Tomato mutants reveal root and shoot strigolactones involvement in branching and broomrape resistance

Uri Karniel
Hebrew University of Jerusalem The Alexander Silberman Institute of Life Sciences
Amit Koch
Hebrew University of Jerusalem - Rehovot Campus
Nurit Bar Nun
Hebrew University of Jerusalem The Alexander Silberman Institute of Life Sciences
Dani Zamir
Hebrew University of Jerusalem - Rehovot Campus
Joseph Hirschberg
hirschu@mai.huji.ac.il

Alexander Silberman Institute of Life Sciences: Hebrew University of Jerusalem The Alexander Silberman Institute of Life Sciences https://orcid.org/0000-0002-4927-178X

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Abstract

The phytohormones strigolactones (SLs) control root and shoot branching and are exuded from roots into the rhizosphere to stimulate interaction with mycorrhizal fungi. The exuded SLs serve as signaling molecules for the germination of parasitic plants. The broomrape *Phelipanche aegyptiaca* is a widespread noxious weed in several crop plants, including tomato (*Solanum lycopersicum*). 'In silico' screening of tomato (M82) mutants identified three lines that showed significantly increased branching. Two mutants, *SHOOT BRANCHING 1* (*sb1*) and *SHOOT BRANCHING 2* (*sb2*), lack SLs due to loss of function mutations in the genes for the carotenoid cleavage dioxygenase enzymes CCD7, and CCD8, respectively. Beyond the increased branching, these mutants were completely resistant to infection by *P. aegyptiaca*. The third branching mutant, *SHOOT BRANCHING 3* (*sb3*), carried a point mutation in the SLs receptor *DWARF14* and was found to be highly susceptible to *P. aegyptiaca.* SL concentration in roots of the *sb3* was two-fold higher than in the wild type due to the upregulation of transcription of SL biosynthesis genes. This phenomenon suggests that the steady-state level of SLs is regulated by a feedback mechanism that involves the SL signaling pathway. Grafting experiments showed that *sb1* and *sb2* rootstocks protected wild-type tomato scions from *P. aegyptiaca* infection without a significant yield loss, offering a solution to the broomrape crisis. These results also demonstrate that strigolactones synthesized in the shoots are involved in the control of shoot branching.

Key Message

Tomato mutations impaired in strigolactone synthesis and perception reveal shoot strigolactones involvement in controlling yield and branching and establishing horticultural utilization by grafting to cope with broomrape infestation of crops.

Introduction

Strigolactones (SLs) are a group of conserved carotenoid-derived hormones present across all land plants. They were first characterized as potent stimulant crystalline compounds that induced the germination of the parasitic weed *Striga lutea* (Cook et al. 1966). Further research showed that SLs are involved in plant development and responses to biotic and abiotic stresses and rhizosphere signaling (Xie et al. 2010; Ruyter-Spira et al. 2012; Al-Babili and Bouwmeester, 2015; Kyozuka et al. 2022). One of the most important roles of SLs is to suppress axillary bud growth and shoot branching (Umehara et al. 2008; Domagalska and Leyser, 2011; Barbier et al. 2019). SLs also affect root architecture (Koltai, 2011; Khuvung et al. 2022) and are involved in other processes, from seed germination to senescence (Al-Babili and Bouwmeester, 2015; Zwanenburg and Blanco-Ania, 2018; Waters et al. 2017; Trasoletti et al. 2022). Recent evidence revealed complicated crosstalk mechanisms between SLs and other phytohormones (Omoarelojie et al. 2019; Wu et al. 2022).

Below the ground, SLs are exuded from roots, stimulating various processes in the rhizosphere. An important role of SLs is the establishment of a beneficial symbiosis with arbuscular mycorrhizal fungi

through promoting root colonization and hyphal branching, which improves the plant's mineral nutrition (Kyozuka et al. 2022). It was shown that a deficiency in the primary nutrients phosphate and nitrogen induces SLs biosynthesis and response (Lopez-Raez et al. 2008; Umehara et al. 2010). SLs promote plant defense against root-knot nematodes in tomato (*Solanum lycopersicum*) by influencing the accumulation of the phytohormones jasmonic acid and abscisic acid in the roots (Xu et al. 2019).

SLs exuded by the roots also stimulate the germination of several parasitic plant species, most of which belong to the *Orobanchaceae* (broomrapes) family. This family comprises the Striga, Orobanche, and Phelipanche genera, among them the numerous holoparasitic weeds responsible for severe damage to crop yield worldwide (Joel et al. 2013). *Phelipanche aegyptiaca spp.* (Egyptian broomrape) is a widespread noxious weed in tomato fields in Africa, the Middle East, and the Mediterranean (Eizenberg and Goldwasser, 2018). This parasitic plant can cause a severe yield loss ranging from 5–100% (Hershenhorn et al. 2009). The life cycle of *P. aegyptiaca* starts with seed germination, followed by the radicle growth of the parasite towards the host root. After that, a constant secretion of SLs stimulates and directs haustorium (a root-like structure) formation toward the vascular system of host roots, resulting in a compatible interaction. The inflorescence of the parasitic plant emerges from the soil and produces flowers that yield a huge number of seeds (Matusova et al. 2005).

The initial steps of SLs biosynthesis in plants occur in the plastids. It starts with the isomerization of the C9-C10 double-bond of all-*trans* β-carotene to produce 9-*cis*-β-carotene, catalyzed by the carotene isomerase DWARF27 (D27) (Jia et al. 2018). Further reactions, catalyzed by Carotenoid Cleavage Dioxygenase 7 (CCD7) and CCD8, convert 9-*cis* β-carotene to carlactone, the common precursor for all the divergent active molecules in the SL family (Alder et al. 2012)(Bonhomme and Guillory, 2022; Yoneyama and Brewer, 2021). Subsequent reactions in the cytosol are catalyzed by cytochrome P450 enzymes, which convert carlactone into functional SLs through hydroxylation and oxidation reactions (Fig. 1, and (Mashiguchi et al. 2020; Jia et al. 2018; Wang et al. 2022).

The perception pathway of SLs starts with their binding by the receptor DWARF14 (D14) (Yao et al. 2016; Seto et al. 2019). This binding leads to the recruitment of the F-box protein MAX2, which targets the repressor proteins, DWARF53 and SMXLs, for ubiquitination and subsequent degradation in the proteasome, resulting in the activation of various SLs downstream target genes (Wang et al. 2020). The receptor D14, which belongs to the α/β-hydrolase enzyme superfamily, is conserved in all land plants (Waters et al. 2017; Bythell-Douglas et al. 2017) and recently has been characterized in tomato (*Solanum lycopersicum*) (Li et al. 2022)

Tomato is a major horticultural crop of global importance, and the parasitic weed *P. aegyptiaca* endangers its cultivation in vast areas worldwide (Joel et al. 2013). Several strategies have been developed to cope with broomrapes (Eizenberg and Goldwasser, 2018). Most of them use chemicals, including the application of herbicides and soil fumigation that kill the parasites, and treatment of the field before cultivation with SL analogs, in a process known as 'suicidal germination' (Fernández-Aparicio et al. 2016; Kountche et al. 2019). Apart from these methods, the breeding of *P. aegyptiaca*-resistant

varieties, based on low exudation of the SLs stimulants by the host, has been endeavored (Aly et al. 2021; Hershenhorn et al. 2009; Dor et al. 2010; Dor et al. 2011; Hasegawa et al. 2018; Bai et al. 2020; Bari et al. 2021; Galili et al. 2021). Some were based on impairing the function of the carotenoid cleavage enzymes CCD7 and CCD8, which are involved in the SLs' biosynthesis pathway.

Here, we describe the identification and characterization of isogenic tomato mutants impaired in the CCD7 and CCD8 enzymes and a mutant in the SL-receptor D14. Molecular and physiological characterization of these mutants shed new light on SLs functioning in tomato, the regulation of their synthesis and their effects on broomrape resistance.

Materials and methods

Plant material and growth conditions.

Tomato cultivar M82 served as a reference 'wild-type.' Seeds from that variety were treated with ethylmethane sulfonate (EMS) or fast neutron bombardment (Menda et al. 2004). Visual screening of M2 plants identified several mutants with over-branching phenotypes. Following measurement of the resistance level against *P. aegyptiaca* infection in an inoculated field was established using the identified over-branching lines. Three mutant lines, *sb1*, *sb2*, and *sb3*, were isolated and further studied in this research. The commercial hybrids sft3 and H4107 used as scions in this study were obtained from Prof. Dani Zamir lab. Root extracts for qPCR and *P. aegyptiaca* germination bioassay were performed from five-week-old hydroponically-grown plants in Hoagland-medium in the greenhouse. For the bioassay analysis, the Hoagland medium was changed to Hoagland without phosphate for four days, followed by a water-only medium for three days to elevate the SLs synthesis in Pi-starved condition.

The field trials presented in this study were performed during three growing seasons. In 2016 and 2017, they were conducted in the Eden research station and the Gadash Ein Harod Ichud. In 2018, the field trials were conducted at the Western Galilee Experimental station in Akko and Eden research station. The Akko experiments were performed in a wide-spacing planting density of one plant per 1 m^2. The plants in the Eden experiment were grown in plots of 27 plants per 5 m^2. Seedlings were grown in the greenhouse for 35 days and then transplanted to the field at the beginning of March in Eden and April in Akko.

Grafting experiments were conducted during the summer of 2018 in the Western Galilee experimental station in Akko, Israel. Grafted plants were prepared by Hishtil Nursery (Ashkelon, Israel, https://www.hishtil.com/) as described (Koltai et al. 2010). Twenty-one days post-germination, sterile-grown seedlings were sectioned within the hypocotyl region, and the combinations of scion and rootstock were aligned to form a graft union. The grafted plants were transplanted to the field in April. The experiment examined individual plants in a completely randomized design in two blocks, and plant experiments were represented by a minimum of 15 replicates in each block. M82, *sb1*, and *sb3* seedlings were used for reciprocal and self-grafting as a control. Two types of measurements were performed. The first, which included vegetative and growth characteristics, was performed about 40 days after planting.

The second, which included yield and fruit traits, was done during harvesting, about 100 days after planting.

DNA extraction and sequencing.

DNA was extracted from young tomato leaves of approximately 15 mg, as previously described (Eshed and Zamir, 1995). Genomic DNA of the genes *Sl*Ccd7 (Solyc01g090660.2), *Sl*Ccd8 (Solyc08g066650.2), and *slDWARF14* (Solyc04g077860.2) was amplified by PCR from total genomic DNA, using a Readymix kit (PCR-Ready High Yield, Syntezza, http://www.syntezza.com/). These genes' whole DNA length was sequenced and compared to the *Solanum lycopersicum* reference genome (build SL2.40 in http://solgenomics.net/). The mutations that located in these genes were identified by sequencing using the following primers: 5'-AGTGTCTTTTAGCACCACATGT-3' (Forward) and 5'-CTTCAAGTCTTGCAACTACTTCA-3' (Reverse) for *Sl*Ccd7; 5'-CAGAACAGGGCCAAATGACC-3' (Forward) and 5'-ACCAAGGTCAGCTTCTTTTCC-3' for *Sl*Ccd8; 5'-CCTTAGTTTATGTTTGACAAAATTCAT-3' (Forward) and 5'-CAAACAATGTTATGTCTGGTCTCA-3' (Reverse) for *slDWARF14*.

RNA extraction, cDNA sequencing, and measurement of mRNA with Quantitative Real-Time RT-PCR.

RNA extraction from hydroponically grown tomato roots was extracted from approximately 200 mg tissue with TRI Reagent RNA isolation reagent (Sigma-Aldrich), according to the manufacturer's protocol. Reverse transcription and DNase treatment were performed with the iScript™ gDNA Clear cDNA Synthesis Kit #172–5035 (Bio-Rad). In order to detect genomic DNA contamination, the cDNA was amplified using ACTIN primers 5'-TTGCTGACCGTATGAGCAAG-3' (Forward) and 5'-GGACAATGGATGGACCAGAC-3' (Reverse) that differentiate between genomic DNA and cDNA sequences. The cDNA was amplified by ProFlex PCR System (Applied Biosystems by Thermo Fisher Scientific). To investigate the effect of the mutations located in exon junctions in the genes *SlCcd7 and SlDWARF14* on the transcript, the cDNA of these genes was amplified and sequenced using the primers 5'-TCTTAACGTTCGGGTCGTCG-3' (Forward) and 5'-GGTACAGAGTGGTCCCTTGC-3' (Reverse) for *slDWARF14*; 5'- TGAATGGAACAAAGCAGCAG-3' (Forward) and 5'- GTTGGTAGGAGCCCAAAAGC-3' for *SlCcd7*.

Quantitative polymerase chain reactions were performed using the Applied Biosystems™ Fast SYBR™ Green Master Mix on a StepOnePlus™ Real-Time PCR System (Applied Biosystems). Cycling conditions were 95°C for 20 sec, followed by 40 cycles of 95°C for 3 sec, 60°C for 30 sec, and fluorescence acquisition at 60°C. The relative mRNA level was determined for each gene in three biological replicates. The gene ACTIN (using the primers described above) served as a control for normalization. *slD27* was amplified using the primers 5'-TCCCTAAGCCTATTCTTTCTCTG-3' (Forward) and 5'-TCACCTCACAAGGTCCAACTA-3' (reverse); *slCcd8* was amplified using the primers 5'-CCAATTGCCTGTAATAGTTCC-3' (Forward) and 5'-GCCTTCAACGACGAGTTCTC-3' (Reverse).

Strigolactone quantification bioassay

The amount of SL in the mutants' roots was estimated in a bioassay based on the germination of *P. aegyptiaca* seeds. Three-week-old seedlings of wild type (M82) and mutants *sb1*, *sb2*, and *sb3* were transferred from soil to a hydroponic growth system on Hoagland medium in the greenhouse. After one week, the medium was changed to Hoagland, lacking Pi to induce SLs synthesis. After one week, the roots were ground in a mortar and pestle with liquid nitrogen and then extracted with ethyl acetate in a glass tube. The tubes were vortexed for 10 minutes and then centrifuged at 12,000 g for 5 minutes, and the organic phase was transferred to a glass vial. The extraction of the root pellet was repeated once more, and the extract was dried under nitrogen gas. The dried SL samples were dissolved in sterile distilled water to adjust the concentrations equivalent to 1 gr root fresh weight per 1 milliliter of water. The vials were stored at -20°C. *Phelipanche aegyptiaca* (Pers.) seeds were collected from fields near Kfar Yehoshua and Mevo Hama, Israel. Dry seeds of *P. aegyptiaca* were surface sterilized in 70% ethanol for 2 min, rinsed three times with distilled sterile water, then sterilized in 3% NaOCl for 3 min, and again rinsed three times with sterile distilled water. Seeds of *P. aegyptiaca* were placed for three days in six-well Elisa plates (Nunc, Roskilde, Denmark), moistened with 0.6 ml of sterile distilled water on a fiberglass filter paper (GF/A Whatman Maidstone, UK) at 25°C in the dark. After three days, the water was removed, and 200 µl of root extracts were applied to the disks. This amount was determined after calibration of the bioassay with root extracts from M82 roots that were applied in different dilutions (Fig. S7). The treated seeds were incubated in the dark at 25°C for two weeks. All the germination treatments were conducted under aseptic conditions. The germination response of the *P. aegyptiaca* seeds was observed 14 days after stimulation by the root extracts. Dry seeds moistened only with deionized water were used as a control. Water alone did not induce any germination. Germinated and non-germinated seeds were counted under a microscope.

Results

Isolation and molecular characterization of SL mutants

The collection of ethylmethane sulfonate (EMS)-mutagenized tomato plants (*Solanum Lycopersicum* cv M82) (Menda et al. 2004) was screened for mutants with alternative growth habits. Three mutants with increased shoot branching were identified (Fig. S1) and were named *SHOOT-BRANCHING1* (*sb1*), *SHOOT-BRANCHING2* (*sb2*), and *SHOOT-BRANCHING3* (*sb3*). The distinctive phenotype suggested these mutants were impaired in strigolactone (SL) functions. The over-branching phenotype caused a significant reduction in total fruit yield due to the decrease in fruit size and fruit set (Fig. 2).

The amount of SL in the mutants' roots was estimated in a bioassay of germination of *P. aegyptiaca* seeds (Materials and methods). The results showed that root extracts from *sb1* and *sb2* plants starved for phosphate did not induce germination of *P. aegyptiaca*, compared with the wild-type line M82 (Fig. 3A). By contrast, the root extract from *sb3* increased the germination rate of *P. aegyptiaca* by two-fold compared to M82 (Fig. 3A). This results explains the high susceptibility of *sb3* plant to infection of *P. aegyptiaca* in the field (Fig. S2). The response of the *sb* mutants to infection by *P. aegyptiaca* was measured in a field infested with seeds of this parasite by the number of broomrape inflorescence per

plot. No broomrape inflorescences were found in mutants *sb1* and *sb2*, indicating that they were resistant to infection by *P. aegyptiaca* (Fig. 3B). These results agree with the finding that *sb1* and *sb2* lack SLs. By contrast, the infection of *sb3* plants was more than 50 percent higher than that of wild-type M82 plants. The increased susceptibility of *sb3* plants to *P. aegyptiaca* corresponds to the higher level of SL in this mutant's roots (Fig. 3A, B).

Mutations in tomato that impair strigolactone biosynthesis have been previously reported in the carotenoid cleavage dioxygenase enzymes *Sl*CCD7 (Solyc01g090660.2) and SlCCD8 (Solyc08g066650.2) (Koltai et al. 2010; Kohlen et al. 2012; Bari et al. 2019; Hasegawa et al. 2018; Aly et al. 2021). The genes *Sl*Ccd7 and *Sl*Ccd8 were sequenced in the *SHOOT-BRANCHING* mutants and compared with M82. The sequence data showed that the mutations in *sb1* and *sb2* are in the *Sl*Ccd7 and *Sl*CcdD8, respectively (Table 1). The gene *Sl*Ccd7 encodes a polypeptide with 663 amino acid residues and a molecular weight of 75 kDa. The gene *Sl*Ccd7 from the mutant *sb1* contains two mutations that alter the splice site of exon #7 (Fig. S3 A). An alternative splicing event in intron #6 creates a seven-nucleotide deletion in the mRNA, leading to a frameshift mutation and a truncated polypeptide (Fig. S3 B). CCD8 in tomato is a 64.7 kDa polypeptide with 579 amino acid residues. A point mutation of G to A in position 2,659 of *sl*Ccd8 from *sb2-1* creates a missense mutation that changes glutamate to lysine in mutant *sb2-1* (Fig. S4 A,B).

Table 1. Mutations identified in the over-branching tomato mutants

Gene	Locus	Mutation	Impact of the mutation
Carotenoid cleavage dioxygenase 7 (*Sl*Ccd7) Solyc01g090660	*sb1-1*	G→C Position 3268 G→T position 3272	Intron/exon #7 junction, deletion of 7bp in the mRNA of *Ccd7* exon #7
Carotenoid cleavage dioxygenase 8 (*Sl*Ccd8) Solyc08g066650	*sb2-1*	G→A Position 2659	A change of Glu529 to Lys
α/β-hydrolase receptor Dwarf14 (D14) Solyc04g077860	*Sb3-1*	G→A position 1582	Intron/exon #2 junction, deletion of 17 bp in the mRNA of *DWARF14* exon #2

In contrast to *sb1* and *sb2*, the third mutant *sb3* exhibited high shoot branching and a higher SL concentration. This finding suggested that *sb3-1* is involved not in the SLs' biosynthesis but in one of the genes that participated in the SLs' signaling pathway. Three major components are involved in the SLs perception pathway: the a/b-fold hydrolase D14, the F-box protein MAX2, and the repressor protein D53 (Bürger and Chory, 2020; Bonhomme and Guillory, 2022). The recessive nature of *sb3-1* eliminated the possibility of gain of function mutation in this mutant's D53 repressor. Furthermore, comparing the two tomato orthologous genes, Solyc07g055120, and Solyc12g010900, from *sb3-1* and M82 indicated no polymorphism in their sequences. However, a mutation in *sb3-1* was discovered in the gene *Sl*Dwarf14 (*sl*D14) (Solyc04g077860), which encodes a 29.8 kDa protein with 267 amino acid residues (Table 1, Fig.

S5 A). The G to A mutation at position 2,582 in the *sl*Dwarf14 gene from *sb3-1* eliminates the splice site in exon #2, resulting in a 17 nucleotides deletion in the mRNA caused by alternative splicing that produces a truncated protein (Fig. S5 B).

The higher concentration of SL in *sb3-1* suggested that strigolactone signaling is involved in regulating the steady-state level of the phytohormone. Therefore, the expression of the genes *SlD27*, the first step in strigolactone biosynthesis, and *SlCcd8* were analyzed in the roots of *sb3-1* and WT (M82). Despite the SLs' abundance, the expression of these genes was higher in *sb3-1* compared with M82 (Fig. 4). Since the mutation in *sb3-1* eliminates the SL receptor D14, this result indicates that the feedback regulation of the SL biosynthesis genes operates via SL perception and signal transduction.

The phenotype of sb mutants in grafting experiments

Different grafting combinations using the *sb* mutants were carried out to estimate the contribution of roots and shoots to the SL in the plant. In these experiments, the SL mutants *sb1* and *sb3* and the wild type (M82) served as rootstocks and scions in all combinations and several morphologic traits were measured (Fig. S6). The parameter found to have the highest correlation to the branching phenotype was the ratio between the number of branches and the stem length (Fig. 5A, Table S1). As expected, grafting of *sb1* scion on *sb1* rootstock exhibited the typical over-branching phenotype. However, reciprocal grafting of the wild type M82 and mutant *sb1* did not significantly alter the branching, indicating that the shoot compensated for the lack of SL in the roots and vice versa (Fig. 5A). The over-branching phenotype due to the inhibition of SL perception seen in the self-grafted *sb3* mutant was restored in when M82 was grafted on *sb3* rootstock but not in the reciprocal grafting. This result indicates that the SL regulation on the branching is confined to the shoot SL signal transduction is lacking in *sb3*. The yield parameters of the grafted plants generally corresponded to the degree of branching, despite some changes that probably reflect other effects of SL on fruit development.

The SL-deficient rootstock of *sb1* renders *P. aegyptiaca* resistant to grafted commercial varieties of tomato (Fig. 6). To determine the utility of *sb1* as a rootstock in processed tomato varieties, scions from the commercial varieties sft3 and H4107 were grafted on *sb1* rootstock, and their yield was tested in field conditions. No yield loss was observed in the grafted plants (Fig. 7).

Discussion

Isogenic mutations in strigolactone synthesis and perception

Strigolactones are a class of plant hormones that regulate various aspects of plant growth and development, including branching and root architecture, and play crucial roles in interactions between soil organisms and roots. In the present study, we identified and characterized loss-of-function mutations in tomato (*S. lycopersicum* cv. M82) that impair strigolactone biosynthesis, *sb1* and *sb2*, and perception, *sb3*. The mutations *sb1* and *sb3* in the *Sl*Ccd7 and *Sl*D14 genes occurred in splicing sites, leading to aberrant transcripts that create early stop codons. The mutation *sb2* in the *Sl*Ccd8 gene

causes a substitution of the negatively charged amino acid glutamic acid to the positively charged lysine at position 529 of the CCD8 protein. This glutamic acid residue is conserved in all CCD8 proteins examined in monocots and dicots (Batra *et al.* 2019), so the substitution likely impairs the enzymatic activity. The original mutants obtained by EMS mutagenesis (Menda *et al.* 2004) were backcrossed with the parental M82 wild-type line, and the characteristic over-branching phenotype was co-segregated with the mutations in the respective genes among all F2 offspring in a typical 3:1 ratio. Mutations in SL biosynthesis genes have been previously described (Dor *et al.* 2010; Kohlen *et al.* 2012; Hasegawa *et al.* 2018; Zhang *et al.* 2018; Bari *et al.* 2021; Bari *et al.* 2019; Li *et al.* 2022). However, these mutants were isolated in different lines with diverse genetic backgrounds, making phenotypic characterizations of quantitative effects impossible to compare. In this study, we analyzed three mutations that affect SL functioning in an entirely isogenic background, allowing accurate comparisons of the specific impact of SL on growth and agronomic traits.

The over-branching in the SL mutants was accompanied by a reduction in total fruit yield, which was partly caused by a significant decrease in fruit size (Fig. 2). It is unclear whether the reduction in fruit size is a direct effect of the lack of SL signaling on fruit development or because of a different allocation of photosynthates between the fruits and the enlarged vegetative organs of the plant. The latter option is supported by the grafting experiments, where the fruit yield was inversely proportional to the branching degree in all grafting combinations (Fig. 5, Table S1).

The mutation *sb3* in the SL receptor D14 exposed differences between the absence of SLs versus impairment in SL perception and signaling. Lack of strigolactones in the mutants *sb1* and *sb2* reduced fruit yield by 33 percent, compared with a 45 percent reduction when the SL perception was eliminated in *sb3*. The more severe effect in the absence of the D14 receptor can be attributed to the distinct influence on gene expression when the D14 protein is present but not induced by SLs, compared to the condition where it is absent. The most likely explanation relates to the fact that perception of strigolactones by D14 requires the interaction with the F-box protein MAX2 to target proteins, such as SMXL/D53 repressor of SL signaling, for ubiquitin-dependent degradation (Stirnberg *et al.* 2007; Al-Babili and Bouwmeester, 2015; Seto *et al.* 2019; Mashiguchi *et al.* 2021). Although strigolactones facilitate the interaction of D14 with MAX2, a residual small interaction may occur in the absence of SL. Other explanations might be feasible if the D14 protein serves other yet unknown functions unrelated to SL, or if the higher SL concentrations in *sb3* (Fig. 3) cause detrimental effects through a D14-independent mechanism.

It was demonstrated that the D14 receptor protein degrades SL upon perception (Seto *et al.* 2019). The elevated concentration of SL in the *sb3* roots, which was also manifested by a higher infestation of *P. aegyptiaca* in the field (Fig. 3), can be attributed in part to the lack of SL hydrolysis by D14. However, as seen in Fig. 4, the transcript levels of the SL-biosynthesis genes SlD27 and SlCcd8 were 5- and 2.5-fold higher, respectively, when the D14 signaling was blocked. These data strongly suggest that increased synthesis of SL underlies the higher SL levels in *sb3* roots. Moreover, this conclusion reveals a feedback mechanism controlling the steady-state level of SL that involves SL signaling and regulation of gene

expression. This conclusion is supported by related phenomena observed in other plant species. In a D14 mutant in rice (*Oryza sativa*), the levels of epi-5-deoxystrigol in the roots were higher than in the wild type (Arite *et al.* 2009). Mutations in the genes for D53, a repressor of SLs signaling, led to the upregulation of D10, an orthologue gene of CCD8 (Zhou *et al.* 2013). In the *rms2* (D53 orthologue) mutant in pea (*Pisum sativum*), the expression of RMS5 (CCD7 orthologue) and RMS1 (CCD8 orthologue) genes was elevated (Johnson *et al.* 2006). In Arabidopsis, mutations in MAX2 (D53 orthologue) and D14 enhanced the expression of the SLs biosynthesis genes, MAX3 (CCD7 orthologue) and MAX4 (CCD8 orthologue) (Mashiguchi *et al.* 2009; Mashiguchi *et al.* 2020).

Graft transmissible effects of SL mutations

The over-branching phenotype of the SL-deficient mutant *sb1* mostly disappeared when it was grafted on a wild-type rootstock (Fig. 5A Table S1), complying with the notion that the roots are the primary site of SL biosynthesis, which are transported acropetally to the shoots through the xylem (Kohlen *et al.* 2011; Kameoka and Kyozuka, 2018; Mashiguchi *et al.* 2020). Similar results were previously reported in other plant species (Beveridge *et al.* 2000; Morris *et al.* 2001; Simons *et al.* 2007; Kameoka and Kyozuka, 2018; Mashiguchi *et al.* 2022; Mashiguchi *et al.* 2020). The over-branching, accompanied by loss of fruit yield, was also recovered in wild-type (M82) scion grafted on the SL-deficient rootstock of *sb1* (Fig 5, Table S2). This result indicates that SLs constitutively synthesized in shoots at > 100-fold lower levels than in the roots (Kameoka and Kyozuka, 2018) can compensate for SL-deficient roots and comply with the finding that SL synthesized in shoots control axillary bud outgrowth in apple (Malus×domestica) (Foster *et al.* 2018).

By contrast, M82 rootstocks did not complement the branching phenotype of *sb3* (Fig. 5), indicating that lack of SL signaling in the shoot determines the over-branching phenotype regardless of SL levels. Grafting experiments in peas showed that the shoot branching phenotype of a scion lacking D14 was partially rescued by WT rootstocks (Beveridge *et al.* 1996; Kameoka *et al.* 2016). It was demonstrated that the D14 protein in pea is transmissible from roots to shoots (Kameoka *et al.* 2016). Our data indicate that similarly to petunia and Arabidopsis (Simons *et al.* 2007; Chevalier *et al.* 2014), this phenomenon is not observed in tomato.

The branching index of the grafting combination *sb1*/*sb3* was lower than in each mutation individually despite the higher SL concentration in the roots (Fig. 5). However, the fruit yield loss was the same (Fig. 5, Table 2). A possible explanation for this inconsistency is that the higher SL concentration in the *sb3* roots also exists in the *sb1* lower parts of the grafted shoots, where the wild-type D14 receptor properly transduces it to limit branching. However, SL involved in fruit development are solely provided by the shoots and are deficient in *sb1*.

A genetic solution to broomrape infestation

The Egyptian broomrape (*Phelipanche aegyptiaca*) is a highly damaging parasitic weed that attacks the roots of various crops, including tomato. *P. aegyptiaca* infestations severely damage tomato plants and

lead to devastating yield losses (Joel *et al.* 2013). Various ways to cope with *P. aegyptiaca* infections in tomato have been used (Eizenberg and Goldwasser, 2018; Hershenhorn *et al.* 2009), but at an economic and environmental cost involved in employing chemicals. Developing tomato varieties resistant to *P. aegyptiaca* is a promising approach to improving tomato agriculture by reducing yield losses, minimizing environmental impacts, and promoting sustainable and efficient farming practices.

Strigolactones are critical to parasitic weed infestation. Broomrape seeds require the presence of strigolactones in the soil to trigger their germination and subsequent attachment to the host plant's roots. Plant mutants lacking SL are less susceptible or even resistant to broomrape (Dor *et al.* 2011; Galili *et al.* 2021; Gobena *et al.* 2017; Li *et al.* 2023; Bari *et al.* 2021; Bari *et al.* 2019; Pavan *et al.* 2016) and Fig. 3). Since SL in the soil is produced by the plant and exuded into the soil from the roots, SL-deficient roots can be used as rootstocks for plants of elite varieties. However, SL deficiency also causes severe yield loss due to the over-branching phenotype. The grafting experiments indicated that SL-deficient rootstock provide P. aegyptiaca resistance while the adverse influence on branching and fruit yield are compensated by a wild-type shoot. The field trial of commercial elite tomato varieties grafted on the *sb1* rootstock (Fig. 6, 7) unequivocally prove this method an effective solution to growing tomato in *P. aegyptiaca*-infested fields.

Declarations

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Author contribution

AK, UK, DZ and JH conceived and designed the research plan. AK isolated and characterized the SL mutants and carried out the field trials. UK carried out the genetic and molecular analyses. NB analyzed the root SLs. The manuscript was written by UK and AK and edited by JH and DZ.

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Table 2

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Figures

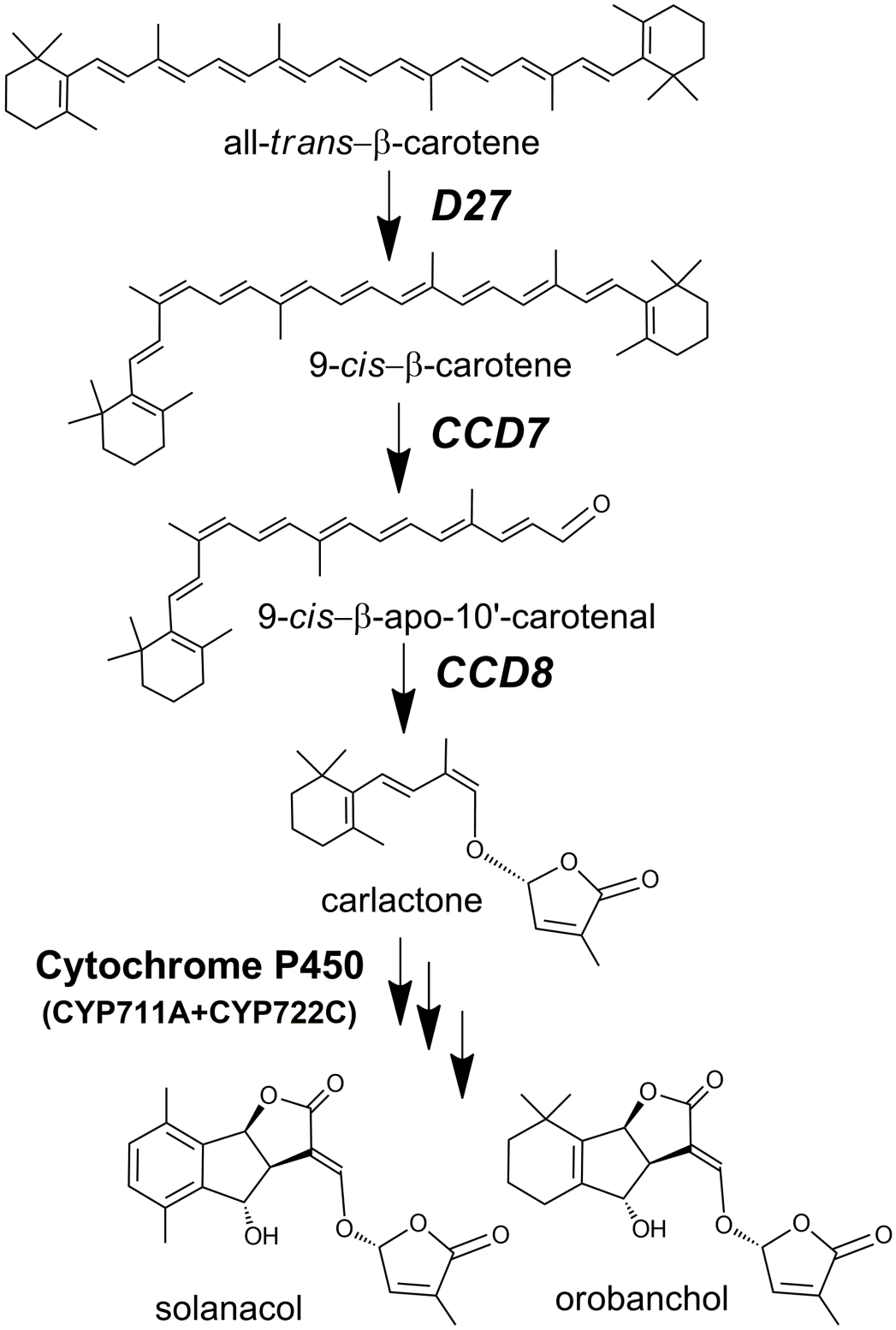

Figure 1

The strigolactone biosynthesis pathway. D27- β-carotene isomerase; CCD7- carotenoid cleavage dioxygenase 7; CCD8- carotenoid cleavage dioxygenase 8. Cytochrome P450, CYP722C, CYP712G1.

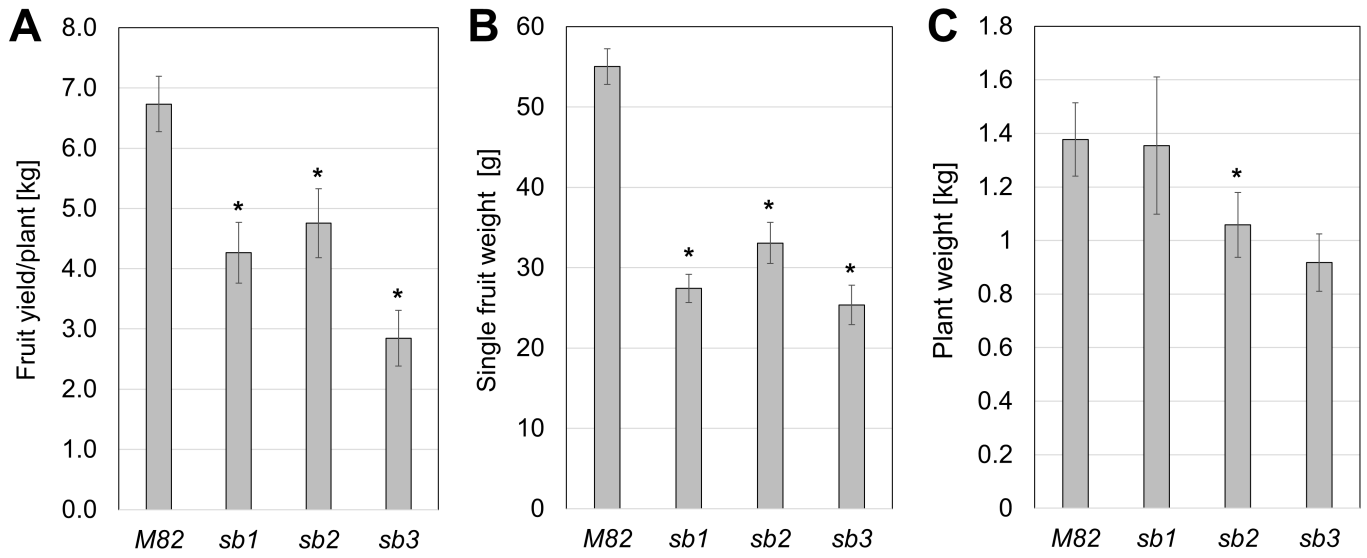

Figure 2

Yield parameters in field-grown SL mutants. Fruit yield (kg/plant) (**A**), single fruit weight (**B**) and plant size at time of harvest (kg/plant) (**C**) of wild type (M82) and the isogenic mutants *sb1*, *sb2*, *sb3*(n≥10, ±SE, p<0.05).

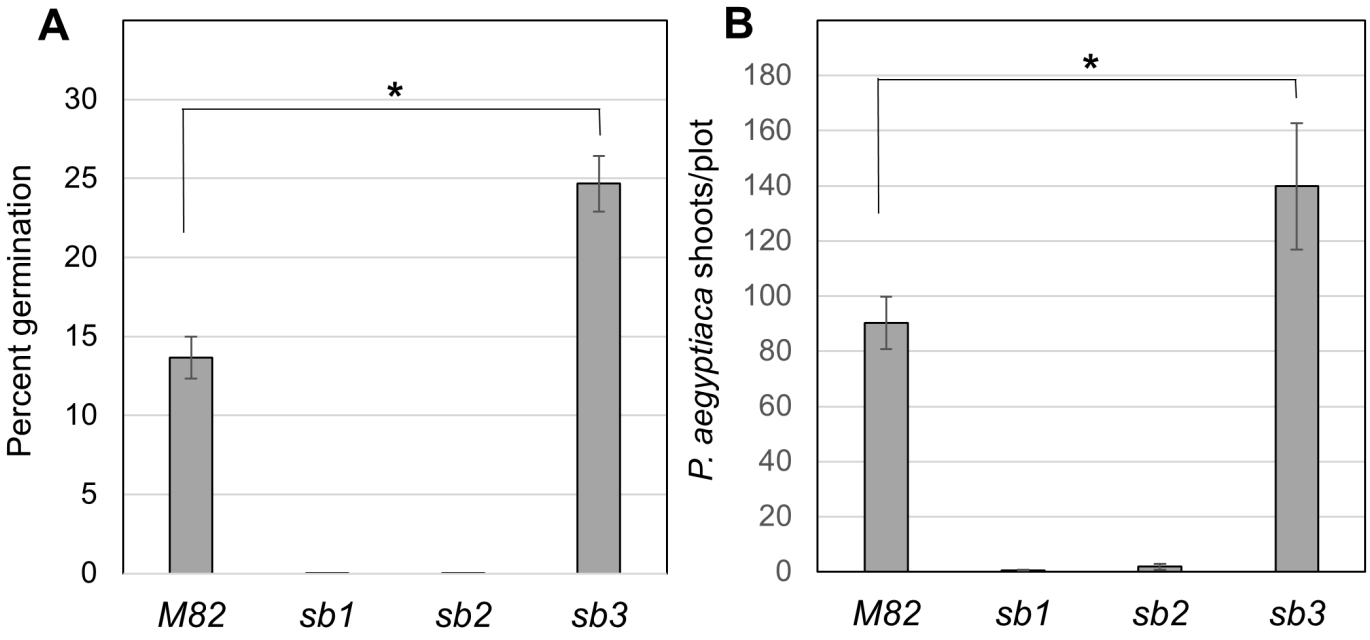

Figure 3

Root strigolactones and broomrape susceptibility of SL mutants. A. Quantification of SL in roots of M82 and the mutants *sb1*, *sb2,* and *sb3* based on the germination rate of *P. aegyptiaca* seeds induced by root extracts. Germination was recorded after 14 days. There was no germination in *sb1*and *sb2* assays. Data

represent an average of three independent replications (n=3, ±SE); B. Infection by *P. aegyptiaca* of field-grown tomato mutants *sb1*, *sb2* and *sb3*, and the WT (M82) (Eden research station) (n=5, ±SE, p<0.05).

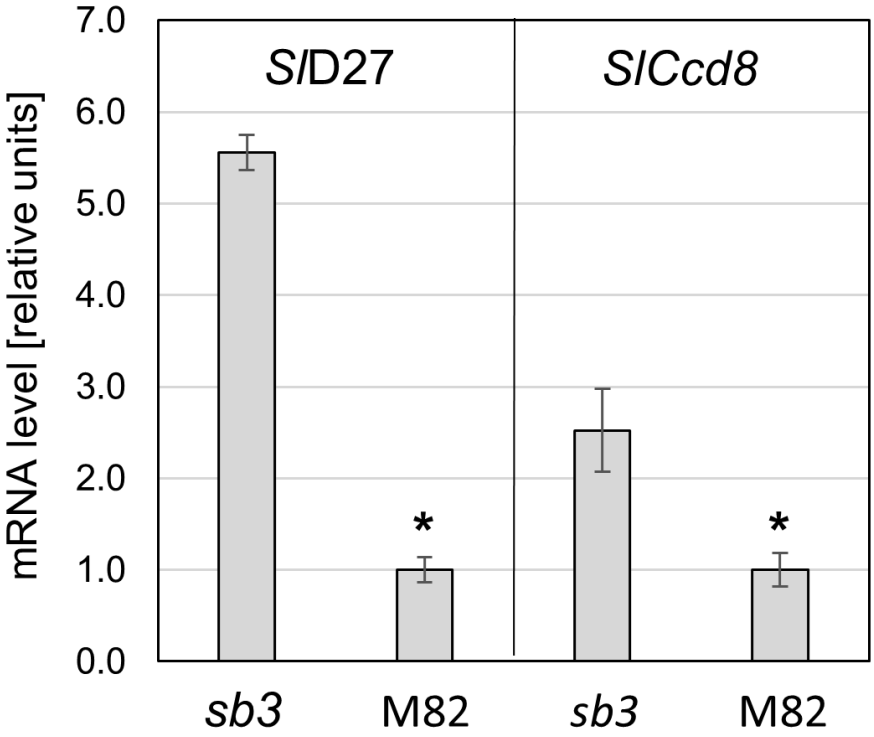

Figure 4

Quantification of mRNA of the genes encoding slD27 and SlCCD8 in roots of the mutant *sb3* and the wt, M82. (n=3, ±SE, * P<0.05,).

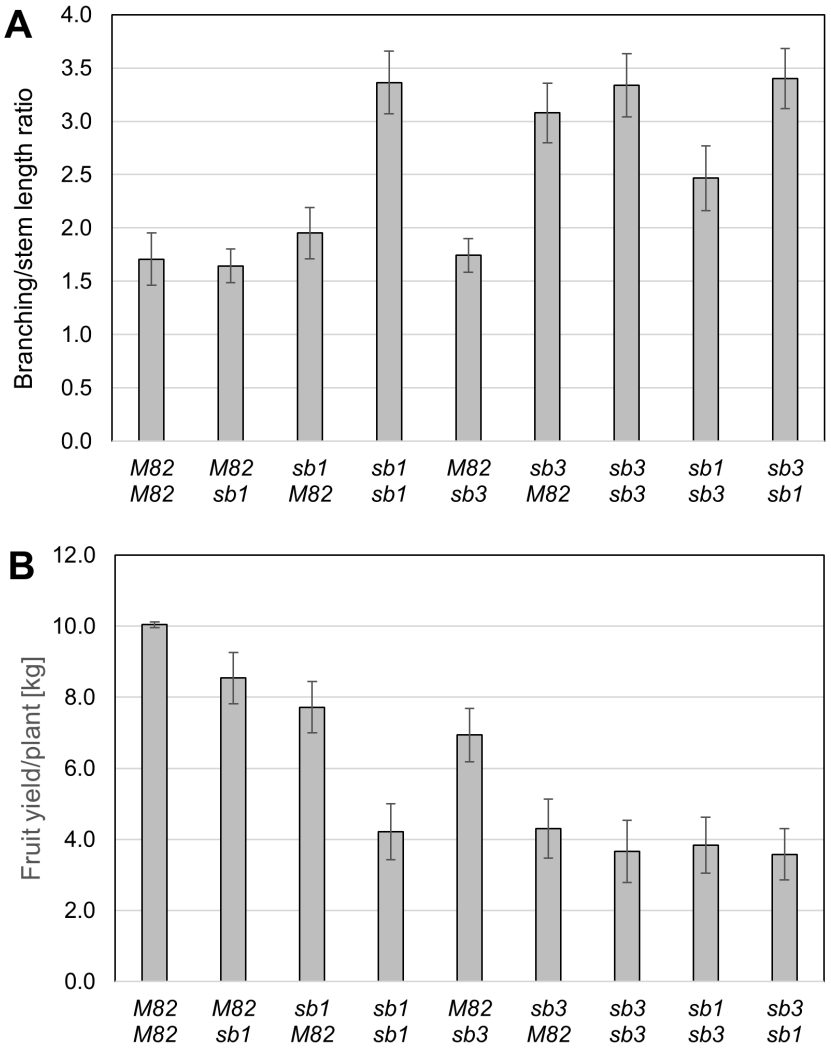

Figure 5

Root and shoot contribution to branching and fruit yield in SL mutants. Rate of branching (A) and fruit yield (B) of plants with different grafting combinations (scion/rootstock) of mutants *sb1*, *sb3,* and the wild type M82. At least three plants were characterized for branching and ten for yield (n=12, ±SE).

Figure 6

Resistance to *P. aegyptiaca* of tomato plants grown in the field. The cultivated tomato variety SFT1341 was grafted on *sb1* (right) or M82 (left) rootstocks. Plants were grown in a highly infested field in Ein Harod. The picture was taken two weeks before harvest.

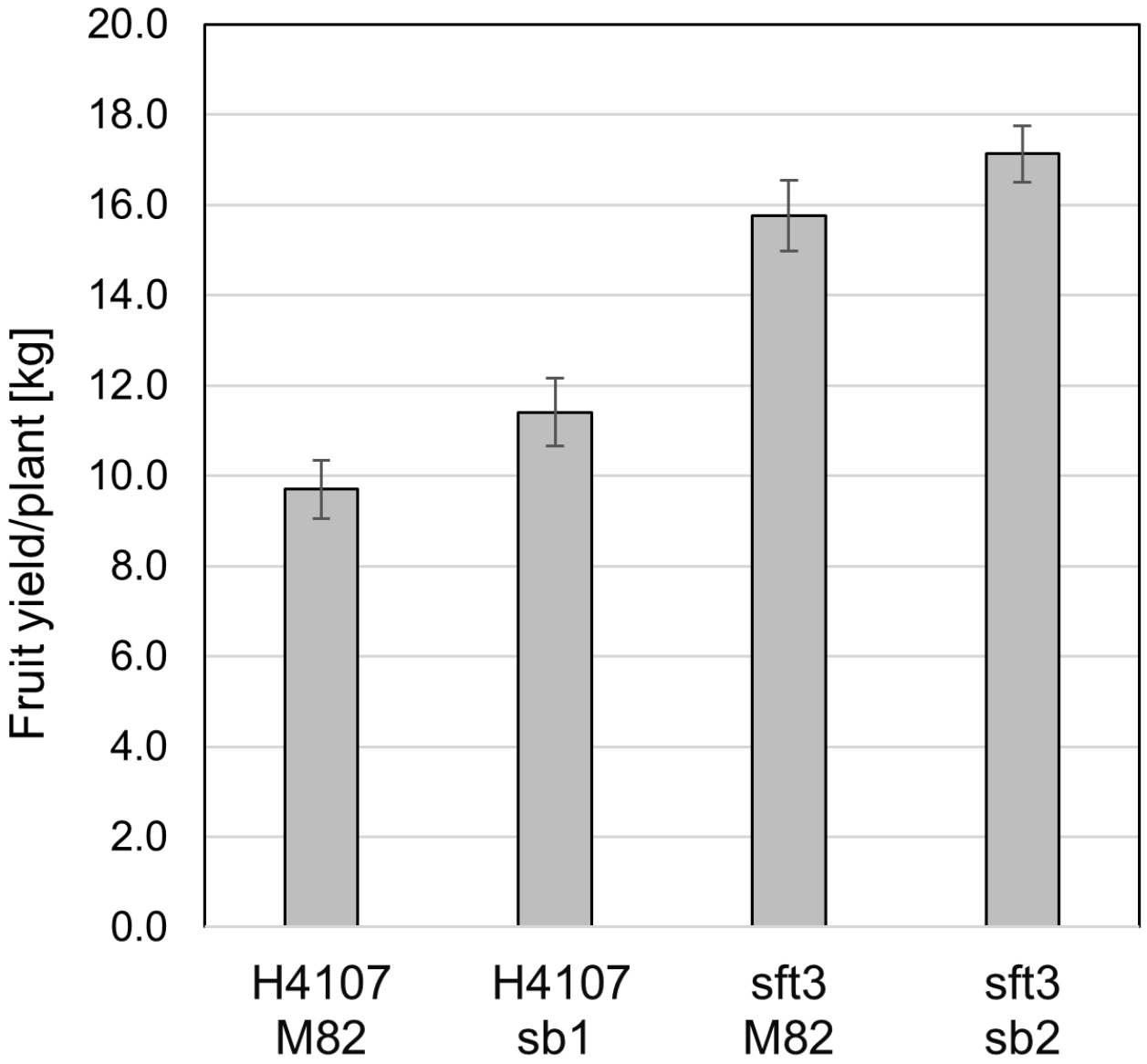

Figure 7

Performance of *sb1* as a rootstock compared with M82 in commercial tomato varieties. Total fruit yield of field-grown commercial varieties sft3 and H4107 grafted on *sb1-1*or M82 rootstock. (n=15, ±SE).

Supplementary Files

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