KERLY JESSENIA MONCALEANO ROBLEDO

THE ROLES OF FLORIGEN AND ANTIFLORIGEN IN PHYSIOLOGY AND DEVELOPMENT OF TOMATO PLANTS

Dissertation submitted to the Plant Physiology Graduate Program of the Universidade Federal de Viçosa in partial fulfillment of the requirements for the degree of Magister Scientiae.

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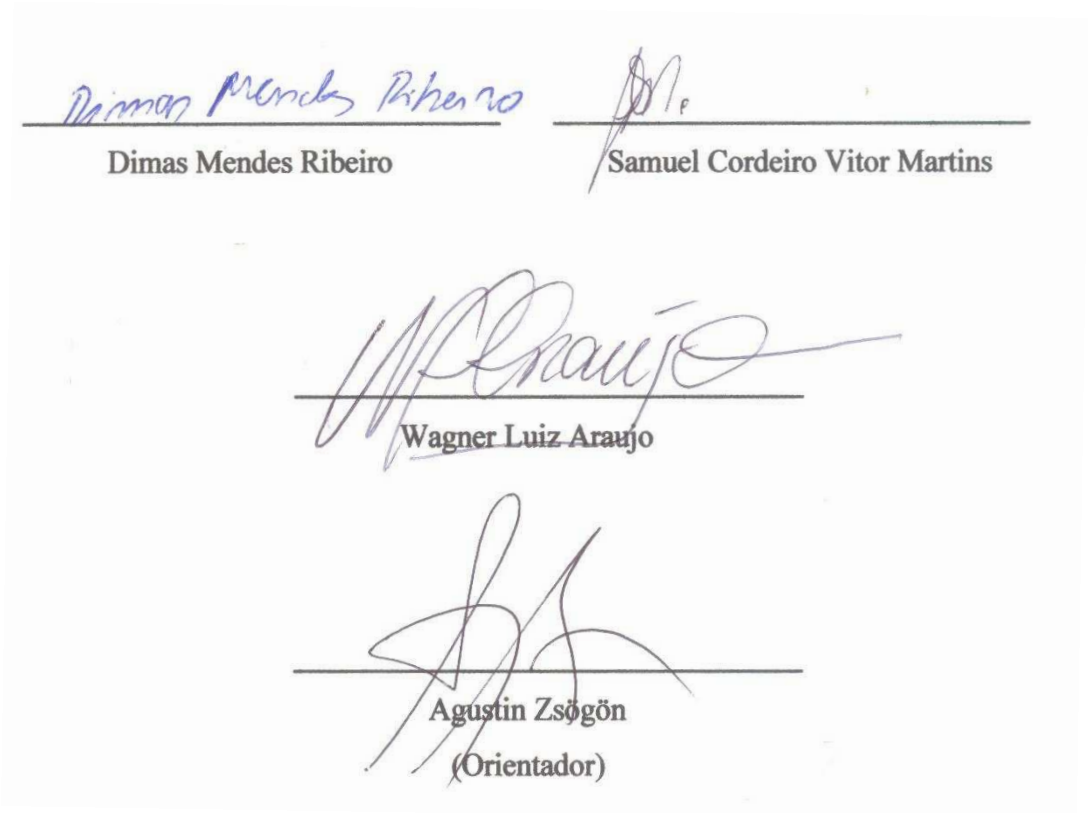

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ABSTRACT

MONCALEANO, Robledo Kerly Jessenia, M.Sc., Universidade Federal de Viçosa, July, 2017. **The roles of florigen and antiflorigen in physiology and development of tomato plants.** Adviser: Agustin Zsögön.

The CETS gene family CENTRORADIALIS / TERMINAL FLOWER 1 / SELF-PRUNING, homologs in Antirrinum, Arabidopsis and tomato have been characterized by the control of the balance and regulation of the determined and indeterminate growth. Twelve paralogous genes were identified in tomatoes, classified into three clades: SELF-PRUNING (SP) –like floral development repressors, SINGLE FLOWER TRUSS (SFT) -like as flowering promoters and the third group MOTHER FLOWER TRUSS (MFT) involved in germination processes. The SP repressor gene of flowering or antiflorigenic is responsible for the growth determined in tomato, but the physiological basis is unknown. On the other hand, the FLOWERING LOCUS T (FT) orthologous SFT in Arabidopsis was described like promoter of flowering with fruit number regulation, growth habit and water use efficiency. However, the mechanism associated with the efficiency of water use has not been explained either. This work aimed at analyze the relationship of SP with auxin hormone associated with growth and development using allelic variations of SP in comparison with Diageotropica (DGT), mutant of auxin polar transport, and also to identify pleiotropic effects of SFT in leaves of tomato from allelic variations to SFT. We found that auxin free levels, auxin polar transport and gravitropic curvature of the shoot apex are all altered by SP. In addition, SP and DGT reciprocally affect AUX/IAA and ARF transcript accumulation in the sympodial meristem. Our results provide evidence of the relation SP and auxin and will allow to increase the understanding of the habit of growth. Subsequently, we show that SFT besides promoting changes in tomato growth and development, has pleiotropic effects on gas exchange and foliar anatomy. These results suggest a new function of the SFT gene and aid to the understanding of the role of SFT in leaf development

RESUMO

MONCALEANO, Robledo Kerly Jessenia, M.Sc., Universidade Federal de Viçosa, julho de 2017. **Efeitos do florigeno e antiflorigeno na fisiologia e desenvolvimento de plantas de tomate.** Orientador: Agustin Zsögön

A família dos genes pertencentes à família CENTRORADIALIS / TERMINAL FLOWER 1 / SELF-PRUNING (CETS), homólogos em Antirrinum, Arabidopsis e tomateiro tem sido caracterizados pelo controle do equilíbrio e regulação do crescimento determinado e indeterminado. Em tomateiro foram identificados 12 genes parálogos, onde foram classificados em três clados: SELF-PRUNING (SP) e similares como repressores do desenvolvimento floral, SINGLE FLOWER TRUSS (SFT) e similares como promotores da floração e o terceiro grupo MOTHER FLOWER TRUSS (MFT) envolvidos em processos de germinação. O gene SP repressor da floração ou antiflorigeno é o responsável do crescimento determinado em tomateiro, porem a base fisiológica é desconhecida. Por outra parte, o SFT ortólogo de FLOWERING LOCUS T (FT) em Arabidopsis foi descrito além de promotor de floração como regulação de número de frutos, hábito de crescimento e eficiência do uso de agua. Porém o mecanismo associado a eficiência do uso de agua também não tem sido explicado. Nesse contexto, nosso primer objetivo foi analisar a relação de SP com auxina hormônio associado a desenvolvimento usando variações alélicas de SP em comparação com Diageotropica, mutante para transporte polar de auxina, e o segundo objetivo foi identificar efeitos pleiotrópicos de SFT em folhas de tomateiro a partir de variações alélicas para SFT. Nós encontramos que níveis livres de auxina, transporte polar de auxina e curvatura gravitropica do ápice caulinar são todos alterados por SP. Além disso, SP e DGT afetam reciprocamente AUX/IAA y acumulo de transcrito ARF no meristema simpodial. Nossos resultados proveem evidencia da relação SP e auxina e vão permitir aumentar a compreensão do habito de crescimento. Posteriormente, mostramos que SFT além de promover mudanças no crescimento e desenvolvimento de tomateiro tem efeito pleiotrópico nas trocas gasosas e anatomia foliar. Estes resultados sugerem uma nova função do gene SFT e ajudam ao entendimento do papel de SFT no desenvolvimento foliar

CONTENTS

GENERAL INTRODUCTION

In crop plants, the architecture is a very important agronomic characteristic, influencing the suitability of the plant yield and the harvest efficiency. In this context, architecture is defined as the three-dimensional organization of the plant body including branching pattern, growth habit and position of leaves and flowers in the branch (Reinhardt and Kuhlemeier, 2002). Plant architecture refers to the height and spread a plant reaches while it is growth can be monopodial or sympodial growth. In plants with monopodial growth, e.g. Arabidopsis, the shoot apical meristem (SAM) is indeterminate, vegetative and reproductive phases are clearly differentiated. In contrast, plants with sympodial growth as tomato present a determinate SAM, where vegetative and reproductive stages are shown in alternate modular units (sympodia). The tomato growth habit can be classified in: determinate, semi-determinate and indeterminate.

Tomato is normally a perennial plant with indeterminate growth habit (Yeager, 1927). The vegetative apical meristem becomes floral after the production 9-12 internodes with leaves. Growth then continues from the nearest axillary meristem below the inflorescence, which generates a new branch with three leaves and an inflorescence (sympodium). However, this pattern is altered in tomato of determinate growth with recessive alleles of the SELF-PRUNING gene (SP), in which, from the first flowering time, the number of leaves per sympodial units is gradually reduced (though the mutation does not accelerate the appearance of the first inflorescence), until the formation of two consecutive inflorescences which terminate vertical growth (MacArthur, 1932a). Finally, all vegetative meristems of the plant, which form leaves and branches become floral meristems (Fridman et al., 2001).

The recessive mutation self-pruning (sp) appeared spontaneously in Florida, USA in 1914 (Rick, 1978). Until then, all tomato cultivars were indeterminate, which is conditioned by the dominant allele SELF-PRUNING (SP) for the locus in question. The sp mutation was subsequently mapped on chromosome 6 as a homozygous recessive allele which causes a "self-pruning", that is, loss of function to continue forming vegetative branches after flowering. In return for this limited growth of the

stem, the plant has a vigorous growth of lateral branches, giving it compact and thick aspect characteristic of processing tomato. The growth of certain sp/sp plants leads to an almost simultaneous ripening of all the fruits, which, combined with the compact growth habit allows mechanical harvesting on a large scale (Stevens and Rick, 1986b). Starting in 1964, the sp mutation has been extensively used in breeding programs for processed tomatoes for the production of purees, sauces, extracts, juices, and others.

The SP gene is an ortholog of CENTRORADIALIS (CEN) and TERMINAL FLOWER 1 (TFL1), that promotes growth and represses flowering in tomato, Arabidopsis and Antirrhinum, respectively (Pnueli et al., 1998). These genes play a critical role in shaping plant architecture that is conserved in among species (Pnueli et al., 2001) and were previously thought to be primarily involved in the control of the switch to reproductive development, recent studies on species with sympodial growth suggest a more general role in controlling the growth and termination of meristems (Lifschitz et al., 2006). The CETS gene family was described with twelve genes members that were mapped on chromosomes 1 (SP1C-A, SP1C-B), 2 (SP2G), 3 (SP3C, SP3D, SP3I), 5 (SP5G), 6 (SP6A, and SP), 9 (SP9D) and 11(SP11B-A, SP11B-B) (Carmel-Goren et al., 2003; Kim et al., 2014) (Carmel-Goren et al., 2003; Kim et al., 2014).

The SP3D locus corresponds to the SINGLE FLOWER TRUSS gene (SFT) which in turn is the FLOWERING LOCUS T ortholog gene (FT) from Arabidopsis (Lifschitz et al., 2006). FT protein, a mobile signal recognized as a major component of florigen, has a central position in mediating the onset of flowering (Corbesier et al., 2007; Wigge, 2011). Regulation of FT expression promotes flowering regardless of the pathway including autonomous promotion, photoperiodic, vernalization, or temperature-dependent pathways (Kardailsky et al., 1999; Kobayashi, 1999; Blázquez et al., 2003). SFT regulates termination of sympodial meristems and leaf architecture in tomato in a SP-dependent manner, indicating that SFT acts not only as a mobile floral signal, but also as a general systemic regulator of growth in tomato (Shalit et al., 2009).

Numerous studies have demonstrated that the proteins encoded by FT-like genes act not only as the mobile flowering signals in plants, FT-like proteins have also

been identified as major regulatory factors in a wide range of developmental processes including fruit set (Lifschitz et al., 2006), tuberization (Navarro et al., 2011), and stomatal aperture (Kinoshita et al., 2011) where was showed that FT to be expressed in guard cells and to influence stomatal opening via activation of the plasma membrane H^+-ATPases.

However, the physiological basis for regulation of growth habit for SP and the effects of SFT in tomato leaf structure and physiology have not been fully explored. Thus, the assays described in this dissertation were designed to test the hypotheses, Florigen and antiglorigen have pleiotropic effects in growth and development in tomato.

Accordingly, this dissertation was divided in two chapters, where our objectives were to analyze the relationship of SP with auxin transport and responsiveness and moreover identify pleiotropic roles of SFT in development and water relations in tomato plants.

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CHAPTER I

SELF-PRUNING affects auxin responses synergistically with the cyclophilin A protein DIAGEOTROPICA in tomato

Running title: SELF-PRUNING regulates auxin responses in tomato

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Key words: shoot architecture, morphogenesis, auxin signaling, mutants, CETS family, Solanum lycopersicum

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SUMMARY

The SELF PRUNING (SP) gene is a key regulator of growth habit in tomato (Solanum lycopersicum). It is an ortholog of TERMINAL FLOWER 1, a phosphatidyl-ethanolamine binding protein with anti-florigenic activity in Arabidopsis thaliana. A spontaneous loss-of-function sp mutation has been bred into most industrial tomato cultivars, as it produces a suite of pleiotropic effects that are favorable for mechanical harvesting, including determinate growth habit, short plant stature and simultaneous

fruit ripening. However, the physiological basis for these phenotypic differences has not been thoroughly explained. Here, we show that the sp mutation alters polar auxin transport as well as auxin responses such gravitropic curvature and root proton extrusion. We further demonstrate that free auxin levels and auxin-regulated gene expression patterns are altered in sp, with epistatic effects of diageotropica, a mutation in cyclophilin A protein encoding gene. Our results indicate that SP impacts growth habit in tomato, at least in part, via changes in auxin transport and responsiveness. These findings hint at novel targets that could be manipulated in the control of growth habit and productivity.

INTRODUCTION

Shoot architecture is a key agricultural trait determined mainly by side branching, internode elongation and shoot determinacy (Wang and Li, 2008). Each of these parameters configures an active research area where considerable theoretical and applied knowledge has been gained over the last decade. Shoot determinacy is a domestication trait in crop species as diverse as soybean (Glycine max), common bean (Phaseolus vulgaris) and tomato (Solanum lycopersicum) (Benfey and Chua, 1990; Tian et al., 2010; Repinski et al., 2012). Tomato is a perennial species cultivated as annual. Wild tomatoes display indeterminate growth, resulting from a sequential addition of modules (sympodial units) formed by three leaves and an inflorescence. The sympodial growth starts in tomato when the vegetative apical meristem is converted into floral after a series of 8-12 internodes with leaves (Samach and Lotan, 2007). Vegetative growth, however, continues through the top-most axillary meristem, which grows vigorously displacing the inflorescence to the side and producing a new sympodial unit with three leaves and an inflorescence. This process is indefinitely iterated by concatenation of sympodial units one on top of the other. In 1914, a spontaneous recessive mutation was discovered, which led to compact, bushy plants with a reduced number of leaves in successive sympodial units (Yeager, 1927; MacArthur, 1934). It was later shown that the mutation is a single nucleotide substitution in the SELF-PRUNING (SP) gene (Benfey and Chua, 1990), which shares sequence similarity with a group of mammalian polypeptides involved in cell signaling, phosphatidylethanolamine binding proteins (PEBPs) (Hengst et al., 2001; Kroslak et al., 2001). Breeding of this mutation into industrial tomato cultivars was

instrumental in the advent of mechanical harvest (Rick, 1978; Stevens and Rick, 1986a). The loss-of-function sp mutant leads to determinate growth habit, as opposed to the indeterminate growth habit of wild-type tomatoes. The determinate growth habit occurs via progressively reduced number of leaves per sympodium until termination in two consecutive inflorescences that top vertical growth of the plant (Samach and Lotan, 2007). Hence, this phenotype leads to simultaneous fruit ripening, therefore allowing mechanical harvest in field-grown processing tomatoes (Stevens and Rick, 1986a).

SP belongs to the CETS gene family, which comprises CENTRORADIALIS (CEN) and TERMINAL FLOWER 1 (TFL1) of Antirrhinum and Arabidopsis, respectively (Benfey and Chua, 1990; Wickland and Hanzawa, 2015). SINGLE FLOWER TRUSS (SFT)/SP3D – a homolog of FLOWERING LOCUS T (FT) and HEADING DATE 3A (HD3A) in Arabidopsis and rice, respectively – is a second CETS gene involved in the control of growth habit in tomato (Alvarez et al., 1992; Kojima, 2002). Unlike sp mutants, which do not affect flowering time, tomato sft loss-of-function mutants are late flowering, and also show a disruption in the sympodial growth pattern: they produce a single and highly vegetative inflorescence, alternating solitary flowers and leaves (Molinero-Rosales et al., 2004). The final phenotypic outcome produced by SP and SFT depends on their local ratio, with the former maintaining meristems in an indeterminate state and the latter promoting the transition to flowering (Park et al., 2014a). Heterozygous sft mutants in a homozygous sp mutant background display yield heterosis in tomato (Krieger et al., 2010). Hence, the SP/SFT genetic module has been proposed as a target to improve plant architecture and thereby crop yield (McGarry and Ayre, 2012; Zsögön et al., 2017). It has also been previously suggested that SP gene function could be linked to auxin (Pnueli et al., 2001), a hormone with strong effects on plant morphogenesis (Berleth and Sachs, 2001).

Auxin is a key controller of plant development; however, its role in the regulation of plant growth habit is still unclear. An aspect that sets auxin apart from the other plant hormones is the relatively well understood nature of its transport through the plant body (Friml, 2003; Petrasek and Friml, 2009). ‘Polar auxin transport’ (PAT), which occurs basipetally from the apical meristem, is critically important for the distribution of this hormone within plant tissues (Rubery and Sheldrake, 1974; Sheldrake, 1974). PAT works as an organizer of apical-basal polarity in the plant body

(Friml et al., 2006), thus controlling a multiplicity of developmental processes (Reinhardt et al., 2003; Blilou et al., 2005; Scarpella et al., 2006). A second, more diffuse, auxin distribution mechanism named connective auxin transport (CAT) was recently proposed (Bennett et al., 2016).

It has recently been shown that the cyclophilin A protein DIAGEOTROPICA (DGT) affects polar auxin transport (PAT) in tomato (Ivanchenko et al., 2015). DGT is a cyclophilin with peptidyl-prolyl trans-cis isomerase activity (Takahashi et al., 1989; Oh et al., 2006). Some of the most significant phenotypic defects caused by the lack of functional DGT protein in tomato are horizontal shoot growth, thin stems, altered secondary vascular differentiation and roots lacking lateral branches. Here, we investigated whether SP affects auxin responses, by itself, and in combination with DGT. We produced four combinations of functional and loss-of-function mutant alleles of SP and DGT (i.e. SP DGT, SP dgt, sp DGT and sp dgt) in a single tomato genetic background (cv. Micro-Tom) and assessed a series of physiological responses to auxin. We found that free auxin levels, polar auxin transport and gravitropic curvature of the shoot apex are all altered by SP. Our results further show that SP and DGT reciprocally affect AUX/IAA and ARF transcript abundances at the sympodial meristem, the key niche of SP function in growth habit.

EXPERIMENTAL PROCEDURES

Plant material

Seeds of the tomato (Solanum lycopersicum, L.) cultivar Micro-Tom (MT) were kindly donated by Dr. Avram Levy (Weizmann Institute of Science, Israel) in 1998 and subsequently maintained (through self-pollination) as a true-to-type cultivar. MT seeds carrying the synthetic auxin-responsive (DR5) promoter fused to the reporter gene uid (encoding a β-glucuronidase, GUS) were obtained from Dr. José Luiz García-Martínez (Universidad Politécnica de Valencia, Spain). The diageotropica (dgt) mutation was introgressed into MT from its original background in cv. VFN8 (LA1529), donated by Dr. Roger Chetelat (Tomato Genetics Resource Center, Davis, University of California, USA). The functional allele of SELF-PRUNING was introgressed from cv. Moneymaker (LA2706). pPIN1:PIN1::GFP MT transgenic line was generated as described by Pino et al. (2010) using the construct donated by Prof.

Cris Kuhlemeier (University of Bern, Switzerland). Homozygous SP pPIN1:PIN1::GFP plants were screened by PCR and progeny tests as described (Sestari et al., 2014).

Introgression of mutations into the MT cultivar was described previously (Carvalho et al., 2011). A comparison between indeterminate (SP/SP) and determinate (sp/sp) plants in the MT background has been published previously (Vicente et al., 2015). Both sp and dgt mutations were confirmed by CAPS marker analyses and sequencing, respectively. All experiments were conducted on BC_6F_3 plants or subsequent generations.

In vitro seedling cultivation was conducted under controlled conditions (16h/8 h day/night, approximately 45 µmol m^{-2} s^{-1} PAR, 25±1ºC) in flasks with 30 ml of MS/2 media gellified with 0.5% agar, pH 5.8. Seeds were surface sterilized by agitation in 30% (v/v) commercial bleach (2.7% sodium hypochlorite) for 15 min followed by three rinses with sterile distilled water.

Growth conditions

Plants were grown in greenhouse in Viçosa (642 m asl, 20º45' S; 42º51' W), Minas Gerais, Brazil, under semi-controlled conditions: mean temperature of 28°C, 11.5 h/13 h (winter/summer) photoperiod, and 250-350 µmol m^{-2} s^{-1} PAR irradiance and irrigation to field capacity twice a day. Seeds were germinated in 350-mL pots with a 1:1 (v/v) mixture of commercial potting mix Basaplant® (Base Agro, Brazil) and expanded vermiculite supplemented with 1g L^{-1} 10:10:10 NPK and 4 g L^{-1} dolomite limestone ($MgCO_3$ + $CaCO_3$). Upon appearance of the first true leaf, seedlings of each genotype were transplanted to pots containing the soil mix described above, except for the NPK supplementation, which was increased to 8 g L^{-1}.

Auxin quantification

Endogenous indole acetic acid (IAA) levels were determined by gas chromatography tandem mass spectrometry-selecting ion monitoring (GC-MS-SIM, Shimadzu model GCMS-QP2010 SE). Samples (50-100 mg fresh weight, FW) were extracted and methylated as described in (Rigui et al., 2015). About 0.25 µg of the

labeled standard [$^{13}C_6$]IAA (Cambridge Isotopes, Inc.) was added to each sample as internal standards. The chromatograph was equipped with a fused-silica capillary column (30m, ID 0.25 mm, 0.50 μm thick internal film) DB-5 MS stationary phase using helium as the carrier gas at a flow rate of 4.5 mL min^{-1} in the following program: 2 min at 100ºC, followed a ramp by 10ºC min^{-1} to 140ºC, 25ºC min^{-1} to 160ºC, 35ºC min^{-1} to 250ºC, 20ºC min^{-1} to 270ºC and 30ºC min^{-1} to 300ºC. The injector temperature was 250°C and the following MS operating parameters were used: ionization voltage, 70 eV (electron impact ionization); ion source temperature, 230°C; interface temperature, 260°C. Ions with a mass ratio/charge (m/z) of 130 and 189 (corresponding to endogenous IAA) and 136 and 195 (corresponding to [$^{13}C_6$]-IAA) were monitored and endogenous IAA concentrations were calculated based on extracted chromatograms at m/z 130 and 136.

Polar auxin transport analysis

Polar auxin transport (PAT) was assayed in hypocotyl segments of 2-week-old seedlings according to the protocol originally described by (Al-Hammadi, 2003) with some modifications. Briefly, 10 mm hypocotyl sections were excised and incubated in 5 mM phosphate buffer (pH 5.8) containing 1 µM IAA for 2 h at 25 ± 2ºC on a rotary shaker (200 rpm). These segments were placed between receiver (1% [w/v] agar in water) and donor blocks (1% [w/v] agar in 5 mM phosphate buffer [pH 5.8] containing 1 µM IAA and 100 nM ^{3}H-IAA) oriented with their physiological tips toward the donor blocks. After 4 h of incubation inside a humid chamber at 25 ± 2ºC, the receiver blocks were removed and stored in 3 mL scintillation cocktail (Ultima Gold™, PerkinElmer, USA). Receiver blocks plus scintillation cocktail were shaken overnight at 100 rpm and 28 ± 2ºC before analysis in a scintillation counter. As negative control, some hypocotyl segments were sandwiched for 30 min between NPA-containing blocks (1% [w/v] agar in water containing 20 µM NPA) prior the auxin transport assays. ^{3}H d.p.m. was converted to fmol auxin transported as described in (Lewis and Muday, 2009).

Rhizospheric pH measurements

Plates containing either 4-day-old or 10-day-old seedlings were used in each of three independent experiments as described in (Zandonadi et al., 2016). Briefly, seedlings were placed over autoclaved filter paper and treated for 60 minutes with distilled water (control) or NAA 1 µM. Subsequently, pretreated seedlings were transferred to a 5 mm agar (1 %) gel layer that contained the pH bromocresol purple indicator (0.04 g dm^{-3}) for an additional 24 h of incubation. Afterwards, the pH was measured in the gel using a flat pH probe in the lateral root formation zone. The specific ATPase activity was secured by using negative control plates with 500 µM sodium orthovanadate (Na_3VO_4), the plasma membrane (PM) H^+-ATPase specific inhibitor.

Histochemical assays

Transgenic DR5::GUS plants were incubated overnight at 37°C in GUS staining solution (100 mM NaH_2PO_4; 10 mM EDTA, 0,5 mM $K_4Fe(CN)_6$; 0,05% Triton X-100, 1mM 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid). Following GUS staining, samples were washed in a graded ethanol series to remove chlorophyll. Samples were then photographed using a Leica S8AP0 (Wetzlar, Germany) magnifying glass set to 80× magnification, coupled to a Leica DFC295 camera (Wetzlar, Germany).

Confocal microscopy

Seedlings of homozygous pPIN1:PIN1::GFP plants either in MT or MT-SP were grown in vitro for two weeks. Root tips were carefully sampled from the main root and mounted on a slide for observation in a LSM510 META (Zeiss, Oberkochen, Germany) laser-scanning confocal microscopy. The observations were repeated three times on different batches of seedlings and representative results are shown.

Gene expression analyses

Total RNA was extracted from approximately 30 mg FW of sympodial meristems following the protocol of the manufacturer (Promega SV total RNA isolation sytem). Four biological replicates were used for subsequent cDNA synthesis.

RNA integrity was analyzed on 1% agarose gel and RNA concentration was estimated before and after treatment with DNase I (Amplification Grade DNase I, Invitrogen). Total RNA was transcribed into cDNA using the enzyme reverse transcriptases, Universal RiboClone® cDNA Synthesis (Promega, Madison, WI, USA) following the manufacturers' protocols.

For gene expression analyses Power SYBR® green PCR Master Mix was used in MicroAmp™ Optical 96-well reaction plates (both from Applied Biosystems, Singapore) and adhesive film MicroAmp™ Optical (Applied Biosystems, Foster City, CA, USA). The number of reactions from the cycle threshold (CT) as well as the efficiency of reaction were estimated using the Real-Time PCR Miner tool (Zhao and Fernald, 2005).

Relative expression was normalized using actin and ubiquitin; actin was used to calculate ΔΔCT assuming 100% efficiency of amplification of genes (2^- ΔΔCT). Primer sequences used are shown in Supplementary Table 1. Melting curves were checked for unspecific amplifications and primer dimerization.

In silico sequence analyses

SP gene alignments was performed using the ClustalW alignment option of the Geneious R9 (Biomatters, Auckland, New Zealand) software package.

Statistical analysis

ANOVA and Tukey HSD tests were performed using Assistat 7.6 beta (http://assistat.com). Percentage data were converted to inverse function (1/X) before analysis.

RESULTS

Comparing the four combinations of homozygous wild-type and mutant lines for the SP and DGT genes generated (i.e., SP DGT, SP dgt, sp DGT and sp dgt), it became clear that the growth habit was affected solely by SP and not by DGT (Fig. 1A). Regardless of their DGT or dgt allele, SP plants showed indeterminate growth whereas sp mutants were determinate (Fig. 1A). Time to flowering, however, was affected by both genes in combinatorial fashion. sp DGT plants flowered earlier, followed by SP DGT plants, whereas both SP dgt and sp dgt were late flowering (Fig.

1B). The number of leaves to the first inflorescence was also affected by the combination of alleles (Fig. 1C), although not reflecting the time to flowering. Thus, although dgt sp flowered later, it formed less leaves until before producing the first inflorescence. This suggests that the flowering inducer signal is low in this genotype and that other factor is controlling leaf emission. Regardless of their SP allele, dgt mutants exhibited markedly reduced transcript abundance of the flowering inducer SINGLE FLOWER TRUSS (SFT) compared to DGT plants (Fig. 1D), which fits with the delayed flowering in these mutants in both SP and sp backgrounds (Fig. 1B). Axillary branching was affected mainly by the SP gene, which led to reduced bud outgrowth in plants carrying the wild-type allele; the dgt mutation, however, exacerbated this repressing effect (Fig. 1E). On the other hand, sp mutants branched more profusely when combined with dgt than DGT (Fig. 1E). Thus, dgt can enhance apical dominance or increase branching, depending on the presence or absence of a functional SP allele, respectively. Other morphological variables, such as stem diameter, were influenced by the combined effects of the SP and DGT alleles (Table 1).

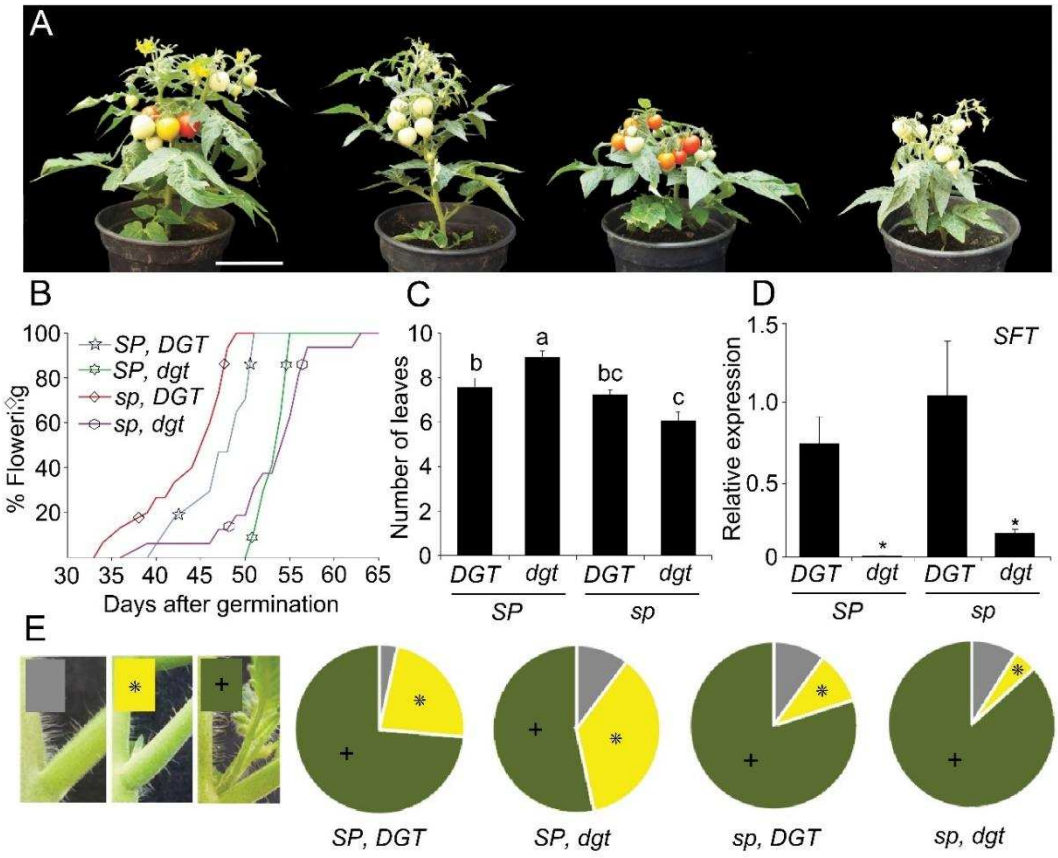

Figure 1. Synergisticallyphenotype of the self-pruning (sp) and diageotropica (dgt) mutations in tomato cv. Micro-Tom. (a) Representative plants of SP DGT; SP dgt; sp DGT (original cv. Micro-Tom) and sp dgt, 90 dag. Note the simultaneous fruit ripening in sp compared to SP, a well-known effect of the sp mutation. The dgt mutation delays fruit ripening (at least in part due to its late flowering, as indicated in b) in either genetic background. Bar=10 cm. **(b) sp and dgt affect chronological time to flowering.** Percentage of plants (n=15) with at least one open flower. Micro-Tom (sp DGT) plants flower earlier than wild type (SP DGT), whereas dgt mutants are late flowering. **(c) sp and dgt influence developmental time to flowering.** The number of leaves produced before the first inflorescence was reduced in sp DGT (Micro-Tom) and increased in genotypes carrying the functional allele of SP. **(d) sp and dgt alter expression of the flowering inducer SINGLE FLOWER TRUSS (SFT).** The dgt mutation leads to lower SFT expression and thus delays flowering. A minor influence from SP reducing SFT levels is also noticeable. **(e) sp and dgt synergistically affect side branching.** Schematic representation of side branching in shoots of SP DGT; SP dgt; sp DGT (Micro-Tom) and sp dgt (n=15). Pizza charts depicting the distribution of side branches in each genotype 60 dag. Grey denotes absence of axillary bud; yellow, a visible bud (>1cm) and dark green, a full branch (with one or multiple leaves). Asterisks indicate statistically significant differences compared with the wild-type SP DGT (Student's t-test, $P<0.05$).

Table 1. Parameters that define growth habit: (i) number of leaves on the primary shoot (PS) (i.e. number of leaves up to the first inflorescence); (ii) number of leaves on the main shoot (MS) (i.e. number of leaves of PS plus leaves on sympodial units (SU) following the first inflorescence); (iii) height of PS, MS and lateral shoot (LS); iv) internode length (cm); v) leaf angle insertion; vi) diameter of stem and vii) number of flowers, fruits and flowers per inflorescence. Measurements performed 60 days after germiantion. Data are mean ± s.e.m. (n = 10 plants).

	SP, DGT	**SP, dgt**	**sp, DGT**	**sp, dgt**
Number of leaves on the primary shoot	9.00±0.36 a	9.09±0.28 a	7.50±0.48 b	7.67±0.58 b
Height of the main shoot	17.45±0.79 a	12.74±0.61 b	13.45±0.49 b	10.70±1.07 b
Length of fourth internode	1.08±0.12 a	1.11±0.07 a	1.28±0.13 a	1.22±0.22 a
Leaf insertion angle	74.81±3.41a	74.87±3.26 a	73.01±4.38 a	63.54±5.22 a

Stem diameter	5.46±0.26 a	4.36±0.12 ab	5.36±0.21 a	4.45±0.1 b
Flowers per inflorescence	7.00±0.71 b	7.80±0.45 a	7.00±0.00 b	7.00±0.5 b
Number of inflorescences	12.4±1.14 a	9.20±1.30 b	7.40±0.89 b	8.80±1.48 b

Different letters indicate statistically significant differences (Tukey's test, $p < 0.05$) among genotypes.

Next, the endogenous levels of free indolyl-3-acetic acid (IAA), which is the most abundant auxin in plants (Bartel and Fink, 1995), was determined in three sections of tomato seedlings: leaves plus cotyledons, hypocotyls and roots (Fig. 2A). In leaves plus cotyledons, IAA concentration was more than twice higher in sp dgt double mutant than in the other three genotypes (Fig. 2B). In the hypocotyl tissues, SP DGT seedlings had the lowest free IAA content, the sp and dgt single mutants presented intermediate values and the double mutant (sp dgt) exhibited the highest IAA levels (Fig. 2C). Although root IAA levels were clearly higher than in the other hypocotyl regions analyzed, no statistically significant differences in root IAA content was observed among the four genotypes (Fig. 2D).

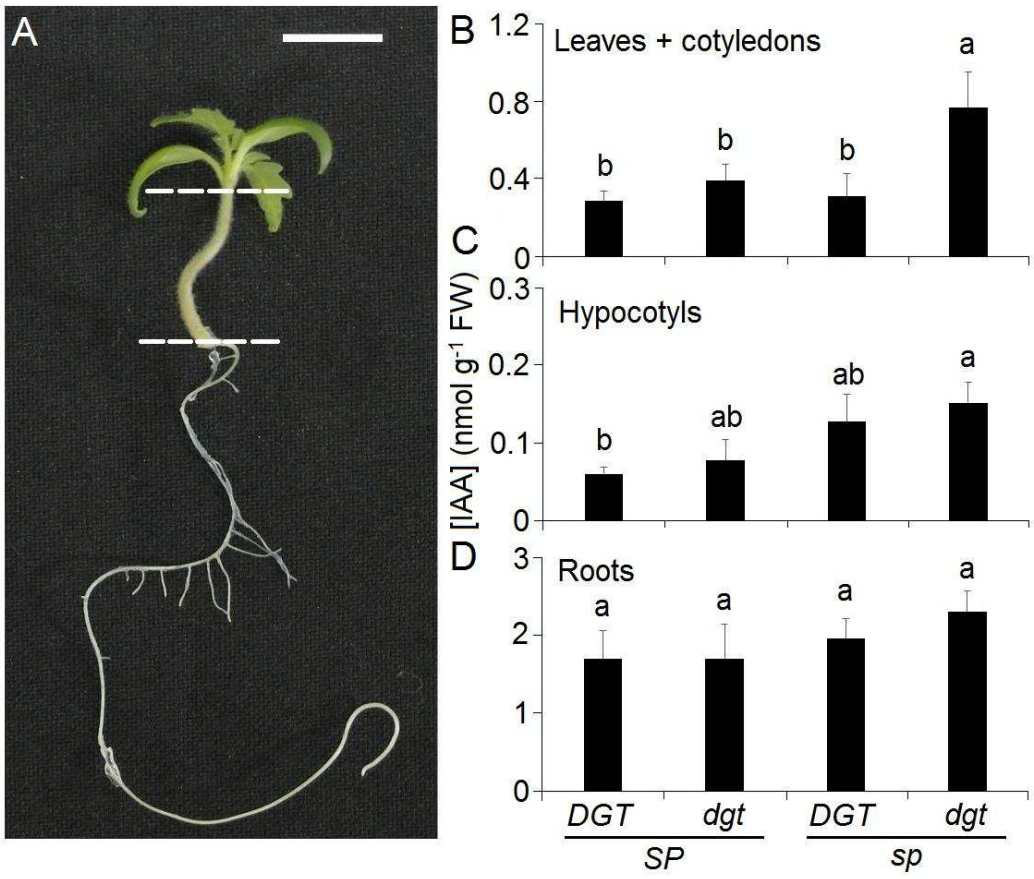

Figure 2. Auxin levels in tomato seedlings are affected synergistically by the self-pruning (sp) and diageotropica (dgt) mutations. (a) Representative 7-day old seedling showing the dissection points for auxin quantitation. Free IAA levels in (b) leaves + cotyledons, (c) hypocotyls and (d) roots. Data are mean±s.e.m. (n=10) Different letters indicate statistically significant differences (Tukey's test, $P < 0.05$) among genotypes.

To understand the variation in endogenous free IAA levels within the seedling tissues and among the four genotypes, we next determined polar auxin transport (PAT) across detached hypocotyls. Whereas SP DGT had the highest PAT values, the sp and dgt single mutants had intermediate ones and the double mutant the lowest values (Fig. 3A). This indicates that both sp and dgt alleles reduces PAT and that their effects can be additive. A strong (R^2=0.75) negative correlation was observed when compared IAA transport rates and IAA concentration in hypocotyls (Fig. 3B). As PAT and auxin concentration are known to influence vascular patterning (Scarpella, 2017), we also analyzed cross-sections of stems in adult plants. Marked differences were observed in both the extent of xylem development and the number of secondary xylem elements among the genotypes (Fig. 3C). Compared to the wild type, the sp dgt double mutant

showed impaired xylem development, both quantitative and qualitatively (Fig. 3C). Another physiological response affected by PAT is negative gravitropism of the shoot (Morita, 2010). The kinetics of gravitropic curvature in seedling shoots was affected by both SP and DGT (Fig. 4A). Loss of SP function decreased gravitropic response in both DGT and dgt backgrounds. Rhyzogenesis from cotyledon explants is an assay to determine auxin sensitivity (Cary et al., 2001). As expected, root formation was reduced in dgt mutants (Coenen and Lomax, 1998), but also in sp compared to SP in the presence of a functional DGT allele (Fig. 4B).

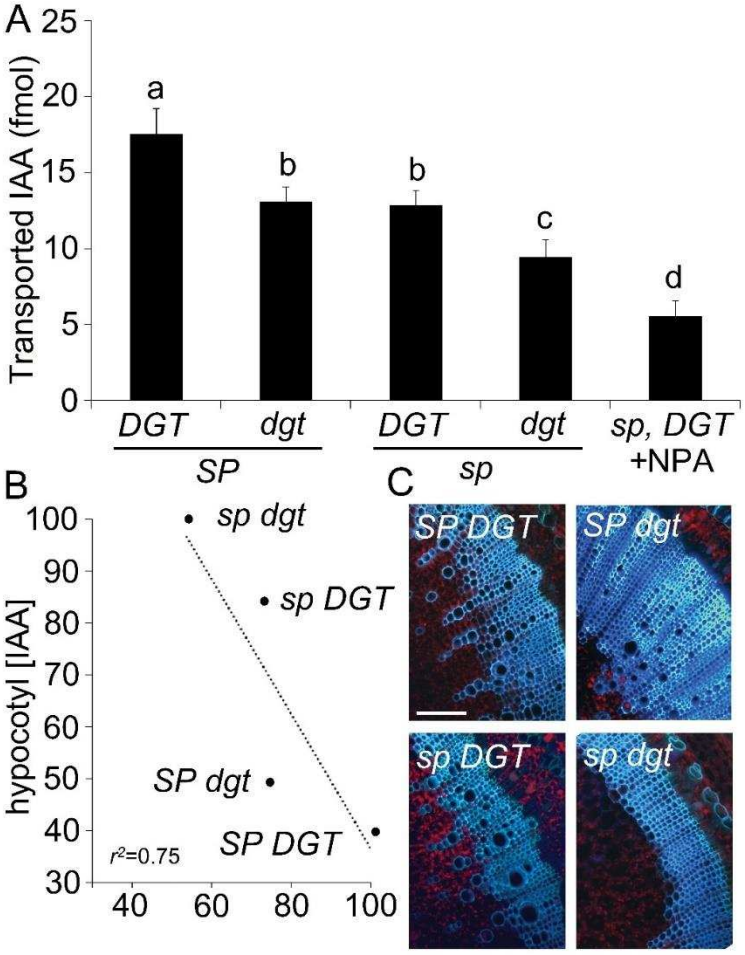

Figure 3. (a) The self-pruning (sp) mutation exacerbates defective polar auxin transport in hypocotyls caused by diageotropica (dgt). Basipetal ^{3}H-IAA transport in 10 mm hypocotyl sections of wild-type (SP, DGT), SP dgt, sp DGT (Micro-Tom; also the negative control treated with NPA) and double mutant sp dgt roots. Data are mean±s.e.m. (n=10). Asterisk indicates statistically significant differences between treatments (ns, non-significant; **P≤0.05; ***P≤0.01, t-test). **(b) ^{3}H-IAA transport intensity and hypocotyl IAA concentration are negatively correlated**. Values for

both parameters were equalized to percentage setting the highest value as =100 **(c) sp and dgt affect vascular patterning in tomato stems**. Cross-sections of the fifth internode taken 45 dag. Bar = 100 µm.

Histochemical analysis of DR5 promoter activity revealed no discernible staining difference in both SP and sp seedlings incubated in water, although roots of the sp mutant showed a shorter trace of GUS precipitate in the vascular bundle (Fig. 4C). Exogenous IAA, however, strongly induced GUS expression in SP compared to sp plants, which was evident both in seedlings and in root tips (Fig. 4C). As PIN-FORMED 1 (PIN1) auxin efflux transporter is a key player determining auxin distribution in plants, we visually assessed its distribution in root tips via a GFP fusion construct. Stronger fluorescence was found in SP roots, both in the periphery and the vascular bundle, compared to very faint signal in sp mutants (Fig. 4D).

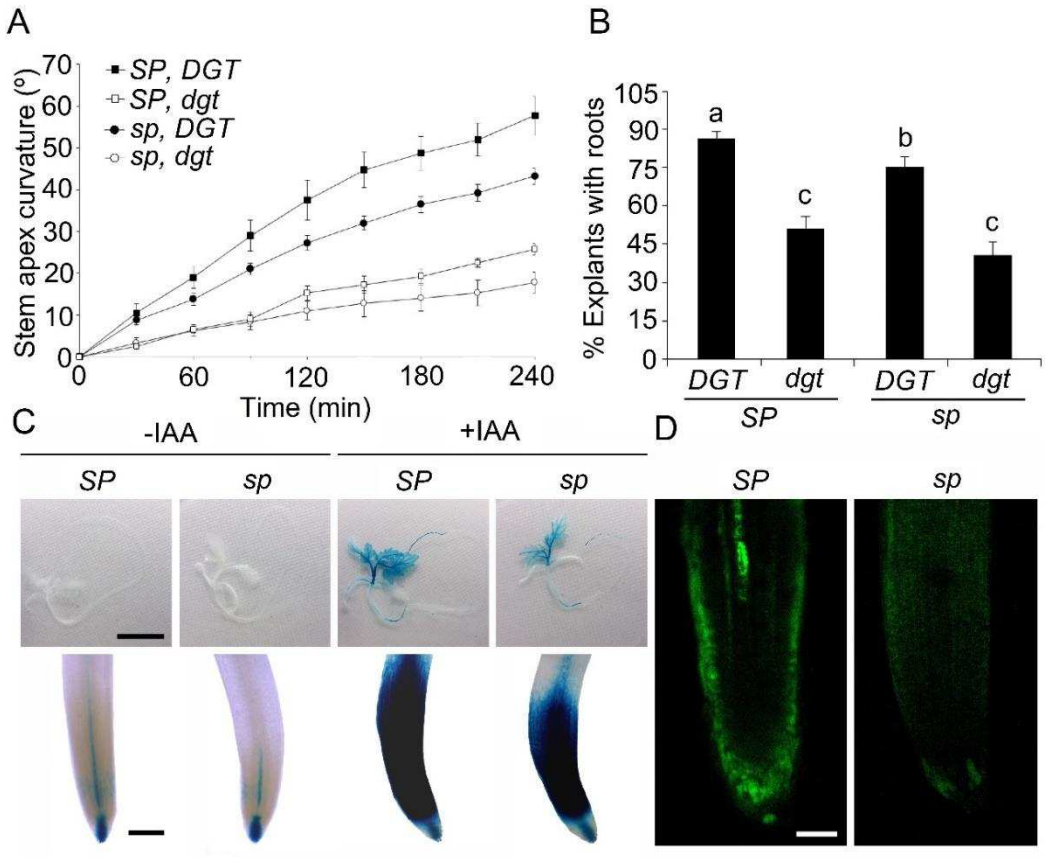

Figure 4. Impact of the self-pruning (sp) mutation on auxin responses in planta. (a) Kinetics of gravitropic response in the shoot (n=4). (b) Expression pattern of the GUS reporter driven by the auxin-inducible DR5 promoter in wild-type (SP) and

mutant (sp) seedlings (bar=2 cm) and their root tips (bar=250 µm) in the absence or presence of exogenous auxin (20 µM, 3h) 15 dag. (c) Fluorescence pattern of PIN1::GFP in root tips of SP and sp plants (bar=100 µm). (d) Auxin-induced rhyzogenesis in cotyledon explants evaluated 12 days after excision of 8-day-old seedlings, grown in MS medium supplemented with 0.4 µM NAA. Values are mean ± sd (n=60 explants per genotype).

As auxin induces rapid changes in the plasma membrane H^+ secretion, through alterations in the concentration or the activity of H^+-ATPases (Gaxiola et al., 2007), we further evaluated the impacts of isolated and combined sp and dgt mutations on this classical auxin-dependent physiological response. In the assay of H^+ extrusion from the roots, IAA-treated seedlings were incubated in agar plates with the pH indicator bromocresol purple and the formation of yellow color indicated the acidification of the medium. The area around the roots of double mutants (sp dgt) was considerably more acidified than in the other genotypes (Fig. 5A). We also used a flat probe to determine H^+ around the first 12 mm of rhyzosphere with or without previous auxin treatment. Comparing wild-type plants for DGT, it is evident that the sp mutation reduced H^+ extrusion in both cases (Fig. 5B). The dgt mutation led to an increase in H^+ extrusion in the control treatment in both SP and sp backgrounds, but in a more dramatic and significant way in the latter. Proton extrusion after auxin treatment, on the other hand, was reduced in dgt mutants regardless of the SP genotype (Fig. 5B). Vibrating-probe analyses offer another way to assess H^+ efflux in roots. Analyzing functional DGT background plants it was observed an expected stimulus of H^+ efflux after IAA addition in the medium (Fig. 5C, E). The sp mutation in the DGT background reduced the H^+ efflux under auxin treatment around 30%. The dgt mutation, on the other hand, impaired the plasma membrane H^+-ATPase activation and no H^+ efflux was recorded (Fig. 5D, F). Prior to adding auxin, all genotypes presented H^+ influx. Interestingly, the sp DGT background showed the lowest H^+ influx.

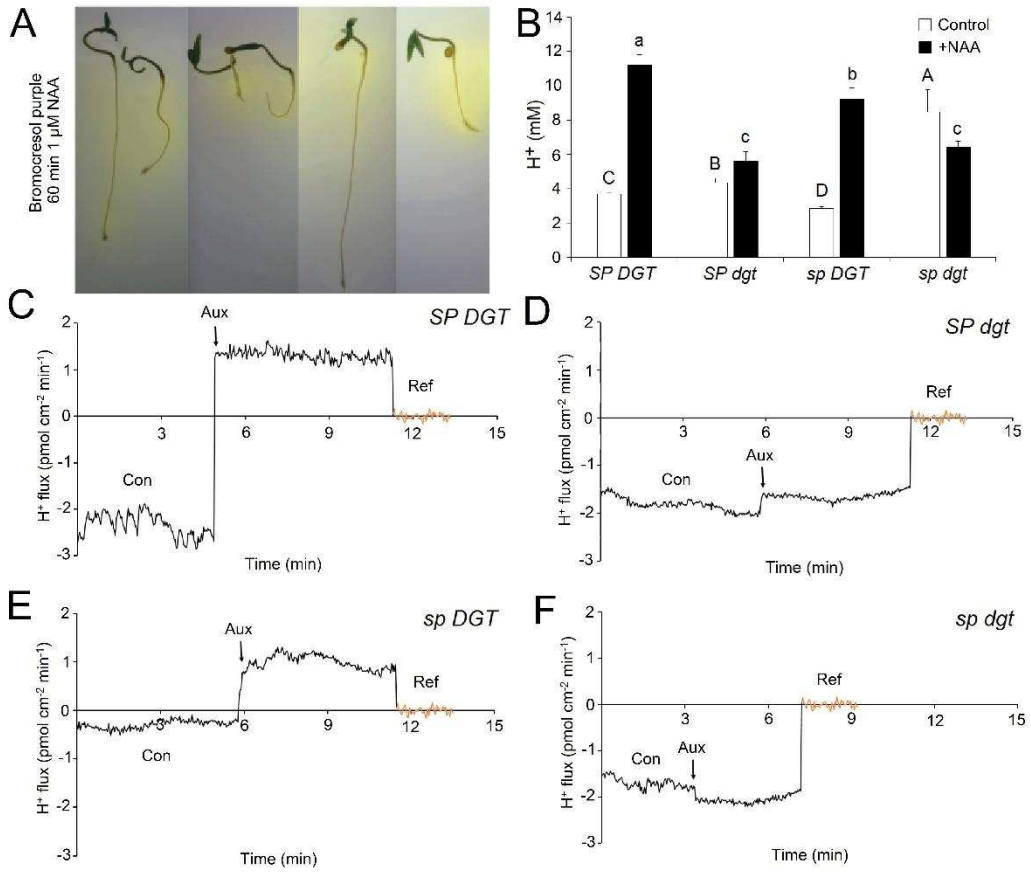

Figure 5. Rhyzospheric proton (H^+) extrusion is affected by the self-pruning (sp) and diageotropica (dgt) mutations. (A) Representative 4-day old seedlings and (B) results of rhizospheric H+ extrusion in seedlings treated for 60 min with 1-naphthaleneacetic acid (NAA) 1 µM or mock-treated (control) and transferred to the agar medium with pH indicator bromocresol purple (yellow indicates medium acidification) for an additional 24 h. Capital letters indicate significant differences ($p< 0.001$), treatment ($p < 0.0001$) and interaction between the two ($p < 0.0001$). (C-F). H+ fluxes determined using the ion-selective vibrating probe system. 10-day old seedlings of each genotype were analysed (n=6). H+ flux at the root elongation zone was determined initially as a control (CON) after 30 min incubation in plates with MS/2 medium. Auxin (1 µM IAA, AUX) was then applied and measurement continued for 5 min. Background reference (REF) measurements were performed at 500 µm from the roots and subtracted from measurements performed near the root surface.

Finally, we determined whether auxin affects SP at the transcriptional level, as suggested by the presence of auxin-response elements (TGTCTC, and their degenerate version, TGTCNC) in the 3´ and 5´ flanking regions of the SP gene in tomato and related Solanaceae species (Fig. 6A). Analyzing SP mRNA levels in seedlings of SP DGT and sp DGT plants sprayed with IAA or a mock solution, revealed that SP

expression was induced by IAA treatment in either genotypes. Importantly, SP transcript levels were significantly higher in dgt mutant plants both in IAA-treated and control seedlings (Fig. 5B). We further assessed the effect of SP and DGT on the mRNA levels of the key players in the auxin signaling cascades, including some members of the AUXIN RESPONSE FACTORS (ARF) and AUXIN/INDOLE-3-ACETIC ACID INDUCIBLE (Aux/IAA) gene families, which were chosen by their high auxin-inducibility (Audran-Delalande et al., 2012). SP and DGT had combinatorial effects in the expression levels of IAA1, IAA2, IAA9, ARF8, and ARF10, whereas functional DGT decreased expression of IAA3 (Fig. 6C).

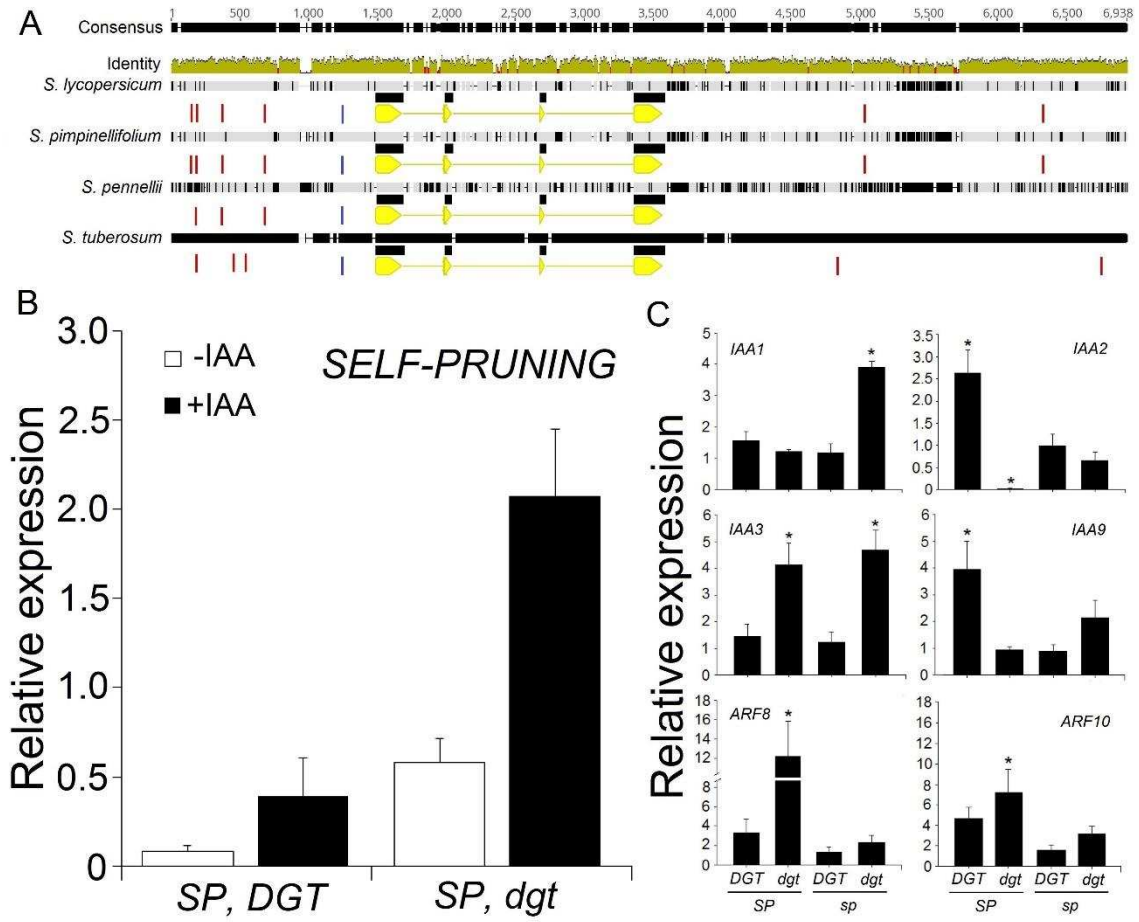

Figure 6. SELF-PRUNING (SP) and auxin-signaling gene expression is altered by the sp and diageotropica (dgt) mutations (a) Genomic structure of the SP gene in various related solanaceous species: tomato (S. lycopersicum), its wild relatives S. pimpinellifolium and S. pennellii and potato (S. tuberosum). The coding sequence is indicated in yellow (exons, thick bars; introns, thin bars). Red blocks indicate the

presence of a conserved or degenerate auxin-response element (AuxRE), TGTCNC. The (b) Relative transcript accumulation of SP in a wild-type or dgt mutant background, 4h after IAA or mock spray (c) Auxin-related gene expression in four genotypes with different SP and DGT alleles.

DISCUSSION

Control of endogenous auxin levels and polar auxin transport by SP and DGT

Endogenous IAA concentration and distribution within tissues determine a wide range of plant developmental processes, including apical dominance, stem growth, vascular patterning, root development and others (Petrasek and Friml, 2009; Ljung, 2013). IAA synthesis is maximal in younger, developing parts of the plant such as leaflets and root apices (Ljung et al., 2001). Previous work revealed no difference in IAA levels in dark-grown seedlings or in roots of sp DGT (tomato cultivar VFN8) and sp dgt plants (Fujino et al., 1988; Muday et al., 1995). Here, we show that synergistic influence of SP and DGT on free IAA levels dependent on plant region, being greater in the aerial parts than in the roots.

The above results could reflect changes in IAA biosynthesis, degradation or transport. The reduction in PAT produced by dgt mutation was described previously (Ivanchenko et al., 2015), but the synergistic effect of the sp mutation described here was unexpected. A fairly strong negative correlation (R^2=0.75) is observed between the intensity of IAA transport and IAA concentration in hypocotyls (Fig 3). Thus, the differences in IAA concentration in the aerial part of the seedlings could be due to altered PAT caused by both the sp and dgt mutations. PAT from the shoot organs to the root tips induces the formation of the entire plant vascular system (Aloni, 2013; Marcos and Berleth, 2014). Increased vessel size and reduced density are associated with decreased auxin concentrations along the longitudinal axis of the plant shoot (Aloni and Zimmermann, 1983). We observed that the alleles sp and dgt clearly reduced xylem vessel density, so the differences could be linked to their different PAT rates along the longitudinal axis.

Impact of SP alleles, and their interaction with auxin, in the control of shoot architecture

Although it has been previously demonstrated that the sp mutation does not alter tomato flowering time or the number of leaves before termination of the shoot (Benfey and Chua, 1990; Shalit et al., 2009), SP orthologs vary in this respect depending on the species. Flowering time is not affected in cen mutants in Antirrhinum (Bradley et al., 1996) whereas, Arabidopsis tfl1 mutants flower earlier and TFL1 overexpression delays flowering by preventing the transition to floral meristem (Ratcliffe et al., 1998). In soybean, where large intraspecific variation exists in days to flowering, association mapping has recently linked this important agronomic trait to the Dt1 locus, a CEN/TFL1/SP ortholog (Zhang et al., 2015). Comparison of 23 determinate and 23 indeterminate near-isogenic soybean lines consistently showed earlier flowering for the former across different locations and planting seasons (Ouattara and Weaver, 1994). Our data show that loss of SP function (sp allele) leads to slightly but consistently earlier flowering in tomato, measured either in days after germination or the reduction of the number of nodes before the first inflorescence. Interestingly, the dgt mutation delays the number of days to flowering in either SP or sp backgrounds, apparently by reducing the expression of SINGLE FLOWER TRUSS (SFT) gene, which encodes the florigen. It does not, however, significantly affect the number of leaves produced before termination, which is a proven effect of SFT and its orthologs in tomato and other species. Hence, the loss-of-function mutant sft produced >130% leaves on the primary shoot than the control MT (Vicente e al., 2015). Conversely, transgenic tomato plants overexpressing SFT flower after only three or four leaves have been produced (Molinero-Rosales et al, 2004; Shalit et al, 2009).

Axillary branching was increased in sp mutants in both DGT and dgt allele combinations. The expression of SP is restricted to the axillary meristems, suggesting a possible role for SP in the control of apical dominance (Thouet et al., 2008). Our results reinforce this notion, as sp mutants are more profusely branched than wild-type plants. This also agrees with the effects of the SP ortholog Dt1 in soybean, where comparison of determinate and indeterminate isogenic lines revealed an increased propensity to side branching in the former (Gai et al., 1984). The dgt mutant responds to auxin treatment of decapitated shoots, which inhibits bud outgrowth to the same extent as in wild-type plants (Cline, 1994). Apical dominance has been reported to be

reduced in intact dgt plants (Coenen, 2003), but caution should be exercised when interpreting these results, as published work on dgt has been conducted in tomato cultivars differing in their SP alleles (Supplementary Table 1). Our results indicate a strong and complex interaction between SP and DGT in the control of apical dominance: the dgt mutation increased it in the wild-type SP background, but also increased axillary bud outgrowth in the sp background, enhancing its branching phenotype.

SP alters gravitropic responses and root H^+ extrusion

Hypocotyl elongation in response to different concentrations of exogenous auxin is a classic assay for auxin sensitivity. The response of dgt has been described in the background of VFN8, which is a mutant for sp. In both intact or excised hypocotyl segments, a significantly reduced response to exogenous auxin was observed for the sp dgt double mutant (Kelly and Bradford, 1986; Rice and Lomax, 2000). Our results, although confirming the reduced response to exogenous auxin for sp dgt (see Fig. S2), show that the response of the dgt mutant in an SP background did not differ from wild-type (SP, DGT). Collectively, it indicates that some compensatory effect can be ascribed to SP on this response. Hypocotyl elongation in Arabidopsis relies on auxin-induced changes in the activity of plasma membrane H^+-ATPases, which leads to increased H^+ extrusion and cell expansion, through expansin-mediated cell wall loosening, as per the acid growth theory (Takahashi et al., 2012). An intact DGT gene product is necessary for auxin-induced H^+ secretion (Coenen et al., 2002) and SP also affects it, as H^+ extrusion is reduced in sp mutants with an intact DGT gene and even more reduced in sp dgt double mutants upon exogenous auxin application (Fig. 5). Both the hypocotyl elongation and H^+ extrusion, upon exogenous auxin application, point to a positive effect of SP on the activity of plasma membrane H^+-ATPases. Interestingly, the activity of both plasma membrane H^+-ATPases and PIN efflux transporters, which are also influenced by SP (Fig. 4C) is regulated by changes in their phosphorylation state (Takahashi et al., 2012; Zourelidou et al., 2014; Weller et al., 2017). This fits with earlier suggestions that SP, which encodes a phosphatidylethanolamine binding protein (PEBP), exerts at least some of its effects on membrane proteins by interacting with kinases (Pnueli et al., 2001).

The Cholodny-Went hypothesis is a classic model suggesting that differential auxin distribution is the cause of directional plant bending with respect to an exogenous stimulus such as light or gravity (Went, 1974). That DGT is required for a correct gravitropic response of roots and shoots has been demonstrated, but the explanation at the molecular level is still lacking (Muday et al., 1995; Rice and Lomax, 2000). To the best of our knowledge, an effect of SP on shoot gravitropism had not been tested before. Functional SP enhances shoot gravitropism in horizontally positioned plants of either DGT or dgt background. Functional SP produces taller plants, so it is likely that they should have a stronger gravitropic response in order to facilitate the bending of a larger stem. In Arabidopsis, the IAA efflux transporter PIN3 mediates lateral redistribution of auxin and is therefore involved in hypocotyl and root tropisms (Friml et al., 2002). It seems reasonable to suggest a link between SP and PIN3 in the face of our results with PIN1 (Fig. 4C). Remarkably, both types of efflux transporters have been shown to relocate at the subcellular level via the same mechanism: vesicle trafficking along the actin cytoskeleton between the plasma membrane and endosomes (Geldner et al., 2001; Friml et al., 2002). Dissecting the intertwined mechanisms involved in this possible co-regulation will be required to fully understand to which extent and how exactly SP affects auxin distribution.

Interactions between SP and the auxin signaling machinery

Auxin signaling output can be estimated by following DR5 promotor activation pattern (Ulmasov et al., 1995; Liao et al., 2015). For example, GUS staining of DR5::GUS line of different species has revealed that auxin flux at the root tips proceeds acropetally up to the root cap, where it is redistributed via lateral efflux transporters toward a peripheral basipetal transport route. Our GUS analyses suggest that SP enhances both pathways, which fits to increased PAT ratios in SP plants (Fig. 3), and whose molecular basis is probably linked to its increased PIN1 expression (Fig. 4C). Exogenous auxin application leads to greater GUS signal in seedlings with a functional SP allele, owing probably to alterations in the auxin signaling machinery produced by SP, such as expression of Aux/IAA and ARF family genes (Fig. 6C).

Auxin signalling is strongly dependent on auxin levels and the responsiveness of target cells. At low IAA levels, a suite of repressor proteins, including Aux/IAA

and TOPLESS, repress ARFs, a group of transcription factors which regulate the expression of auxin-responsive genes (Causier et al., 2012; Bargmann and Estelle, 2014; Chandler, 2016). At high IAA levels, auxin acts as a molecular glue to stabilize the TIR1/AFB receptor binding and tagging of Aux/IAAs for 26S proteasome degradation (Hayashi, 2012). This, in turn, frees ARFs bound to Auxin-Response Elements (AuxRE) in the genome (TGTCTC, or its degenerate, but also functional form, TGTCNC) to activate or repress gene expression (Ulmasov et al., 1995). Our in silico analyses demonstrated the presence of conserved AuxRE elements both 5´ (upstream) and 3´ (downstream) of the SP coding sequence in the genome of tomato and closely related species. It has recently been shown that the 3´ region of TFL1 in Arabidopsis contains multiple auxin cis-regulatory elements key for the control of spatio-temporal expression of the gene (Serrano-Mislata et al., 2016). It remains to be determined whether such cis-regulatory elements are also functional in tomato and if they are involved in the response of SP expression to auxin.

Tomato has 25 Aux/IAA and 22 ARF genes (Audran-Delalande et al., 2012; Zouine et al., 2014, Supplemental Table 2), indicating that auxin signalling is very complex. DGT can alter the expression of genes related to auxin signaling (Mito and Bennett, 1995), including Aux/IAA genes (Nebenführ et al., 2000). It is likely that some transcriptional feedback exists when the right conformers are not produced, as suggested by increased transcript levels of IAA3 and reduced levels of IAA9 in dgt mutants. The mechanisms behind SP effects on Aux/IAA and ARF expression remain to be elucidated.

It was recently discoverd that cyclophilin isomerases (such as DGT) catalyse the cis/trans isomerisation of peptide bonds preceding proline residues of target peptides, including Aux/IAAs (Jing et al., 2015). Only Aux/IAA peptides of the right conformation can bind to the TIR1 receptor and be tagged for degradation, thus peptidyl-prolyl isomerases, such as DGT, are believed to play a key role in auxin perception (Su et al., 2015). On the other hand, our in silico analysis shows that the sp mutation occurs in a highly conserved cis-proline residue in a DPDxPxn10H consensus into its PEBP domain (Fig. S1), which is a potential target for the peptidyl-prolyl cis-trans isomerase encoded by DGT. Whether this putative molecular interaction between SP and DGT could account for the phenotypic interactions shown here, remains to be determined.

CONCLUDING REMARKS

Auxin gradients are critical for organogenesis in the shoot apex, however, the influence of this hormone on shoot determinacy, which is a key determinant of growth habit, has not been addressed in depth. Our data provides the first link between auxin and the anti-florigenic protein SELF-PRUNING (SP), the main switch between indeterminate/ determinate growth habit in tomato. Although it is not clear whether auxin itself can affect growth habit, a physiological interaction between this hormone and members of the CETS family was clearly demonstrated here for the additive response triggered by SP. Hence, SP alleles affected various auxin-related responses (e.g. apical dominance, PIN1-mediated polar auxin transport, vascular differentiation, H^+ extrusion and gravitropism responses), different SP orthologs presented AuxREs, and the auxin mutant dgt downregulated SFT and upregulated SP expression. There are now increasing evidences that SP/SFT genetic module is a hub in crop productivity, affecting heterosis for yield (Krieger et al., 2010) and improving plant architecture and the vegetative-to-reproductive balance (McGarry and Ayre, 2012; Vicente et al., 2015; Zsögön et al., 2017). Our results suggest that at least part of the effect of the SP/SFT module on yield is mediated by auxin. This knowledge may inspire novel and more precise manipulation of this hormone for applications in agriculture.

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SUPPLEMENTAL MATERIAL

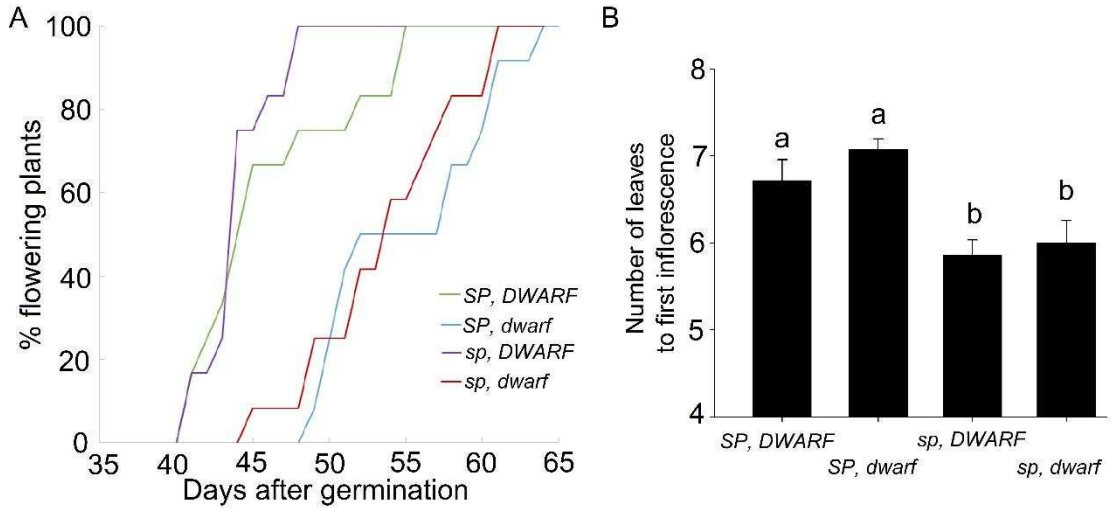

Figure S1. The tomato sp mutation occurs in a highly conserved proline residue. Diagram showing Clustal-X aligment of PEBP proteins with highly conserved cis-proline in DPDxPxn10H consensus into PEBP domain compared with cristalographic structure of Human PEBP1 (Simister et al., 2002). Principal CETS multiple protein aligment for Solanum lycopersicum, other solanaceous species, and Arabidopsis thaliana was performed in Jalview plataform (Waterhouse et al., 2009) and corrected manually.

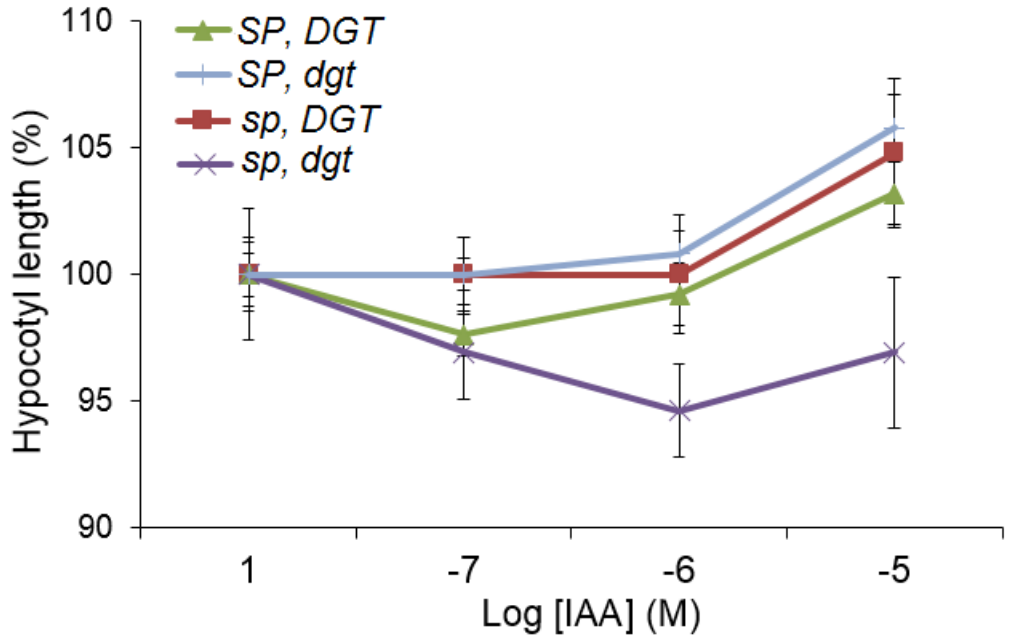

Figure S2. Hypocotyl elongation is synergistically affected by SP and DGT. Change in hypocotyl length after 24 hours of immersion in indole-3-acetic acid (IAA) solutions of different concentrations.

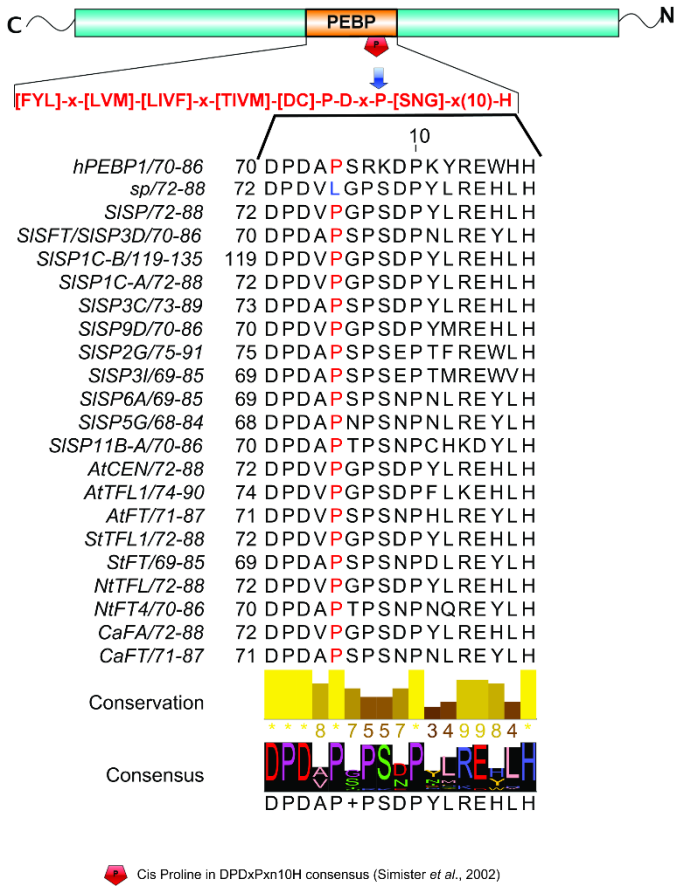

Figure S3. The tomato sp mutation occurs in a highly conserved proline residue. Diagram showing Clustal-X aligment of PEBP proteins with highly conserved cis-proline in DPDxPxn10H consensus into PEBP domain compared with cristalographic structure of Human PEBP1 (Simister et al., 2002). Principal CETS multiple protein aligment for Solanum lycopersicum, other solanaceous species, and Arabidopsis thaliana was performed in Jalview plataform (Waterhouse et al., 2009) and corrected manually.

Supplemental Table 1. List of publications featuring the tomato diageotropica mutant available on PubMed as of January 2017, discriminated by genetic background. The allele of the SELF PRUNING gene of each tomato cultivar is also detailed

Reference	**Cultivar (alleles of the SP gene)**
Bradford and Yang, 1980	VFN8 (sp/sp)
Caderas et al., 2000	

Cline, 1996	
Daniel et al., 1989	
Fujino et al., 1988	
Hicks et al., 1989	
Kelly and Bradford, 1986	
Muday et al., 1995	
Nebenführ et al., 2000	
Park, 1998	
Peres et al., 2005	
Rice and Lomax, 2000	
Shi and Cline, 1992	
Scott, 1988	
Ursin and Bradford, 1989b, 1989a	
Zobel, 1972, 1973, 1974	
Zurek et al., 1994	
Coenen et al., 2002	Ailsa Craig (SP/SP)
Coenen et al., 2003	
Hanlon and Coenen, 2011	
Ivanchenko et al., 2013, 2015	
Jackson, 1979	
Jackson and Campbell, 1975	
Konings and Jackson, 1979	
Kraepiel et al., 2001	
Madlung et al., 1999	
Mignolli et al., 2012	
Mito and Bennett, 1995	
Vidoz et al., 2010	
Goverse et al., 2000	Moneymaker (SP/SP)
Coenen and Lomax, 1998	Ailsa Craig (SP/SP)

Coenen et al., 2003	VFN8 (sp/sp)
Balbi and Lomax, 2003	Ailsa Craig (SP/SP) Chatham (SP/SP) VFN8 (sp/sp)
Ivanchenko et al., 2006 Oh et al., 2002, 2006	Ailsa Craig (SP/SP) Chatham (SP/SP) VF36 (sp/sp)
Canellas et al., 2011 Carvalho et al., 2010 Gratão et al., 2009, 2012 Lima et al., 2009 Lombardi-Crestana et al., 2012 Zandonadi et al., 2016 Zsögön et al., 2008	Micro-Tom (sp/sp)

Supplemental Table 2. Primers used for qPCR.

Gene	**Locus ID[1]**	**Primer sequence[2]**
Actin	Solyc03g078400	Fwd 5'-GGTCCCTCTATTGTCCACAG-3' Rev 5'-TGCATCTCTGGTCCAGTAGGA-3'
SP	Solyc06g074350	Fwd 5'-CATTCCAGGCACTACAGATTGC-3' Rev 5'-TGGTGAGCCAAGTTCATTTTCTTC-3'
SFT	Solyc03g063100	Fwd 5'-GACCCTGATGCTCCAAGTCC-3' Rev 5' – GTGACCAACCAGTGAAGGTATTC-3'

IAA1	Solyc06g053840	Fwd 5'-GAAAATGTTCAAGCTGAGTATC-3'
		Rev 5'-CTGATCCTTTCATTATCCTTAG-3'
IAA2	Solyc06g084070	Fwd 5'-TGATGAACCACCACAAAAAGCAC-3'
		Rev 5'-GATCGAACCGGTGGCCATC-3'
IAA3	Solyc09g065850	Fwd 5'- TGGCCACCAGTTCGATCATAC-3'
		Rev 5'- GGTGCTCCATCCATGCTAACT-3'
IAA9	Solyc04g076850	Fwd 5'-GTTGTCAAGTGTGTGACAGCC-3'
		Rev 5'-TGTCACTTACACATAGGGCCA-3'
DGT	Solyc01g111170	Fwd 5'-GAGTCGCCGTTTTAGGCTTT-3'
		Rev 5'-GCAACACAACAACCAATTACG-3'
ARF8	Solyc02g037530	Fwd 5'- CTGCTCAAACCCAAATGCTGTC-3'
		Rev 5'-GGTAAGTGTGTTGGTGAGCCTG-3'
ARF10	Solyc11g069500	Fwd 5'- CAGGTCCAGCAGTCCTTTCT-3'
		Rev 5'- CGCTGGAAACTTGGTGGTAA-3'

[1]Locus according to the Sol Genomics Network database (http://solgenomics.net/).

[2]Fwd: forward, Rev: reverse

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CHAPTER II

Pleiotropic effects of SINGLE FLOWER TRUSS in tomato leaf development and water relations

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ABSTRACT

The CETS gene family includes SP (SELF-PRUNING), CENTRORADIALIS (CEN) and TERMINAL FLOWER 1 (TFL1), which are flowering regulators in tomato, Arabidopsis and Antirrhinum, respectively. These genes play a critical role in the control of plant architecture. In addition to the SP gene, the 12 CETS paralogs in tomato include SINGLE FLOWER TRUSS (SFT), the ortholog of FLOWERING LOCUS T (FT) in Arabidopsis. SP and SFT have antagonistic roles as suppressor and promoter of flowering, and therefore, in the control of shoot architecture in tomato. FT-like proteins are also regulatory factors in a wide range of developmental processes including fruit set, vegetative growth and water use efficiency (WUE). The aim of this work was to identify the mechanisms responsible for alterations in WUE associated with the SFT gene. We analyzed wild-type, mutant (sft) and overexpression (35S::SFT) lines in the background of the tomato cultivar Micro-Tom (MT). We show that loss of function in sft decreases photosynthesis rate (A) and stomatal conductance (g_s) compared to MT, whereas A and g_s were higher in 35S::SFT. Furthermore, leaf anatomy analyses also revealed striking differences between the SFT genotypes in anatomical features such as leaf thickness, palisade parenchyma cell, size and stomatal

density. We discuss a potentially broad role for SFT linked to pleiotropic control of leaf development and function in tomato.

INTRODUCTION

One of the greatest successes of the Green Revolution, which led to major increases in productivity, was the genetic manipulation of plant architecture (Peng et al., 1999; Spielmeyer et al., 2002), which is defined as the three-dimensional organization of the plant body (Wang and Li, 2006). In the shoot, architecture is determined by branching pattern, growth habit, shape and position of leaves and flowers (Wang and Li, 2008). In crop plants, shoot architecture is a key agronomic trait, influencing plant yield and harvest efficiency. Depending on their pattern of shoot development plants can be classified as monopodial or sympodial (Reinhardt and Kuhlemeier, 2002). In plants with monopodial growth, e.g. Arabidopsis, the shoot apical meristem (SAM) is indeterminate, i.e. plants show vegetative and reproductive phases clearly differentiated (Alvarez et al., 1992). In contrast, the SAM of plants with sympodial growth is determinate, so vegetative and reproductive stages occur in alternate modular units. Many Solanaceae species, including tomato (Solanum lycopersicum), show sympodial growth (Samach and Lotan, 2007).

The tomato is a perennial plant with indeterminate growth habit, in which the vegetative and reproductive phases alternate indefinitely. The vegetative apical meristem transitions to inflorescence meristem after production 9-12 internodes with leaves (Samach and Lotan, 2007). Vertical growth then continues from nearest axillary meristem below the inflorescence, which generates a new branch with three leaves and an inflorescence (sympodium). This pattern, however, is altered in determinate tomatoes that harbor a recessive allele of the SELF-PRUNING gene (SP) (Yeager, 1927; MacArthur, 1932b). In sp plants, the number of leaves in sympodial units is gradually reduced until two consecutive inflorescences terminate vertical growth, usually after formation of five or six inflorescences on the main stem (MacArthur, 1932b). Growth then continues from axillary meristems, which confers sp plants a short, bushy habit (Fridman et al., 2001). A variation on this theme is semi-determinate growth habit, where vegetative growth is extended before termination, usually after the eight inflorescences. This growth pattern has been described for sp mutant plants

harboring the SP5G and SP9D alleles of the wild tomato relative Solanum pennellii (Fridman et al., 2001; Jones et al., 2007).

Cloning and molecular characterization of the SP gene revealed it to be the ortholog of CENTRORADIALIS (CEN) and TERMINAL FLOWER 1 (TFL1), which regulate flowering in Arabidopsis and Antirrhinum, respectively (Alvarez et al., 1992; Bradley et al., 1996). These genes and their orthologs constitute the CETS gene family, whose products are similar to phosphatidyl ethanolamine-binding proteins (PEBP) in animals (Hengst et al., 2001; Kroslak et al., 2001). In addition to SP gene, paralogs gene were mapped to chromosomes 1 (SP1C-A e SP1C-B), 2 (SP2G), 3 (SP3C, SP3D e SP3I)), 5 (SP5G), 6 (SP6A e SP), 9 (SP9D) e 11(SP11B-A e SP11B-B) (Carmel-Goren et al., 2003; Kim et al., 2014). The SP3D locus corresponds to SINGLE FLOWER TRUSS (SFT), the ortholog of the flowering inducer FLOWERING LOCUS T (FT) in Arabidopsis (Koornneef et al., 1991; Kardailsky et al., 1999; Molinero-Rosales et al., 2004). The polypeptide encoded by SFT has therefore been equated with the "florigen", a compound originally proposed for photoperiodic plants that is produced in the leaves and moves through the phloem to the shoot apex to induce flowering (Evans, 1971; Zeevaart, 2006). SFT has a complex interaction with SP because SFT promotes systemic termination signals that induce the transition from vegetative to reproductive growth, while SP promotes maintenance of the indeterminate state of the apical meristem (Lifschitz and Eshed, 2006). They thus represent ‘florigenic’ and ‘anti-florigenic’ molecular signals (Matsoukas et al., 2012). In Arabidopsis, both FT and TFL1 bind to the same receptor, a bZIP transcription factor (Abe, 2005; Wigge, 2011) and their antagonistic effects of are caused by a switch in a single amino acid (Hanzawa et al., 2005).

Interest in understanding the function of SFT has been growing since it was shown that it can drive yield heterosis in an sp mutant (i.e. determinate) tomato background (Krieger et al., 2010). Subsequent work proved that this effect is controlled by a ‘fine-tuning’ of vegetative to reproductive growth in the tomato plant, caused by the dosage of florigenic SFT and anti-florigenic SP signals (Jiang et al., 2013; Park et al., 2014b). Recent work showed that SFT alleles can influence water use efficiency (WUE), a key agronomical parameter in many crops, particularly in arid and semi-arid regions (Condon et al., 2004; Zsögön, 2017). Semi-determinate sft/+ plants have higher WUE for yield (Vicente et al., 2015). Tomato introgression lines harboring the SP5G allele of S. pennellii are also semi-determinate and have higher

WUE (Barrios-Masias et al., 2014). This suggests an association between growth habit and WUE, although the mechanistic explanation is still lacking. Interestingly, in Arabidopsis the FT gene is expressed in guard cells and regulates stomatal opening via activation of the H+-ATPases (Kinoshita et al., 2011). Stomatal activity can drive increases in WUE by altering the ratio of CO_2 assimilation to transpirational H_2O loss. It is therefore not clear whether SFT produces such an alteration in tomato via gross morphological changes in growth habit, directly at the molecular level through regulation of stomatal aperture, or both. Thus, for test the hypothesis that SFT regulate the stomatal response and leaf development altering the water relations. For this, our aim was to clarify this issue and identify the effects of SFT in tomato leaf structure and physiology. We analysed genotypes with functional, overexpression and loss-of-function SFT alleles in tomato cultivar Micro-Tom background.

MATERIAL AND METHODS

Plant material and growth conditions

Tomato (Solanum lycopersicum L.) cv. Micro-Tom (MT) plants were used in all experiments with reduced and over expression of SFT and additionally, plants with superexpression and reduced expression of ABA biosynthesis were used as controls in analyzes of water relations. MT seeds were provided by Dr. Avram Levy (Weizmann Institute of Science, Israel). The single flower truss (sft) and notabilis (not) loss-of-function mutant were introgressed into MT as described by Carvalho et al. (2011) from their original backgrounds (LA2460, probably cv. Ailsa Craig and LA1607, cv. Lukullus, respectively), whose seeds were donated by Dr. Roger Chetelat (Tomato Genetics Resource Center, Davis, California). The tomato cv. Ailsa Craig line harboring the Sp12::NCED (9-cis-epoxycarotenoid dioxygenase tomato gene driven by the Gelvin Superpromoter, described originally in Thompson et al. (2000) was donated by Dr. Andrew Thompson (Cranfield University, UK). The transgene was introgressed in MT as described above. The 35S::SFT construct was a kind gift of Prof. Eliezer Lifschitz (Technion-Israel Institute of Technology, Haifa). All genotypes are described in Table 1. Transgenic plants were generated following the protocol of Pino et al., (2010).

Seeds were surface-sterilized in 30% sodium hypochlorite solution for 15 min, and then rinsed with deionized water. Seeds were germinated and grown in a greenhouse of the Universidade Federal de Viçosa – UFV, during the months of November to January 2016/2017, photoperiod of 13-h/11-h light/dark with 850-950 µmol m^{-2} s^{-1} white light, 25ºC/18ºC throughout the day/night cycle and relative humidity of 71,5±10. Seeds were sown in polyethylene trays containing commercial substrate Tropostrato® and germination occurred about seven days after planting. After of appearance of the true first leaf, the plants were transplanted to pots of 1.5 L, containing commercial substrate Tropostrato® and NPK supplementation was 8 g L^{-1} and 4 g L^{-1} dolomite limestone ($MgCO_3$ + $CaCO_3$). Fortnightly foliar fertilization was carried out using 10 mL of Biofert® leaf fertilizer.

Growth analyses

Immediately after anthesis, which occurred at variable times for each genotype, we determined branching pattern, length of third, fourth and fifth internodes, number of leaves up to the first inflorescence and on the main shoot (MS) (leaves of primary shoot plus leaves on sympodial units following the first inflorescence) and height of the plant on the primary shoot (MS).

Whole plants were harvested and the dry weight (DW), total leaf area (LA) and specific leaf area (SLA) were evaluated. LA was measured by digital image analysis using a scanner (Hewlett Packard Scanjet G2410, Palo Alto, California, USA) and the images were later processed using the ImageJ® software. SLA was calculated using the following equations:

$$SLA\ (m^2.\ kg^{-1}) = \frac{LA}{Leaves\ dry\ weight} \qquad (Eq.1)$$

Water loss determinations

For water loss measurements, 3rd terminal leaflets detached from six different plants of 42-day old were floated in MES-KCl buffer (5mM KCl, 10 mM MES, 50 µM $CaCl_2$, pH 6.15) with the abaxial side down in Petri dishes and incubated under

Table 1. Description of the genotypes in the Micro-Tom background used in this work

Genotype	Gene function	Reference
Micro-Tom (MT)	Harbors the recessive allele self-pruning (sp), which leads to a determinate growth and uniform fruit ripening; the recessive allele dwarf (d), which leads to reduced brassinosteroid biosynthesis.	Meissner et al., 1997; Bishop et al., 1999; Martí et al., 2006
single flower truss (sft)	Reduced flowering induction. The locus is on chromosome 3. sft was introgressed into cv Micro-Tom (MT) from its original background (LA2460, possibly cv. Ailsa Craig).	Kerr EA, 1982; Molinero-Rosales et al., 2004
SFT-ox	Plants with overexpression of SFT have a high flowering induction. The locus is on chromosome 3	Lifschitz et al., 2006
notabilis (not)	Abscicic acid deficiency. Defective for 9-cis-epoxycarotenoid dioxygenase (NCED) key for oxidative cleavage of 9-cis-epoxycarotenoids in ABA biosynthesis.	Tal, 1966; Carvalho et al., 2011
NCED-ox	Overproduction of abscisic acid. Overexpression of NCED carotenoid cleavage enzyme	Thompson et al., 2007

continuous illumination (120 μE m^{-2} s^{-1}) at 25ºC for 2 h to open stomata. Following water loss was determined gravimetrically over 4 h at the indicated time points. Water loss was then calculated as a percentage of the initial fresh weight (Araújo et al., 2011b).

Thermal image

For thermal imaging analysis, five plants of each genotype were analyzed. Thermal images were obtained from 42-day old plants at 12:00 in the greenhouse using an infrared camera (FLIR systems T360, Nashua, USA) and leaf temperature was measured by FLIR Tools + version 5.2 software.

Gas exchange and chlorophyll fluorescence measurements

Gas exchange parameters were determined simultaneously with chlorophyll a (Chl a) fluorescence measurements using an open-flow infrared gas exchange analyzer system (LI-6400XT; LI-COR Inc., Lincoln, NE) equipped with an integrated fluorescence chamber (LI-6400-40; LI-COR Inc.). Instantaneous gas exchange were measured in 2nd leaf from top to the base in four plants from 42 days old after 1 h illumination during the light period under photon flux density (1000 μmol m^{-2} $s^{-1)}$. The reference CO_2 concentration was set at 400 μmol CO_2 mol^{-1} air. All measurements were performed using the 2 cm^2 leaf chamber at 25 °C, and the leaf-to-air vapor pressure deficit was kept at 1.2-2.0 kPa, while the amount of blue light was set to 10% PPFD to optimize stomatal aperture. Dates obtained were analyzed in the Curve Expert (Version 1.4 Image Pro-Plus® software (version 4.5, Media Cybernetics, Silver Spring, USA)

The initial fluorescence (F_0) was measured by illuminating dark-adapted leaves (1 h) with weak modulated measuring beams (0.03 μmol m^{-2} s^{-1}). A saturating white light pulse (8000 μmol m^{-2} s^{-1}) was applied for 0.8 s to obtain the maximum fluorescence (F_m), from which the variable-to-maximum Chl fluorescence ratio, was then calculated: $F_v/F_m=[(F_m - F_0)/F_m)]$. In light-adapted leaves, the steady-state fluorescence yield (F_s) was measured with the application of a saturating white light pulse (8000 μmol m^{-2} s^{-1}) to achieve the light-adapted maximum fluorescence ($F_m´$). A far-red illumination (2 μmol m^{-2} s^{-1}) was applied after turn off the actinic light to measure the light-adapted initial fluorescence ($F_0´$). The capture efficiency of excitation energy by open photosystem (PS) II reaction centers ($F_v´/F_m´$) was estimated following Logan et al. (2007) and the actual PSII photochemical efficiency (φ_{PSII}) was estimated as $\varphi_{PSII} = (F_m´ – F_s)/F_m´$ (Genty et al., 1989).

As the φ_{PSII} represents the number of electrons transferred per photon absorbed in the PSII, the electron transport rate (J_{flu}) was calculated as $J_{flu} = \varphi_{PSII}. \alpha. \beta.$ PPFD, where α is leaf absorptance and β reflect the partitioning of absorbed quanta between PSII and PSI, and the product $\alpha\beta$, was adopted of the literature to C3 plants (Flexas et al., 2007).

Dark respiration (R_d) was measured using the same gas exchange system as described above after at least 1 h during the dark period and it was divided by two ($R_d/2$) to

estimate the mitochondrial respiration rate in the light (R_L) (Niinemets et al., 2005, 2006; Niinemets et al., 2009).

Determination of mesophyll conductance (g_m), maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}) and photosynthetic limitations

The responses of A_N to C_i (A_N/C_i curves) were performed at 1000 μmol m^{-2} s^{-1} at 25 °C under ambient O_2. Briefly, the measurements started at the ambient CO_2 concentration (C_a) of 400 μmol mol^{-1} and once the steady state was reached, C_a was decreased stepwise to 50 μmol mol^{-1}. Upon completion of the measurements at low C_a, C_a was returned to 400 μmol mol^{-1} to restore the original A_N. Next, C_a was increased stepwise to 1600 μmol mol^{-1} for a total of 13 different C_a values (Long and Bernacchi, 2003). Corrections for the leakage of CO_2 into and water vapor out of the leaf chamber of the LI-6400 were applied to all gas exchange data as described by Rodeghiero et al. (2007). A_N/C_i curves were obtained using the terminal leaflet of the 2nd fully expanded leaf from the top to the base in four different plants per genotype from 42 days old. The CO_2 concentration in the carboxylation sites (C_c) was calculated according to Harley et al. (1992) as:

$$C_c = \Gamma^* [J_{flu}+8 (A_N + R_L)]/ [J_{flu} – 4(A_N + R_L)] \qquad \text{(Eq 2)}$$

where the conservative value Γ* for C3 plants was taken from (Harley et al. 1992; Wullschleger 1993). So, the g_m was estimated according to Ethier and Livingston (2004).

From A_N/C_i and A_N/C_c curves, the maximum carboxylation velocity (V_{cmax}) and the maximum capacity for electron transport rate (J_{max}) were calculated by fitting the mechanistic model of CO_2 assimilation (Farquhar et al. 1980), using the C_i and C_c based on temperature of kinetic parameters of Rubisco (K_c and K_o) (Walker et al. 2013), While, V_{cmax}, J_{max} and g_m were normalized to 25ºC using the temperature response equations from Sharkey et al. (2007).

To further investigate the photosynthetic responses we calculated the limitations based on the approach described by Grassi and Magnani (2005). Thus, these methods use the values of A_N, g_s, g_m, V_{cmax}, Γ^*, C_c, K_m and $K_m=K_c$ (1+ O/K_o) and

permits the partitioning into the functional components of photosynthetic constraints related to stomatal (l_s), mesophyll (l_m), and biochemical (l_b) limitations as bellow:

$$l_s = \frac{(\frac{g_{tot}}{g_s} x \frac{\partial A_N}{\partial A_N})}{(g_{tot} + \frac{\partial A_N}{\partial C_c})}$$

$$l_{\mathrm{m}} = \frac{\left(\frac{g_{tot}}{g_{\mathrm{m}} x} x \frac{\partial A_N}{\partial C_c}\right)}{g_{tot} + (\frac{\partial A_N}{\partial C_c})}$$

$$l_b = \frac{g_{tot}}{(g_{tot} + \partial A_N / \partial C_c)}$$

g_{tot} is the total conductance to CO_2 from ambient air to chloroplasts: (g_{tot} = $1/[(1/g_s)+(1/g_m)]$). The fraction $\partial A_N/\partial C_c$ was calculated as:

$$\frac{\partial A_N}{\partial C_c} = ([V_{\mathrm{cmax}}(I^* + K_m)])/(C_c + K_m)$$

Stomatal opening and closing kinetics

Stomatal conductance (g_s) values were recorded at intervals of 30 s using the same gas exchange system described above. The g_s responses to dark/light/dark transitions were measured in 2nd leaf from top to the base in four plants from 42 days old adapted to the dark for at least two hours. Light in the chamber was kept turned off, and then turned on/turned off for 2/30/30 min. The CO_2 concentration in the chamber was 400 μmol mol^{-1} air. For responses to CO_2 concentration transitions leaves were exposed to 400/800/400 μmol mol^{-1} CO_2 air for 10/30/30 min under PPFD of 1000 μmol m^{-2} s^{-1}.

Epidermal features

Lateral leaflets of the 3rd leaf from five plants 42 days old were cleared in 95% methanol and transferred to 100% lactic acid, conditioned in a water bath at 95 °C until the leaf presented a completely translucent appearance. Sections were mounted on glass slides, and the samples of adaxial and abaxial epidermis were analyzed with photomicroscope Zeiss AxioScope A1 model (Thornwood, NY, USA) with an attached Axiovision® 105 color image capture system. Images obtained in the photomicroscope were evaluated in the Image Pro-Plus® software (version 4.5, Media Cybernetics, Silver Spring, USA). Stomatal density and stomatal index of the adaxial and abaxial faces (the ratio of stomata to stomata plus other epidermal cells) were determined in at least 6 fields of 0.05 mm^2 per leaf from five different plants.

Leaf Anatomy

A fragment of approximately 1 × 0.5 cm of 2nd central leaflet from the top to the base of four different plants from 42 days old was fixed in FAA 70%, posteriorly were dehydrated in ethanolic series (70%, 85% and 95%), and infiltrated in historesin (Leica microsystems, Wetzlar, Germany). Cross-sections of 5 μm thickness were stained with 0.05% toluidine blue and analyzed in light microscope (Zeiss AxioScope A1 model Thornwood, NY, USA) with image capture system Axiovision® 105 coupled color. Images were processed in Image Pro-Plus® software (version 4.5, Media Cybernetics, Silver Spring, USA) for quantify abaxial and adaxial epidermal thickness, whole leaf thickness, palisade parenchyma thickness, spongy parenchyma thickness and percentage de intercellular spaces analyzing nine fields per repetition.

Stomatal aperture bioassay

The response of intact leaves to the exogenous application of a range of physiologically relevant substances (ABA, sucrose, malate, and fumarate) was evaluated to assess stomatal function. From five different plants from 42 days old, the 3rd leaf was detached and floated in MES-KCl buffer medium under light, for 2.5 h to open the stomata, then the compounds were added to the buffer solution. Leaves with the abaxial side up on open Petri dishes under a direct light source (120 μE m^{-2} s^{-1} at 25ºC and 70-80% relative humidity in the plant growth chambers for 2 h. After,

mannitol, control (solvent control) and ABA was added to the opening buffer to final concentration of 20 mM, 0.1 %, 5 µM respectively. After more 2 h of incubation the stomatal aperture was evaluated. Leaf impressions were taken from the abaxial surface of the leaf with dental resin imprints (Berger and Altmann, 2000). Nail polish copies were made using a colorless glaze (Von Groll et al., 2002). The samples were analyzed with the aid of a Zeiss AxioScope A1 photomicroscope with an attached Axiovision® 105 color image capture system. The images obtained in the photomicroscope were evaluated in the Image Pro-Plus® software (version 4.5, Media Cybernetics, Silver Spring, USA). At least 100 stomata per genotype were analyzed.

Gene expression quantification

Quantitative real-time PCR (qRT-PCR) analysis was performed as described by (Zanor et al. 2009) with total RNA isolated from 3rd terminal leaflet of plants after anthesis in four biological replicates and at least two technical replicates, harvesting and immediately snap-freezing the samples in liquid nitrogen. For RNA extraction, we used TRIzol® (Ambion, Life Technology, Waltham, USA) following the manufacturer's manual. Digestion with DNase I (Ambion; was performed according to the manufacturer's instructions. The integrity of the RNA was checked on 1% (w/v) agarose gels, and the concentration was measured after DNase I digestion using a Multiskan GO^{TM} spectrophotometer (Thermo scientific, Massachusetts, USA) cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen, Carsbad California) according to the manufacturer's instructions.

For gene expression analyses Power SYBR$^{®}$ green PCR Master Mix was used in MicroAmp^{TM} Optical 96-well reaction plates (both from Applied Biosystems, Singapore) and adhesive film MicroAmp^{TM} Optical (Applied Biosystems, Foster City, CA, USA). The number of reactions from the cycle threshold (CT) as well as the efficiency of the reaction was estimated using the Real-Time PCR Miner tool (Zhao and Fernald, 2005). Relative expression was normalized using actin, one constitutively expressed gene; actin was used to calculate ΔΔCT assuming 100% efficiency of amplification of genes (2^- ΔΔCT). The relative transcript abundance of SFT was analyzed to confirme the genotypes. The primer sequences for actin (endogenous) and SINGLE FLOWER TRUSS (SFT) are shown in supplemental table S1.

Experimental design and statistical analysis

The data were obtained from the experiments using a completely randomized design using MT, sft and SFT-ox. Data are expressed as the mean ± standard error (SE). Data were submitted to analysis of variance and tested for significant differences ($P < 0.05$) using Student's t-tests from MT. All the statistical analyses were performed using the Assistat version 7.7 (Campina Grande, Brasil)

RESULTS

Mutant sft plants exhibit greater vegetative growth that plants harbouring a functional SFT allele

The genotypes used in this worked have been described before in different tomato backgrounds. Here, we compared a set of transgenic and near-isogenic lines all in the cultivar Micro-Tom (MT) tomato background (Figure 1A). Since the focus of our work was on the analysis of the phenotypic effects of the SFT gene, we first assessed its expression levels in the leaves of all genotypes. Transcript accumulation was similar in MT as in the ABA-deficient mutant notabilis (not) and the ABA-overproducing transgenic NCED-ox (Figure 1B). The SFT transcript was detected in the sft mutant, whereas the SFT-overexpressing line, as expected, was orders of magnitude higher the control genotypes.

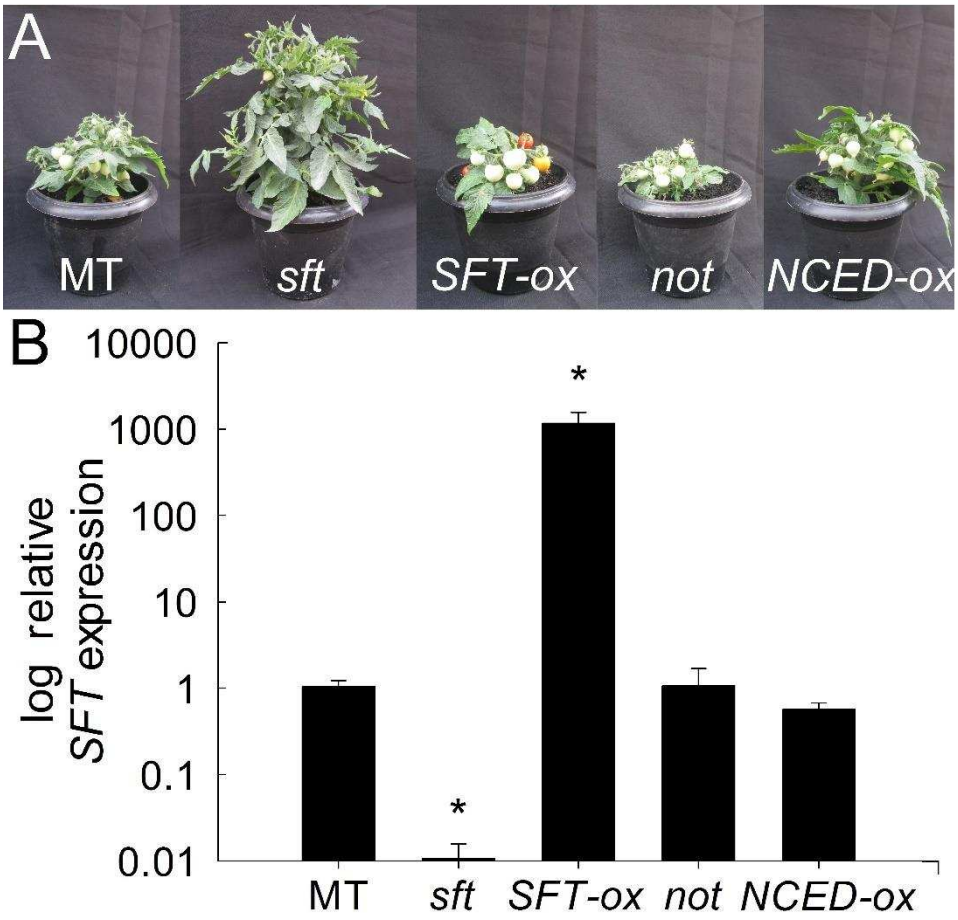

Figure 1. SFT affect the growth habit of tomato. A) Representative plants of MT; sft and SFT-ox, not and NCED-ox 60 days after germination. Note the differences of growth habit in sft and SFT-ox compared with MT. B) Histogram showing the relative transcript accumulation of SFT determined through Real Time PCR (qRT-PCR). The reference gene was actin. Asterisk indicates statistically significant differences compared with MT

Tomato has a sympodial growth habit and it was proposed that SP and SFT local ratios control the balance of determinate and indeterminate growth in the primary shoots and the sympodial secondary shoots (Shalit et al., 2009). Our results showed that plants with basal expression of SFT are very different in the whole plant compared with MT. sft delayed the flowering with increase of number of leaves, greater total leaf area and greater height. In contrast, SFT-ox accelerated the flowering with corresponding increasing of dry matter of flowers and fruits, reduced plant height, the leaf area was lower as well as dry matter of leaves, stem and roots, while that the specific leaf area was similar for all the genotypes (Table 1). On the other hand, the pattern branching in anthesis was predominant reproductive in SFT-ox plants with 92%

conversely, in sft 38,9% of lateral shoot was absent and just 18,9% showed lateral buds (Fig. 2).

Table 1. SFT regulate the sympodial development of tomato. Parameters in MT, sft and SFT-ox mutants after anthesis. Values are presented as means ± SE (n=6). Values in bold in mutants plants were determined by the Student's t-test to be significantly difference (P< 0.05) from MT.

	MT	sft	SFT-ox
Number of days until anthesis	43.5 ± 1.8	**57.3 ± 0.7**	**19 ± 0.8**
Number of leaves to first inflorescence	6.17 ± 0.31	**12.17 ± 0.17**	**2.83 ± 0.4**
Height to first inflorescence (cm)	6.13 ± 0.23	**15.07 ± 0.21**	**2.77 ± 0.21**
Stem diameter (cm)	5.3 ± 0.24	5.33 ± 0.09	**3.81 ± 0.17**
2nd internode length	0.88 ± 0.05	1.10 ± 0.04	0.77 ± 0.02
Total leaf area (cm^2)	449.92 ± 63.76	**848.56 ± 18.26**	**135.87 ± 34.57**
Specific leaf area ($cm^2\ g^{-1}$)	350.76 ± 32.3	353.84 ± 28.62	353.61 ± 27.2
Leaves mass fraction	0.60 ± 0.04	0.49 ± 0.03	0.54 ± 0.01
Stem mass fraction	0.18 ± 0.02	0.3 ± 0.02	0.25 ± 0.01
Root mass fraction	0.22 ± 0.02	0.21 ± 0.02	0.21 ± 0.01

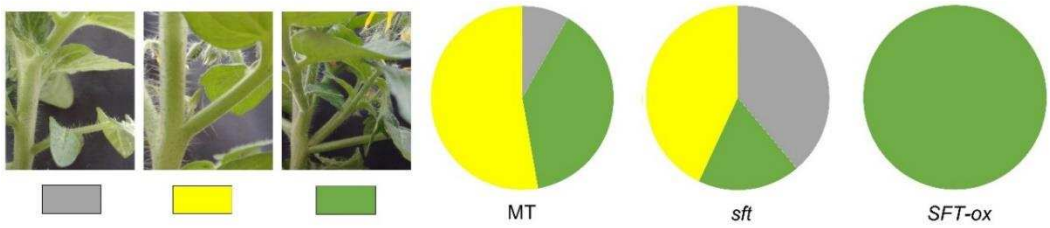

Figure 2. The florigen alters the lateral branching. Schematic representation of side branching in shoot of MT, sft and SFT-ox in 42 days after germination (n=6). Pizza charts depicting the distribution of side branches in each genotype after anthesis. Grey denotes absence of axillary bud; yellow represents primordia; dark green, fully formed side branches.

Water loss and leaf temperature is affected by SFT

We found that SFT-overexpressing (ox) tomato plants exhibited a phenotype characterized by low leaf temperatures and high water loss in leaves detached compared with MT exhibited significant differences after 60 min (P<0,005), after 240 min the difference was 26% greater loss of fresh weight in SFT-ox that MT. In contrast, plants carrying the allele sft showed high leaf temperature and 20% fresh weight loss,

as expected, not and NCED-ox showed low and high water loss respectively. (Fig. 3). Consequently, sft and NCED-ox presented higher temperature and SFT-ox and not lower temperature proportionally, this possibly related to lower transpiration rates that facilitate foliar cooling (Gates, 1968).

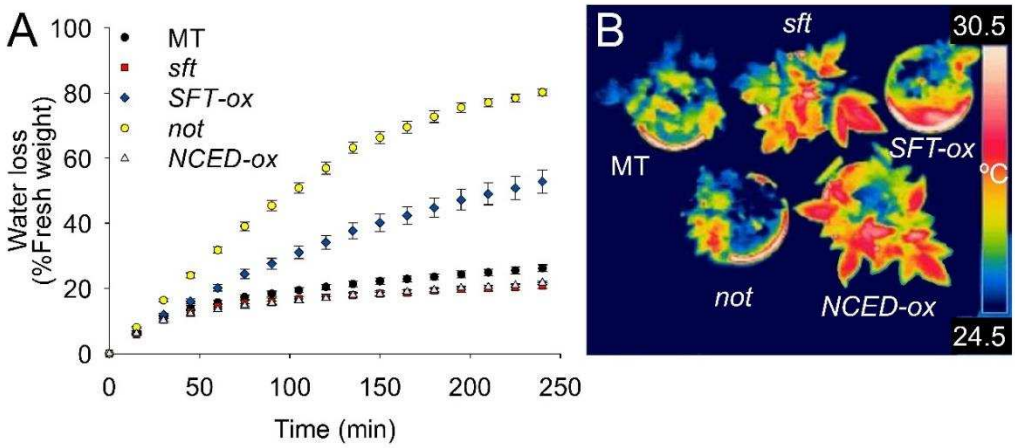

Figure 3. SFT mutant plants affected lost water with consequent alterations of leaf temperature. A) Water loss rate in detached terminal leaflet of plants MT, sft, SFT-ox, not and NCED-ox. Measurements obtained from the third fully expanded leaf with 42 DAG. Data show percentage of initial fresh weight loss from detached leaves. Values are means ± s.e.m. (n=6). B) Infrared thermal imaging of MT, sft, SFT-ox, not and NCED-ox.

Photosynthesis and stomatal conductance are increased when SFT is overexpressed while that A_N/C_i and A_N/C_c curve showed limitation of Jmax

Considering that the leaf temperature is related to stomatal conductance, we fully characterized the photosynthetic performance by analyzing diffusional, photochemical and biochemical constraints to photosynthesis.

The hyperbolic relationship between A_N and g_s measured at ambient CO_2 concentration, is strongly relationed with the genotype, where, sft and NCED-ox showed decrease in g_s associated with low photosynthetically rates while that high g_s not promove increase in A_N (Fig. 4).

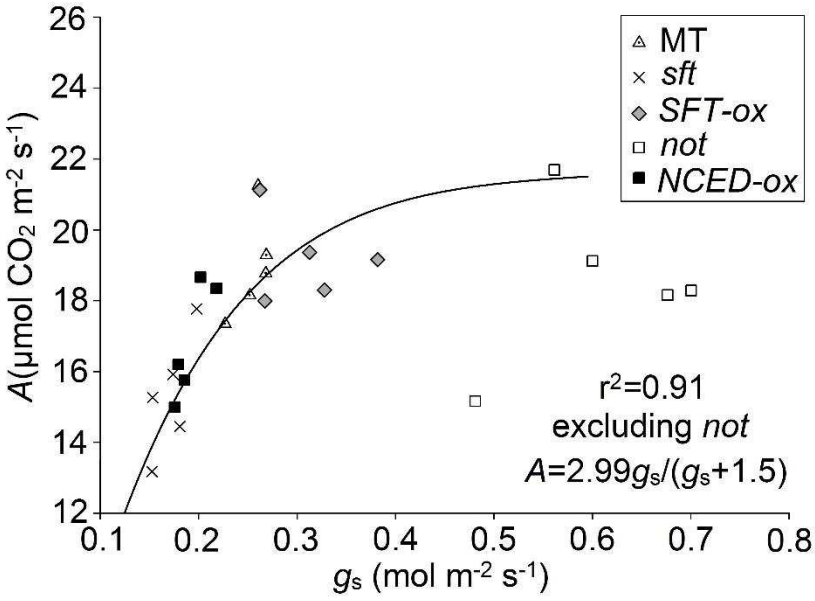

Figure 4. The florigen influence the stomatal conductance under optimal conditions. Net photosynthesis (A_N) curves in response to stomatal conductance (g_s) in MT, sft, SFT-ox, not and NCED-ox plants under saturing light. Values obtained using the second leaf from top to the base fully expanded. The solid line indicates a hyperbolic function was fit to the data and points indicate values of A_N/g_s for five plants of each genotype.

Compared with MT, SFT-ox displayed higher stomatal conductance and net photosynthetic rates (A_N) whereas, same low intrinsic water use efficiency (WUE_i) were observed. Dark respiration (R_d) was as high in sft as MT, conversely, SFT-ox presented low respiration (approximately 50% less that MT). The differences in A_N were unlikely to have been related to photochemical constraints given that both the maximum quantum efficiency of PSII (F_v/F_m) and capture efficiency of excitation energy (F_v'/F_m') remained invariant. Additionally, the electron transport rate (J_{flu}) was marginally decreased only in SFT-ox (Table 2).

Table 2 Gas exchange and chlorophyll a fluorescence. Parameters in MT, sft and SFT-ox, obtained from second leaf from top to the base fully expanded of 42 DAG. Values are presented as means ± SE (n=5). Values in bold in mutants plants were determined by the Student's t-test to be significantly difference ($P < 0.05$) from MT.

Parameters	MT	sft	SFT-ox
A_N (μmol CO_2 m^{-2} s^{-1})	18.98 ± 0.72	**15.31 ± 0.76**	19.18 ±0.55
g_s (mol H_2O m^{-2} s^{-1})	0.25 ± 0.01	**0.17 ± 0.01**	**0.31 ±0.02**

E (mmol H_2O m^{-2} s^{-1})	3.7 ± 0.39	**2.92 ± 0.17**	4.31 ± 0.25
WUE_i (A/g_s)	76.49 ± 1.37	89.42 ± 3.22	63.19 ± 5.23
R_d (umol CO_2 m^{-2} s^{-1})	2.08 ± 0.1	2.5 ± 0.46	**1.12 ± 0.41**
F_v/F_m	0.79 ± 0	0.77 ± 0.01	0.77 ± 0
F_v'/F_m'	0.56 ± 0.03	0.6 ± 0.02	0.54 ± 0.02
J_{flu} (umol m^{-2} s^{-1})	181.73 ± 4.65	195.28 ± 8.52	**163.81 ± 3.81**

*R_d: dark respiration; F_v/F_m: maximum quantum efficiency of PSII photochemistry; F_v'/F_m': actual PSII photochemical efficiency; q_P: photochemical quenching coefficient; NPQ: non-photochemical quenching; J_{flu}: rate of linear electron transport estimated by chlorophyll fluorescence parameters. Actual PSII efficiency (Φ_{PSII}); (**B**) efficiency of excitation capture by open PSII reaction centres (F'_v/F'_m)

Additionally, the response of A_N to the internal CO_2 concentration - A_N/C_i curves (Fig. 5A and Fig. 5B) was obtained, which were further converted into responses of A_N to chloroplastic CO_2 concentration - A_N/C_i curves (Supplemental Figure 1). Interestingly, under ambient CO_2 concentration (400 µmol mol^{-1}) and less, A_N estimations were lower in sft than in MT plants and under higher CO_2 concentrations the A_N was similar, in contrast, SFT-ox presented higher A_N than MT and under CO_2 concentrations higher 400 µmol mol^{-1} A_N was limited. In this way, the maximum carboxylation velocity (V_{cmax}) did not show significant differences between genotypes while maximum capacity for electron transport rate (J_{max}) was lower in SFT-ox only when estimated on a C_i basis. (Table 3).

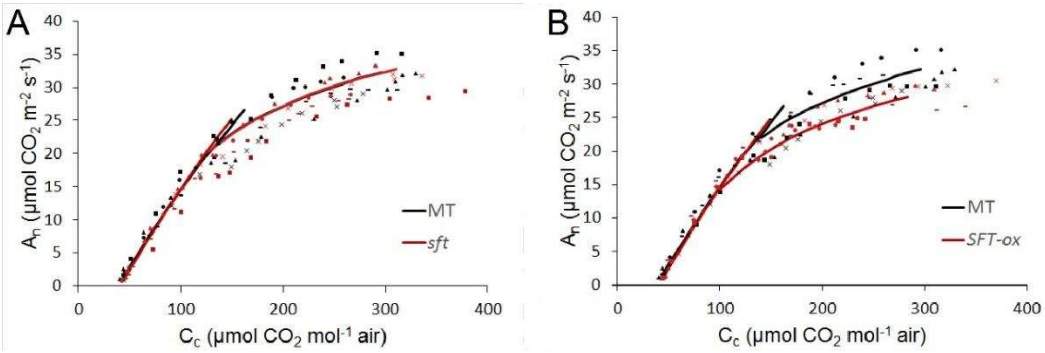

Figure 5. SFT influence CO_2 assimilation rate Net photosynthesis (A_n) curves in response to chloroplastidic (C_c) CO_2 concentration in MT and sft or SFT-ox plants. Values are presented as means ± SE (n=5) obtained using the second leaf from top to the base fully expanded. The solid line indicates the curve based on leaf biochemistry parameters, dotted line indicate the C_i obtained under atmospheric CO_2. The biochemically based leaf photosynthesis model (Farquhar et al., 1980) was fitted to the

data based on C_c, values of A_n/C_c for five plants of MT (black points) and sft or SFT-ox (red points).

We did not observe variation on mesophilic conductance (g_m); g_m was estimated by a combination of gas exchange and chlorophyll a fluorescence parameters, using (Harley et al. 1992) method. The overall photosynthetic limitations were next partitioned into their functional components: stomatal (l_s), mesophyll (l_m), and biochemical (l_b) (Table 3). The photosynthetic rates were mainly constrained by ls (37% and 43% in MT and sft plants, respectively. These analyses demonstrated that sft plants exhibit higher ls compared with MT however is not significant.

Table 3. Photosynthetic characterization of MT and sft and SFT-ox plants. Values are presented as means ± SE (n=5) obtained using the second leaf from top to the base fully expanded. Values in bold in mutants plants were significantly different of MT determined by the Students's t-test ($P < 0.05$)

Parameters*	**MT**	**sft**	**SFT-ox**
C_i (umol CO2 mol^{-1})	255.59 ± 6.74	248.41 ± 6.31	260.12 ± 4.43
C_c (umol CO2 mol-1)	132.9 ± 2.39	**119.67 ± 4.66**	137.64 ± 3.57
g_m Harley (mol CO2 m^{-2} s^{-1} bar)	0.16 ± 0.01	0.19 ± 0.03	0.17 ± 0.01
V_{cmax}_C_i (umol m^{-2} s^{-1})	82.63 ± 3.61	93.15 ± 9.32	86.01 ± 2.38
V_{cmax}_C_c (umol m^{-2} s^{-1})	165.76 ± 5.34	176.24 ± 4.46	173.63 ± 2.02
J_{max}_C_i (umol m^{-2} s^{-1})	153.46 ± 6.68	166.96 ± 14.37	**130.05 ± 5.76**
J_{max}_C_c (umol m^{-2} s^{-1})	184.04 ± 5.07	194.11 ± 6.88	170.28 ± 2.15
J_{max}_C_i : V_{cmax}_C_i	1.86 ± 0.04	1.82 ± 0.08	**1.51 ± 0.06**
J_{max}_C_c : V_{cmax}_C_c	1.11 ± 0.01	1.1 ± 0.02	**0.98 ± 0.02**
Stomatal limitation	0.37 ± 0.02	0.43 ± 0.02	0.37 ± 0.01
Mesophyll limitation	0.36 ± 0.01	0.31 ± 0.03	0.35 ± 0.02
Biochemical limitation	0.28 ± 0.02	0.26 ± 0.02	0.29 ± 0.01

* Ci: sub-stomatal CO_2 concentration; Cc : Chloroplastic CO2 concentration; gm: mesophyll condutance to CO_2 estimated according to the Harley; Vcmax_Ci or Cc: maximum carboxylation capacity based or Ci or Cc ; Jmax_Ci or Cc: maximum capacity for eléctron transport rate based on Ci or Cc.

SFT alters leaf anatomical traits

Epidermal features of adaxial side did not exhibited significant differences in sft or SFT-ox when compared with MT while that in the abaxial side sft plants showed high pavement cells density and SFT-ox presented high stomatal density but the relationship between the number of stomata and the number of pavement cells was similar for both genotypes (Table 4), however, anatomical traits were dramatically affected by allelic variation of SFT. Total thickness, palisade and spongy parenchyma, intercellular space and adaxial epidermal were significantly higher in sft and in contrast, SFT-ox presented lower values for parameters cited (Fig. 6).

Table 4. Epidermal features MT, sft and SFT-ox. Measurements obtained from the third fully expanded leaf with 42 DAG. Values are presented as means ± SE (n=5). Values set in bold in mutants plants were significantly different from MT according to the Students's t-test ($P < 0.05$)

Parameters*		**MT**	**sft**	**SFT-ox**
SD (mm^{-2})	Adaxial	44.7 ± 2.7	48.6 ± 10.7	51.5 ± 5.3
	Abaxial	93.9 ± 3.6	99.7 ± 6.1	**109.8 ± 3.1**
PCD (mm^{-2})	Adaxial	290.9 ± 13.2	293.5 ± 18.8	293.2 ± 11.9
	Abaxial	284.8 ± 9.5	**333.4 ± 22.1**	301.5 ± 5.6
SI (%)	Adaxial	13.4 ± 0.7	14.2 ± 2.8	15.3 ± 1.7
	Abaxial	24.9 ± 0.8	23.2 ± 1.2	26.7 ± 0.5

* SD: Stomatal density; PCD: Pavement cell density; SI: Stomatal index.

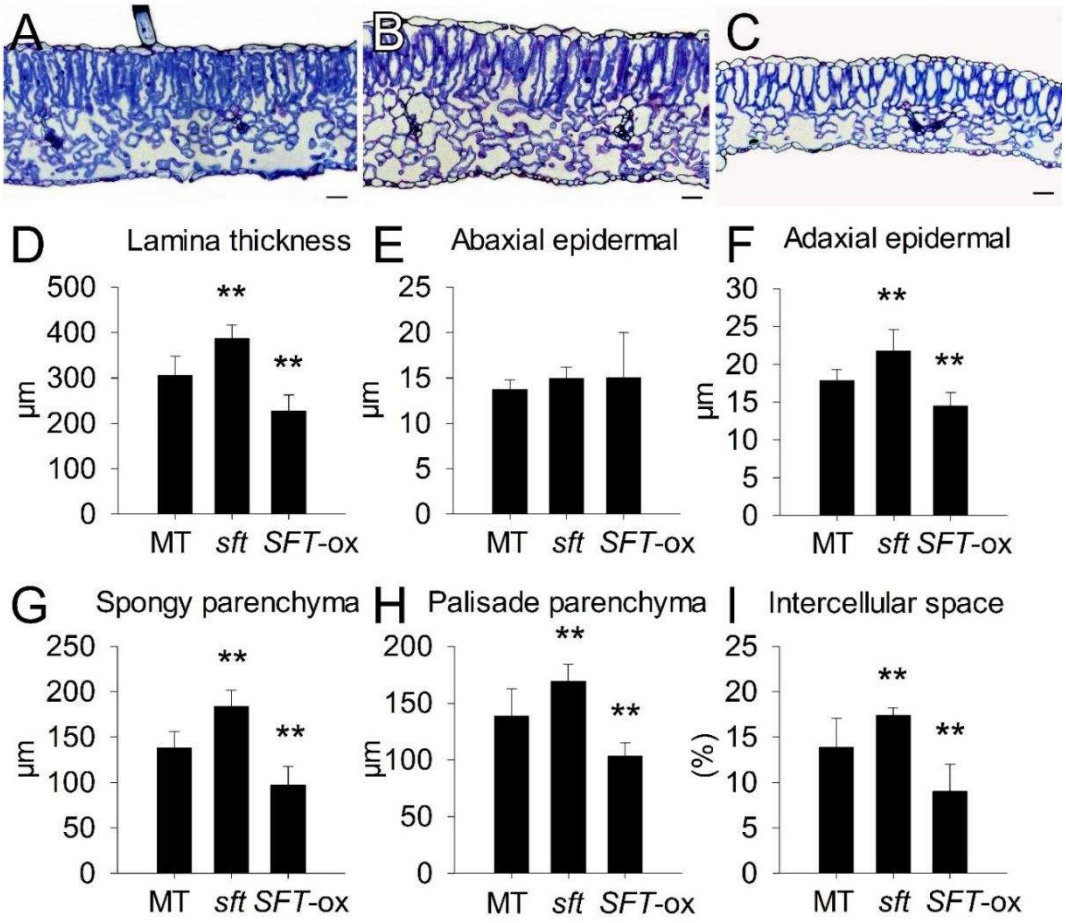

Figure 6. SFT triggered anatomical modifications in leaves of tomato. Representative cross sections of fully-expanded terminal leaflets obtained from the second leaf from top in 42-day old plants Samples stained with toluidine blue. Scale bars = 50 µm A) MT, B) sft and C) SFT-ox. **Leaf anatomical parameters in MT, sft and SFT-ox**. D) Lamina thickness E) Abaxial epidermis thickness F) Adaxial epidermis thickness G) Spongy parenchyma thickness H) Palisade parenchyma thickness and I) relative volume of intercellular air spaces. Measurements obtained from the second leaf from top to the base fully expanded in plant with 42 DAG. Data are mean ± s.e.m. (n=4) Asterisks indicate differences from MT according to Student's t-test($P<0.05$).

Stomatal conductance in response to light levels and CO_2 concentrations was affected for allelic variations of SFT

The allelic variations of sft triggered differences in stomatal conductance, thus providing an excellent system to evaluate the effects of SFT defective or overexpression and stomatal conductance. Were determinate kinetics of MT and sft or SFT-ox in dark-adapted leaves and saturating light 1000 µmol $m^{-2}s^{-1}$ or ambient CO_2 concentration (400 µmol mol^{-1}) and high CO_2 concentration 800 µmol mol^{-1} for either opening or closure. In light response, sft and MT presented similar conductance in

dark, but in saturating light sft exhibited lower stomatal conductance that MT, besides SFT-ox in dark showed higher stomatal conductance in all light intensity until saturating light. On the other hand, sft had minor stomatal conductance in 400 umol mol^{-1} and presented less stomatal closing that MT in high CO_2 concentrations, while SFT-ox was indistinguishable.

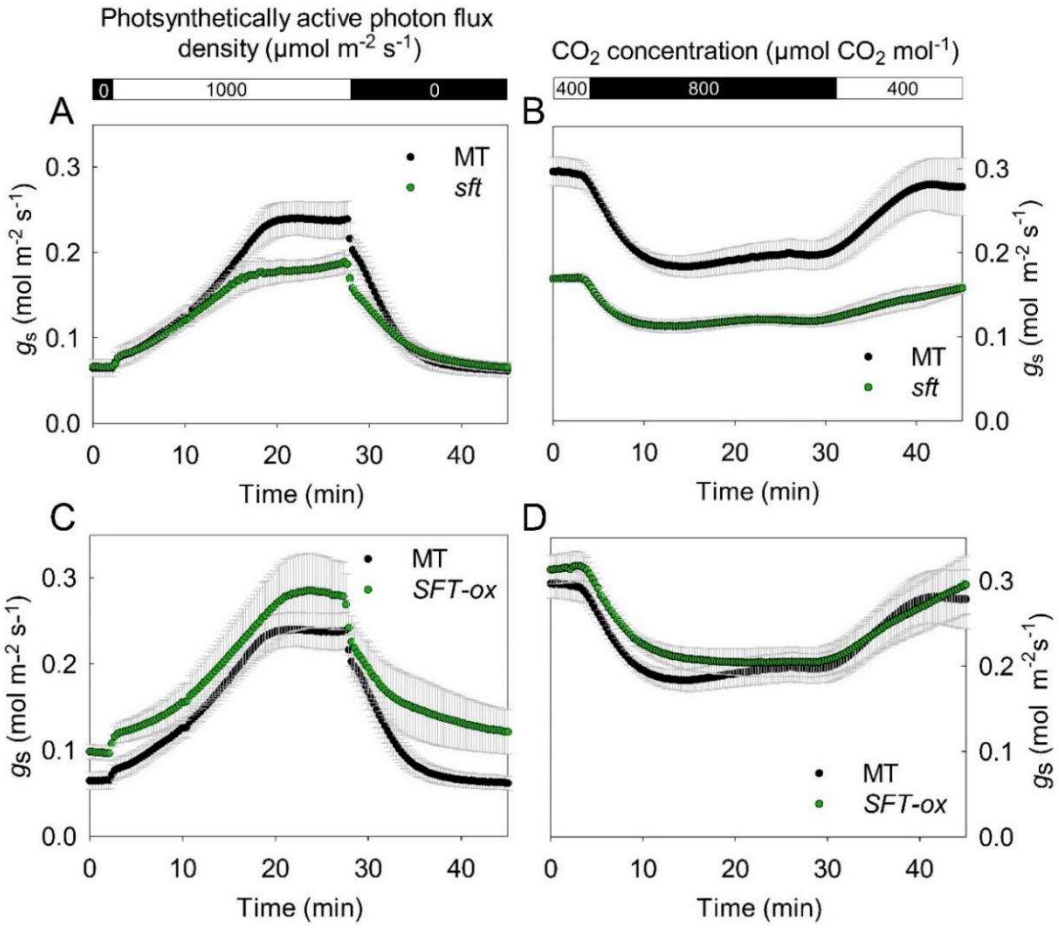

Figure 7. Stomatal kinetics in response to both light to-dark and normal-to high CO_2 is modify for SFT . Stomatal conductance (g_s) was evaluated in sft and SFT-ox compared with MT in response to step-changes in light intensity (A and C, respectively) and ambient CO_2 levels (B e D). Measurements obtained from the second leaf from top to the base fully expanded with 42 DAG. Values are means ± s.e.m.(n=4). Asterisks indicate differences from MT according to the Student's t-test to be significantly different ($P<0.05$).

Stomatal bioassays

When leaves of MT, sft, SFT-ox, not and NCED-ox were exposed to aperture buffer, significant differences were showed for all genotypes compared with MT. To control for possible osmotic effects, 20 mM mannitol was supplied to the medium, however, no apparent effect on guard cell movement was observed. On the other hand,

application of ABA induced stomatal closure in all genotypes compared with the respective control, providing evidence that the effects observed in sft and SFT-ox are ABA independent.

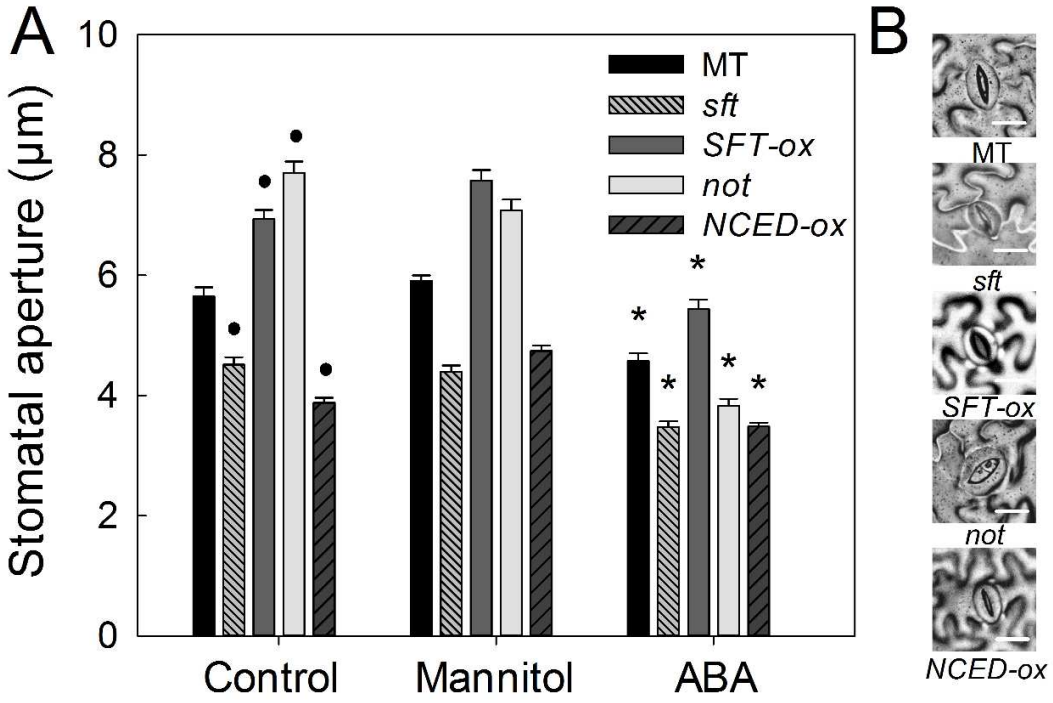

Figure 8. Stomatal conductance by SFT independent of abscisic acid (ABA) signal transduction pathway. A) Stomatal response of leaves of tomato plants MT, sft, SFT-ox, not and NCED-ox in the presence of mannitol (Man) and abscisic acid (ABA). Measurements obtained from the third fully expanded leaf of 42 day old plants. Values are means ± s.e.m (n=100 stomata). Black points represent differences of each genotype compared with MT. Asterisks show differences of treatments from its corresponding control according to the Student's t-test ($P<0.05$). B) Representative stomatal images in opening buffer (Control).

DISCUSSION

Here, we used MT as genetic background compared with genotypes carrying allelic variations for SFT and two controls of stomatal behaviour notabilis and NCED-ox ABA deficient and ABA overproduction respectively. FT exhibit multifaceted roles in plant development, among them triggered flowering in angiosperms (Karlgren et al., 2011) In tomato, SFT has been described by controls floral meristem identity and the sympodial development of tomato, this feature allows that SFT is directly related with growth habit (Molinero-Rosales et al., 2004). Our results showed that transcript levels were consistent for each genotype, highlighting that not and NCED-ox, not

showed differences in relative transcript accumulation, which suggests that SFT expression is independent of ABA.

Moreover allele loss function sft delay flowering and increased number of leaves as described for Molinero-Rosales (2004), conversely SFT-ox induce early flowering and decreased number of leaves without effects in root or stem as shown for (Lifschitz et al., 2006). On the other hand, the pattern branching was drastically altered in SFT-ox can be attributed to constant development of sink organs (Shalit et al., 2009).

FT is involved in the regulation of H^+-ATPase by blue light in guard cells controlling the stomatal opening. We analysed fresh weight loss and leaf temperature showing differential weight loss between genotypes as well as clear differences in leaf temperature. However, given that fresh weight loss in detached leaflet might not reflect the true state of the plant and also the general difficulty with thermal methods because the plant temperature is affected not only by g_s and transpiration, but also factors such as air temperature, humidity, radiation and wind speed (Leinonen et al., 2006), we decided to analyse the gas exchange. This clearly indicate differences in water relations between evaluated genotypes.

The photosynthesis rate, dark respiration and stomatal conductance were altered for SFT, The enhanced carbon dioxide assimilation was a function of enhanced stomatal conductance facilitated by the wider stomatal aperture likewise leaf cross section shown that anatomical features were affected, sft presented greater leaf thickness and low photosynthesis rate, while SFT-ox exhibited thinner leaves and greater photosynthesis rate in accordance with Terashima et al., (2001) who showed the same relation in hypostomatous leaves. Furthemore Vicente et al., (2015) showed a relationship between water use efficiency (WUE) and growth habit in SFT mutants in tomato, pointing out the superiority of plants with allele recessive sft in the water use efficiency for the production of biomass. This evidence suggests that WUE may be related to changes in the vegetative/reproductive balance or pleiotropic effect of SFT gene.

It is widely accepted that leaf dark respiration is a determining factor for the growth and maintenance of plant tissues and the carbon cycle, sft showed greater respiration in leaf and growth while SFT-ox was characterized for a decreased rate of

leaf respiration, and an enhanced photosynthetic rate explained possibly by strong sink.

The analysis of A/C_i and A/C_c curves reveals that the maximum carboxylation velocity of Rubisco (V_{cmax}) and maximum electron transport rate (J_{max}) was similar between sft and MT, in the same way, sft not showed significant differences in C_i, g_m, Vcmax, J_{max} or TPU compared with MT, in contrast, SFT-ox exhibited lower $J_{max}\_C_i$ and decreased the ratio J_{max}:V_{cmax} for C_i and C_c Also stomatal, mesophyll or biochemical limitation was not evidenced.

Having established that the low photosynthetic rates and increased growth rates were, at least, predominantly due to the altered stomatal function of the genotypes, we concentrated on trying to understand the mechanisms underlying this change. To further assess the impact of the lack of a functional SFT on stomatal conductance (g_s) and water loss in Tomato plants, we next adopted two complementary approaches. First, we confirmed the duration of stomatal responses following dark-to-light and light-to-dark transitions as well as normal-to-high and high-to-normal CO_2 concentrations. Our results confirmed differences in optimal conditions, sft and SFT-ox exhibited lower and greater stomatal response in saturate light respectively with no differences in response speed, conversely, sft started with lower stomatal conductance but was less responsive to high CO_2 concentrations, and on the other hand, SFT-ox showed similar behaviour to MT.

Subsequently, was analysed the stomatal response to ABA. In this work was demostrate that the allelic variations of SFT gene triggered alterations in the stomatal conductance of tomato plants independent of abscicic acid same that demonstred in Arabidopsis where the stomatal aperture in response to ABA promoved closure in all genotypes (Kinoshita et al., 2011).

CONCLUSION

Here, we provide evidence that SFT have pleiotropic effects in tomato mainly playing a role in leaf structure and physiological traits where both as aperture as closure stomatal are altered. However the signaling for module anatomical features is unknown. Our approach can be extended to add further mutants to the collection,

generated either by conventional crossing or by genetic transformation and explore the link between SFT and stress tolerance.

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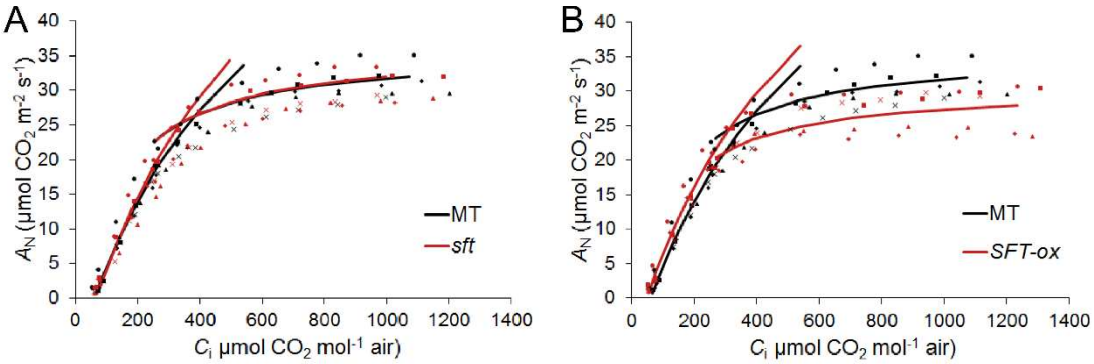
SUPPLEMENTAL MATERIAL

Gene	Locus ID	Primer sequence
Actin	Solyc03g078400	Fwd 5'-GGTCCCTCTATTGTCCACAG-3'
		Rev 5'-TGCATCTCTGGTCCAGTAGGA-3'
SFT	Solyc03g063100	Fwd 5'-GACCCTGATGCTCCAAGTCC-3'
		Rev 5' – GTGACCAACCAGTGAAGGTATTC-3'



Supplemental figure 1 Net photosynthesis (A_N) curves in response to sub-stomatal (C_i) CO_2 concentration in MT and sft or SFT-ox plants under saturing light. Values obtained using the second leaf from top to the base fully expanded. The solid line indicates the curve based on leaf biochemistry parameters, dotted line indicate the C_i obtained under atmospheric CO_2. The biochemically based leaf photosynthesis model (Farquhar et al., 1980) was fitted to the data based on C_i, values of A_N/C_i for five plants of MT (black points) and sft or SFT-ox (red points).

GENERAL CONCLUSION AND PERSPECTIVES

Our data provides the first link between auxin and the anti-florigenic protein SELF-PRUNING (SP), the main switch between indeterminate/ determinate growth habit in tomato. A physiological interaction between auxin and CETS for control of growth habit was demonstrated here. Our results suggest that at least part of the effect of the SP/SFT balance on yield is mediated by auxin. On the other hand, were identified pleiotropic effects in tomato for SFT, however, for alters anatomical and physiological features of tomato plants, this functional diversity raises intriguing questions, especially regulatory mechanisms. Research is needed to identify proteins that interact directly with FT, like so characterization of other paralogs genes in tomato. That said the concepts outlined here do not merely reflect the challenges presented in understanding the interplay between plant and auxin, and flowering and co2 conductance in a crop species such as tomato but have broader implications for understanding these trade-offs in any plant species.