantly observed over a diel course. Both L3 and L13 showed reduced WPT in all intervals analyzed (Figure S7A), although relatively greater differences in early hours of the photoperiod were observed (Figure S7B). In summary, L3 and L13 transpired far less than WT plants under fluctuating environmental conditions.

For the reason that leaf area and stomatal density also exert great influence on plant transpiration (NILSON; ASSMANN, 2006), we have also determined these parameters and expressed the WPT data per leaf area. The leaf area was initially smaller in L3 and L13 compared to WT. However, after eleven days only L13 leaf area remained smaller (Figure S8). WPT per leaf area was also lower in L3 (28%) and L13 (22%) than WT (Figures 3D,E), which leads to lower accumulated water loss per leaf area (Figure 3F). The differences in WPT are thus not associated to leaf area and also to changes in leaf stomatal density, given that this parameter showed no difference among the genotypes (Figures S8 and S9A). We further investigate whether these differences could be associated to a disrupted stomatal signalling mechanism. For this, we have analyzed water loss on detached leaves and no difference among the genotypes was observed (Figure S9B).

At the end of the experiment, plants were harvested and growth parameters determined. Both L3 and L13 presented reduced accumulated total biomass (Table 1). By contrast, all transgenic lines showed higher % DW leaves, % DW shoots and shoot-to-root and leaves-to-root ratios, although only L3 was statistically different from WT in all these parameters. It is noteworthy that the leaves are the harvestable part of tobacco plants and the harvest index of L3 reached 0.58 g g^{-1}, significantly higher than WT. RGR, total leaf number, specific leaf area and stem length were not significantly altered in the transgenic lines (Table 1). These results

highlight the priority of transgenic lines in allocating carbon resources for the development of the aerial part.

Whole plant transpiration under cycles of drought stress

Given that the transgenic plants have demonstrated great differences under interruption of irrigation compared to WT plants, we next subjected the plants to grown under growth chamber condition under three consecutive cycles of drought. Slight differences in relative water content (RWC) among the genotypes were observed on the second and third cycle (Figure S10). In the first days of the first cycle of drought stress, the transgenic lines behaved similarly to that previously shown in well-watered conditions, in which the WPT was lower in L11 and L13 compared to WT plants (Figures 4A,B). Given the milder conditions of the growth chamber, reductions in WPT were observed only at the day 5 (second day without irrigation). Similar to that observed under greenhouse conditions, WT transpiration dropped (10 g H_2O d^{-1} $plant^{-1}$) whilst transgenic lines tended to transpire slightly more than day 4, with a significant increase of 8 g H_2O d^{-1} $plant^{-1}$ in L13 (Figure 4A). From day 5 to 8, all genotypes decreased transpiration, but the differences among L13 and WT continued until day 8. Interestingly, L11 and L13 transpired 85% and 166% more than WT at the day 8, which highlights the capacity of these plants to keep transpiring despite the low availability of water (Figure 4B). This leads to a higher accumulated WPT in L13 (16.25% more) over the days (Figure 4C). At the day 3, we have also evaluated whether the transpiration ranges through the diel course and no statistical difference was observed among WT and transgenic lines (Figure S11). In the second cycle, L13 transpired about 59% more than WT after four days without irrigation (day 29) (Figure 4B). After seven days under drought stress, all genotypes reduced transpiration, but without significant differences between them. After second recovery, the third drought cycle was initiated and at day 43 all transgenic lines transpired less than WT, with L3 transpiring 11 g H_2O d^{-1} $plant^{-1}$ less (Figure 4A). Interestingly, until four days without water, all genotypes transpired more than WT in the second cycle, but in the third cycle, the opposite behavior was observed. At the third cycle, no visual differences between transgenic lines and WT over ten days of drought were observed (Figure S12). These results contrast with those observed under greenhouse conditions, given that the line L13 lost 48 g H_2O $plant^{-1}$ more accumulated water than WT under growth chamber at the end of drought cycle experiment (Figure 4C).

We next investigated the growth phenotype of the transgenic lines grown under controlled growth chamber condition with drought cycles. The leaf area was initially lower in

L13 compared to WT at day 1 of drought cycle, but there was no difference between the genotypes at day 5 (Figure S13). Also, the transpiration per leaf area followed the same pattern as on greenhouse, in which transgenic lines transpiration is not associated to leaf area (Figure S14). After all drought cycles, the differences in growth between transgenic lines and WT have almost disappeared compared to those previously observed under greenhouse condition. However, some significant differences were observed in L11, such as lower stem DW and higher % DW roots (Table 2). Because the small stem DW, the DW shoots was also significantly reduced in L11 by 49.2%. Overall, the growth parameters of the transgenic lines did not show any difference from WT in the growth chamber after three drought cycles.

Stomatal opening kinetics in response to light

The dynamic of g_s and A was performed in plants that has experienced drought cycles under growth chamber condition. Transgenic lines showed a different pattern of A and g_s in response to light (Figures 5A,B). While WT reached maximum g_s (g_{smax}) at 2000 sec and then decreased until steady state, lines L11 and L13 kept rising and took longer to reach g_{smax}. The L3 g_{smax} was the smallest one but was equal to WT at steady state. By remaining longer with high g_s, L13 reached elevated A than the other genotypes. The highest light-induced stomatal opening velocity was observed in WT, evidenced by its tendency to has higher Sl_{max} and lower $t_{50\%}$ (Figures 6A,B), suggesting that guard cell *NtSUS2* and consequently sucrose breakdown within guard cells is an important mechanism to regulate stomatal speediness.

Changes in water use efficiency

The *NtSUS2* gene silencing had significant impact in $_y$WUE under greenhouse conditions. All transgenic lines reached higher $_y$WUE than those observed in WT, with line L3 presenting 3.47 g kg^{-1}, approximately 0.61 g kg^{-1} more than WT (Table 1). $_{sl}$WUE showed no significant difference between transgenic lines and WT. However, $_i$WUE was lower in L3 than WT (Table 1). Under growth chamber conditions and after drought cycles, slight differences in $_i$WUE between transgenic lines and WT during dark-to-light transition were observed, in which L3 and L13 showed higher values than WT at the first 2700 sec of light (Figure S15). Taken together, these results highlight that the decrease in *NtSUS2* expression leads to increased $_y$WUE under well-watered and fluctuating environmental conditions, which means higher productivity per transpirational water loss.

Metabolic alterations in leaves and guard cells induced by NtSUS2 silencing

The analysis of metabolite profiling was carried out in leaves and guard cell enriched epidermal fragments in order to observe the metabolic changes induced by guard cell *NtSUS2* silencing. This analysis was performed only in lines L3 and L13, since L11 did not show significant differences in whole plant transpiration and stomatal conductance. We construct heat maps to visualize the relative changes in all metabolites in relation to WT (Figure S16A), which presented statistically lower relative changes in the metabolites glycerol, citrate, isocitrate, idoise and glucose in L3 and L13. Also, trehalose was found to have higher relative changes in both transgenic lines compared with WT. Regarding metabolite content, we were able to see the metabolic differences in light compared with dark of each genotype (Figure S16B). Some metabolites significantly increased/decreased from dark to light, such as fructose and glucose, which increased in WT but not in L13 and mannitol and fumarate, which decreased in L13 and L3, respectively, but none were altered in WT (Figure S16B). The data was also subjected to multivariate analysis such as OrthoPLS-DA and PLS-DA. Metabolites with variable importance in projection (VIP) scores more than 1 are the mainly responsible for changes observed in the PLS-DA model. These multivariate analyses are useful to understand metabolomics data, since it can identify the metabolites that contribute most to variation and/or separation. OrthoPLS-DA (Figures 7A,B) and PLS-DA (Figures 8A,B) demonstrated that the relative metabolic changes during dark-to-light transition were significantly different between the transgenic lines and WT. A total of 24 metabolites (e.g. carbohydrates, amino acids and tricarboxylic acid (TCA) cycle intermediates) have VIP score higher than 1, in which six (trehalose, mannitol, glycerol, alanine, lysine and dehydroascorbate) of them were observed in both lines (Figures 8C,D). Several amino acids had low relative content compared with WT (e.g. serine, alanine, methionine, isoleucine). A considerable number of sugars had low relative content in the lines, such as glucose and mannitol. Glycerate also had low relative content in both lines. The sucrose/malate ratio was also higher in lines than WT in dark-to-light transition. Every TCA cycle intermediates (e.g. citrate, aconitate, isocitrate, fumarate) found presented low relative content. Taken together, these differences in relative changes may explain the low transpiration of transgenic lines by low energy availability provided by respiration in guard cells.

Differences were also found in leaf relative changes of transgenic lines compared with WT and metabolite content in light compared with dark, with great variability even between

transgenic lines (Figure S17). Following these results of leaves, we have analyzed relative changes during dark-to-light transition through multivariate analysis (Figures S18 and S19). OrthoPLS-DA and PLS-DA analyzes suggest that the changes in guard cell sucrose metabolism also affected leaf metabolism. Similar to that observed in guard cells, several leaf metabolites with VIP score > 1 can be attributed to the differences found in the two transgenic lines, such as cellobiose, rhamnose, sucrose, glucose, proline and tyamine. On the other hand, this time, the differences are characterized by higher relative metabolite content in the lines than in WT.

Discussion

Sucrose-mediated stomatal movement regulation is related to NtSUS2 expression

Sucrose has long been pointed out as an important molecule that regulates stomatal movements. The SUS enzyme is known as one of the key enzymes that regulate plant sucrose metabolism (BIENIAWSKA et al., 2007), in which SUS3 isoform has been shown to be highly expressed in guard cells (BATES et al., 2012; DALOSO; DOS ANJOS; FERNIE, 2016; KOPKA; PROVART; MULLER-ROBER, 1997). Taking this in consideration, we generated tobacco plants antisense for the *StSUS3* gene under control of KST1 promoter, which reduced the expression of guard cell *NtSUS2* in tobacco guard cells (Figure 1B). Reductions in *NtSUS2* gene expression decreased g_s in both L3 and L13 with minor impact on *A* (Figures 2 and S5). These results are in agreement with those observed in potato plants expressing an antisense construct targeted against *SUS3* (ANTUNES et al., 2012). Here, reduced guard cell *NtSUS2* expression leads to substantial changes in the metabolite profile of guard cells (Figures 7 and 8), most probably due to a decreased sucrolytic capacity of transgenic guard cells, as evidenced by the lower increase in fructose and the lower sucrose/fructose ratio during dark-to-light transition observed in L13 and L3, respectively (Figure S16). In turn, changes in sucrolytic activity of guard cells lead to decreased g_s.

The light-induced stomatal opening is an ATP-dependent process. Given the low photosynthetic capacity (GOTOW; TAYLOR; ZEIGER, 1988; LAWSON, 2009) and the high respiration rate (ARAÚJO et al., 2011; WILLMER; FRICKER, 1996) found in guard cells, it has been increasingly recognized that the mitochondrial metabolism would be the main source of ATP for guard cells (ARAÚJO; NUNES-NESI; FERNIE, 2014; SANTELIA; LAWSON, 2016). This idea is supported by the fact that the C derived from sucrose breakdown is used to sustain mitochondrial and glutamine metabolism (DALOSO et al., 2015; MEDEIROS et al.,

2018) and by other studies that confirm the link between sucrose breakdown and g_s *in planta* by characterizing transgenic plants with altered guard cell sucrolytic activity (ANTUNES et al., 2012, 2017; DALOSO et al., 2016; NI, 2012). Notably, previous studies showed that increased expression of *hexokinase* specifically in guard cells decreased considerably stomatal aperture and g_s in Arabidopsis and citrus (KELLY et al., 2013, 2019; LUGASSI et al., 2015). Furthermore, decreasing the expression of a guard cell plasma membrane sucrose transporter (*SUT1*) led to reduced guard cell sucrose content and lower g_s and net CO_2 assimilation rate compared with WT plants (ANTUNES et al., 2017), whilst the overexpression of acid invertase in potato by cell type-specific expression in guard cells led to increased g_s and photosynthesis (ANTUNES et al., 2012). Taken together, these studies highlight that sucrose breakdown is an important mechanism to sustain the energetic demand of guard cells during stomatal opening.

We postulate that the low substrate availability for glycolysis due to low sucrose degradation capacity in guard cells resulted in a decreased content of amino acids and TCA cycle intermediates in the transgenic guard cells (Figures 8 and S16). This in turn has several consequences for g_s regulation given that organic acids are key for regulation of stomatal movements (ARAÚJO et al., 2012; FERNIE; MARTINOIA, 2009; NUNES-NESI et al., 2007). Thus, our results strengthen the idea that the guard cell sucrose metabolism is closely linked to organic acids biosynthesis and is key for the regulation of g_s and consequently plant transpiration.

NtSUS2 regulates whole plant transpiration

Beyond the surrounding environment, plant transpiration rate depends on stomatal movements, stomatal density and leaf area (FRANKS; BEERLING, 2009; HETHERINGTON; WOODWARD, 2003; VERTESSY et al., 1995). Plants with closer stomata, lower stomatal density and smaller leaf area generally transpire less (PUTNIK-DELIC et al., 2019). Thus, identifying the mechanisms that regulates stomatal movements and stomatal development is pivotal for molecular breeding toward WUE improvement. In this context, the mechanisms that regulates stomatal development have been identified (BERGMANN; SACK, 2007; CHATER et al., 2017; TORII et al., 2007; ZOULIAS et al., 2018). However, the key regulators of WPT have remained elusive. Here, we demonstrated that *NtSUS2* is pivotal for WPT regulation. Transgenic lines with reduced expression of *NtSUS2* have strong reductions in WPT. In certain days, both L3 and L13 lost 40% less water than WT plants (Figures 3A-C), meaning that the transgenic lines conserved up to 44% water compared to WT. No change in the number of

stomata per leaf was observed (Figure S9A), demonstrating that the lower transpiration of transgenic lines was not influenced by changes in stomatal density. Furthermore, although leaf area of L3 was smaller than WT in certain days (Figure S8), transpiration per leaf area remained significantly lower than WT (Figures 3D-F), indicating that the smallest leaf area was also not the cause of the decreased transpiration. It is interesting to note that with less transpiration, the transgenic lines conserved up to 44% water compared to WT.

It has been shown that plants behave differently according to different drought cycles (MENEZES-SILVA et al., 2017), in which priming mechanisms and a hypothetical biochemical memory would anticipate a new stress period by inducing genetic and biochemical mechanisms responsible for plant stress acclimation (ALVES DE FREITAS GUEDES et al., 2019). It has been postulated that such stress memory is a physiological adjustment that helps the plant to overcome successive drought periods (FLETA-SORIANO; MUNNÉ-BOSCH, 2016). Evaluating drought cycles are thus a great strategy to investigate plant stress acclimation (CYRIAC et al., 2018; FLEISHER et al., 2014; SAINT PIERRE et al., 2012). Here, the experiment in which drought cycles were imposed on the plants showed exactly this stress memory pattern reflected in changes of transpiration behavior in WT plants (Figure 4). In short recovery periods (7 days), WT plants adjusted the transpiration to transpire less under drought conditions. However, given its water conservative phenotype, the transgenic lines do not change the transpiration behavior. By contrast, in longer recovery periods of (17 days), the WT seems to lose the drought memory and quickly depletes the water in the soil, while the transgenic lines remain transpiring less. Interestingly, at the end of the drought cycles, the transgenic lines lost more water than WT, but no differences in the RWC between WT and transgenic lines was observed (Figure S10). The fact that transgenic plants transpire more in drought leads to a greater effective use of water (EUW) that aims to optimize the water consumption by the plant, which is the opposite of WUE (BLUM, 2009). Some authors believe that increased production under limited water conditions should be reached by high EUW and not high WUE (BLUM, 2009; POLANIA et al., 2016). Given the water conservative phenotype of the transgenic lines, it is reasonable to hypothesize that the constitutive lower WPT leads to less water used from soil, which thus enable the plants to transpire more under drought episodes.

Guard cell metabolic engineering toward water use efficiency improvement

Plant scientists are looking to understand the dynamic of plant cells under increasing global environmental changes in order to improve yield, WUE and/or stress tolerance through

genetic manipulation of plant metabolism. This is extremely important given that the current scenario of climate changes is likely to lead to increased incidence of a number of stresses including drought, temperature extremes and flooding that are in turn expected to reduce plant productivity. Plant stress responses represent therefore a high priority area in plant science research. In this context, several strategies have been adopted to improve WUE, in which stomata has received great attention. The major challenge to improve WUE by changing stomatal movements is the direct effect on photosynthesis. When transpiration is decreased by lowering g_s, it also commonly leads to decreased photosynthesis (ARAÚJO et al., 2011; FLEXAS et al., 2013; FRANKS et al., 2015). For this reason, different non-stomatal approaches have been also used to increase WUE, such as mesophyll conductance and those involved with photosynthetic capacity such as rubisco and light use efficiency (GŁOWACKA et al., 2018; SIMKIN et al., 2015). Nevertheless, it is important to highlight that guard cell metabolic engineering is still a possible approach to be used for WUE improvement without strongly impacting photosynthesis. For instance, the expression of a synthetic light-induced K^+ channel in Arabidopsis guard cells accelerated the stomatal opening in light with increased biomass without impairment of water use (PAPANATSIOU et al., 2019). Furthermore, increasing *hexokinase* expression within tomato and Arabidopsis guard cells reduced transpiration and elevated the WUE (KELLY et al., 2019). The expression of maize NADP-malic enzyme in guard cells and companion cells of tobacco plants resulted in reduced stomatal aperture and enhanced WUE, along with increased net CO_2 fixation rate (MÜLLER et al., 2018). Similarly, here we demonstrate that reducing guard cell *NtSUS2* expression leads to a water conservative phenotype without severe impact on photosynthesis which leads to a higher $_y$WUE in the transgenic plants.

The stomatal opening kinetics experiment (Figures 5B and 6) evidenced lower stomatal velocity response of transgenic lines, which were slightly slower than WT after drought cycles. The rapidity of g_s is influenced by the anatomy, structure and biochemistry of the stomata (LAWSON; VIALET-CHABRAND, 2019) and can contribute to $_i$WUE (MCAUSLAND et al., 2016). Given that sucrose breakdown increase the glycolytic fluxes toward mitochondrial metabolism in guard cells during dark-to-light transition (DALOSO et al., 2015; MEDEIROS et al., 2018) and that this response is exacerbated in tobacco transgenic lines overexpressing *StSUS3* (DALOSO et al., 2016), it seems likely that antisense inhibition of *NtSUS2* (*StSUS3*) reduce the amount of substrate for glycolysis, which in turn would lead to lower H^+-ATPase activity due to lower mitochondrial ATP production. It is well-known that H^+-ATPase is key to induce stomatal opening (ANDO; KINOSHITA, 2018; KINOSHITA; DOI; SUETSUGU,

2001; TOMINAGA; KINOSHITA; SHIMAZAKI, 2001). Light-induced stomatal opening is delayed in mutants with defected triacylglycerol catabolism due to low supply of ATP for H^+-ATPase (MCLACHLAN et al., 2016). Recently, it has been shown that guard cell starch degradation plays an important role in providing ATP for blue light-induced stomatal opening (HORRER et al., 2016). The overexpression of H^+-ATPase in Arabidopsis increased the stomatal response with g_s reaching higher values and lower WUE than WT when increase of light intensities (WANG et al., 2014). Thus, it is reasonable to suggest that a low H^+-ATPase activity in our transgenic lines would decrease stomatal opening speed and limit transpiration. However, there is no evidence in literature that connects sucrose breakdown and H^+-ATPase activity; a topic that deserves special attention in future studies. Notably, the *NtSUS2* gene appears to be linked to stomatal opening speediness but probably not to stomatal closure, given that no differences in stomatal closure in detached leaves were observed (Figure S9B). It has been shown that mutants of the ABA-signaling pathway are unable to close their stomata in detached leaves (LEUNG; MERLOT; GIRAUDAT, 1997; SCHROEDER et al., 2001) and the decrease in WUE is the expected effect (GONZALEZ-GUZMAN et al., 2012). Thus, the fact that transgenic stomata take longer to close during drought periods is probably not involved with an ABA signaling mechanism.

Here, decreased saccharolytic activity of guard cells by inhibition of *NtSUS2* expression increased $_y$WUE, which led to higher productivity in relation to water consumed by the plant (Table 2). The small g_s values of transgenic lines influenced the $_i$WUE in greenhouse conditions, with line L3 showing lower $_i$WUE than WT (Table 1). However, the line L13 kept $_i$WUE similar to WT, even though it transpired less. Most studies use $_i$WUE as a measurement factor in water use efficiency and extrapolate to the entire plant (DA SILVA BRANCO et al., 2017; FLEXAS et al., 2010; TOMÁS et al., 2012), but leaf level values do not always represent what happens in whole plant level (MEDRANO et al., 2015; PONI et al., 2009). This can be seen on the line L3, which, despite the low $_i$WUE, achieved significantly higher $_y$WUE than WT as a result of its high harvest index. These results confirm that changing guard cell metabolism may be an effective option for increasing WUE.

Concluding remarks

Tobacco transgenic plants with antisense inhibition of the guard cell *NtSUS2* had decreased stomatal conductance, whole plant transpiration and increased $_y$WUE, presenting a drought avoidance phenotype. Our results indicate that *NtSUS2* is a key regulator of guard cell

metabolism and whole plant transpiration and strengthen the idea that engineering guard cell metabolism is a promising strategy to decrease crop water consumption and increase WUE.

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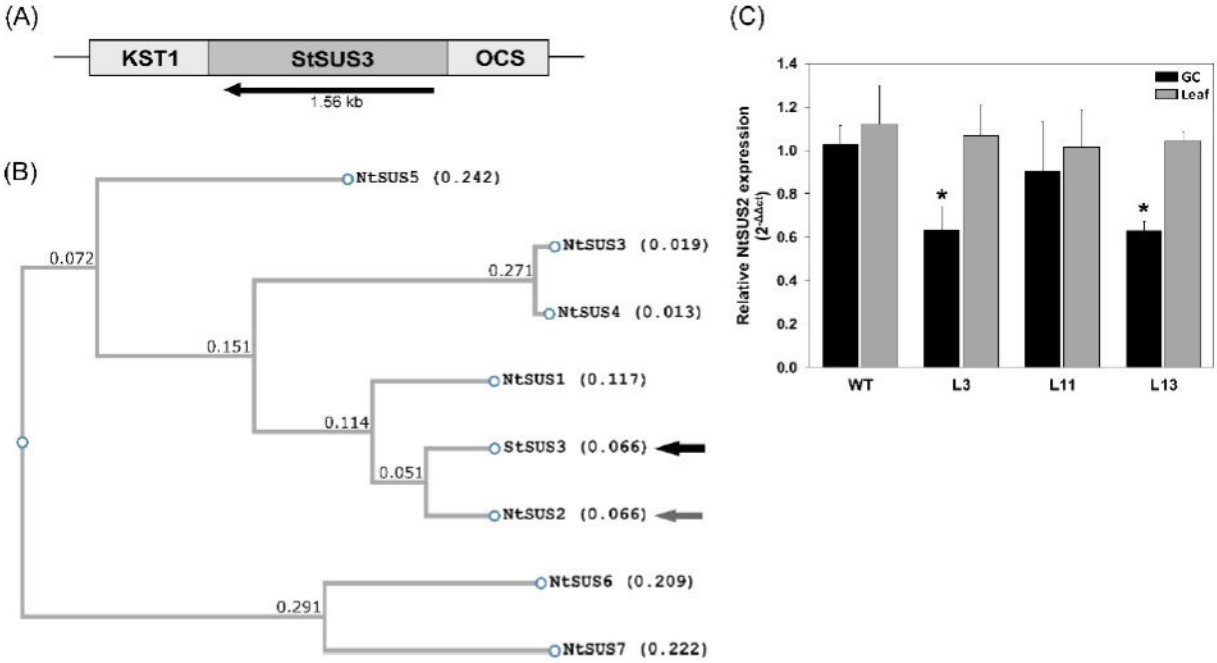

Figure 1. Initial characterization of *Nicotiana tabacum* transgenic lines antisense for the potato *SUS3* gene under control of KST1 promoter. (A) Construction used in this study with KST1 promoter, potato *SUS3* isoform (antisense direction) and OCS terminator. (B) Phylogenetic tree of seven tobacco SUS isoforms (*NtSUS1-7*) (*NtSUS2,* grey arrow) against potato SUS isoform 3 (*StSUS3,* black arrow). (C) Gene expression in leaves and guard cell-enriched epidermal fragments of wild-type (WT) and transgenic lines (L3, L11, L13) plants grown under greenhouse conditions. *NtSUS2* gene expression was analyzed by qRT-PCR normalized by protein phosphatase 2A (*PP2A*) gene expression as internal control. Data are shown as relative expression normalized to WT (n = 3 ± SE). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

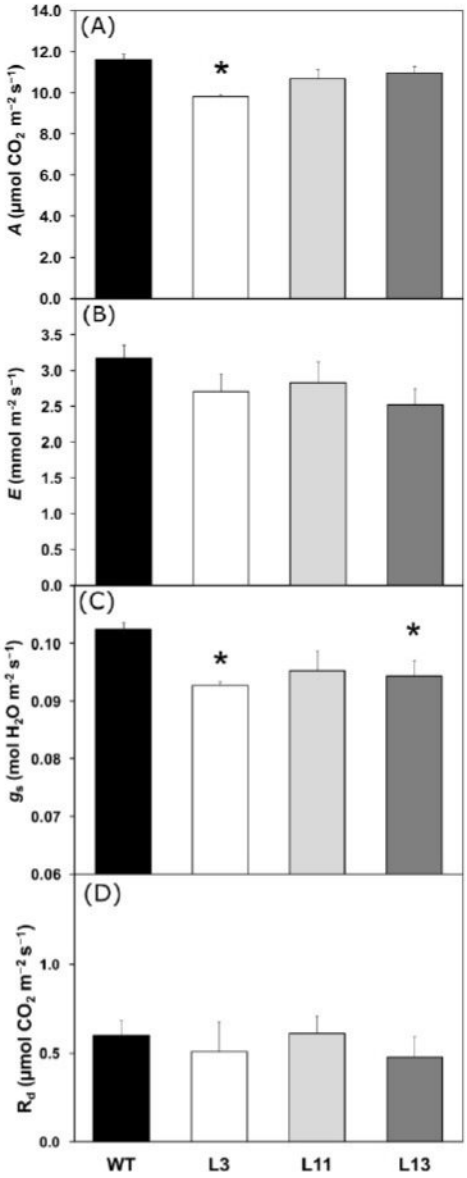

Figure 2. Gas exchange measurements in completely expanded leaves of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13). (A) *A*, net photosynthetic rate. (B) *E*, transpiration rate. (C) g_s, stomatal conductance. (D) R_d, dark respiration. Plants were grown under greenhouse conditions and measurements were taken at morning (n = 5 ± SE). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

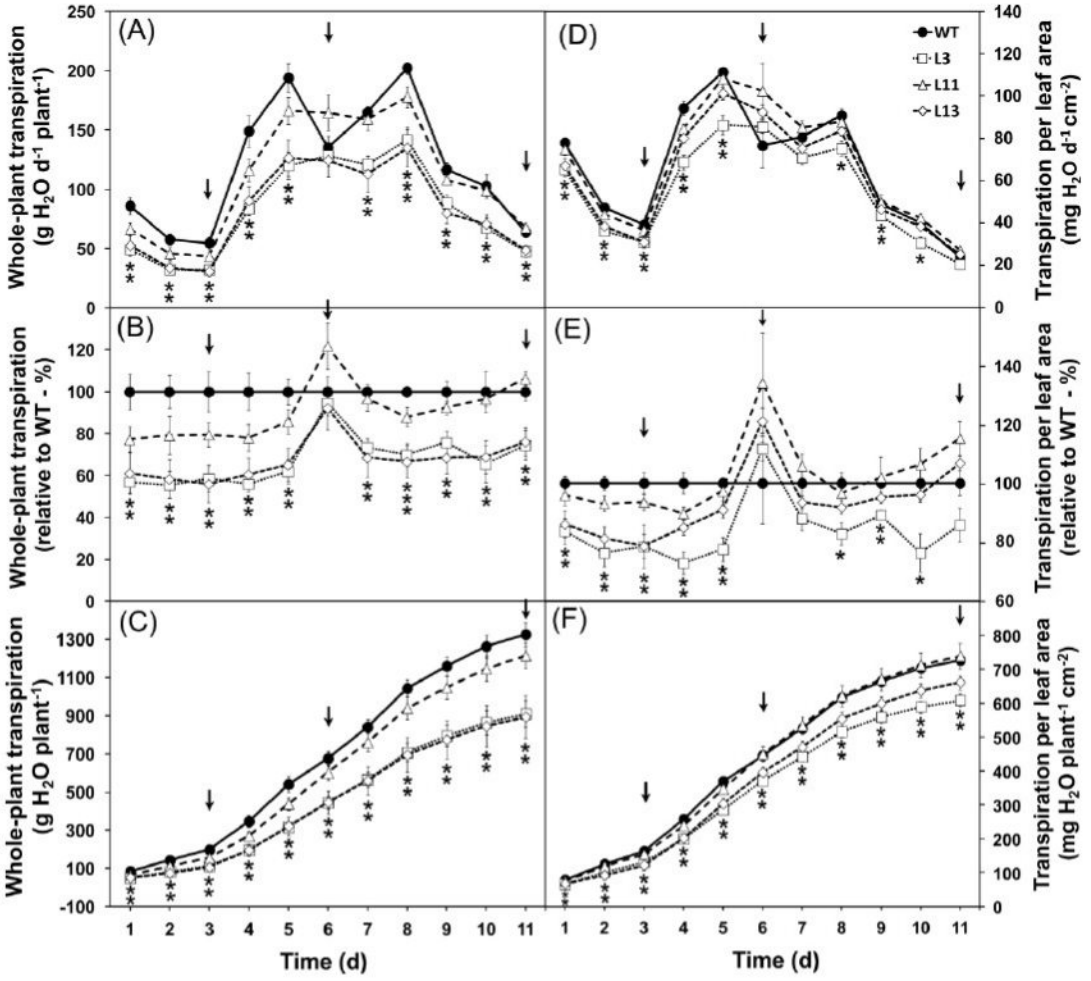

Figure 3. Effect of antisense inhibition of the *NtSUS2* gene in guard cells on whole plant transpiration in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13). (A-C) Whole plant transpiration as water loss. (D-F) Whole plant transpiration as water loss per leaf area. (A,D) Absolute values per individual day. (B,E) Values relative to WT. (C,F) Accumulated absolute values. Plants were grown under greenhouse and well-watered conditions and all measurements were taken for eleven days (n = 5 ± SE). Black arrows indicate days of no watering (see the Materials and Methods section for details). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

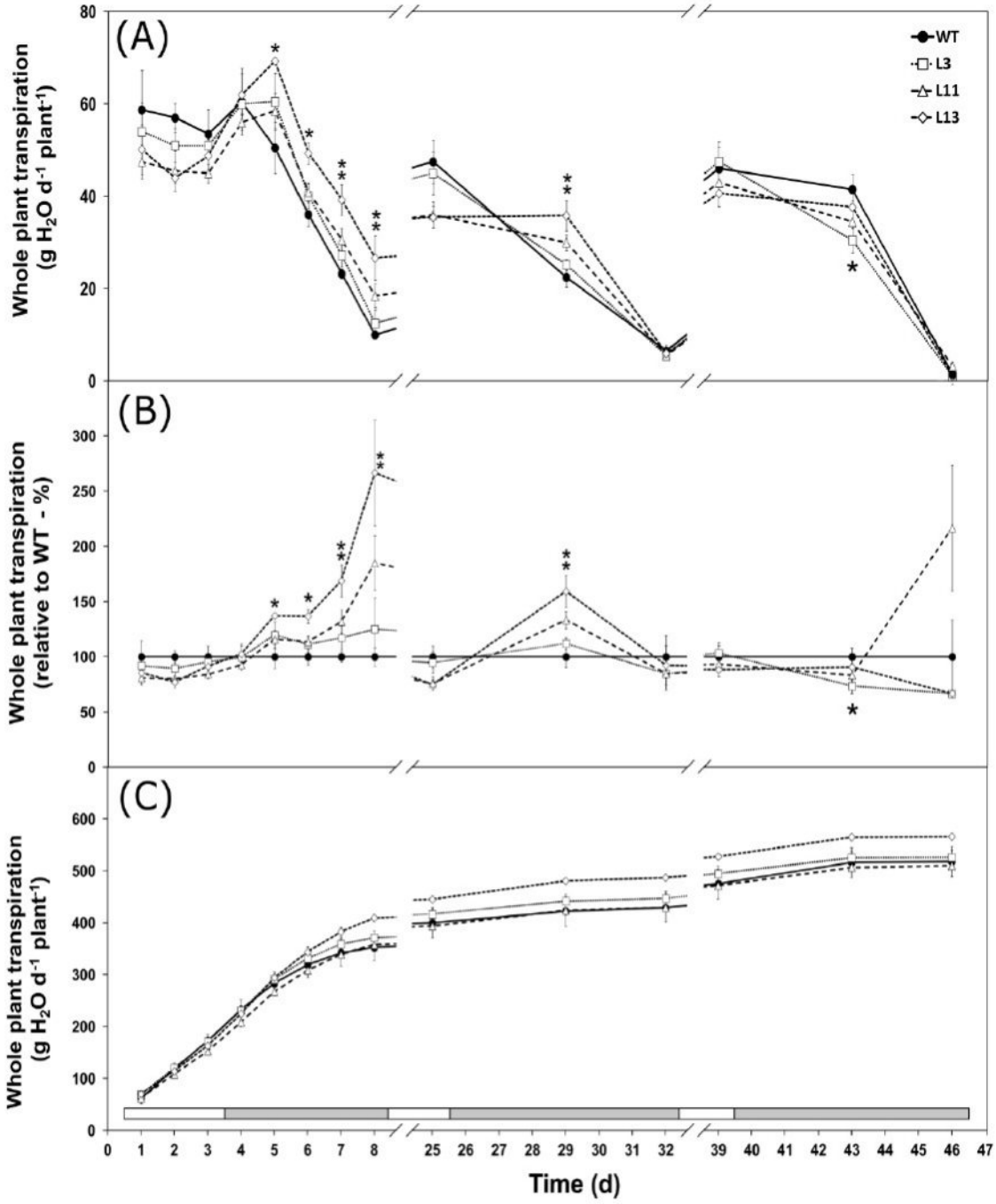

Figure 4. Whole plant transpiration of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells under drought cycles. (A-C) Whole plant transpiration as water loss. (A) Absolute values per individual day. (B) Values relative to WT. (C) Accumulated absolute values. Plants were grown under growth chamber and drought cycles conditions and all measurements were taken for forty six days (n = 4 ± SE). White and grey bars on the x-axis indicate well-watered and no watered days, respectively (see the Materials and Methods section for details). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

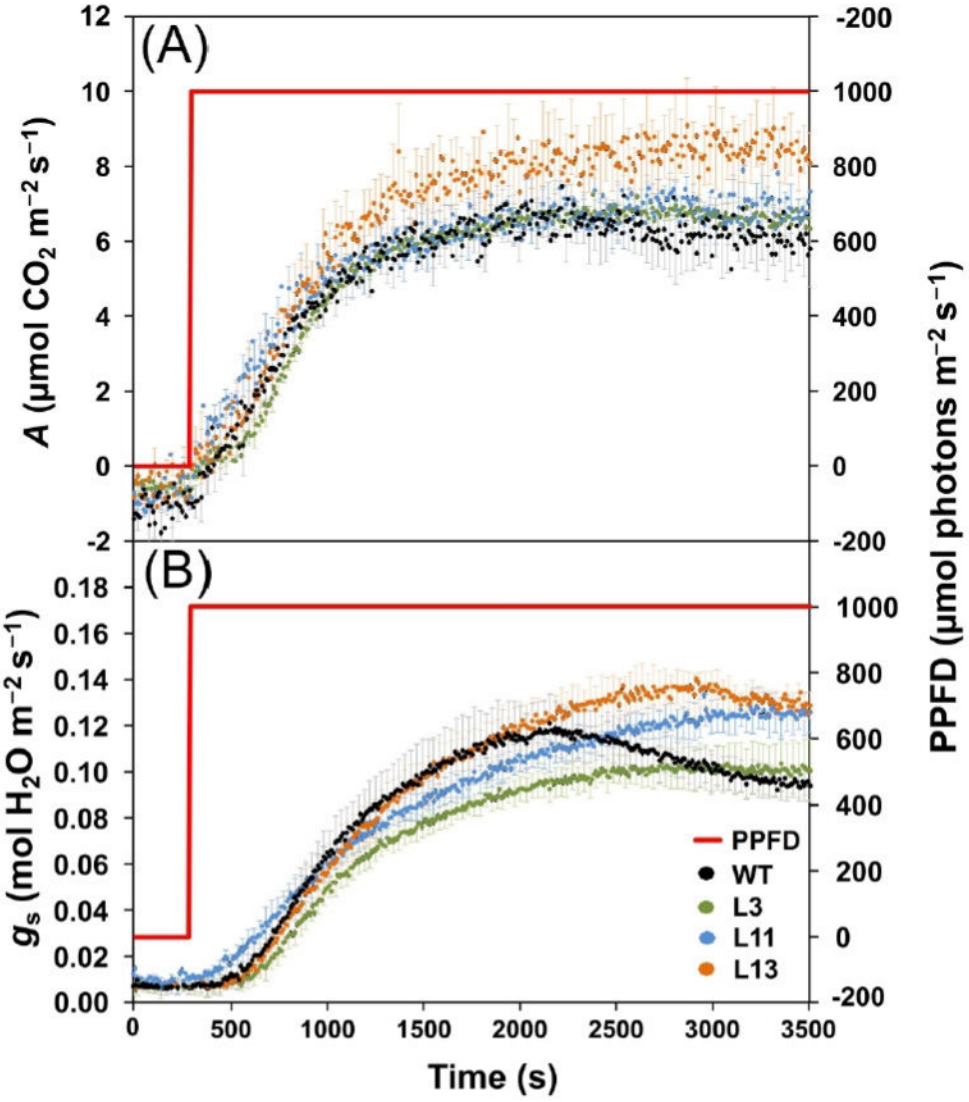

Figure 5. Stomatal opening kinetics during dark-to-light transition of *Nicotiana tabacum* plants with antisense inhibition of the *NtSUS2* gene in guard cells (L3, L11, L13) and wild-type (WT). (A) A, net photosynthetic rate. (B) g_s, stomatal conductance. Plants were grown under growth chamber conditions and all measurements were taken in dark adapted plants ten days of recuperation after drought cycles at morning for 300 sec of dark and 3200 sec of light (n = 4 ± SE).

Fonte: dados da pesquisa.

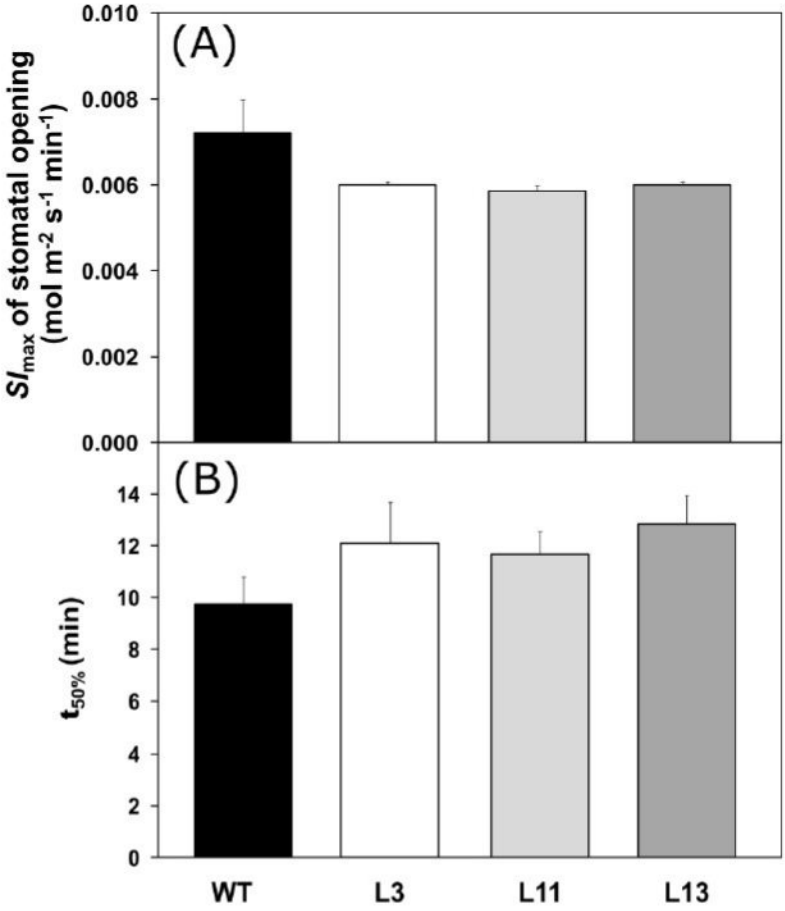

Figure 6. Rapidity of light-induced stomatal opening in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells. (A) The maximum slope (Sl_{max}) obtained through the linear step of light-induced stomatal opening. (B) Half-time ($t_{50\%}$) needed to g_s reach steady state. Plants were grown under growth chamber conditions and all data were plotted using stomatal opening kinetics measurements in dark adapted plants after drought cycles (n = 4 ± SE). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

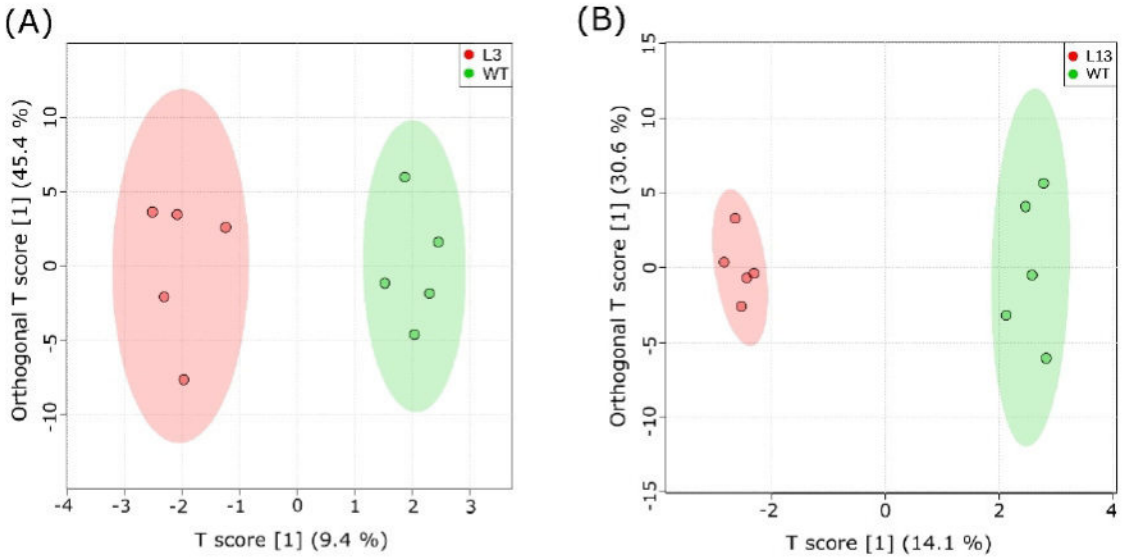

Figure 7. Orthogonal partial least squares-discriminant analysis (orthoPLS-DA) from guard cell metabolite profile of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L13). (A-B) OrthoPLS-DA carried out using the relative guard cell-enriched epidermal fragments metabolic changes observed during dark-to-light transition. (A) OrthoPLS-DA line L3 against WT. (B) OrthoPLS-DA line L13 against WT. Plants were grown under greenhouse conditions and guard cell-enriched epidermal fragments were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). These analyses were performed using Metaboanalyst platform.

Fonte: dados da pesquisa.

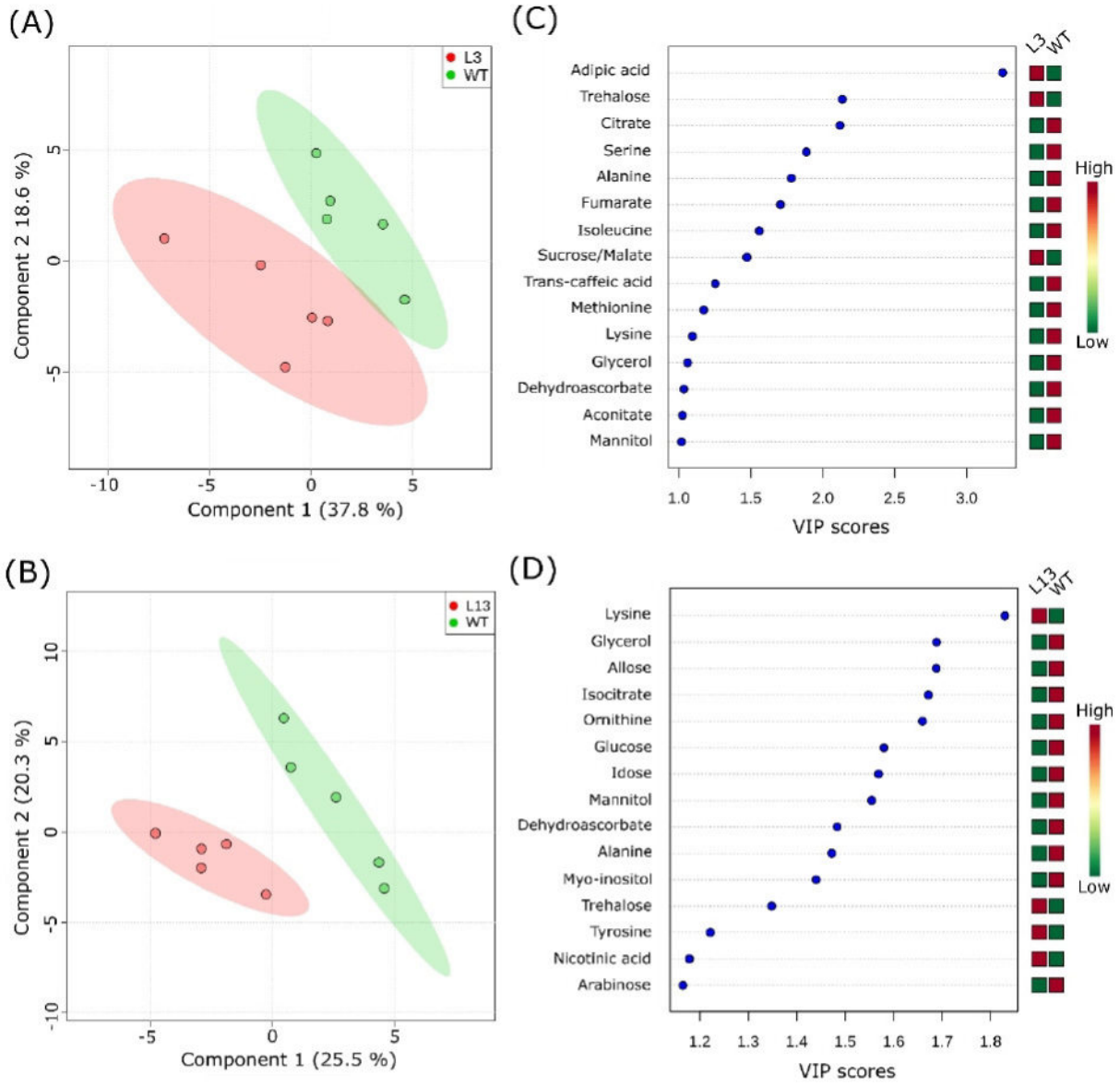

Figure 8. Partial least square-discriminant analysis (PLS-DA) from guard cell metabolite profile of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L13). (A) PLS-DA was created using the relative guard cell-enriched epidermal fragments metabolic changes observed during dark-to-light transition. (A) PLS-DA line L3 against WT. (B) PLS-DA line L13 against WT. (C-D) Variable importance in projection (VIP) scores of the respective PLS-DA with metabolite list ranked from top to down for more important metabolites in PLS-DA models. (C) L3 against WT. (D) L13 against WT. Plants were grown under greenhouse conditions and guard cell-enriched epidermal fragments were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). These analyses were performed using Metaboanalyst platform.

Fonte: dados da pesquisa.

Table 1. Biometric analysis of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells grown under greenhouse conditions. Plants were harvested with 60 days-old (n = 5 ± SE). Average points in bold and underlined indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Parameter	WT	S.E.	L3	S.E.	L11	S.E.	L13	S.E.
Total biomass DW (g)	11.64	1.22	**7.71**	0.68	10.34	0.71	**8.01**	0.94
Leaves DW (g)	5.93	0.51	4.50	0.40	5.48	0.36	**4.29**	0.50
% DW leaves	51.52	1.34	**58.38**	0.66	53.08	0.77	53.68	1.63
Stem DW (g)	3.01	0.27	**2.02**	0.14	2.79	0.26	2.33	0.25
% DW stem	26.16	0.94	26.31	0.70	26.89	1.08	29.36	1.33
Roots DW (g)	2.69	0.45	**1.19**	0.17	2.07	0.20	**1.39**	0.24
% DW roots	22.32	2.07	**15.31**	1.08	20.04	1.73	**16.96**	0.95
Shoots DW (g)	8.95	0.77	**6.52**	0.53	8.27	0.62	6.62	0.71
% DW shoots	77.68	2.07	**84.69**	1.08	79.96	1.73	**83.04**	0.95
Shoot DW/root DW ($g\ g^{-1}$)	3.69	0.56	**5.68**	0.62	4.13	0.40	4.97	0.33
Leaves DW/root DW ($g\ g^{-1}$)	2.44	0.37	**3.91**	0.41	2.73	0.25	3.22	0.25
Harvest index ($g\ g^{-1}$)	0.52	0.01	**0.58**	0.01	0.53	0.01	0.54	0.02
Plant leaf area/roots DW ($cm^2\ g^{-1}$)	1257.81	340.17	2136.12	265.85	1292.05	111.24	1498.65	93.35
Total leaf number (> 5 cm length)	13.40	0.40	14.75	0.85	13.20	0.20	13.60	0.98
Plant leaf area (cm^2)	2791.72	118.50	2422.40	183.93	2596.96	175.53	**2004.09**	227.40
Specific leaf area ($cm^2\ g^{-1}$)	487.53	52.01	541.39	18.75	473.93	10.54	468.30	13.88
Stem length (cm)	45.60	1.94	41.88	1.19	45.50	1.82	43.70	2.11
Transpiration* (g)	1326.50	59.71	**910.25**	67.88	1214.30	66.73	**893.90**	111.53
RGR ($g\ g^{-1}\ day^{-1}$)	0.07	0.00	0.05	0.00	0.07	0.00	0.06	0.00
$_{y}$WUE ($g\ kg^{-1}$)	2.86	0.13	**3.47**	0.12	3.14	0.25	3.32	0.17
$_{sl}$WUE ($g\ kg^{-1}$)	4.41	0.48	3.74	0.32	4.51	0.16	4.02	0.36
$_{i}$WUE ($\mu mol\ mmol^{-1}$)	115.01	2.74	**104.15**	2.09	115.73	6.04	116.49	5.67

*It was not computed the water transpired between 17:00h until 06:00h on the following days. Accumulated in eleven days. DW: dry weight; RGR: relative growth rate; $_{y}$WUE: yield water use efficiency; $_{sl}$WUE: season-long water use efficiency; $_{i}$WUE: intrinsic water user efficiency.

Fonte: dados da pesquisa.

Table 2. Biometric analysis of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells grown under growth chamber conditions. Plants were harvested after drought cycles with 100 days-old (n = 4 ± SE). Average points in bold and underlined indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Parameter	WT	S.E.	L3	S.E.	L11	S.E.	L13	S.E.
Total biomass DW (g)	15.92	1.01	16.03	0.51	16.64	0.82	15.56	1.31
Leaves DW (g)	6.21	0.26	6.13	0.20	6.13	0.20	5.63	0.38
% DW leaves	39.25	1.51	38.42	2.21	36.97	1.05	36.29	0.71
Stem DW (g)	2.76	0.25	2.69	0.25	**2.02**	0.09	3.16	0.48
% DW stem	17.34	1.13	16.75	1.41	**12.22**	0.75	20.92	3.89
Roots DW (g)	6.95	0.63	7.22	0.65	8.49	0.64	6.78	1.29
% DW roots	43.41	1.64	44.83	3.14	**50.80**	1.51	42.79	4.60
Shoots DW (g)	8.98	0.44	8.82	0.40	8.15	0.21	8.79	0.49
% DW shoots	56.59	1.64	55.17	3.14	**49.20**	1.51	57.21	4.60
Shoot DW/root DW ($g\ g^{-1}$)	1.31	0.09	1.26	0.16	**0.97**	0.06	1.41	0.30
Leaves DW/root DW ($g\ g^{-1}$)	0.91	0.07	0.88	0.11	0.73	0.04	0.88	0.13
Harvest index ($g\ g^{-1}$)	0.39	0.02	0.38	0.02	0.37	0.01	0.36	0.01
Plant leaf area/roots DW ($cm^2\ g^{-1}$)	304.21	28.09	268.07	20.03	245.02	24.65	287.28	30.03
Total leaf number (> 5 cm length)	12.00	0.41	12.75	0.75	11.75	0.25	13.33	0.33
Plant leaf area (cm^2)	2062.24	54.80	1896.63	62.30	2034.52	57.87	1857.62	160.37
Specific leaf area ($cm^2\ g^{-1}$)	332.86	9.77	310.85	17.16	333.68	18.90	329.38	10.50
Stem length (cm)	7.25	0.25	7.50	0.29	7.25	0.63	9.67	1.76
Transpiration* (g)	517.79	29.24	525.99	13.77	509.77	6.35	566.21	6.49
¡WUE (µmol $mmol^{-1}$)	62.51	5.06	67.09	5.99	55.09	2.17	64.40	4.80

*It was not computed the water transpired between 16:00h until 08:00h on the following days. Accumulated in fourteen days. DW: dry weight; ¡WUE: intrinsic water user efficiency.

Fonte: dados da pesquisa.

Supplemental material

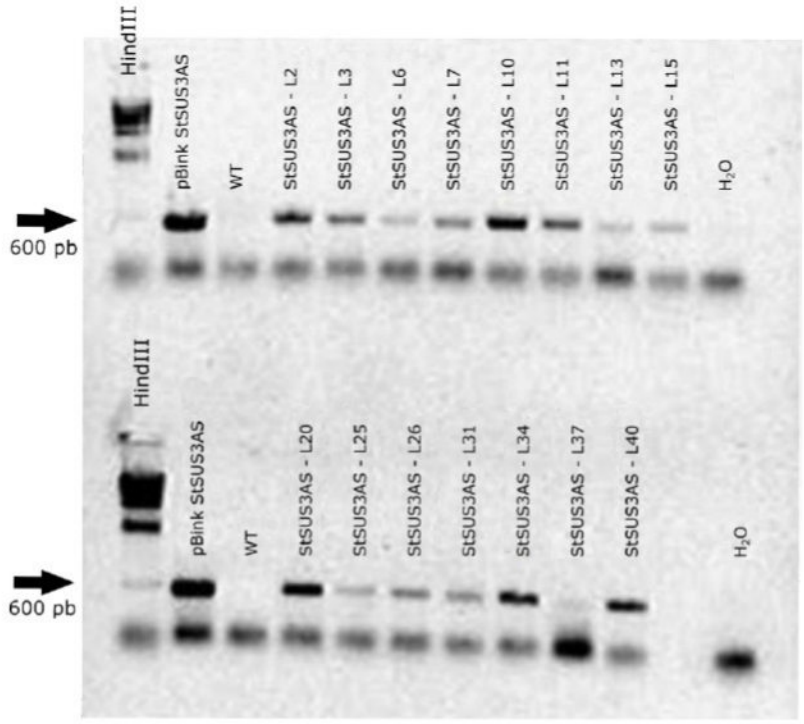

Figure S1. PCR products (600 bp) in agarose gel evidencing the insertion of the exogenous marker gene *NPTII* (kanamycin resistance) in several transgenic lines with antisense construction for the potato *SUS3* gene under control of KST1 promoter.

Fonte: dados da pesquisa.

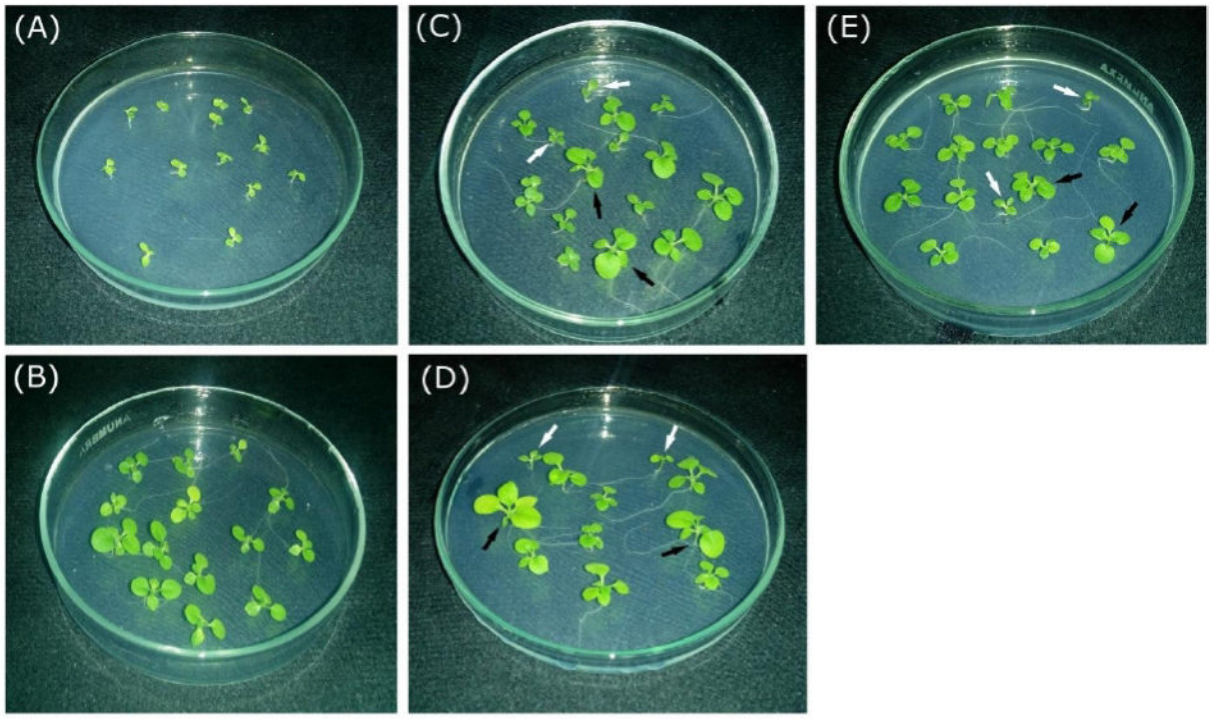

Figure S2. Seedlings *in vitro* selection of *Nicotiana tabacum* plants with antisense construction for the potato *SUS3* gene under control of KST1 promoter (L3, L11, L13) showing kanamycin (KAN) resistance compared to wild-type (WT). (A) WT plants in MS medium + 50 µm KAN. (B) WT plants in MS medium. (C) L3 plants in MS medium + 50 µm KAN. (D) L11 plants in MS medium + 50 µm KAN. (C) L13 plants in MS medium + 50 µm KAN. Black arrows show KAN resistant plants and white arrows show plants without KAN resistance.

Fonte: dados da pesquisa.

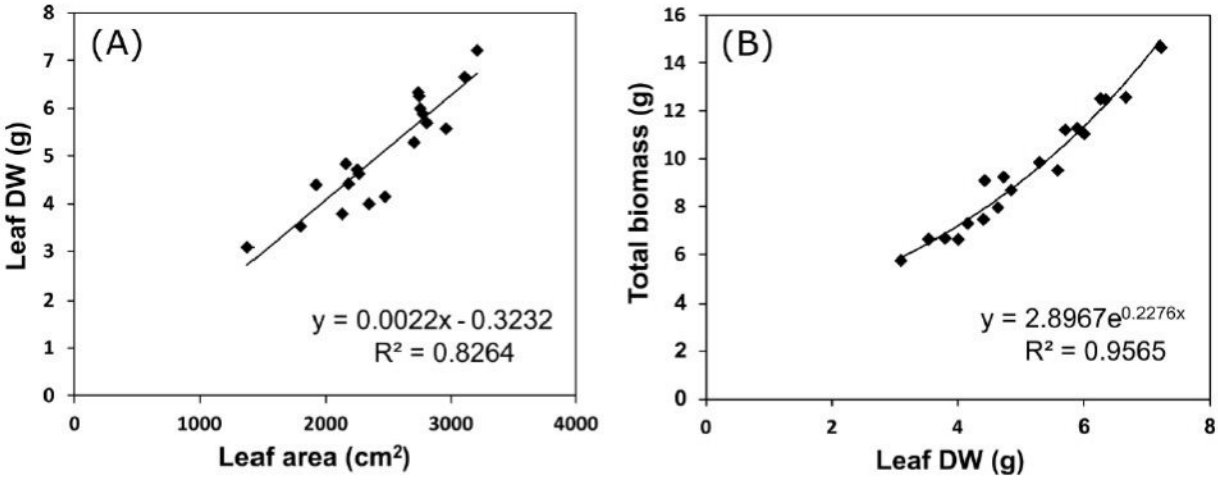

Figure S3. Linear (A) and exponential (B) regression to estimate leaves dry weight (DW) and total biomass, respectively, at first day of the whole plant transpiration experiment under greenhouse conditions of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells.

Fonte: dados da pesquisa.

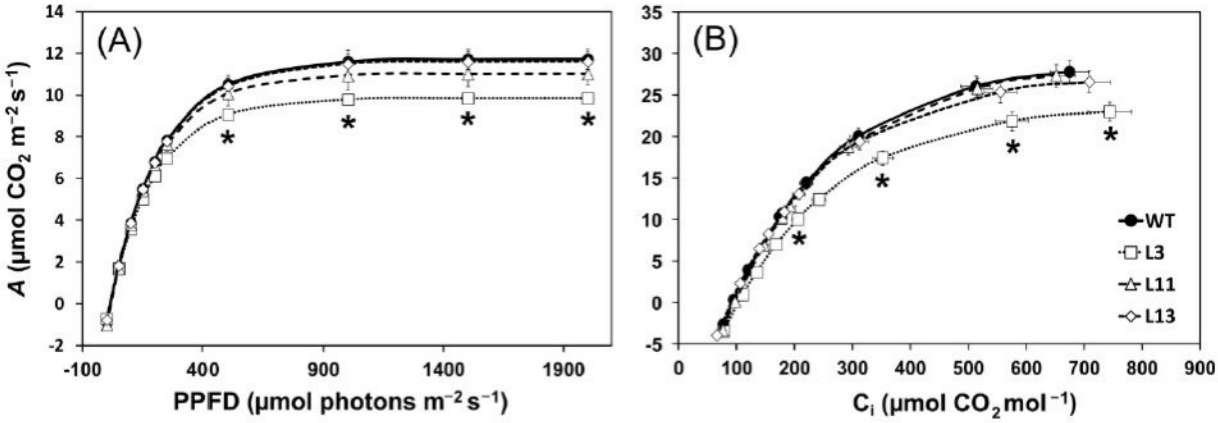

Figure S4. Photosynthetic light (*A*-PPFD) and CO_2 response curves (*A*-C_i) curves of fully expanded leaves of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells. (A) *A*-PPFD. (B) *A*-C_i. Plants were grown in substrate under greenhouse conditions and measurements were taken at morning (n = 4 ± SE). The regression line was determined using the equation $A = a\ (1 - e^{-bx}) + c$ (Watling et al, 2000). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

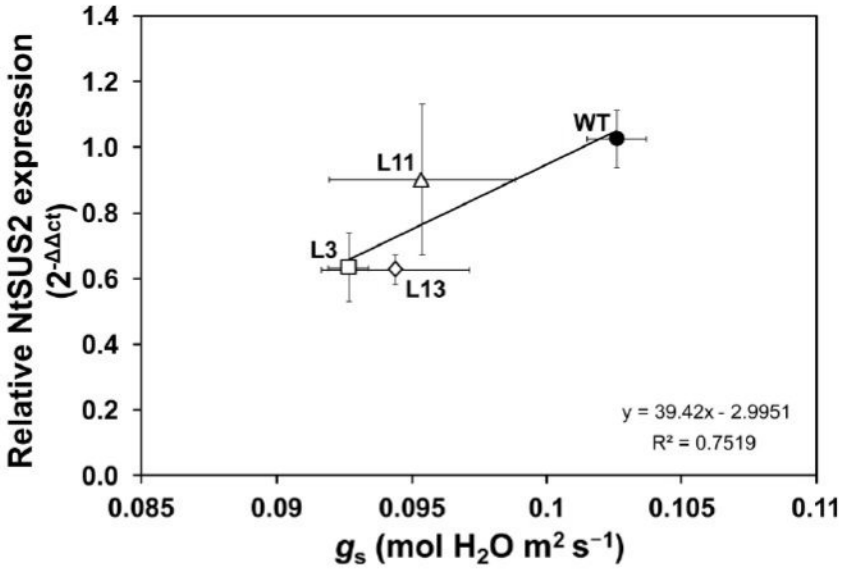

Figure S5. Relationship between relative *NtSUS2* expression versus stomatal conductance (g_s) using data from transgenic lines and WT. Plants were grown in substrate under greenhouse conditions (n = 3 ± SE). The regression line was determined using Microsoft Excel (Microsoft, Redmond, WA, USA).

Fonte: dados da pesquisa.

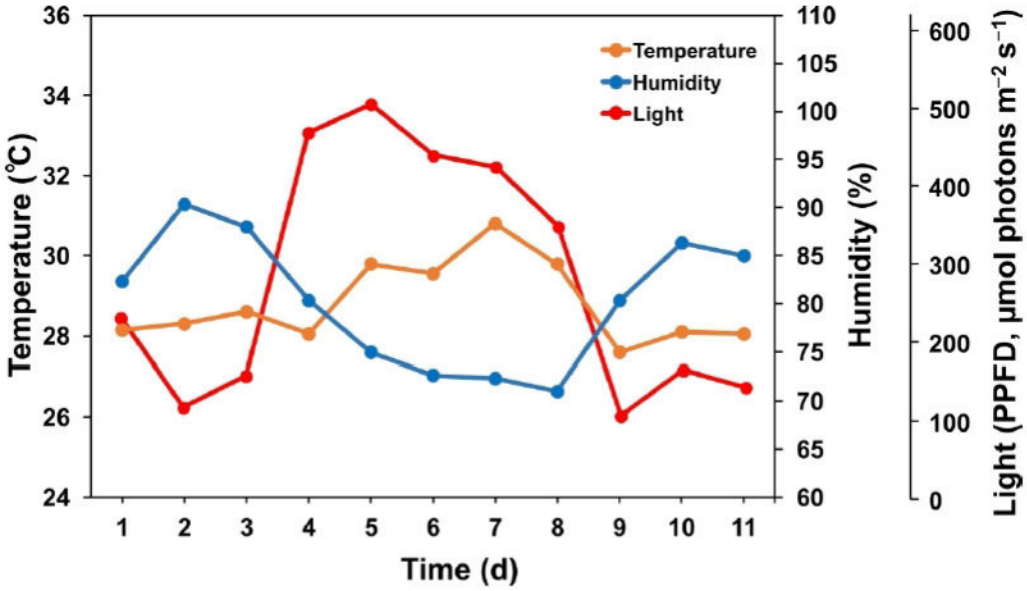

Figure S6. Variation of environmental parameters under greenhouse conditions. Data are average of light, temperature and humidity throughout eleven days during the whole plant transpiration experiment under greenhouse conditions.

Fonte: dados da pesquisa.

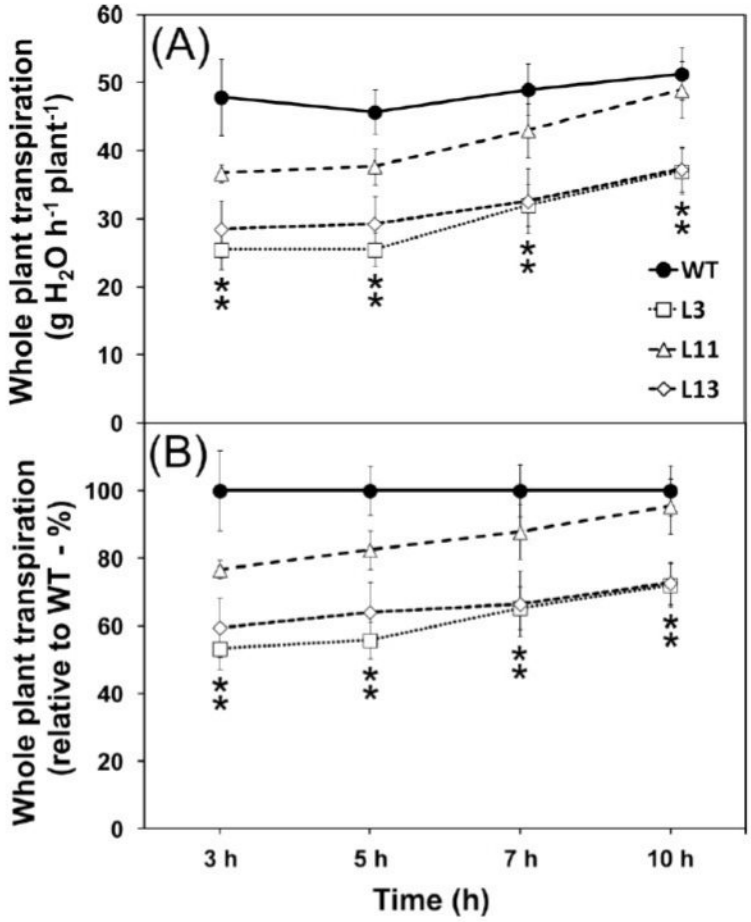

Figure S7. Diel course of whole plant transpiration in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13) at different time intervals under greenhouse conditions. (A) Whole plant transpiration as water loss. (B) Whole plant transpiration as water loss (%) relative to WT. The measurements were determined in different times after the beginning of the light period (n = 5 ± SE). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

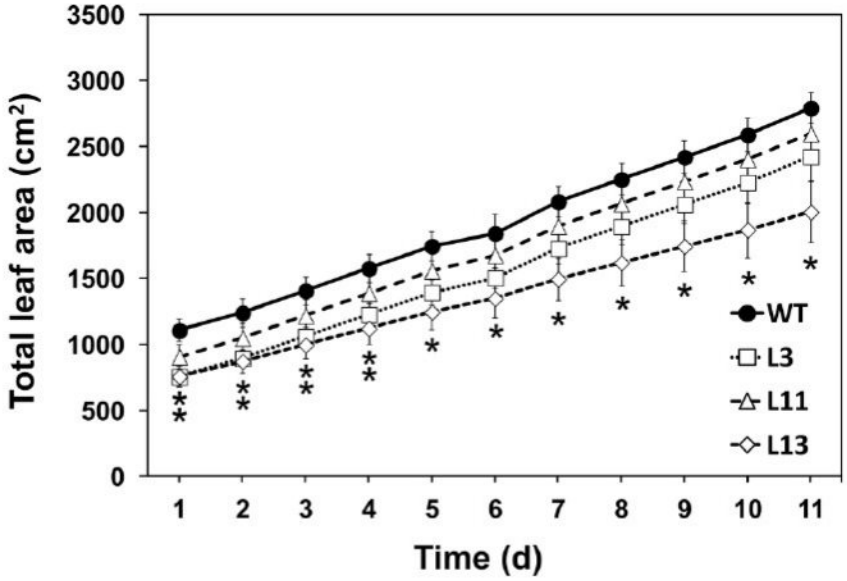

Figure S8. Total leaf area of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells cultivated in substrate under greenhouse conditions. The measurements were taken during the whole plant transpiration experiment (n = 5 ± SE) (see the Materials and Methods section for details). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

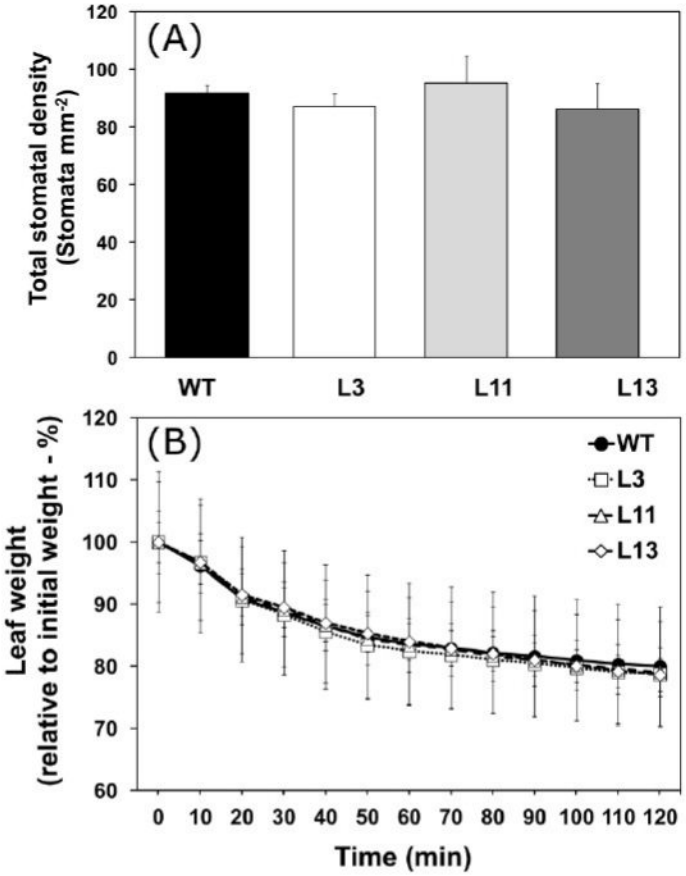

Figure S9. Total stomatal density (A) and weight loss of detached leaves (B) of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells. Both measurements were taken in fully expanded leaves grown in substrate under greenhouse conditions (n = 5 ± SE).

Fonte: dados da pesquisa.

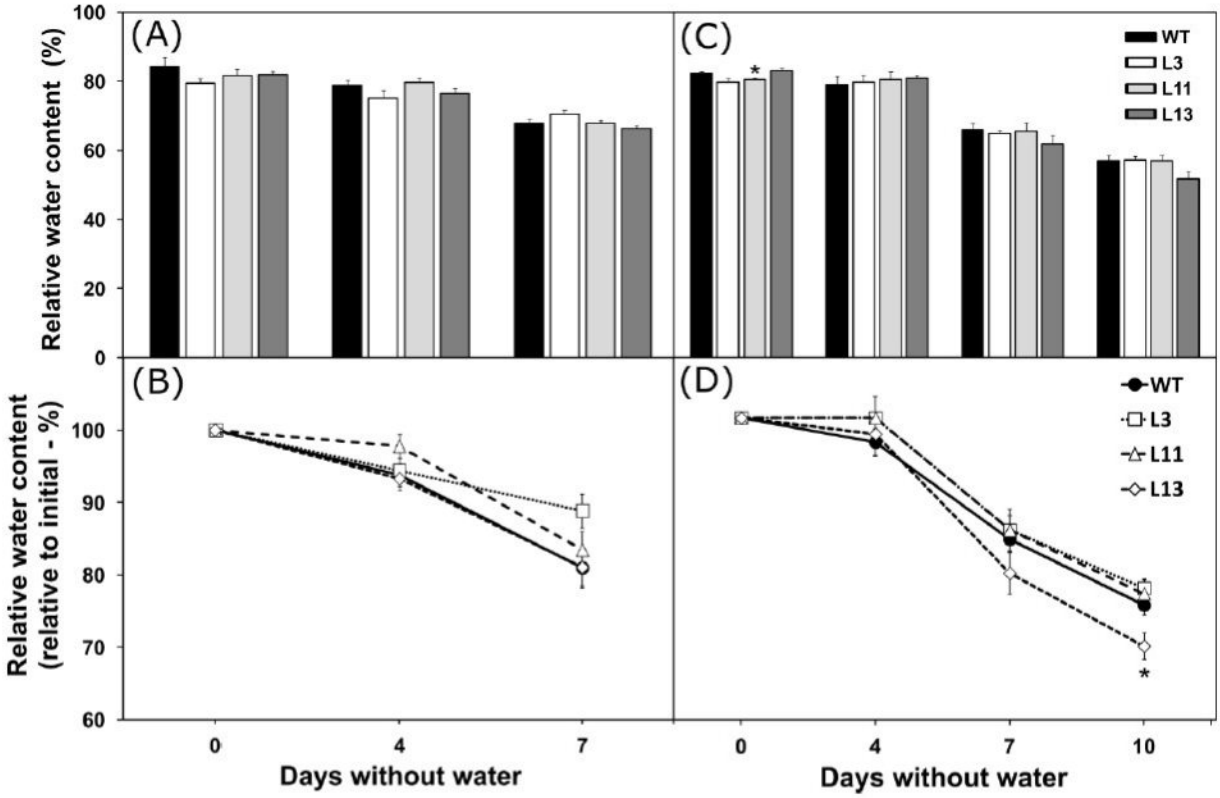

Figure S10. Relative water content of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) during drought cycles. (A-B) Second drought cycle. (C-D) third drought cycle. (A-C) Relative water content. (B-C) Relative water content relative to initial value at day 0. Plants were grown under growth chamber conditions and the measurements were taken in leaf discs (n = 4 ± SE) (see the Materials and Methods section for details). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

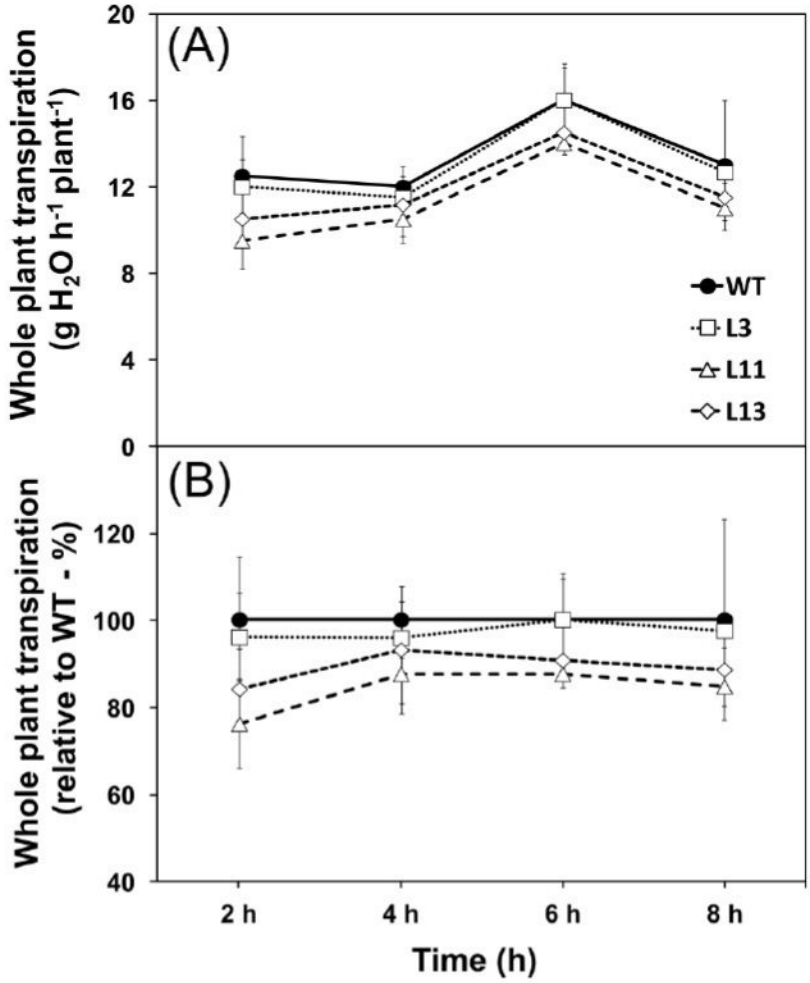

Figure S11. Diel course of whole plant transpiration in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13) at different time intervals under growth chamber conditions. (A) Whole plant transpiration as water loss. (B) Whole plant transpiration as water loss (%) relative to WT. The measurements were determined in different times after the beginning of the light period (n = 4 ± SE). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

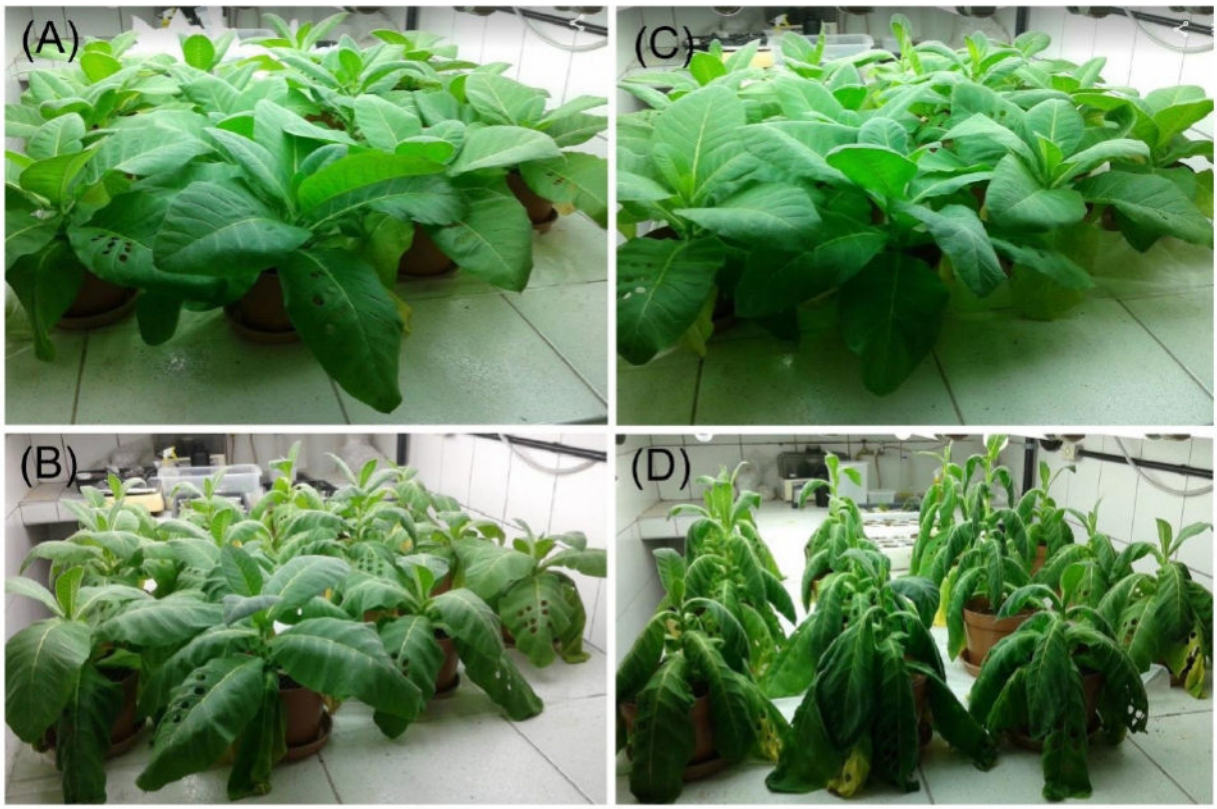

Figure S12. Visual phenotype of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells during the first cycle of drought stress under growth chamber conditions. (A) Well-watered (day 0). (B) Day 4 of no watering. (C) Day 7 of no watering. (D) Day 10 of no watering.

Fonte: dados da pesquisa.

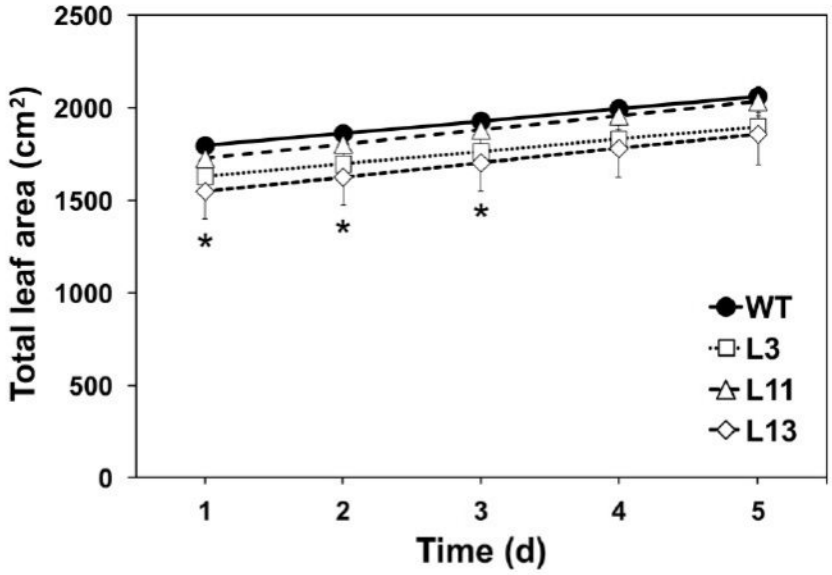

Figure S13. Total leaf area of *Nicotiana tabacum* plants wild-type (WT) and transgenic lines (L3, L11, L13) with antisense inhibition of the *NtSUS2* gene in guard cells under growth chamber conditions. The measurements were taken during the first cycle of the drought cycle experiment (n = 4 ± SE) (see the Materials and Methods section for details). Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

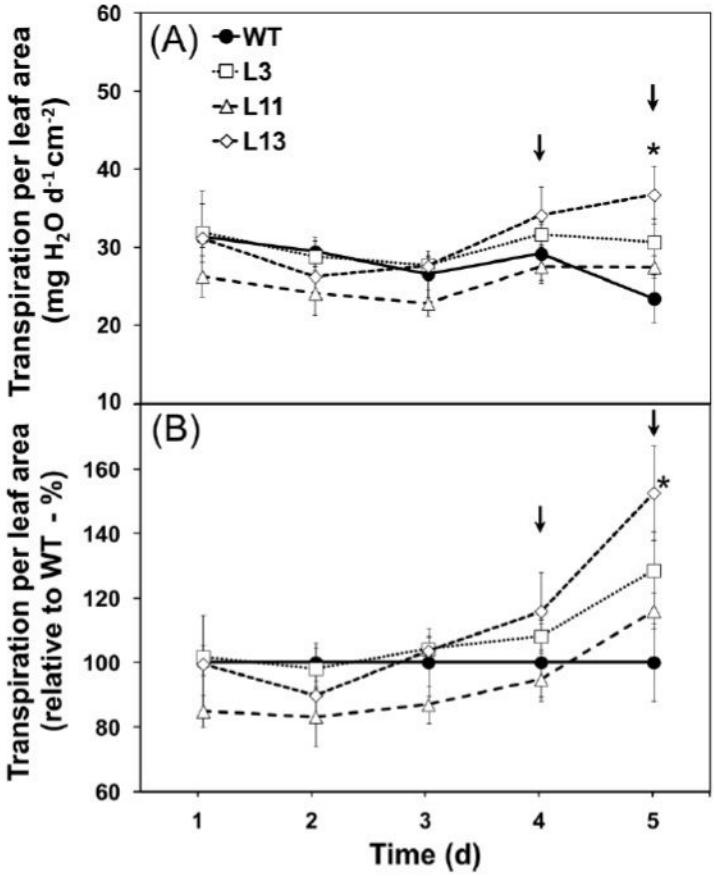

Figure S14. Whole plant transpiration per leaf area in *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L11, L13) at different time intervals under growth chamber conditions. (A) Whole plant transpiration as water loss per leaf area. (B) Whole plant transpiration as water loss per leaf area (%) relative to WT. The measurements were taken during the first cycle of the drought cycle experiment (n = 4 ± SE). Black arrows indicate days of no watering. Asterisks (*) indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$).

Fonte: dados da pesquisa.

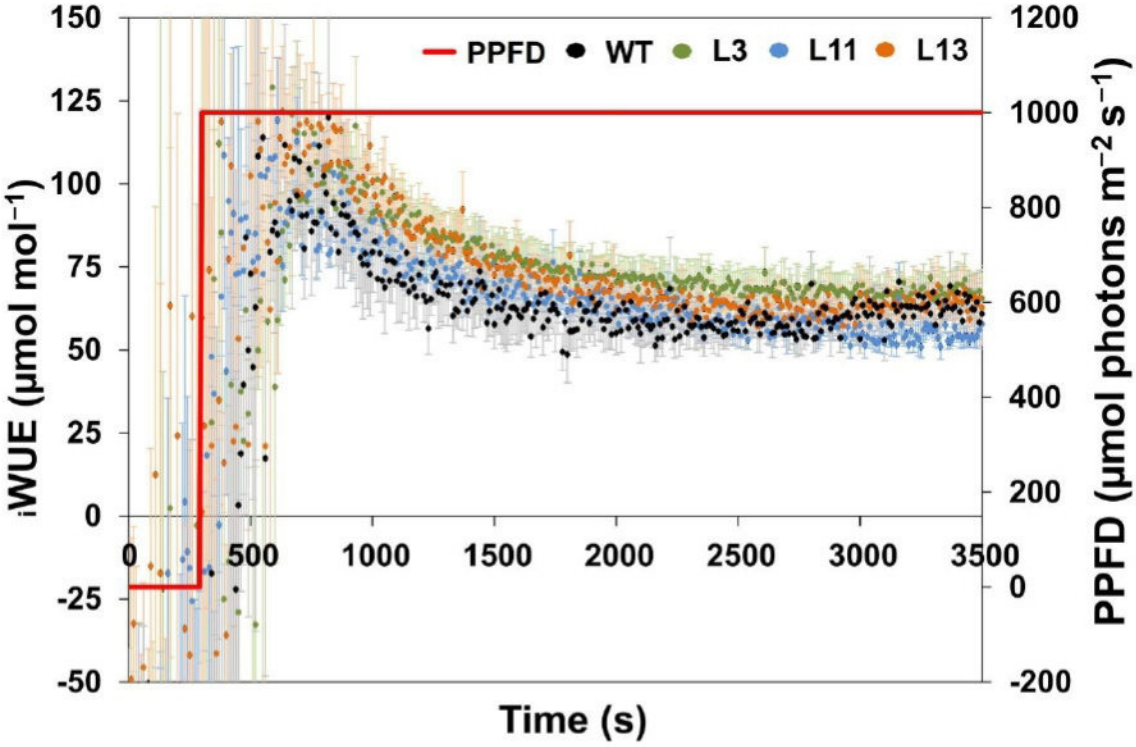

Figure S15. Intrinsic water use efficiency (iWUE) during stomatal opening kinetics through dark-to-light transition of *Nicotiana tabacum* plants with antisense inhibition of the *NtSUS2* gene in guard cells (L3, L11, L13) and wild-type (WT). Plants were grown under growth chamber conditions and all data were plotted ten days after recuperation of drought cycles using stomatal opening kinetics measurements (n = 4 ± SE).

Fonte: dados da pesquisa.

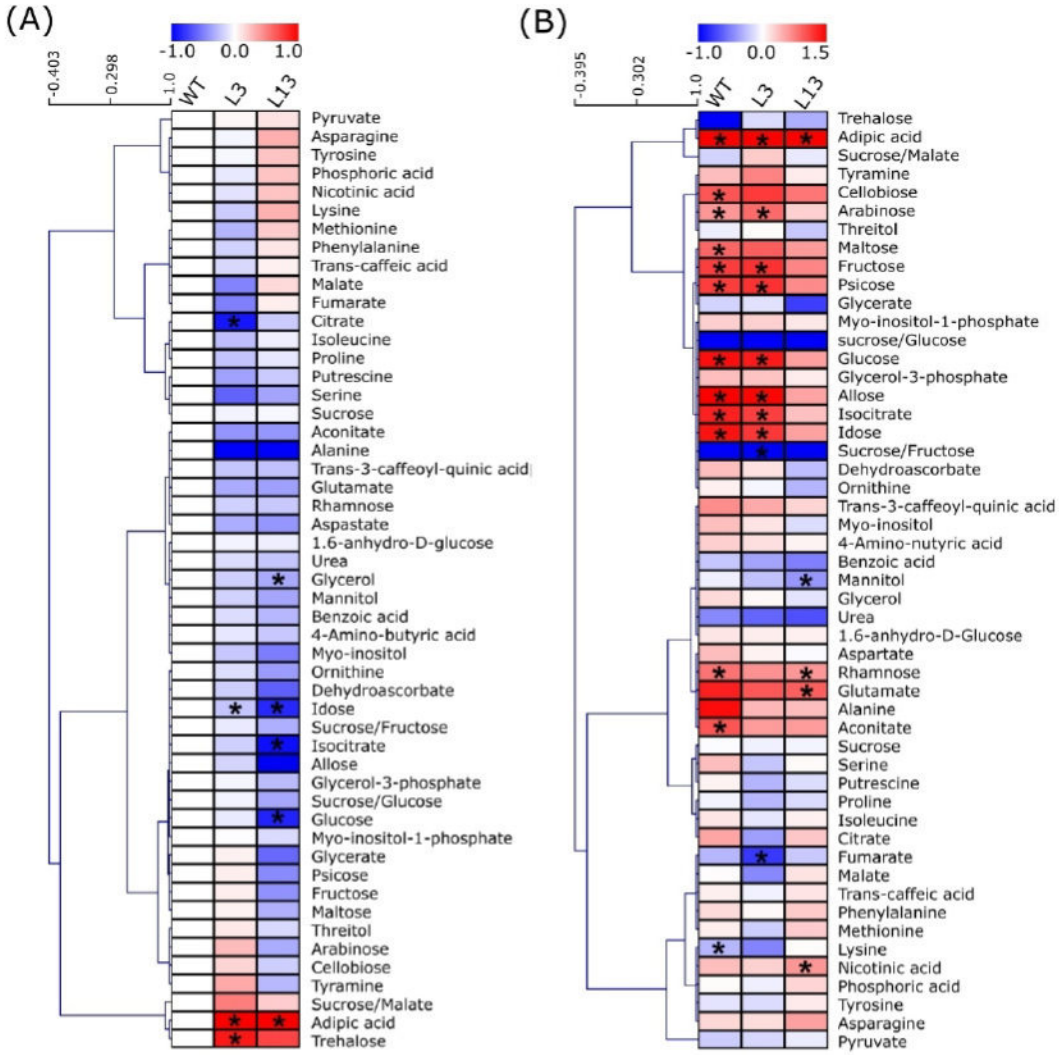

Figure S16. Guard cell metabolite profile representing the absolute differences in the content of metabolites by clusted heat maps of *NtSUS2* gene antisense inhibited guard cells of *Nicotiana tabacum* plants (L3, L13) and wild-type (WT). (A) Dark-to-light transition relative changes in metabolite levels of guard cell-enriched epidermal fragments of transgenic lines compared to WT. The heat map was created by dividing each content value in light by the average of respective content value in dark, then dividing each value of transgenic lines and WT by WT values and then log2 transformed. (B) Light metabolite content of guard cell-enriched epidermal fragments of transgenic lines and WT compared to dark. The heat map was created by dividing the average of each content value in light by the average of respective content values of transgenic lines and WT in dark and then $\log_2$ transformed. Plants were grown in hydroponic system under greenhouse conditions and guard cell-enriched epidermal fragments were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). Asterisks (*) on the left indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$). Asterisks (*) on the right indicate significant difference from dark by Student's *t* test at 5% of probability ($P < 0.05$). These heat maps were created using MeV software.

Fonte: dados da pesquisa.

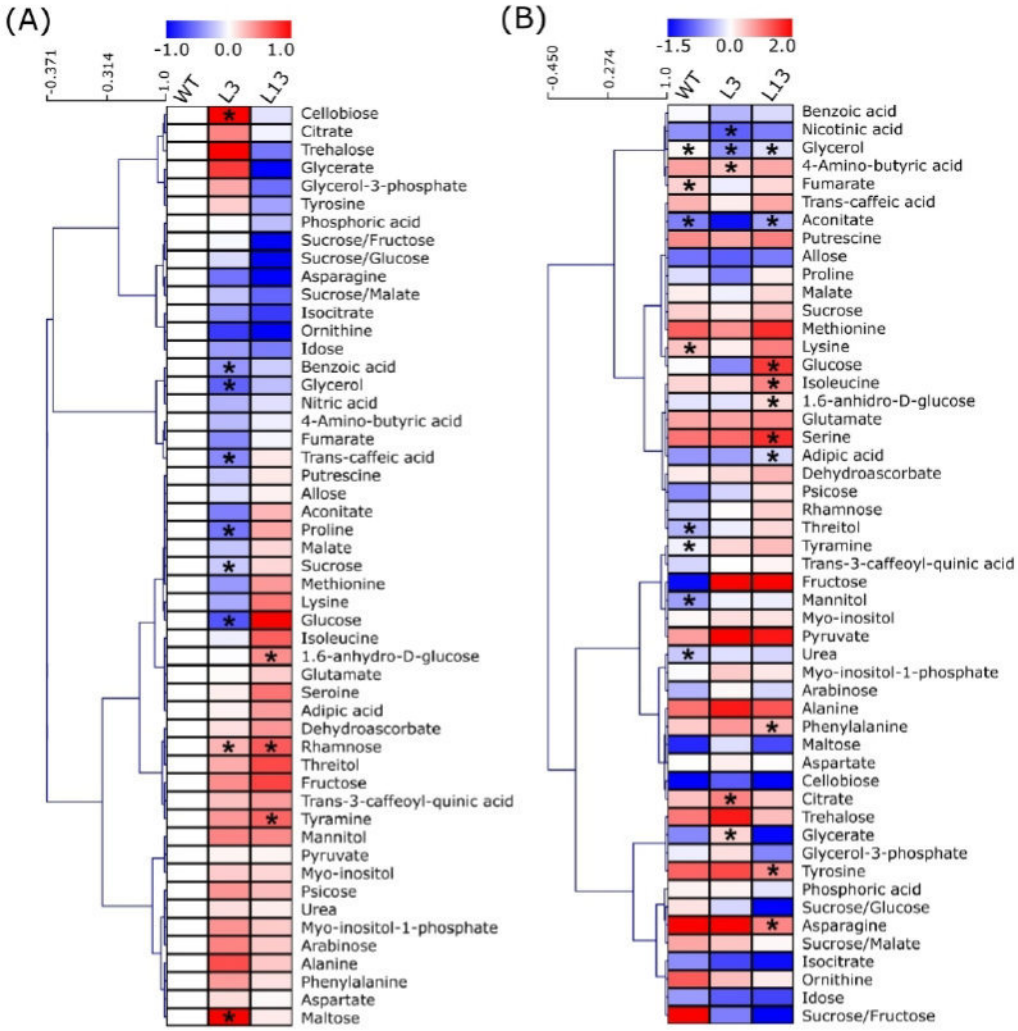

Figure S17. Leaf metabolite profile representing the absolute differences in the content of metabolites by clusted heat maps of *NtSUS2* gene antisense inhibited guard cells of *Nicotiana tabacum* plants (L3, L13) and wild-type (WT). (A) Dark-to-light transition relative changes in metabolite levels of leaves of transgenic lines compared to WT. (B) Light metabolite content of leaves of transgenic lines and WT compared to dark. Plants were grown in hydroponic system under greenhouse conditions and leaves were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). Asterisks (*) on the left indicate significant difference from WT by Student's *t* test at 5% of probability ($P < 0.05$). Asterisks (*) on the right indicate significant difference from dark by Student's *t* test at 5% of probability ($P < 0.05$). These heat maps were created using MeV software.

Fonte: dados da pesquisa.

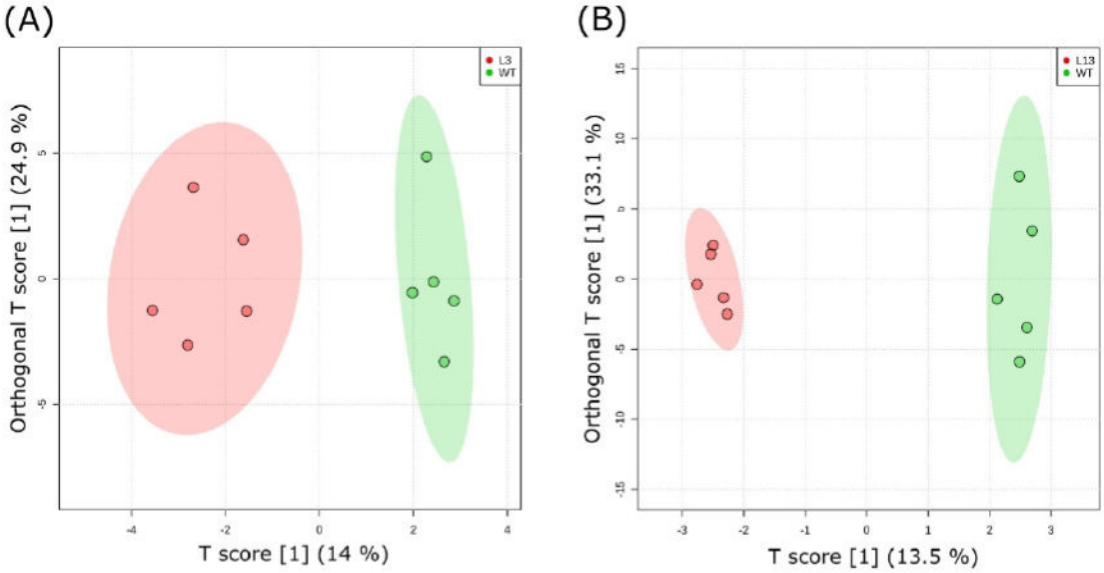

Figure S18. Orthogonal partial least squares-discriminant analysis (orthoPLS-DA) from leaves metabolite profile of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L13). (A-B) OrthoPLS-DA carried out using relative leaf metabolic changes observed during dark-to-light transition. (A) OrthoPLS-DA line L3 against WT. (B) OrthoPLS-DA line L13 against WT. Plants were grown under greenhouse conditions and leaves were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). These analyses were performed using Metaboanalyst platform.

Fonte: dados da pesquisa.

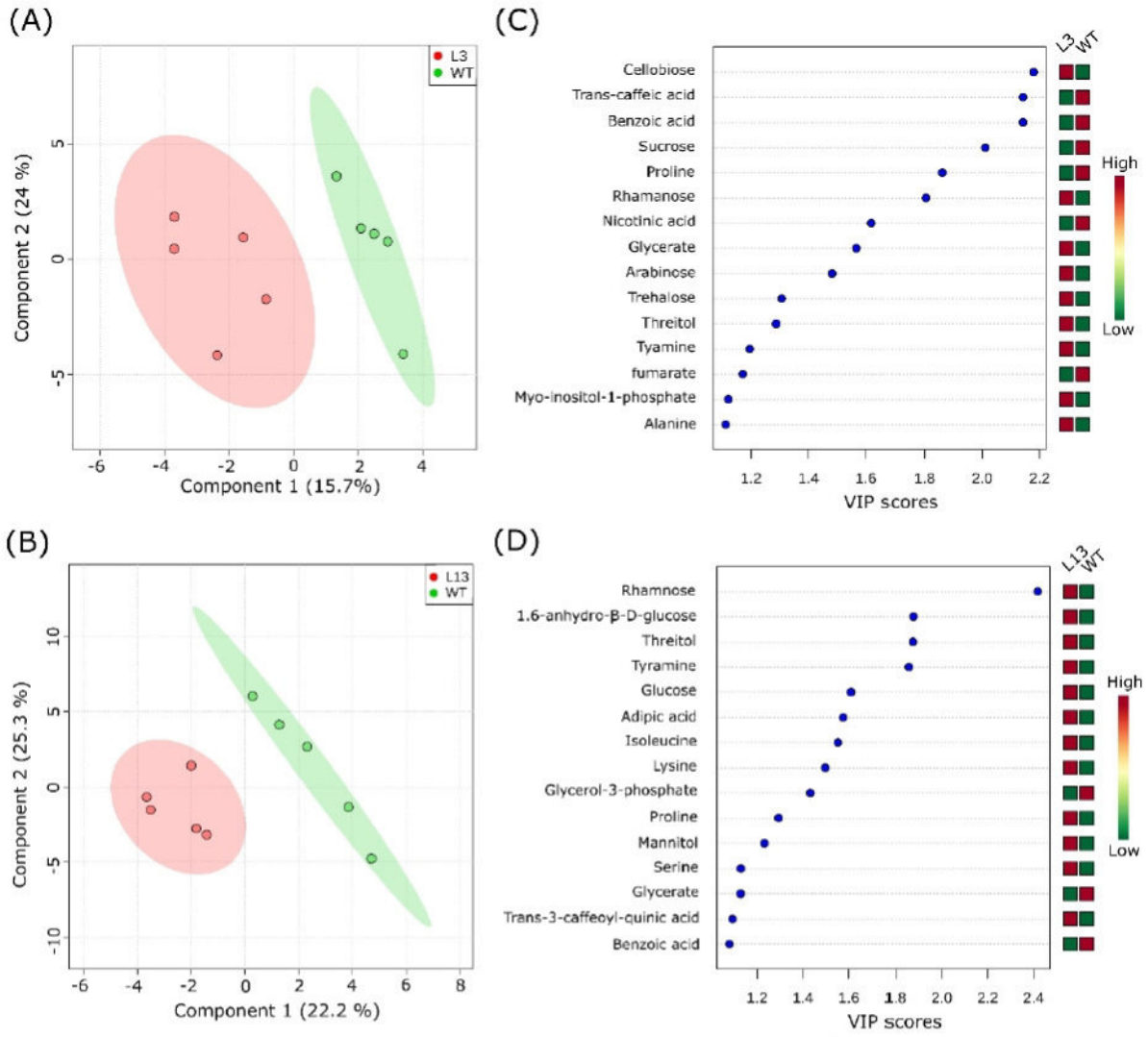

Figure S19. Partial least square-discriminant analysis (PLS-DA) from leaves metabolite profile of *Nicotiana tabacum* wild-type (WT) and transgenic lines (L3, L13). (A) PLS-DA was created using relative leaf metabolic changes observed during dark-to-light transition. (A) PLS-DA line L3 against WT. (B) PLS-DA line L13 against WT. (C-D) Variable importance in projection (VIP) scores of the respective PLS-DA with metabolite list ranked from top to down for more important metabolites in PLS-DA models. (C) L3 against WT. (D) L13 against WT. Plants were grown under greenhouse conditions and leaves were harvested between 3-5 a.m. (dark) and 7-9 a.m. (light) (n = 5). These analyses were performed using Metaboanalyst platform.

Fonte: dados da pesquisa.

5 CONCLUSIONS

In summary, tobacco transgenic plants with antisense inhibition of the guard cell *NtSUS2* gene under control of KST1 promoter showed decreased whole plant transpiration, stomatal conductance and increased $_{y}$WUE, presenting a phenotype more related to drought avoidance. Our results suggest that *NtSUS2* is a key regulator of guard cell metabolism and controls stomatal movements. Overall, our findings add more steps toward understanding the regulation of stomatal movements and indicate that engineering the guard cell metabolism is a promising strategy to decrease crop water consumption and increase WUE.

6 FUTURE PERSPECTIVES

The characterization presented in this work led to promising results regarding the development of varieties with lower water consumption, maintenance of productivity and drought avoidance phenotype by engineering the guard cell metabolism. Further analysis of *N. tabacum* transgenic lines antisense for the potato SUS3 gene and others transgenic plants regarding alterations on the guard cell carbohydrate metabolism are necessary to complement our current knowledge about the role of sucrose in stomatal movements. In addition, it is still necessary to analyze the economic and commercial viability of such cultivars with high water use efficiency until the development of plants that can be used by companies and farmers in field.

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