

# A *Solanum lycopersicum* polyamine oxidase contributes to the control of plant growth, xylem differentiation, and drought stress tolerance

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## SUMMARY

Polyamines are involved in several plant physiological processes. In *Arabidopsis thaliana*, five FAD-dependent polyamine oxidases (AtPAO1 to AtPAO5) contribute to polyamine homeostasis. AtPAO5 catalyzes the back-conversion of thermospermine (T-Spm) to spermidine and plays a role in plant development, xylem differentiation, and abiotic stress tolerance. In the present study, to verify whether T-Spm metabolism can be exploited as a new route to improve stress tolerance in crops and to investigate the underlying mechanisms, tomato (*Solanum lycopersicum*) AtPAO5 homologs were identified (*SIPAO2*, *SIPAO3*, and *SIPAO4*) and CRISPR/Cas9-mediated loss-of-function *slpao3* mutants were obtained. Morphological, molecular, and physiological analyses showed that *slpao3* mutants display increased T-Spm levels and exhibit changes in growth parameters, number and size of xylem elements, and expression levels of auxin- and gibberellin-related genes compared to wild-type plants. The *slpao3* mutants are also characterized by improved tolerance to drought stress, which can be attributed to a diminished xylem hydraulic conductivity that limits water loss, as well as to a reduced vulnerability to embolism. Altogether, this study evidences conservation, though with some significant variations, of the T-Spm-mediated regulatory mechanisms controlling plant growth and differentiation across different plant species and highlights the T-Spm role in improving stress tolerance while not constraining growth.

**Keywords:** Thermospermine, polyamine oxidase, drought stress tolerance, genome editing for crop improvement, leaf transpiration rate, relative water content, *Solanum lycopersicum*, xylem hydraulic conductivity.

## INTRODUCTION

Plants need large amounts of water to live and grow. Vascular plants evolved the xylem for long-distance transport of water and nutrients (Lucas et al., 2013; Sperry, 2003; Venturas et al., 2017). In angiosperms, xylem vessels can

be several centimeters long, consisting of coordinated chains of vertically aligned vessel elements that have partially or entirely lost their common end-walls. Xylem vessels are highly interconnected through pits forming a complex network. Pit membranes play a crucial role in

xylem safety, as they can prevent air propagation between conduits, which can disrupt water transport and affect plant growth and survival (Brodersen et al., 2019; Venturas et al., 2017). Water transport through the xylem depends on numerous parameters, such as the anatomical characteristics and spatial distribution of xylem conduits, as well as the number and frequency of network connections. There is a general agreement that wider and longer vessels conduct water more efficiently, but they are also more susceptible to embolism, suggesting a possible trade-off between hydraulic safety and efficiency, although the topic is debated (Brodersen et al., 2019; Lens et al., 2022; Venturas et al., 2017). The understanding of soil-to-leaf water transport can lead to innovative directions to optimize plant water use through breeding programs and biotechnological strategies.

Xylem development is controlled by the integrated action of several endogenous and environmental signals (Aliche et al., 2020; Bollhöner et al., 2012; Jang & Choi, 2018; Milhinhos & Miguel, 2013; Pagliarani et al., 2019; Růžička et al., 2015). Indeed, hormones, small peptides, reactive oxygen species, and  $\text{Ca}^{2+}$  are essential regulators of this process. Polyamines are also implicated in several processes during xylem differentiation (Fraudentali et al., 2021; Tavladoraki et al., 2016; Tisi et al., 2011; Vera-Sirera et al., 2015). In particular, thermospermine (T-Spm) contributes to the control of xylem differentiation by interfering with auxin and cytokinin signaling pathways (Milhinhos et al., 2020; Milhinhos & Miguel, 2013; Tiburcio et al., 2014; Vera-Sirera et al., 2015). Arabidopsis mutants for *thermospermine synthase* (*ACL5*; Solé-Gil et al., 2019) and *S-adenosyl-methionine decarboxylase 4* genes involved in T-Spm biosynthesis exhibit over-proliferation of xylem vessels and impaired stem elongation and (Cui et al., 2010; Ge et al., 2006; Muñoz et al., 2008), while increased *ACL5* expression levels or exogenous T-Spm suppress xylem differentiation (Baima et al., 2014; Kakehi et al., 2010; Mai et al., 2023; Milhinhos et al., 2013). Furthermore, it has been shown that T-Spm suppresses auxin signaling and auxin-inducible xylem differentiation and that auxin induces *ACL5* expression through a mechanism that maintains T-Spm steady-state levels and finely controls xylem development (Baima et al., 2014; Clay & Nelson, 2005; Milhinhos et al., 2013; Yoshimoto et al., 2012a, 2012b). Genetic studies of suppressor mutants that rescue the dwarf phenotype of *acl5* mutants revealed that a key component of T-Spm function is the basic helix–loop–helix transcription factor SUPPRESSOR OF ACAULIS 51 (*SAC51*; Imai et al., 2006; Nishii et al., 2023). *SAC51* can interact with LONESOME HIGHWAY (*LHW*), thus inhibiting the formation of heterodimers between *LHW* and TARGET OF MONOPTEROS 5, which play a central role in xylem formation (Katayama et al., 2015; Vera-Sirera et al., 2015). In particular, T-Spm is believed to activate the translation of *SAC51* mRNA by

inhibiting the negative effect of an evolutionarily conserved upstream open reading frame (uORF) of this gene (Cai et al., 2016; Ishitsuka et al., 2019). Mutations of the ribosomal protein genes *RPL10A*, *RACK1A*, and *RPL4A* were also reported to attenuate the inhibitory effect of the *SAC51* uORF in the absence of T-Spm, further supporting T-Spm involvement in ribosomal function (Imai et al., 2008; Kakehi et al., 2015). Recently, it was additionally shown that mutation of a gene encoding a D6-class Jumonji C protein (*JMJ22*) suppresses the *acl5* phenotype and that *JMJ22* stabilizes *SAC51* transcripts through a hitherto unknown mode of action (Matsuo et al., 2022). Furthermore, small nucleolar ribonucleoproteins were suggested to be essential for T-Spm-mediated biological processes (Li et al., 2023).

Numerous reports support the contribution of the different polyamines in plant stress responses through a variety of mechanisms (Montesinos-Pereira et al., 2014; Wang & Liu, 2016; Liu et al., 2015; Pál et al., 2018; Alcázar et al., 2020; Li et al., 2020; Hasan et al., 2021; Upadhyay et al., 2021; Blázquez, 2024). As considered T-Spm, exogenous application induced the expression of pathogenesis-related proteins and improved tolerance to biotic and abiotic stresses (Alcázar et al., 2020; Mo et al., 2015; Sagor et al., 2012; Shinohara et al., 2019). Furthermore, *ACL5* overexpression increased resistance to *Pseudomonas viridiflava* and *Botrytis cinerea*, while on the contrary reduced *ACL5* expression levels increased susceptibility to the fungal pathogen *Verticillium dahliae* and salt stress (Marina et al., 2013; Mo et al., 2015; Shinohara et al., 2019; Zhu et al., 2021). Also, in the non-vascular plant *Marchantia polymorpha*, loss-of-function mutation of *MpACL5* altered responses to heat and salt stress (Furumoto et al., 2024).

Polyamine homeostasis is controlled not only at the level of biosynthesis but also by catabolism, which involves two classes of amine oxidases, the copper amine oxidases and the FAD-dependent polyamine oxidases (PAOs; Tavladoraki et al., 2016; Tiburcio et al., 2014). Plant PAOs display high diversity in substrate specificity, catalytic mechanism, and subcellular localization and belong to three distinct clades (Bordenave et al., 2019; Fincato et al., 2011, 2012; Salvi & Tavladoraki, 2020; Tavladoraki et al., 2016). Clade I includes plant PAOs similar to the intracellular *Arabidopsis thaliana* PAO1 (AtPAO1; Tavladoraki et al., 2006) and the extracellular maize PAO (ZmPAO1; Tavladoraki et al., 1998; Polticelli et al., 2005), together with fungal and algae PAOs. Clade II includes plant PAOs similar to the *Arabidopsis* polyamine oxidase/dehydrogenase 5 (AtPAO5; Ahou et al., 2014) and animal PAOs (Cervelli et al., 2003; Vujcic et al., 2003). Clade IV includes PAOs similar to the peroxisomal AtPAO2, AtPAO3, and AtPAO4.

AtPAO5 is a cytosolic enzyme catalyzing the back-conversion of spermine (Spm),  $N^1$ -acetyl-Spm, and T-Spm to spermidine (Ahou et al., 2014). Furthermore, AtPAO5 is involved in plant development and xylem

differentiation interfering with the cytokinin and auxin signaling pathways by controlling T-Spm levels (Alabdallah et al., 2017; Kaszler et al., 2023). Indeed, loss-of-function *atpao5* mutants and Arabidopsis transgenic plants ectopically expressing *AtPAO5* exhibit differences compared to wild-type plants in T-Spm levels, stem and root elongation, xylem differentiation, and expression levels of auxin- and cytokinin-related genes. In addition, *atpao5* mutants display increased tolerance to drought and salt stress (Sagor et al., 2016; Zarza et al., 2017).

The present study further investigates the contribution of T-Spm metabolism to plant development and drought stress responses. In particular, the aim of this study was to obtain information on the underlying mechanisms and to verify whether strategies based on polyamine metabolism can be designed to transfer stress tolerance traits into crops. To this end, tomato (*Solanum lycopersicum*) CRISPR/Cas9 mutants for one of the three *AtPAO5*-like genes (*SIPAO3*) were analyzed under well-watered and drought stress conditions. The data obtained allow to hypothesize a role for *SIPAO3* in tomato plant growth and xylem differentiation through the control of T-Spm and auxin homeostasis. Furthermore, they evidence the conservation, though with some significant variations, of the T-Spm-mediated regulatory mechanisms across different plant species. The data suggest also that loss-of-function *SIPAO3* mutations affect plant ability to tolerate drought stress via effects on xylem efficiency (through reducing xylem hydraulic conductivity and thus limiting water loss under water-deficit conditions) and safety (by diminishing vulnerability to embolism).

## RESULTS

### Three *AtPAO5* homologs in *S. lycopersicum*

Sequence homology searches identified three *AtPAO5*-like genes in *S. lycopersicum*, named *SIPAO2* (*Solyc05g018880*), *SIPAO3* (*Solyc07g039310*), and *SIPAO4* (*Solyc12g008850.1.1*). The three genes are intron-less, similar to *AtPAO5* and most other plant *AtPAO5*-like genes that belong to the distinct clade II (Salvi & Tavladoraki, 2020). *SIPAO2*, *SIPAO3*, and *SIPAO4* share a high overall amino-acid sequence identity with *AtPAO5* and with each other (Figure 1a). Furthermore, amino acid sequence alignments revealed that *SIPAO4* differs from *AtPAO5*, *SIPAO2*, and *SIPAO3* in the presence of an additional domain of about 150–160 amino acids (Figure 1a). *SIPAO4* homologs were identified in several plants (e.g., *Solanum tuberosum*) but not all (e.g., *Nicotiana tabacum*), of the Solanaceae family, the length of the extra domain being variable (Figure S1). Notably, MEME and associated MAST analysis (Bailey et al., 2015; <https://meme-suite.org/>) identified the presence of multiple tandem repeats (TRs) of a highly conserved motif of 17 amino acids in the extra domain of

*SIPAO4* homologs (Figure 1b,c; Figure S1), these TRs being distinct from the common TRs known to occur in plant proteins (Kamel et al., 2021; Sharma & Pandey, 2016). The identification of *SIPAO4* homologs only in a subgroup of Solanaceae suggests the recent evolution of this type of PAO.

### *SIPAO2*, *SIPAO3*, and *SIPAO4* expression pattern

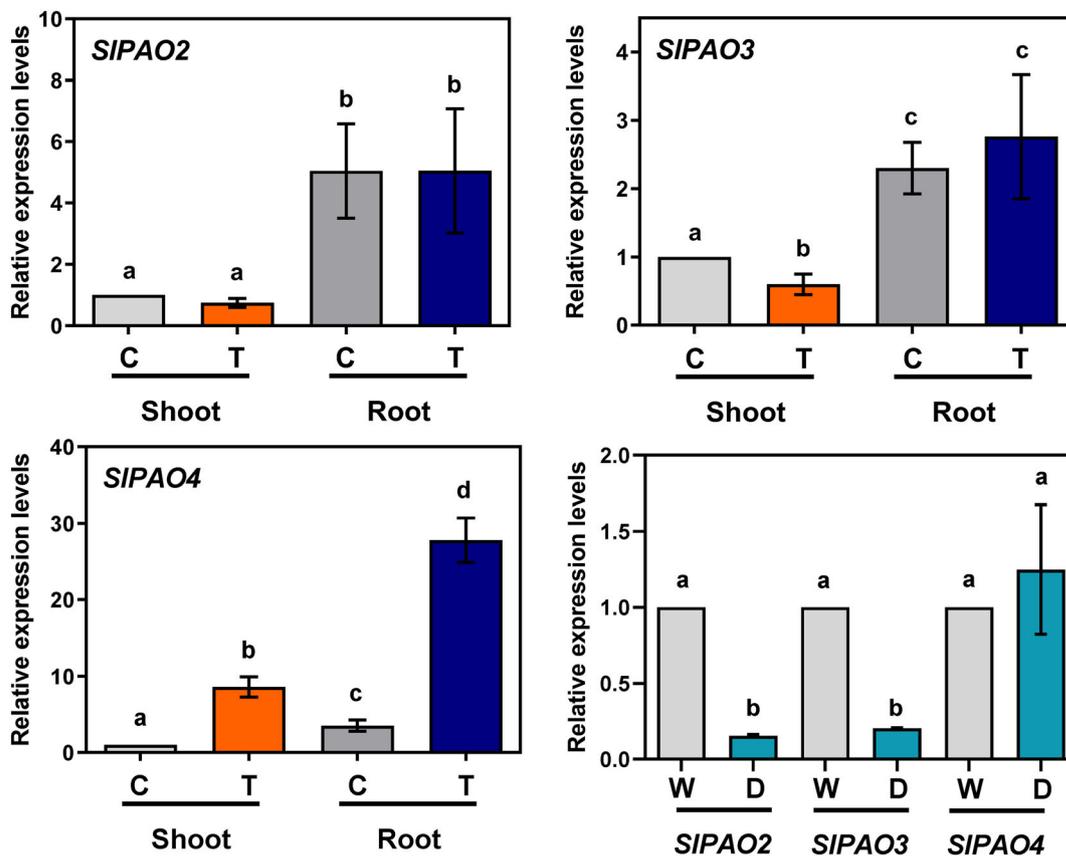
It was previously shown that *AtPAO5* expression levels are higher in roots than in leaves and stems (Alabdallah et al., 2017). Similarly, in the present study, quantitative real-time RT-PCR (RT-qPCR) analysis showed higher *SIPAO2*, *SIPAO3*, and *SIPAO4* expression levels in roots than in shoots (Figure 2). Moreover, it showed that T-Spm treatment upregulates *SIPAO4* in shoots and roots (Figure 2). Conversely, *SIPAO2* and *SIPAO3* are not significantly regulated by T-Spm, *SIPAO3* being only slightly downregulated in shoots (Figure 2). On the other hand, *SIPAO2* and *SIPAO3*, but not *SIPAO4*, are downregulated by drought stress (Figure 2). The gene expression data, together with the amino acid sequence analysis, suggest functional similarities among the three genes (mainly between *SIPAO2* and *SIPAO3*), but also functional divergence (mainly of *SIPAO4* from *SIPAO2* and *SIPAO3*).

### Generation of CRISPR/Cas9-mediated *slpao3* mutant lines

To study the contribution of T-Spm metabolism to tomato developmental and defense processes, *SIPAO3* was chosen as a representative member of the putative T-Spm specific *SIPAO2*-*SIPAO4* gene family. To obtain *slpao3* mutants through CRISPR/Cas9 technology, two *SIPAO3*-specific *gRNAs* (*gRNA1* and *gRNA2*; Figure S2) were designed and assembled into a construct adopting a polycistronic *tRNA-gRNA* (PTG) strategy (Figure S2b; Xie et al., 2015). Several tomato plants mutated in *SIPAO3* target 2, but not in target 1, were recovered (Table S1), and two lines (*slpao3-a* and *slpao3-b*) were selected for further studies.

The *slpao3-a* line brings a mutated *SIPAO3* gene with a homozygous in-frame deletion of 24 nucleotides resulting in a deletion of eight amino acids (amino acids 426 to 433; Figure S2d), which is expected to result in a non-functional enzyme, as evidenced by the structural models of the wild-type protein and the *SIPAO3-a* mutant obtained using trRosettaX (Figure S3). In detail, the overall fold of these models is essentially identical and well superimposable to that of the hSMOX crystal structure (PDB code 7OXL; Diaz et al., 2022) taken as a reference (Figure S3). Furthermore, the 426–433 segment, deleted in the *SIPAO3-a* mutant, is located at the level of a surface loop and thus could appear not detrimental to the overall fold of the protein. However, a detailed comparative analysis of the structural models revealed that this loop is an essential part of the FAD binding domain (Figure S4, top panels). In particular, the 426–433 segment acts as a plug of the cavity hosting the FAD cofactor, with residues Leu432,





**Figure 2.** Regulation of *SIPAO2*, *SIPAO3*, and *SIPAO4* gene expression by T-Spm and drought stress.

For analysis of the effect of T-Spm on gene expression, shoots and roots of 7-day-old tomato seedlings were treated (T) or not (C) with 100  $\mu$ M T-Spm for 24 h and analyzed by RT-qPCR. Values are means of three independent repetitions  $\pm$  SE. For drought stress, leaves of 4-week-old tomato plants grown in soil under well-watered conditions (W) or following water withholding for 12 days (D). Leaves from three different plants were used for each analysis. A representative experiment out of three independent repetitions is shown. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta C_t}$ ) from controls. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P < 0.05$ ).

1 nt (at position 1299 of the *SIPAO3* gene), which is also expected to encode a non-functional enzyme, truncated at amino acid position 432 and bearing an extra domain of seven amino acids at the C' terminus (Figure S2d).

The *slpao3-a* and *slpao3-b* mutants were analyzed for potential off-target mutations. Sequencing analysis of PCR products from six selected potential off-target sites presenting the highest probability score ( $\geq 0.1$ ; Table S2) evidenced no mutation or indel in the two mutants as compared to the wild-type plants (Figure S5). These results indicate high *gRNA2* target specificity.

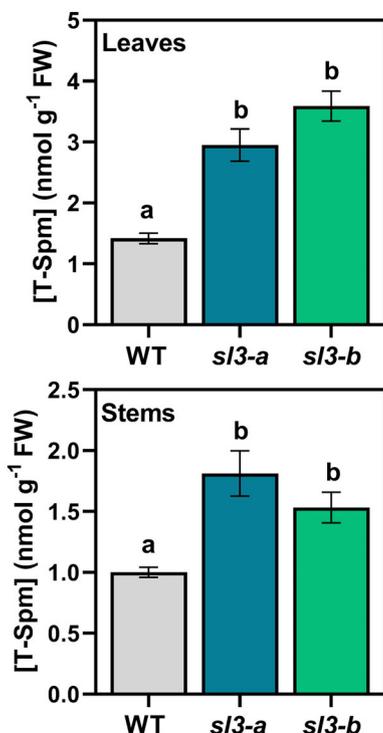
### ***SIPAO3* contributes to T-Spm, auxin, and gibberellin homeostasis in tomato plants**

Analysis of polyamine levels in both leaves and stems evidenced that *slpao3-a* and *slpao3-b* mutants have increased T-Spm levels compared to wild-type plants (Figure 3). On the contrary, no statistically significant difference in putrescine (Put), spermidine (Spd), and Spm levels was observed among the different tomato genotypes (Figure S6). These

data suggest that *SIPAO3* specifically contributes to T-Spm homeostasis in tomato plants, as *AtPAO5* does in Arabidopsis plants (Alabdallah et al., 2017).

In Arabidopsis and *Populus* plants, it was shown that the *thermospermine synthase* gene (*ACL5*; Knott et al., 2007; Takano et al., 2012) is regulated by T-Spm through a negative feedback loop (Baima et al., 2014; Kakehi et al., 2008, 2010; Milhinhos et al., 2013). A similar regulatory mechanism operates in tomato plants as T-Spm treatment downregulates two *ACL5* homologs (*SIACL5-1* and *SIACL5-2*; Figure 4). On the contrary, a third tomato *ACL5* homolog (*SIACL5-3*) is upregulated by T-Spm (Figure 4), probably for fine regulation of T-Spm levels. Of note, *SIACL5-1*, *SIACL5-2*, and *SIACL5-3* expression levels are higher in roots than in shoots (Figure 4), similar to *SIPAO2*, *SIPAO3*, and *SIPAO4* expression levels (Figure 2).

*SIPAO3*, contributing to T-Spm homeostasis, participates in the feedback mechanism regulating *SIACL5-1* and *SIACL5-2*. Indeed, RT-qPCR analyses showed that *SIACL5-1* and *SIACL5-2* expression levels are downregulated in the



**Figure 3.** T-Spm levels in *slpao3-a* and *slpao3-b* mutants. T-Spm levels were determined in leaves and stems of the third internode (from above) of 6-week-old *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, and wild-type (WT) tomato plants. Values are means of three independent repetitions  $\pm$  SE ( $n=5$ ). Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P < 0.05$ ).

*slpao3-a* and *slpao3-b* mutants in shoots (Figure 5) and roots (Figure S7). On the contrary, no difference in *SIACL5-3* expression levels was observed between *slpao3* mutants and wild-type plants (Figure 5). In addition, no difference was observed between *slpao3* mutants and wild-type plants in *SIPA02*, *SIPA03*, and *SIPA04* expression levels (Figure 5). These data suggest that *SIPA03* gene disruption does not affect *SIPA03* transcription and mRNA stability. They also indicate that the increase in T-Spm levels in *slpao3* mutants compared to wild-type plants is not sufficient to upregulate *SIPA04* and *SIACL5-3* expression, whereas it appears high enough to downregulate *SIACL5-1* and *SIACL5-2*.

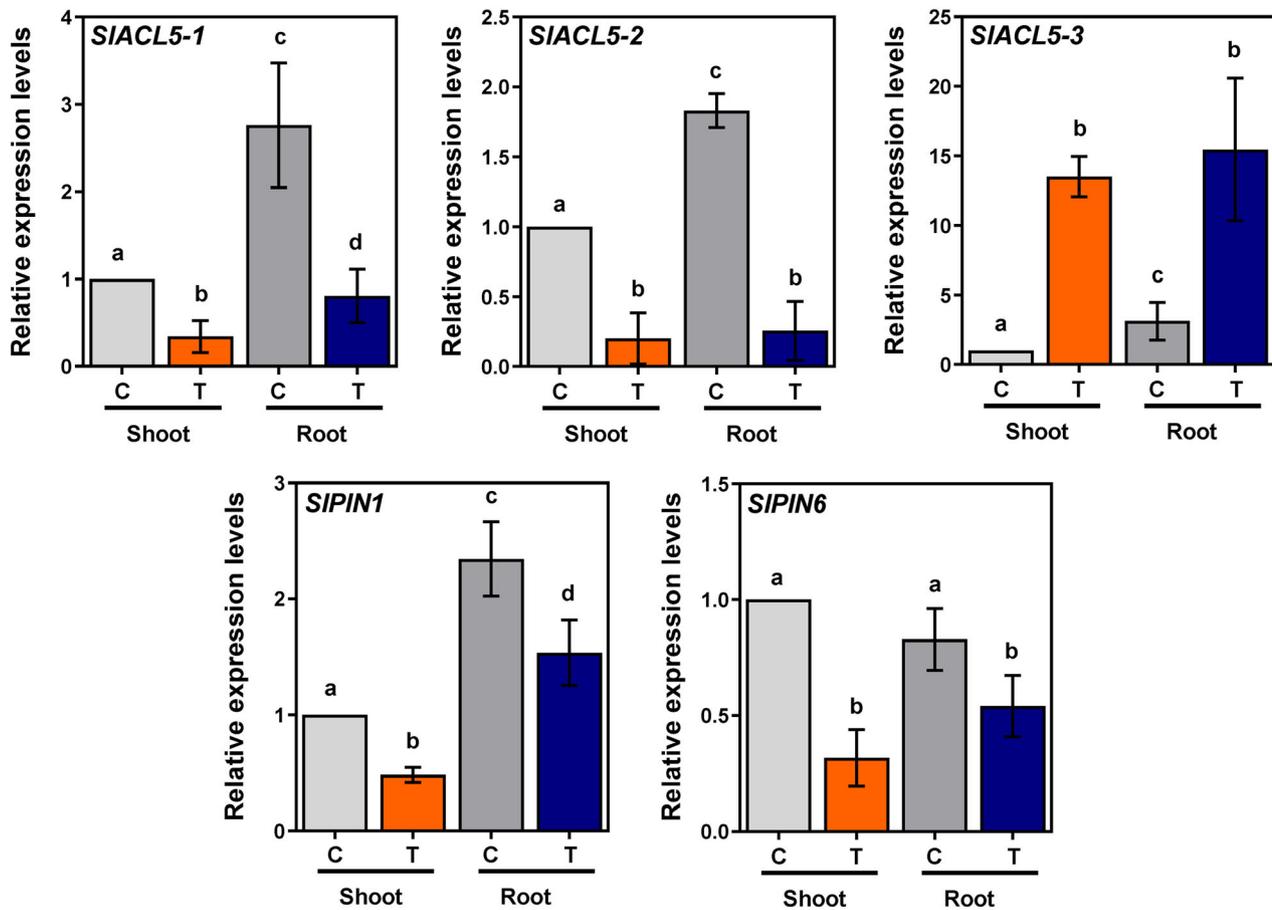
RT-qPCR analyses were also performed on the tomato homologs of the Arabidopsis *PIN1* and *PIN6* auxin efflux carrier genes (*SIPIN1* and *SIPIN6*, respectively) that play an important role in auxin homeostasis and distribution (Blilou et al., 2005; Cazzonelli et al., 2013; Sawchuk et al., 2013; Verna et al., 2015) and are regulated by T-Spm (Tong et al., 2014). Data evidenced that *SIPIN1* and *SIPIN6* genes are downregulated by T-Spm (Figure 4) and that they display lower expression levels in *slpao3* mutants than in

wild-type plants in both shoots and roots (Figure 5; Figure S7). These data suggest that T-Spm and its metabolism interfere with auxin homeostasis in tomato plants, as in Arabidopsis and *Populus* plants (Clay & Nelson, 2005; Milhinhos et al., 2013; Takano et al., 2012; Tong et al., 2014; Vera-Sirera et al., 2010; Yoshimoto et al., 2012a, 2012b).

The RT-qPCR analyses also showed that T-Spm treatment decreases the expression levels of the gibberellin (GA) biosynthetic gene *SIGA20ox1* and increases those of the GA deactivation gene *SIGA20ox4* (Yamaguchi, 2008; Figure S8), suggesting that T-Spm also interferes with GA homeostasis. This may be due to the effect of T-Spm in auxin homeostasis, the auxin and GA signaling pathways being highly interconnected (Yamaguchi, 2008). However, a direct effect of T-Spm on GA biosynthesis and signal transduction cannot be excluded. Despite the increased T-Spm levels, the *slpao3* mutants present increased *SIGA20ox1* expression levels compared to the wild-type plants (Figure S8), which indicates perturbations in GA homeostasis and probable activation of the feedback homeostatic mechanisms controlling it (Yamaguchi, 2008) in the mutants. On the contrary, no significant difference in *SIGA20ox4* expression levels was observed in the *slpao3* mutants compared to the wild-type plants (Figure S8).

### **SIPA03 is involved in the control of plant growth and xylem differentiation**

Morphological observations evidenced that the *slpao3-a* and *slpao3-b* mutants have longer stems than the wild-type plants, due to increased internode elongation (Figure 6a,b), which may reflect the observed perturbations in auxin and GA homeostasis. Instead, no difference was observed in the number of internodes and the total leaf area between *slpao3* mutants and wild-type plants (Figure 6c). Transverse sections of stems (Figure 7a,b) and roots (Figure S9) showed that the *slpao3* mutants have a lower number of xylem elements than wild-type plants. In addition, the *slpao3* mutants display thinner cell walls in xylem elements than wild-type plants, as suggested by the differences in the intensity of the cell-wall lignin autofluorescence among the different genotypes (Figure 7a). These differences in xylem differentiation among the different genotypes are probably due to differences in T-Spm levels and consequent alterations in auxin and GA homeostasis. Indeed, T-Spm treatment reduced the number of xylem elements and reverted the observed differences in root xylem differentiation between *slpao3* mutants and wild-type plants (Figure S9a,b). The *slpao3* mutants also display a smaller proportion of large vessels ( $>1200 \mu\text{m}^2$ ) than wild-type plants (Figure 7c). These data suggest that hydraulic conductivity may be affected in *slpao3* mutants compared to wild-type plants. Indeed, both theoretical calculation and experimental data evidenced lower hydraulic conductivity in *slpao3* mutants than in wild-type plants (Figure 8). These data are consistent with



**Figure 4.** T-Spm mediated regulation of genes involved in T-Spm biosynthesis and auxin transport.

Expression levels were analyzed by RT-qPCR in shoots and roots of 7-day-old tomato seedlings treated (T) or not (C) with 100  $\mu$ M T-Spm for 24 h. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta Ct}$ ) from the shoots of control plants. Values are means of three independent repetitions  $\pm$  SE. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P < 0.05$ ). Similar results were obtained in the leaves of 4-week-old plants.

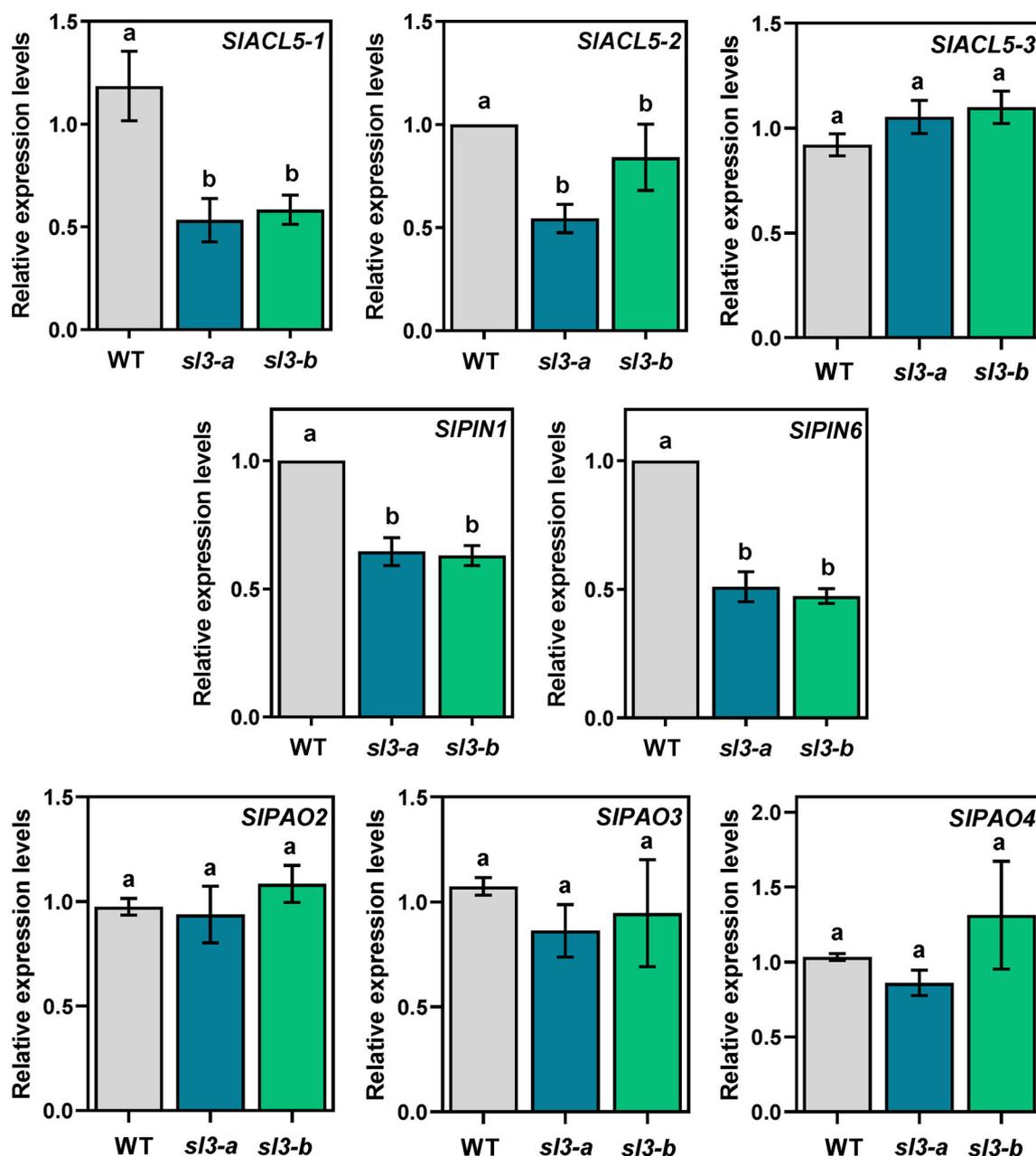
previous reports correlating perturbations in auxin transport and signaling with changes in xylem differentiation and hydraulic conductivity (Andrade et al., 2022; Fàbregas et al., 2015; Johnson et al., 2018).

#### The *slpao3* mutant lines exhibit improved tolerance to drought stress

The role of T-Spm metabolism and *SIPAO3* in drought stress responses was investigated by completely interrupting irrigation of *slpao3* mutants and wild-type plants. Of note, drought stress does not affect T-Spm levels, only the amount of Put being increased under these conditions (Figure S10). In this way, the observed differences in T-Spm levels between the wild-type and mutant plants under well-watered conditions are maintained also under drought stress conditions. Plant observation during drought evidenced that wild-type plants present more severe wilting symptoms than *slpao3-a* and *slpao3-b* mutants (Figure 9a; Figure S11). In addition, measurements of relative water content (RWC; Figure 9b) and leaf

surface temperature (Figure 9c,d) at various percentages of soil humidity showed that leaf water loss under drought conditions was slower in *slpao3* mutants than in wild-type plants. These data demonstrate that *slpao3* mutants experience less water stress than wild-type plants.

Under well-watered conditions, *slpao3* mutants exhibit lower stomatal conductance (Figure 10a) than wild-type plants, as observed by leaf gas exchange measurements. This trend is inverted under water-limited conditions, with the *slpao3* mutants exhibiting a slower reduction in stomatal conductance with decreasing soil humidity compared to the wild-type plants (Figure 10a). The lower stomatal conductance rate under well-watered conditions and the higher one under drought stress observed in *slpao3* mutants compared to wild-type plants translated into parallel changes in transpiration rates (Figure 10b; Figure S12), as expected due to similar environmental evaporative conditions (temperature and relative humidity) to which plants were exposed during



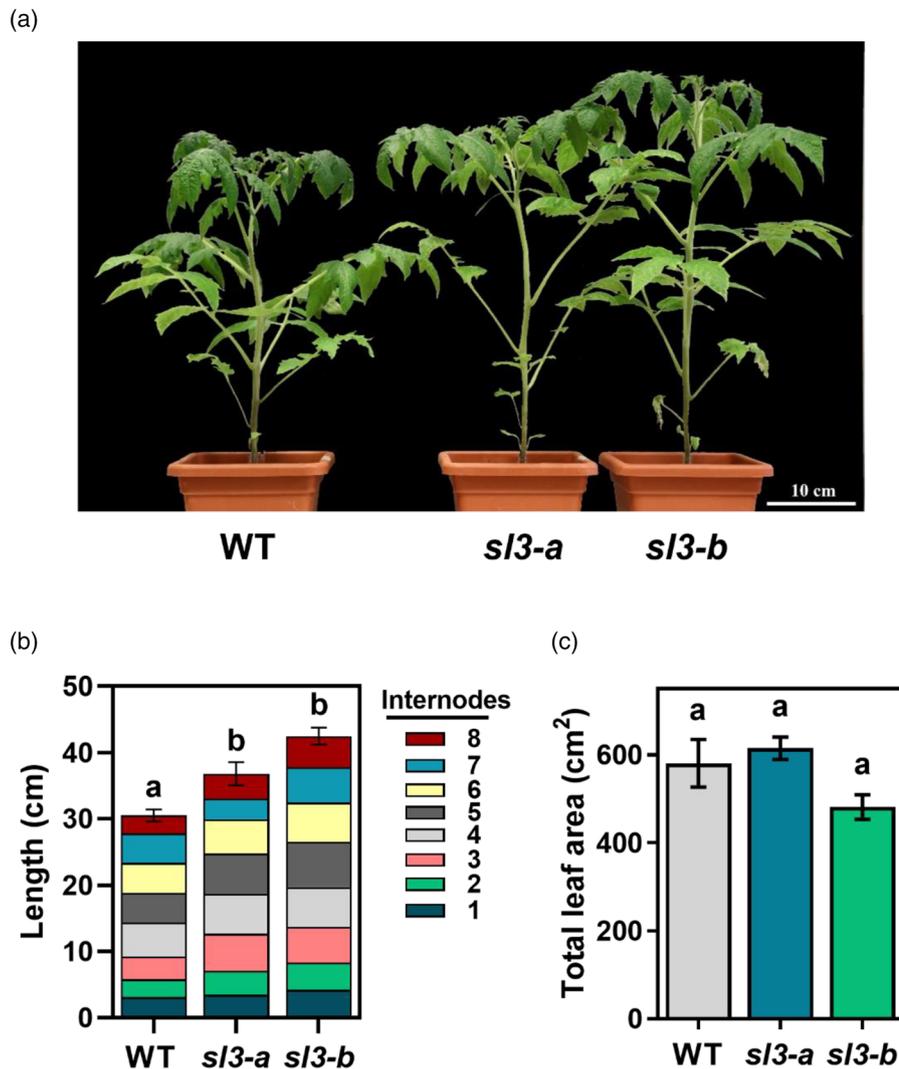
**Figure 5.** Expression levels of genes involved in T-Spm metabolism and auxin transport in *slpao3* mutants.

Expression levels were analyzed by RT-qPCR in shoots of 9-day-old *slpao3-a* (*sl3-a*), *slpao3-b* (*sl3-b*), and wild-type (WT) tomato genotypes. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta C_t}$ ) from WT plants. Values are means of three independent repetitions  $\pm$  SE. Different letters above the columns indicate statistically significant differences ( $P < 0.05$ ; one-way ANOVA).

experiments. Additionally, *slpao3* mutants maintained higher (i.e., less negative)  $\Psi_{leaf}$  values than wild-type plants under water-deficit conditions (Figure 10c). These differences in stomatal conductance, transpiration rate, and water potential between wild-type and mutant plants cannot be attributed to differences in stomatal density or index among genotypes (Figure S13). Furthermore, it was shown that exogenous T-Spm does not affect stomatal

conductance (Figure S14). Hence, these data overall suggest that the differences between the wild-type plants and the *slpao3* mutants in stomatal conductance and transpiration rate may be related to hydraulic limitations imposed on stomatal aperture by differences in stem hydraulic conductivity (Figure 8).

To verify whether the *slpao3* mutants display differences from wild-type plants in morpho-anatomical traits



**Figure 6.** Phenotypal analysis of *slpao3* mutants.

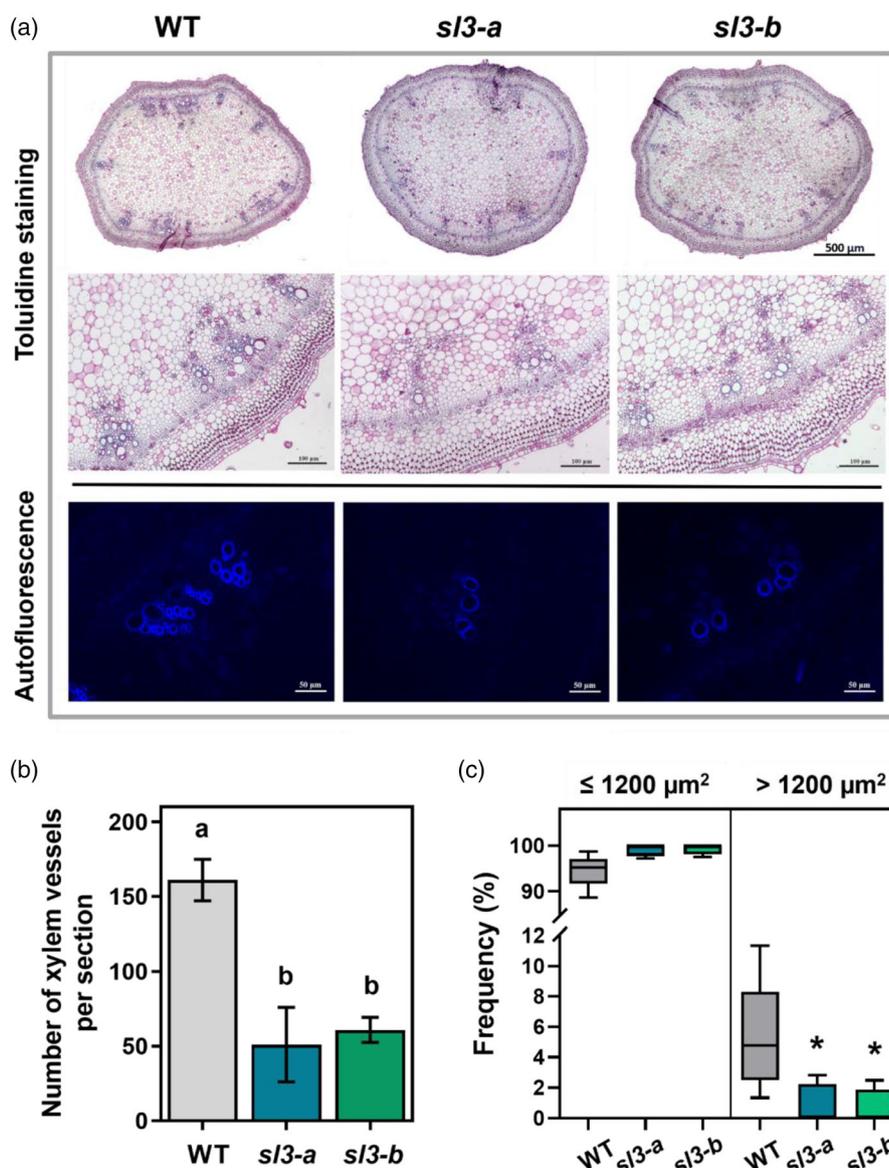
(a) Representative photos of the different tomato genotypes.

(b) Length of the different internodes in the various tomato genotypes. Internode numbering starts from the base of the stem. Bars indicate SEM of the total stem length values.

(c) Total leaf area of the different tomato genotypes. A representative experiment is shown. Different letters above the columns indicate statistically significant differences ( $P < 0.05$ ; one-way ANOVA). WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutant; *sl3-b*: *slpao3-b* mutant.

that can contribute to the reduced water-loss rate under drought stress conditions, leaf dry matter content (LDMC) and root surface area were determined. These analyses showed no significant difference between *slpao3* mutants and wild-type plants (Figure S13). The leaf osmotic potential at full turgor ( $\pi_0$ ) was also measured and the leaf water potential at turgor loss point ( $\Psi_{tlp}$ ) was calculated (Figure S13), as these physiological traits are strongly linked to drought tolerance (Bartlett et al., 2012; Petruzzellis et al., 2019). These analyses evidenced no statistically significant difference between *slpao3* mutants and wild-type plants and suggested that the increased tolerance to water deficit of *slpao3* mutants does not involve

mechanisms related to osmotic adjustments. This conclusion is further supported by data from analysis of the levels of the osmoprotectant proline (Alvarez et al., 2022), which is known to accumulate in response to water or salt stress and is also accepted as a marker of the level of stress perceived by the plants (Le et al., 2022; Pagliarani et al., 2019; Verslues & Sharma, 2010). This analysis revealed that the increase of proline levels during water-deficit progression was lower in *slpao3* mutants than in wild-type plants (Figure 10d). These data further support the notion that *slpao3* mutants undergo less stress than wild-type plants while confirming that *slpao3* stress tolerance is likely not correlated to osmoregulation.



**Figure 7.** Morphological characteristics of *slpao3* mutants.

(a) Histological analysis of stem transverse sections (at the second internode from the apex) of 6-week-old *slpao3* mutants. Samples were examined under bright-field illumination (toluidine blue staining) and UV light (autofluorescence of cell-wall phenolics). All images were obtained under the same conditions.

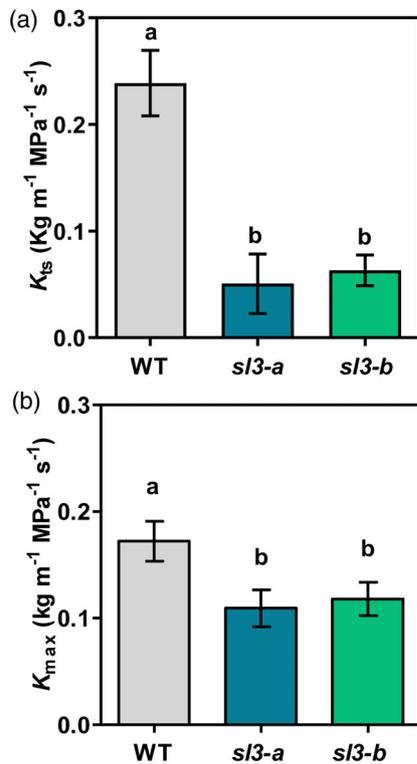
(b) Number of xylem elements in stem transverse sections of *slpao3* mutants. Different letters above the columns denote statistically significant differences ( $P < 0.05$ ; one-way ANOVA).

(c) Frequency distribution of cross-sectional area of the xylem elements in the stems of the different tomato genotypes. The xylem elements of the first internode from above were divided into two classes according to their cross-sectional area ( $\leq 1200 \mu\text{m}^2$ ,  $> 1200 \mu\text{m}^2$ ) and the percentage of xylem elements in each class (frequency) was calculated. Over 25 bundles from minimum of five different plants of each genotype were analyzed. Asterisks indicate statistically significant differences from the wild-type plants ( $P < 0.05$ ; one-way ANOVA and Student's *t*-test). WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutants; *sl3-b*: *slpao3-b* mutants.

Drought stress can induce xylem embolism, which disrupts the root-to-leaf water supply. Measurements of the percentage loss of hydraulic conductivity (PLC) evidenced that *slpao3* mutants are significantly less vulnerable to xylem embolism than wild-type plants, at least under moderate stress conditions (Figure 11), thus confirming a higher degree of drought tolerance.

## DISCUSSION

Drought stress affects plant growth and survival, reducing crop yield. Plants cope with drought using different strategies, which can be divided into three main classes: (i) drought escape through anticipation of transition from the vegetative to the reproductive stage; (ii) dehydration



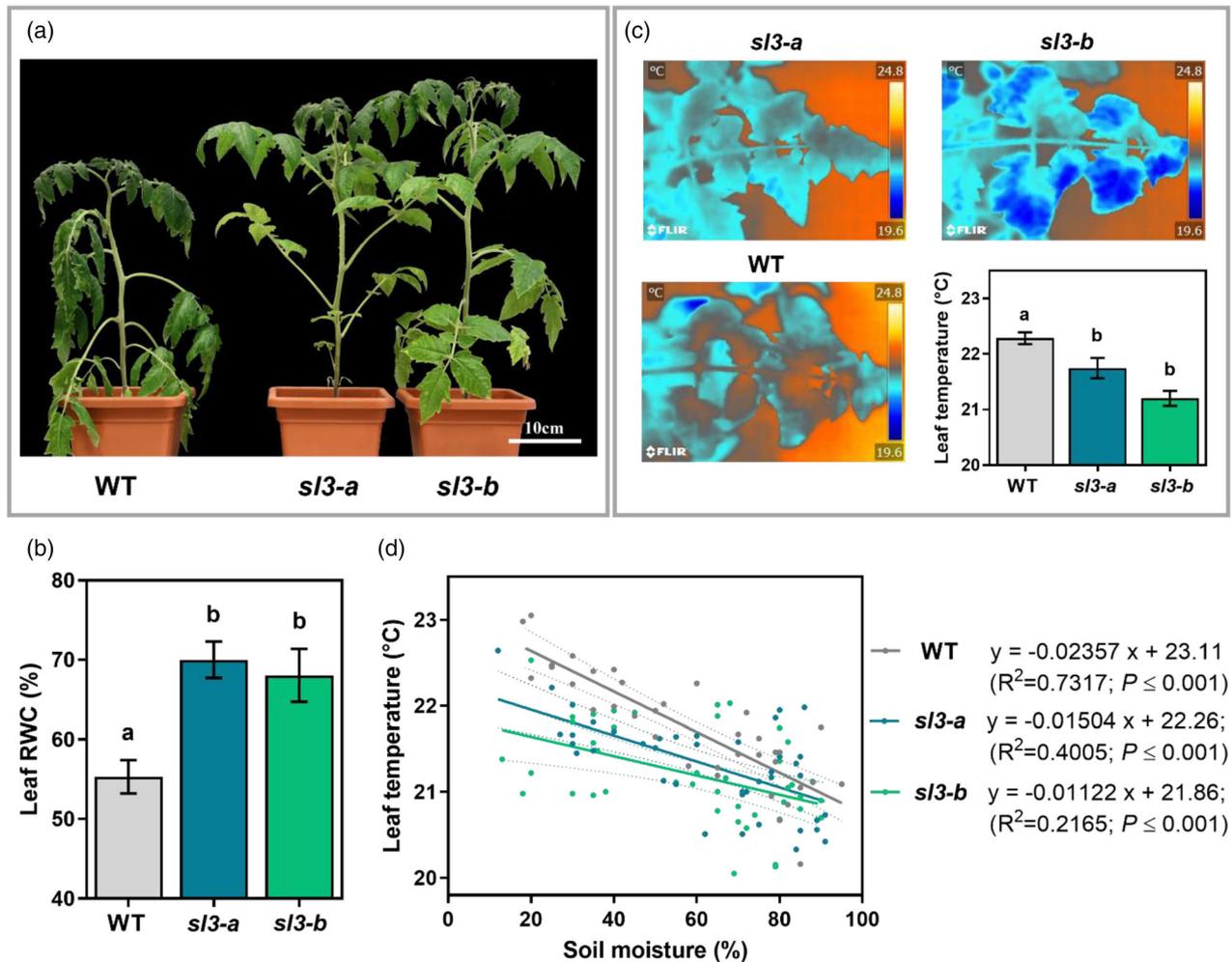
**Figure 8.** Xylem hydraulic conductivity in *slpao3* mutants. (a) Theoretical specific hydraulic conductivity ( $K_{ts}$ ) of the xylem vessels. To calculate  $K_{ts}$ , theoretical hydraulic conductivity ( $K_t$ ) obtained from a modified Hagen–Poiseuille equation (Tyree & Ewers, 1991) was normalized to the area of the stem cross section ( $n \geq 5$  per genotype). (b) Actual hydraulic conductivity ( $K_{max}$ ) of the xylem vessels under well-watered conditions. Different letters above the columns indicate statistically significant differences ( $P < 0.05$ ; Student's *t*-test;  $n \geq 5$  per genotype). WT: wild-type tomato plants; *slpao3-a*: *slpao3-a* mutants; *slpao3-b*: *slpao3-b* mutants.

tolerance through metabolic adjustments; and (iii) dehydration avoidance through maintenance of a relatively high water content even under low soil water availability by reducing transpiration, limiting shoot growth, and/or increasing root elongation (Blum & Tuberosa, 2018; Kooyers, 2015; Martignago et al., 2020).

Here, the contribution of SIPAO3 and T-Spm to plant responses to drought was investigated. Data show that loss-of-function mutations of *SIPAO3* result in improved drought tolerance. Indeed, *slpao3* mutants showed less severe wilting symptoms, higher water content, and less negative  $\Psi_{leaf}$  values than wild-type plants under water shortage (Figure 9; Figure 10; Figure S11). Furthermore, *slpao3* mutants displayed a slower drought-induced reduction in stomatal conductance and transpiration rate, together with a slower drought-induced increase in leaf temperature and proline levels than wild-type plants (Figure 9; Figure 10; Figure S12), suggesting that these mutants undergo less stress than wild-type plants. The drought

tolerance of *slpao3* mutants, which is likely correlated with the drought-induced downregulation of *SIPAO3* (Figure 2), maybe a consequence of the reduced xylem hydraulic conductivity (Figure 8) that limits stomatal conductance and transpiration rate (Figure 10; Figure S12). In turn, the lower xylem hydraulic conductivity can be attributed to the reduced number of xylem elements (Figure 7; Figure S9) and the smaller proportion of large vessels in *slpao3* mutants (Figure 7c) compared to wild-type plants. Instead, the increased drought tolerance of *slpao3* mutants likely does not involve mechanisms related to the regulation of stomatal movement by T-Spm, which was ineffective in promoting stomatal closure (Figure S14) and cannot be explained by differences in stomatal density or index, the two parameters being identical in the different genotypes (Figure S13). As a result of this hydraulic limitation effect, the lower transpiration rate of *slpao3* mutants apparently limits excessive water loss under drought conditions, delaying stress symptoms as compared to wild-type plants (Figure 12). Thus, *SIPAO3* mutation mediates increased tolerance to water shortage by favoring a dehydration avoidance strategy (Fang & Xiong, 2015; Kooyers, 2015; Martignago et al., 2020). Notably, drought tolerance mechanisms related to xylem differentiation and water transport efficiency were also observed in previous studies (Illouz-Eliaz et al., 2020; Zhang et al., 2018), in agreement with the central role of xylem in plant growth and survival. Here, it is further shown that *slpao3* mutants present reduced vulnerability to xylem embolism compared to wild-type plants (Figure 11), which may also contribute to the improved drought tolerance (Figure 12). The reduced vulnerability to xylem embolism of *slpao3* mutants is likely associated with the narrower xylem conduits, without excluding the involvement of other structural and/or metabolic alterations.

Plants function under resource limitations and biophysical constraints imposed by external and/or internal molecular and biochemical networks. These constraints lead to trade-offs whereby one trait depends on and is constrained by others (Karasov et al., 2017; Laitinen and Nikoloski, 2022). For example, growth-defense trade-offs associated with defense mechanisms against pathogens and abiotic stresses often occur at the expense of growth and yield. Interestingly, drought tolerance mediated by *SIPAO3* mutation is not associated with growth penalties, at least under growth chamber conditions. Indeed, the *slpao3* mutants exhibit only a mild long stem phenotype (probably due to the diminished xylem differentiation and/or the altered auxin and GA homeostasis) while maintaining an unaltered leaf and root area (Figure 6; Figure S13). Thus, the reduction of the hydraulic conductivity in the *slpao3* mutants that likely contributes to the mechanism determining the improved stress tolerance is sufficient to limit excessive water loss under drought stress conditions but not to impair growth and/or to induce a wilted phenotype under



**Figure 9.** Phenotypic analysis of *slpao3* mutants under drought stress conditions.

(a) Photos of representative 6-week-old plants taken 10 days after the onset of drought stress by completely interrupting irrigation.

(b) Leaf relative water content (RWC) was determined 10 days after the onset of drought stress.

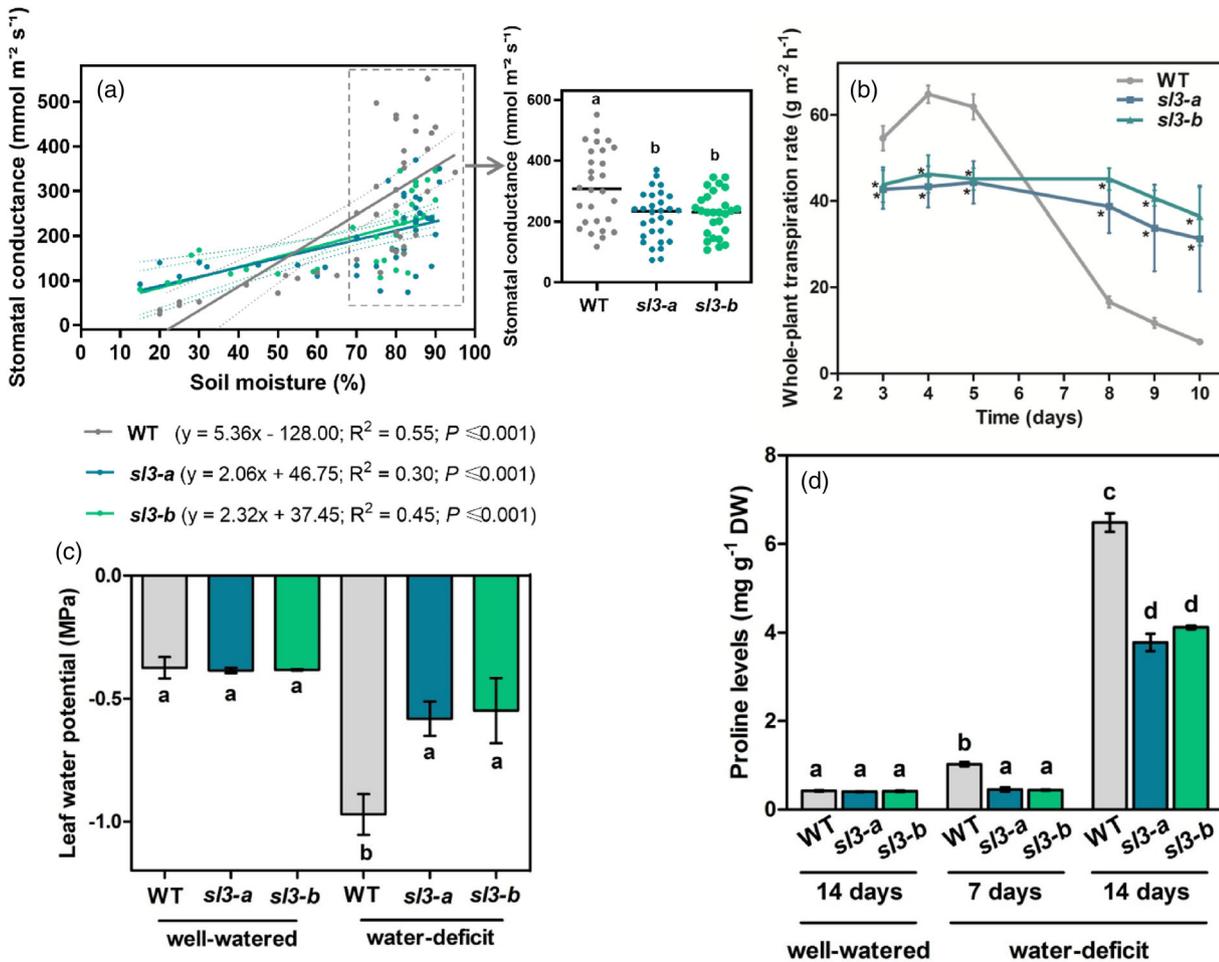
(c) Thermographic pictures of leaves obtained by a thermal camera (FLIR E75) 10 days after the onset of drought stress and respective mean temperatures.

(d) Kinetics of leaf temperature increase with decreasing soil humidity as determined through thermographic pictures. Thermal images were acquired on the third leaf of each plant, before the onset of water stress and then every 2 days, until the end of the experiment. The slope of the correlation curve of the WT plants is statistically different from those of *slpao3-a* and *slpao3-b* mutants, as evidenced by ANCOVA analysis. Different letters above the columns denote statistically significant differences ( $P < 0.05$ ; one-way ANOVA). WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutants; *sl3-b*: *slpao3-b* mutants.

well-watered conditions, as observed in some *wilty* mutants (Dodd et al., 2008; Koizumi et al., 2007).

The results presented in this study show that *slpao3* mutants display increased T-Spm levels compared to wild-type plants in both leaves and stems (Figure 3), suggesting that SIPAO3 is involved in T-Spm catabolism, similar to its Arabidopsis homolog AtPAO5. The increased T-Spm levels in *slpao3* mutants are probably responsible for the differences from wild-type plants in xylem differentiation observed throughout the entire plant. Indeed, treatment of *slpao3* mutants and wild-type plants with exogenous T-Spm reverted these differences. The effect of T-Spm on xylem differentiation can be attributed to the effect of T-Spm on auxin distribution and GA homeostasis, as

indicated by the altered expression levels of *SIPIN1* and *SIPIN6*, as well as of *SIGA20ox1* and *SIGA20ox4*, in *slpao3* mutants and tomato wild-type plants treated with T-Spm compared to control plants (Figure 4; Figure 5; Figure S7; Figure S8). The effect of T-Spm on auxin transport and xylem differentiation of tomato plants is similar to that observed in Arabidopsis and *Populus* (Alabdallah et al., 2017; Milhinhos et al., 2013). Furthermore, similar to Arabidopsis, tomato displays a feedback mechanism regulating T-Spm homeostasis and *SIACL5* expression levels, in which *SIPAO3* participates. Indeed, *slpao3* mutants and T-Spm-treated wild-type tomato plants present a reduction in *SIACL5-1* and *SIACL5-2* expression levels compared to the controls (Figure 4; Figure 5) which, however, is not



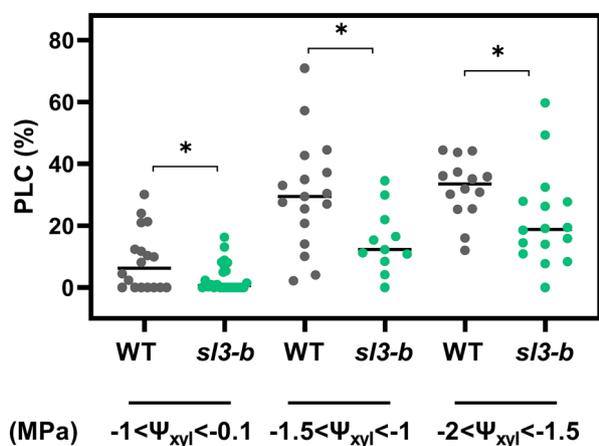
**Figure 10.** Hydraulic parameters and proline content in *slpao3* mutants under well-watered and drought stress conditions.

(a) Stomatal conductance of the different tomato genotypes at various soil humidity conditions. Stomatal conductance was determined through measurement of  $\text{CO}_2/\text{H}_2\text{O}$  gas exchange in leaves of the different tomato genotypes. The slope of the correlation curve of WT plants is statistically different from those of *slpao3-a* and *slpao3-b* mutants, as evidenced by ANCOVA analysis. The inset shows stomatal conductance under conditions of high soil humidity ( $\geq 70\%$ ). (b) Whole-plant transpiration rates through gravimetric determination of water-loss kinetics. Asterisks indicate statistically significant differences from the wild-type plants at the corresponding time point ( $P < 0.05$ ; one-way ANOVA). (c) Leaf water potential of the different tomato genotypes under well-watered and water-stress conditions (10 days after onset of water-withholding). (d) Proline content in the different tomato genotypes under well-watered and water-stress conditions. Different letters above or below the columns and the scatter dot blots denote statistically significant differences ( $P < 0.05$ ; one-way ANOVA). Representative experiments out of at least three repetitions are shown. WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutants; *sl3-b*: *slpao3-b* mutants.

sufficient to restore T-Spm levels in the *slpao3* mutants. These observations suggest conservation of the regulatory mechanisms controlling T-Spm homeostasis, auxin transport, and xylem differentiation across different plant species. Nevertheless, unlike Arabidopsis which has a single *ACL5* gene, tomato has three *SIACL5* genes with high sequence similarity to each other and the Arabidopsis *ACL5* (58% - 72%), the *SIACL5-3* being differently regulated by T-Spm from *SIACL5-1* and *SIACL5-2* (Figure 4). Similarly, while Arabidopsis has a single *AtPAO5* gene, tomato has three *AtPAO5* homologs that display differences among them in the regulation of gene expression. These differences between Arabidopsis and tomato are probably

due to recent events of gene duplication and change of function and may suggest different physiological roles for the three *SIACL5* and the three *AtPAO5*-like *SIPAO* genes. They also suggest the need for extremely fine regulation of T-Spm levels in tomato plants, considering the relevant role of this polyamine in xylem differentiation and hormone signaling.

Analysis of polyamine levels in tomato plants under well-watered and drought stress conditions showed that drought stress increases Put levels but does not affect those of Spd, Spm, and T-Spm (Figure S10). These data seem to partially contradict previous data indicating variations of Spm and Spd levels under drought stress



**Figure 11.** Percentage loss of hydraulic conductivity (PLC) in *slpao3* mutant plants under drought stress conditions. PLC was determined in stems of 6-week-old *slpao3* mutants (*sl3-b*) and wild-type plants (WT) subjected to drought stress by completely interrupting irrigation. PLC was calculated from hydraulic conductivity measurements at different stem water potential ranges ( $\Psi_{xyl}$ , MPa). Asterisks indicate statistically significant differences with respect to the corresponding values of the WT plants (Student's *t*-test,  $P < 0.05$ ).

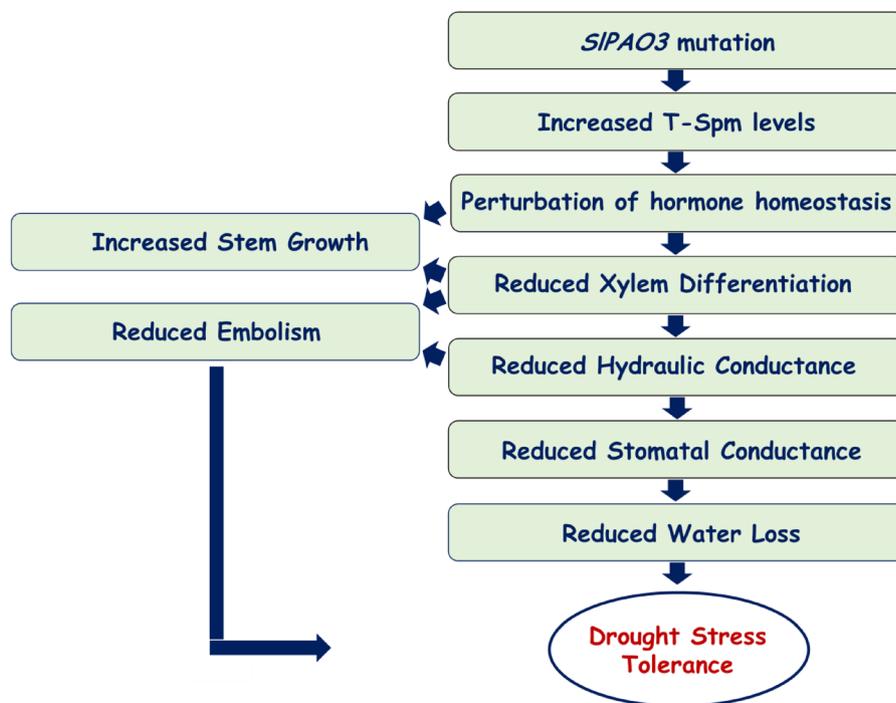
(Montesinos-Pereira et al., 2014; Upadhyay et al., 2021; Wang et al., 2023). These differences may depend on the plant species and genotype analyzed, as well as on the duration, severity, and mode of drought stress (Montesinos-Pereira et al., 2014; Wang et al., 2023). It was further shown here that T-Spm levels are not affected by

drought stress (Figure S10) even though *SIPAO2* and *SIPAO3* genes are downregulated under these conditions (Figure 2) and T-Spm levels were previously shown to be reduced under salt stress conditions (Naka et al., 2010). These data reflect the complex regulatory mechanisms controlling polyamine homeostasis.

All three AtPAO5-like *SIPAO* genes, similar to the three *SIACL5* genes and the *SIPIN1* and *SIPIN6* genes, exhibit higher expression levels in roots than in shoots (Figure 2), suggesting an important role of these genes in root physiology. However, except for differences in xylem differentiation, and despite these differences, no significant change in root growth was observed in *slpao3* mutants compared to wild-type plants. This may be due to gene redundancy and/or the contribution of these genes to different aspects of root physiology.

No information on the tissue-specific expression pattern of *SIPAO3* is available so far. However, the effect of *SIPAO3* gene disruption on xylem differentiation allows hypothesizing expression in xylem cells during vascular differentiation as was previously reported for *AtPAO5* and *ACL5* in Arabidopsis plants (Alabdallah et al., 2017; Clay & Nelson, 2005; Muñiz et al., 2008).

*SIPAO2*, *SIPAO3*, and *SIPAO4* share high overall amino-acid sequence identity. Despite this, *SIPAO4* displays an additional domain characterized by the presence of TRs. The function of these TRs has yet to be discovered, but they may have a regulatory role (Kajava, 2012; Sharma & Pandey, 2016). Biochemical studies on the catalytic



**Figure 12.** Model of the underlying mechanisms in the *SIPAO3*-mediated improved tolerance to drought stress.

properties of the three tomato AtPAO5 homologs are necessary to determine substrate specificity and catalytic mechanism, as well as to verify the TR effect on the catalytic reaction.

*SIPAO2*, *SIPAO3*, and *SIPAO4* present similarities to each other in the regulation of gene expression, but also important differences. In particular, in agreement with previous reports (Hao et al., 2018), both *SIPAO2* and *SIPAO3* genes were shown here to be downregulated by drought stress and not significantly controlled by T-Spm, *SIPAO3* being only slightly downregulated by T-Spm in shoots (Figure 2). *SIPAO2* and *SIPAO3* are also similarly regulated by various stimuli (e.g., various abiotic stresses, polyamine, and hormones), except by cold treatment under which only *SIPAO3* is upregulated (Hao et al., 2018). Unlike *SIPAO2* and *SIPAO3*, *SIPAO4* expression is not affected by drought stress while being upregulated by T-Spm (Figure 2). These data suggest that *SIPAO2* and *SIPAO3* may have similar, though likely not identical, physiological roles, but different from those of *SIPAO4*. This needs to be addressed by characterization of *slpao2* and *slpao4* mutants to determine the specific contribution of each of the three genes in the control of polyamine levels, xylem differentiation, and different developmental and defense processes. Characterization of *slpao2slpao3* and *slpao3slpao4* double mutants is also necessary to analyze gene redundancy effects and verify whether it is possible to further increase T-Spm levels in tomato plants, despite the homeostatic mechanisms, and further improve plant ability to tolerate drought stress.

In conclusion, the present study suggests that genetic manipulation of polyamine metabolism may provide crops with improved tolerance to drought stress without growth penalties. Further studies are in progress to better understand the underlying mechanisms, analyze gene redundancy effects, and unravel the contribution of T-Spm metabolism to different developmental and stress response processes.

## EXPERIMENTAL PROCEDURES

### Plant material and growth conditions

Tomato (*Solanum lycopersicum*) plants cv Money Maker were used throughout this study. Plants were grown in a controlled growth chamber at 23°C under a 16-h/8-h light/dark photoperiod with a photosynthetic photon flux density (PPFD) of 100–150  $\mu\text{mol m}^{-2} \text{sec}^{-1}$ . Seeds were first sterilized and stratified for 4 days at 4°C and then placed on agar plates (0.8% w/v) containing Murashige and Skoog basal medium with Gamborg's vitamins and 3% (w/v) sucrose (MS). For RT-qPCR analysis following T-Spm treatment, 5-day-old seedlings grown on MS agar plates were transferred to MS liquid medium for 24 h. After the addition of fresh medium, seedlings were treated with 100  $\mu\text{M}$  T-Spm. For drought stress, plants were grown in pots of 400 ml or 2.5 L until they produced two (c. 4-week-old plants; RT-qPCR analysis) to five (c. 6-week-old plants; phenotypical analyses) expanded leaves. Drought stress was imposed by

withholding irrigation. Progression of drought stress was monitored on the basis of severity of wilting symptoms. Wilting was scored for 15–30 days after onset of drought on a scale from 10 to 100: 10–20%, very mild wilting in basal leaves; 20–40%, mild wilting in basal internodes; 40–60%, moderate turgor loss in lower internodes; 60–80%, high turgor loss in upper and lower internodes; 80–100%, very high to complete turgor loss of all leaves.

### Generation of CRISPR/Cas9-mediated mutagenesis constructs, tomato plant transformation, and mutant selection

Two single-guide RNAs (*gRNA1* and *gRNA2*) targeting the *SIPAO3* gene were designed using the ChopChop CRISPR web tool (<https://chopchop.cbu.uib.no/>). The two *gRNAs* were assembled in one construct applying a PTG strategy (Xie et al., 2015) and the Golden Gate cloning system (Engler et al., 2008; Weber et al., 2011) and using the *pGTR* plasmid (Xie et al., 2015; Table S3). The assembled *SIPAO3*-PTG cassette was put under the promoter of Arabidopsis *U6 snRNA* gene (*AtU6p*; *AtU6p::PTG* construct) obtained from the *pICSL01009::AtU6p* vector (Nekrasov et al., 2013) and introduced inside the *pICH47751* vector (Weber et al., 2011; Table S3). The *AtU6p::PTG* construct was assembled with *2x35S-5'UTR::hCas9::NOST* and *NOSp::NPTII* constructs within the *pAGM4723* binary vector using the *pICH47742::2x35S-5'UTR-hCas9(STOP)-NOST* (Belhaj et al., 2013) and *pICSL11024 (pICH47732::NOSp-NPTII-OCST)* plasmids, respectively, as well as the *pICH50892* plasmid (Weber et al., 2011) to provide a linker sequence (Table S3). The final binary vector was introduced into *Agrobacterium tumefaciens* strain *C58C1 (pCH32)* (Hellens et al., 2000; Wroblewski et al., 2005) by electroporation. This strain was used to transform tomato plants (Van Eck et al., 2019; van Roekel et al., 1993) and several independent kanamycin-resistant T0 transgenic lines were selected. Transgenic lines were analyzed by PCR for the presence of *Cas9* and/or *neomycin phosphotransferase (NPTII)* genes. *Cas9*-positive lines were further genotyped by *SIPAO3* sequencing. Several homozygous *slpao3* mutant lines were identified in T1 and T2 generations (Table S1), and two independent mutant lines (*slpao3-a* and *slpao3-b*) were selected for further analyses. In the case of the *slpao3-b* line, the *Cas9* construct was segregated out in the T2 generation. All oligonucleotides used for construct preparation and molecular characterization of the transgenic plants are listed in Table S4.

### Extraction of genomic DNA from plant tissue

Genomic DNA was extracted from young leaves following homogenization with 200  $\mu\text{l}$  of extraction buffer (2% SDS, 250 mM NaCl, 25 mM EDTA, 200 mM Tris pH 7.5). The homogenate was heated for 10 min at 60°C and centrifuged at 13 000 *g* to remove debris. Then, an equal volume of isopropanol was added to the supernatant and, after 5 min of incubation, the mix was centrifuged for 15 min at 13 000 *g*. The resulting pellet was washed with cold 70% ethanol, dried, and resuspended in water.

### Identification of the repeated motifs in SIPAO4 homologs

Xstream (<https://amnewmanlab.stanford.edu/xstream/>) analysis was initially conducted on each of the nine *SIPAO4* homologs of Solanaceae found by BLASTp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) and related repeated motifs of 17 amino acids were identified. MEME analysis (<https://meme-suite>)

[org/meme/tools/meme](http://org/meme/tools/meme)) was then conducted to evaluate the presence of a common repeated motif among the nine SIPAO4 homologs. MEME was run with the option *anr*, which assumes that each sequence may contain any number of non-overlapping occurrences of each motif. MEME identified two closely related repeated motifs of 17 amino acids that MAST (<https://meme-suite.org/meme/tools/mast>) analysis merged into one.

### RT-qPCR analyses

Total RNA was extracted using the Trizol reagent (Qiagen S.r.l., Milan, Italy) according to the manufacturer's protocol. Genomic DNA was degraded using the RNase-Free DNase Set (Qiagen). For RT-qPCR, cDNA synthesis and PCR amplification were carried out using the GoTaq 2-Step RT-qPCR System (Promega Corporation, Madison, WI, USA). The PCR reactions were run in a Corbett RG6000 (Corbett Life Science, Qiagen) as follows: at 95°C for 2 min, followed by 40 cycles of 95°C for 3 sec and 60°C for 30 s. At least three independent biological replicates were performed for each experiment, to obtain mean values of relative expression levels from the different biological replicates. Relative expression levels were presented as fold-changes ( $2^{-\Delta\Delta C_t}$ ) from the controls. The genes for actin (Solyc04g011500.3.1) and SGN-U316474 (*SISAND*; Expósito-Rodríguez et al., 2008) were used as reference genes. Gene-specific oligonucleotides used are listed in Table S4.

### Analysis of potential off-target mutations

Potential off-target loci associated with *gRNA2* were identified using CRISPR-P 2.0 (<http://crispr.hzau.edu.cn/CRISPR2/>) and CRISPOR (<http://crispor.org>) website tools and those with a high probability score ( $\geq 0.1$ ) were selected for further analyses (Table S2). The genomic regions flanking the selected putative off-target sites (510–590 bp) were PCR amplified using specific primers (Table S4) and the resulting amplicons were subjected to sequencing analysis.

### Determination of polyamine levels

Polyamine levels were determined in leaves and stems by gas chromatography–mass spectrometry (Ahou et al., 2014).

### Histological analyses

Root and stem segments were embedded in Technovit 7100 resin (Heraeus Kulzer GmbH, Wehrheim, Germany), and transverse sections (10  $\mu$ m) were made using a Microm HM330 microtome (Bio Optica Milano S.p.A., Milano, Italy). For histochemical localization of lignin, sections were stained in 0.05% (w/v) toluidine blue solution and then observed under a Zeiss Axiophot 2 microscope equipped with a Leica DFC450C digital camera. Sections were also examined under UV light to observe the autofluorescence of cell-wall phenolics. Images were used to quantify the number and cross-sectional area of the xylem elements.

### Measurement of leaf and root surface area

Total leaf and root surface area was calculated by photographing the whole foliage and root apparatus (in the latter case after thorough washing) of 6-week-old tomato plants and analyzing the images by ImageJ (<http://rsb.info.nih.gov/ij/>).

### Thermal image acquisition and processing

Thermal images were acquired using a thermal camera (FLIR E75 series; FLIR Systems, Inc.) with a reading accuracy of  $\pm 2\%$ , reading temperature from  $-20$  to  $+650^\circ\text{C}$ , image resolution of  $320 \times 240$  pixels, and thermal sensitivity  $< 0.03^\circ\text{C}$ . Images were acquired by

photographing the third composite leaf (starting from the apex) every 2 days, before and after the onset of drought until the end of the experiment. The false-color images indirectly show the leaf water status (Costa et al., 2013). Images were analyzed using proprietary software (FLIR Thermal Studio Suite). Each composite leaf was subdivided into smaller areas, using dedicated software options, and the temperature of each area was measured. The temperature of the surface of each leaf was calculated as the average of all areas.

### Determination of stomatal conductance and transpiration rate

Leaf stomatal conductance and transpiration rate were recorded by CI-340 Handheld Photosynthesis System (CID Bio-Science, Washington, DC, USA). All measurements were done 8–10 h after the beginning of the light cycle under a PPFD of  $120 \mu\text{mol m}^{-2} \text{sec}^{-1}$ .

### Whole-plant transpiration measurements

Whole-plant transpiration rates were determined using 4-week-old potted plants. Pots were sealed to prevent evaporation from the surface of the growth medium and their weight was monitored using a balance every 1 h (during the light period) for a period of 10 days after the onset of drought. The daily plant transpiration rate (weight loss in 1 h) was calculated from the difference in whole-plant weight between two time points (8–10 h after the beginning of the light cycle) normalized to total leaf area.

### Determination of leaf relative water content

Leaf relative water content (RWC) was measured on the third leaf (from the apex) of each plant under well-watered and drought conditions as previously described (Sade et al., 2009). Immediately after leaf detachment, fresh weight (FW) was measured. Leaves were rehydrated by immersion of petioles in water for 18 h at room temperature under low light conditions, and turgid weight (TW) was recorded. Dry weight (DW) was measured after drying the leaves at  $90^\circ\text{C}$  for 3 days. RWC was calculated as:

$$\text{RWC} = \frac{(\text{FW} - \text{DW})}{(\text{TW} - \text{DW})} \times 100$$

### Calculation of theoretical specific hydraulic conductivity

The theoretical hydraulic conductivity ( $K_t$ ;  $\text{mmol m MPa}^{-1} \text{sec}^{-1}$ ) was calculated based on the modified Hagen–Poiseuille equation (Tyree & Ewers, 1991):

$$K_t = \left( \frac{\pi \rho}{128 \mu} \right) \sum_{i=1}^n (d_i^4)$$

where  $d_i$  is the diameter (m) of the *i*th xylem vessel,  $\rho$  is the water density in  $\text{kg m}^{-3}$  ( $998.2 \text{ kg m}^{-3}$  at  $20^\circ\text{C}$ ),  $\mu$  is the water viscosity in  $\text{MPa sec}$  ( $1.002 \cdot 10^{-9} \text{ MPa sec}$ ) and  $n$  is the number of xylem vessels in the stem section.  $K_t$  was normalized by the area of the cross sections obtained between nodes 5 and 6 (from the apex) to obtain theoretical specific hydraulic conductivity ( $K_{ts}$ ;  $\text{mmol m}^{-1} \text{MPa}^{-1} \text{sec}^{-1}$ ).

### Estimation of leaf and xylem water potential

Leaf water potential measurements were performed using a pressure chamber (mod. 600 D, PMS Instrument Company, Albany, OR, USA) (Scholander et al., 1965). To estimate xylem water potential ( $\Psi_{xyl}$ ), leaves from the third or fourth internode (from above) were covered with plastic film and silver foil under dark.

Leaves were then detached from the plant by cutting the petiole with a sharp blade and the water potential was measured.

### Estimation of maximum vessel length and mean vessel length

To avoid artifacts due to spurious air entering xylem conduits during sample preparation for hydraulic measurements, maximum vessel length was estimated. This was done by the "air-injection method" as previously described (Wang et al., 2014). In detail, stems of 6-week-old plants were cut at the basal end with a sharp razor blade and connected to a Tygon tubing system. Following re-cutting of the stems at the apical end, air delivered from a pressure chamber instrument (mod. 600 D, PMS Instrument Company) was perfused from the basal end of the stem segments to the apical cut sections while immersed in water at an air pressure of 200 kPa. The stem was progressively shortened by 1 cm cuts at the apical end until the first bubbles emerged from the cut sections, indicating that at least one vessel was cut open at both ends. The length of the remaining stem was recorded as the maximum length of the vessels. The stem was then cut systematically and the air flow rate, which is proportional to the number of vessels extending from one cut end to the apical end of the plant, was measured at different stem lengths. To this end, bubbles flowing out during a time interval were collected in a volumetric cylinder by the water displacement method, and the air volume was measured gravimetrically. Flow rate data were used to calculate mean vessel length (Pan et al., 2015; Figure S15).

### Measurement of stem hydraulic conductivity and xylem embolism

Stem hydraulic conductivity was measured in 6-week-old plants either well-watered or dehydrated at different xylem water potentials, as described by Tomasella et al. (2019). Briefly, the main stem was cut at the basal end (1 cm above the soil) and immediately immersed in clean tap water. Following a second short cut made under water next to the previous one, a third cut was made at a distance 1.5-fold longer than the mean vessel length (i.e., 15 cm; Figure S15) to minimize any possible cutting artifact (Torres-Ruiz et al., 2015). After several consecutive cuts made under water at both ends, a 70-mm-long stem segment between nodes 5 and 6 (from above) was obtained and connected to a custom-made hydraulic apparatus. The sample was perfused with a reference 10 mM KCl solution (Nardini et al., 2007) previously degassed and filtered at 0.2 μm. Native stem specific hydraulic conductivity ( $K_i$ ) was measured gravimetrically under low water pressure conditions (5 kPa, generated by a water head) by collecting the solution at the extremity of the stem segment for 30- to 60-sec intervals in 1.5-ml Eppendorf tubes filled with a piece of sponge.  $K_i$  was calculated as:

$$K_i = \frac{M \times L}{A \times t \times P}$$

where M is the mass of the collected perfusion solution (kg), L is the stem segment length (m), A is the area of stem cross section (m<sup>2</sup>), t is the duration of solution collection (s), and P is water pressure applied at the sample (MPa).

The stem segment was then flushed for 5 min at high pressure (0.15 MPa) generated in a captive air water tank connected to the apparatus. The duration of the flush was previously checked to be sufficient to remove embolism and to reach the maximum stem hydraulic conductivity ( $K_{max}$ ), measured as for  $K_i$ . Xylem embolism was quantified as percentage loss of hydraulic conductivity (PLC):

$$PLC = 100 \left[ 1 - \left( \frac{K_i}{K_{max}} \right) \right]$$

### Water potential at turgor loss point

Water potential at the turgor loss point ( $\Psi_{tip}$ ) was estimated in leaves of 6-week-old, well-watered, plants as described by Petruzzellis et al. (2019). Two leaves were detached from each plant, one to determine the leaf dry matter content (LDMC; mg g<sup>-1</sup>) and the other to measure the osmotic potential. LDMC was determined by measuring leaf fresh weight after complete hydration (i.e., turgid weight, TW), through petiole immersion in water for 18 h, and then dry weight (DW) after oven-drying for 24 h at 70°C. LDMC was calculated as:

$$LDMC = \frac{DW}{TW}$$

For osmotic potential measurement, the second leaf was immersed for 2 min in liquid nitrogen while bagged in cling film and then ground into fine pieces. The water potential of the ground leaf material that corresponds to its incorrect osmotic potential at full turgor ( $\pi_{0,incorr}$ ) was measured with a dewpoint hygrometer (Model WP4, Decagon Devices Inc., Pullman, USA). The corrected osmotic potential at full turgor ( $\pi_0$ ) was calculated from  $\pi_{0,incorr}$  taking into account LDMC as follows:

$$\pi_0 = (0.506 \times \pi_{0,incorr}) - (0.002 \times LDMC)$$

$\Psi_{tip}$  was finally calculated as:

$$\Psi_{tip} = 1.312 \times \pi_0 - 0.032$$

### Determination of proline levels

Proline content was measured as previously described (Bates et al., 1973) and using L-proline as the standard. Samples from first and second leaves (from the apex) of control and stressed plants were collected and dried at 90°C for 3 days to eliminate water and inactivate enzymes. Then, samples were homogenized in 3% (w/v) 5-sulfosalicylic acid and the absorbance was read at 520 nm. The proline content was expressed as mg/g of DW.

### Measurements of stomatal index and density

Stomatal index (stomata number/total number of epidermal cells) and stomatal density were determined by the imprinting technique (Nir et al., 2017). Dental resin (eliteHD+; Zhermack Clinical) was attached to the leaf abaxial side and then removed after drying. The resin epidermal imprints were covered with transparent nail polish, which served as a mirror image of the resin imprint. The nail polish imprints were photographed under a Zeiss Axiolab 5 microscope equipped with a Zeiss Axiocam 208 digital camera.

### Measurement of stomatal response to T-Spm treatment

To investigate possible direct effects of T-Spm on stomatal aperture, stomatal conductance was measured as described above following T-Spm treatment. For the treatment, all leaves of 4-week-old plants were sprayed with 1 mM T-Spm and stomatal conductance was registered at various time intervals.

### Molecular modeling

The structural models of SIPAO3 wild-type protein and SIPAO3-a mutant were obtained using trRosettaX, an algorithm for protein structure prediction based on a supervised transformer

protein language model used to predict inter-residue two-dimensional geometry. This, in turn, was used to reconstruct the three-dimensional structure of the protein of interest via energy minimization (Wang et al., 2022). The crystal structure of human spermine oxidase (hSMOX; PDB code 7OXL; Diaz et al., 2022) was used as a reference.

### Statistical analyses

Statistical analysis was performed by the Microsoft Excel software together with Real Statistics Resource Pack Add-in (<https://real-statistics.com/free-download/real-statistics-resource-pack/>), and the GraphPad Prism software (version 6.01). Experiments were repeated at least three times and results are expressed as the mean value  $\pm$  standard error of the mean (SEM). Statistical comparison between groups was performed by unpaired Student's *t*-test, and/or analysis of variance (one-way ANOVA). Statistical analysis of slopes of correlation curves was performed by ANCOVA. *P* values  $\leq 0.05$  were regarded as significant.

### ACCESSION NUMBERS

The Accession number of genes analyzed in the present study are as follows: AT5g13700 (*AtPAO5*; *Arabidopsis thaliana*), Solyc05g018880 (*SIPAO2*; *S. lycopersicum*), Solyc07g039310 (*SIPAO3*; *S. lycopersicum*), Solyc12g008850.1.1 (*SIPAO4*; *S. lycopersicum*), Solyc08g061970 (*SIACL5-1*; *S. lycopersicum*), Solyc09g075900 (*SIACL5-2*; *S. lycopersicum*), Solyc07g041300 (*SIACL5-3*; *S. lycopersicum*), Solyc03g118740 (*SIPIN1*; *S. lycopersicum*), Solyc06g059730 (*SIPIN6*; *S. lycopersicum*), Solyc04g011500.3.1 (*SIACTIN*; *S. lycopersicum*), Solyc03g115810.2.1 (SGN-U316474; *SISAND*; *S. lycopersicum*), Solyc03g006880.2.1 (*SIGA20ox1*; *S. lycopersicum*), Solyc07g061720.2.1 (*SIGA20ox4*; *S. lycopersicum*), Sopen12g003780 (*SpPAO4*; *Solanum pennellii*), SOLCI007272600\_T001 (*ScPAO4*; *Solanum chilense*), Sopim12g008850 (*SpimPAO4*; *Solanum pimpinellifolium*), PGSC0003DMP400000657 (*StPAO4*; *Solanum tuberosum*), KAG5569611.1 (*ScmPAO4*; *Solanum commersonii*), PHT73535.1 (*CaPAO4*; *Capsicum annuum*), PHT52116.1 (*CbPAO4*; *Capsicum baccatum*), PHU08139.1 (*CcPAO4*; *Capsicum chinense*).

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### AUTHOR CONTRIBUTIONS

PT designed the research plan with input from MT<sup>2</sup> and AN. RD, RM, RT, MT<sup>2</sup>, AM, AI, MT<sup>3</sup>, and PT performed the experiments. DM made analyses of mutant sequences. FP performed the protein structural analyses. PT, RD, RM, and MT<sup>3</sup> wrote the manuscript. DM, IF, AC, RA, MT<sup>2</sup>, and AN critically reviewed the manuscript.

### CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

### DATA AVAILABILITY STATEMENT

All data supporting the findings of this study are available within the paper and within its Supporting information published online.

### SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

**Figure S1.** Amino acid sequence of plant SIPAO4 homologs. The additional protein domain in SIPAO4 homologs is highlighted in italic and bold characters. Tandem repeats are indicated in red and green colors.

**Figure S2.** Schematic diagram of *SIPAO3* gene, vector construction, and CRISPR/Cas9-mediated mutagenesis. (a) Schematic diagram of *SIPAO3* gene and position of target sites 1 and 2. (b) Scheme of the binary vector constructed for CRISPR/Cas9-mediated *SIPAO3* mutagenesis in which the relative position of the *NOSp::NPTII*, *2x35S::hCas9* and *AtU6p::tRNA-gRNA1-tRNA-gRNA2* cassettes is shown. The design is not in scale. (c) *SIPAO3-a* and *SIPAO3-b* genotypes. Sequence of target site 2 is indicated in blue and protospacer adjacent motif (PAM) in red. Black dashed lines indicate base pair deletions. (d) The *SIPAO3-a* and *SIPAO3-b* protein isoforms. The deleted sequence in *SIPAO3-a* isoform is indicated in green, while the extra amino acids at the C terminus of the *SIPAO3-b* are shown in red. *AtU6p*: promoter of *Arabidopsis U6 snRNA* gene; *2x35S*: doubled enhanced *CaMV 35S* promoter; *hCas9*: human codon-optimized *Cas9* nuclease gene; *NOSp*: promoter of *nopaline synthase* gene; *NPTII*: *neomycin phosphotransferase II* gene.

**Figure S3.** Structural models of *SIPAO3* wild-type protein and *SIPAO3-a* mutant. Ribbon representation of the crystal structure of human spermine oxidase (hSMOX; PDB code 7OXL; Diaz et al., 2022) and of the structural models of *SIPAO3* and *SIPAO3-a*. Structural models were obtained by trRosettaX using hSMOX as a reference. The FAD cofactor is represented as in "spacefill" and colored in magenta.

**Figure S4.** Comparison of *SIPAO3* and *SIPAO3-a* structural models showing the changes affecting the FAD cofactor molecular environment. Top panels, ribbon representation; central panels, molecular surface representation; bottom panels, enlarged view showing the hydrophobic plug of the FAD binding pocket, formed by Leu432, Phe433, Leu434 residues, present in *SIPAO3* and absent in *SIPAO3-a* mutant. The green arrows highlight the deleted region.

**Figure S5.** Sequence analysis of the top six potential genome-wide off-target sites in the *slpao3* mutants. The genomic regions flanking the selected putative off-target sites (OFF-A to OFF-F; Table S2) were amplified from *slpao3-a* and *slpao3-b* mutants, and wild-type plants (WT). Resulting amplicons were sequenced and chromatograms at the putative off-target sites are shown. Mismatch nucleotides with respect to the *SIPA03 gRNA2* (GGAAACGATCCACTCTTCTGGG) are marked in red, and PAM sites are shown in blue.

**Figure S6.** Putrescine, spermidine, and spermine levels in *slpao3-a* and *slpao3-b* mutants. Polyamine levels were determined in leaves and stems of the third internode (from above) of 6-week-old *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, and wild-type (WT) tomato plants. Values are means of three independent repetitions  $\pm$  SE ( $n=3$ ). Letters above the columns indicate the statistical analysis (one-way ANOVA;  $P<0.05$ ). Put: putrescine; Spd: spermidine; Spm: spermine.

**Figure S7.** Expression levels of genes involved in T-Spm biosynthesis and auxin transport in the roots of *slpao3* mutants. Expression levels were analyzed by RT-qPCR in roots of 9-day-old *slpao3-a* (*sl3-a*), *slpao3-b* (*sl3-b*), and wild-type (WT) tomato genotypes. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta Ct}$ ) from the control plants. Values are means of three independent repetitions  $\pm$  SEM. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P<0.05$ ).

**Figure S8.** Effect of T-Spm on the expression levels of genes involved in gibberellin biosynthesis (*SIGA20ox1*) and deactivation (*SIGA2ox4*). Expression levels were analyzed by RT-qPCR in shoots of 7-day-old tomato wild-type seedlings treated (T) or not (C) with  $100\ \mu\text{M}$  T-Spm for 24 h. Expression levels were also analyzed in leaves of 4-week-old *slpao3-a* (*sl3-a*), *slpao3-b* (*sl3-b*), and wild-type (WT) tomato genotypes. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta Ct}$ ) from the control plants. Values are means of three independent repetitions  $\pm$  SEM. Different letters above the columns indicate statistically significant differences (Student's *t*-test,  $P<0.05$ ).

**Figure S9.** Morphological characteristics of roots of *slpao3-b* mutants and effect of T-Spm. (a) Histological analysis of root transverse sections under bright-field illumination (toluidine blue staining) and UV light (autofluorescence of cell-wall phenolics). (b) Number of xylem elements in root transverse sections of *slpao3-b* mutants. Root segments from 1 cm below the root-hypocotyl junction of 10-day-old seedlings were analyzed. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P<0.05$ ). WT: wild-type tomato plants; *sl3-b*: *slpao3-b* mutants.

**Figure S10.** Polyamine levels in *slpao3* mutants and wild-type plants under drought stress conditions. Polyamine levels were determined in the second and third leaves from the apex of 4-week-old *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, and wild-type (WT) tomato plants. Samples were collected from well-irrigated plants ( $-0.3\ \text{MPa} < \Psi_{\text{xy}} < -0.1\ \text{MPa}$ ) and plants for which irrigation was interrupted for 12 days ( $-1.0\ \text{MPa} < \Psi_{\text{xy}} < -0.7\ \text{MPa}$ ). Values are means of at least three different plants  $\pm$  SE. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P<0.05$ ). Put: putrescine; Spd: spermidine; Spm: spermine; T-Spm: thermospermine.

**Figure S11.** Levels of leaf wilting in *slpao3* mutants under drought stress conditions. For drought stress, irrigation of 6-week-old plants was completely interrupted and the severity of wilting symptoms was monitored for 15–30 days after onset of drought. A scale of leaf wilting was arbitrarily set up: 10–20%, very mild

wilting in basal leaves; 20–40%, mild wilting in basal internodes; 40–60%, moderate turgor loss in lower internodes; 60–80%, high turgor loss in upper and lower internodes; 80–100%, very high to complete turgor loss of all leaves. A representative experiment is shown. WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutants; *sl3-b*: *slpao3-b* mutants. The wilting levels of the two *slpao3* mutants were statistically different from those of the wild-type (WT) plants for at least 26 days after the start of water withholding (one-way ANOVA;  $P<0.05$ ;  $n\geq 5$  per genotype).

**Figure S12.** Transpiration rate of the different tomato genotypes at various soil humidity conditions. Transpiration rate was determined through measurement of  $\text{CO}_2/\text{H}_2\text{O}$  gas exchange in leaves of the different tomato genotypes. The slope of the correlation curve of wild-type plants (WT) is statistically different from those of *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, as evidenced by ANCOVA analysis. The insert shows transpiration rate under conditions of high soil humidity ( $\geq 70\%$ ).

**Figure S13.** Stomatal index, stomatal density, root surface area, leaf dry matter content (LDMC), and leaf water potential at turgor loss point ( $\Psi_{\text{tip}}$ ) in leaves of *slpao3* mutants and wild-type (WT) tomato plants. Letters above the box and whiskers plots indicate the statistical analysis (one-way ANOVA;  $P<0.05$ ).

**Figure S14.** Measurement of stomatal response to T-Spm treatment. All leaves of 4-week-old tomato plants were sprayed with  $1\ \text{mM}$  T-Spm and stomatal conductance was measured through measurement of  $\text{CO}_2/\text{H}_2\text{O}$  gas exchange. Values obtained after 1 h of treatment are shown. Values are means from three different plants  $\pm$  SEM.

**Figure S15.** Estimation of maximum and mean vessel length in the different tomato genotypes. Letters above the scatter dot blots indicate the statistical analysis (one-way ANOVA;  $P<0.05$ ). WT: wild-type tomato plants; *sl3-a*: *slpao3-a* mutants; *sl3-b*: *slpao3-b* mutants.

**Table S1.** Mutated sequence in *slpao3* mutants.

**Table S2.** Putative genome-wide off-target sites.

**Table S3.** Plasmids used for construct preparation and plant transformation.

**Table S4.** Sequence of oligonucleotides used in this study.

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