

**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 colour.

**>SlPAO4 (*Solanum lycopersicum*)**

MVSQKPKIVIIGAGIAGLTAACKLYTTQNTNELFEVCVVEGGNRIGGRIFTTEFCGDRVEMGATWIHGIEGNP  
IYKIAQEINGFETDKPWDSMGGKVDKCLTITEEGHEVHSSFVNSISNFFNNLLEFSSGEGDFDGGVGRKIVES  
LNLEENGRVDKISMGSYLRKGLFYGWGLKK**DQGNVESLNLENDQENVESFNLEDGGIEKEQENVESFNLES**  
**VENDQEKVEGFNLEHAGTEKDQENVESFNLGHVGIKQENVESFNLD****DDDDVEEDQENVESFNLDGGVEKDQ**  
**ENVESFNLDGGVEKDQENVESFNLD****SGSTEKDGENVEVFENWS**SRKALEEGI FAMFENIHRHYSSAGDLGTLT  
FNGESEYCNFPGDEITIAKGYSSIVESLASVLPGLIQLGRKVKIEWQLETS DGNKPKVLHFS DGSVMYADH  
VIVTVSLGVLKQGIREDSLSFSPPLPKFKTEAISRLGFGVVDKVFQLPTPHHDGMNFPNMMMFVHQSNACKL  
NPKIPLWIRRTTLTHPVYPESRVVVSWFAGEEALKVETLDDDEIIEGVSITMSEFLSNTKHYKNSIKFSKVLK  
CKWGTDPFLFSGSYTHIAVGSSGDDLDAMAEPLPKEISDDKNSKKS PRLQVLFAGEATSRNYYSTTHGAYLTGL  
REANRLLEYFQCVDV

**>SpimPAO4 (*Solanum pimpinellifolium*)**

MVSQKPKIVIIGAGIAGLTAACKLYTTQNTNELFEVCVVEGGNRIGGRIFTTEFCGDRVEMGATWIHGIEGNP  
IYKIAQEINGFETDKPWDSMGGKVDKCLTITEEGHEVHSSFVNSISNFFNNLLEFSSGEGDFDGGVGRKIVES  
LNLEENGRVDKISMGSYLRKGLFYGWGLKK**DQGNVESLNLENDQENVESFNLEDGGIEKEQENVESFNLES**  
**VENDQEKVEGFNLEHAGTEKDQENVESFNLGHVGIKQENVESFNLD****DDDDVEEDQENVESFNLDGGVEKDQ**  
**ENVESFNLDGGVEKDQENVESFNLD****SGSTEKDGENVEVFENWS**SRKALEEGI FAMFENIHRHYSSAGDLGTLT  
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NPKIPLWIRRTTLTHPVYPESRVVVSWFAGEEALKVETLDDDEIIEGVSITMSEFLSNTKHYKNSIKFSKVLK  
CKWGTDPFLFSGSYTHIAVGSSGDDLDAMAEPLPKEISDDKNSKKS PRLQVLFAGEATSRNYYSTTHGAYLTGL  
REANRLLEYFQCVDV

**>SpPAO4 (*Solanum pennellii*)**

MVSEKPKIVIIGAGIAGLTAACKLYTTQNSNELFEVCVVEGGNRIGGRIFTTEFCGDRVEMGATWIHGIEGNP  
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**VENDQEKVESFNLEHVGIKQENVESFNLEHVGIKQENVESLNLD****DSGVEKDQEKVKSFYLDGGIEYDQ**  
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**>ScPAO4 (*Solanum chilense*)**

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CKWGTDPFLFSGSYTHIAVGSSGDDLDAMAEPLPKDISDDKNSKRSRRLQVLFAGEATTRNYYSTTHGAHLTGI  
REANRLLEYFQCVDV

**>StPAO4 (*Solanum tuberosum*)**

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YPESRVVVSWFAGEEAMKVETLDDEEIEIGVSI TMSEFLANTEHFKNSIKFSKVLKCKWGTDPFLFLGSYTHIA  
VGSSGNDLDAMAEPLPKEISDDKNSKKSRLQVLFAGEATSRNYYSTH GAYLTGLREANRLLEHFQCVDV

**>ScmPAO4 (*Solanum commersonii*)**

MVSQKPKIVIIGAGIAGLTAACKLYTTQNSNELFELCVVEGGNRIGGRIFTTEFCGDRVEMGATWIHGIEGNP  
IYNIAQEI NGFETDKPWDSMGGKVDKVTIT EDGHEVHSSFVNSVSNFFSNLLEFSSDEGDFDGGVGRKIVES  
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**LESGGVEN****DQENVEFN****LEDRCFN****LDNVGVGK****DKENVESFN****LDG****GVVEVQENVESFN****LDG****SGIEK****DQENIET**  
**FNLDG****SGIEK****DQDNVESFN****FENVGIEK****DGENVDV****FENWS****SRKALEE****GI****FAMFENI****HRHYSS****SAGDL****GT****LAFNGES**  
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DPLFLGSYTHIAVGSSGDDLAMAEPLPKEISDDKNSKKSRLQVLFAGEATSRNYYSTH GAYLTGLREANR  
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**>CaPAO4 (*Capsicum annum*)**

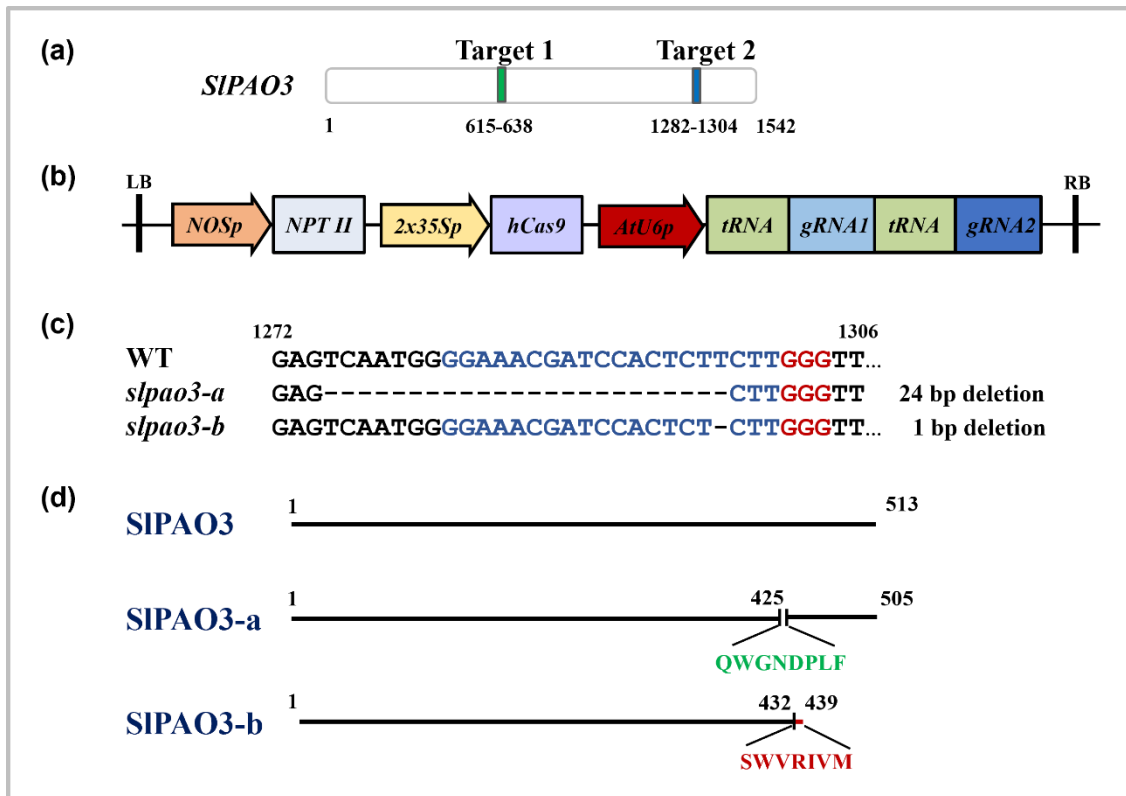
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**SEK****DHENDESFN****LDY****GGSEK****DQENVEV****FENWS****SRK****S****LE****EGV****FAMFENI****HRHYSS****SAGDL****GT****LD****FNGHSEY****ID****FP****G**  
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**G****V****S****T****T****M****S****E****I****L****A****N****A****K****H****F****K****N****S****N****A****A****A****E****S****E****S****F****I****K****F****T****K****V****L****K****C****K****W****G****T****D****P****L****F****L****G****S****Y****T****H****I****A****V****G****S****S****G****D****D****L****D****A****M****A****E****P****L****P****E****K****S**  
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**>CbPAO4 (*Capsicum baccatum*)**

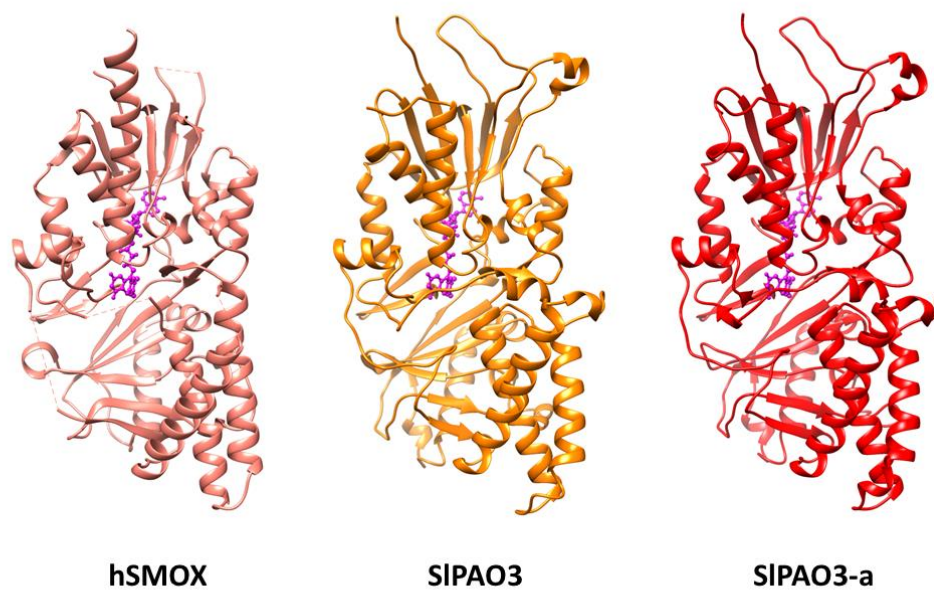
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LRIENGGGI ERVSI GSFLR KGLFEFYWGLR**KDQENVESFN****LDG****GSEK****DHKNGEI****FNLD****NGGSEK****DQEHVKSFN**  
**LDDG****GSEK****DHENDESFN****LDK****GENVESFN****LDG****GSEK****DHENDESFN****LDY****GGSEK****DQENVEV****FENWS****SRK****S****LE****E**  
**V****F****A****M****F****E****N****I****H****R****H****Y****S****S****A****G****D****L****G****T****L****D****F****N****G****H****S****E****Y****I****D****F****P****G****D****D****I****T****I****A****K****G****F****S****S****V****I****E****S****L****G****S****V****L****P****S****G****L****I****Q****L****G****R****K****V****T****K****I****E****W****Q****L**  
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**>CcPAO4 (*Capsicum chinense*)**

MLSQKPKIVIIGAGIAGLAAACKLYTTQNFNELFELCVVEGGNRIGGRIFTNEFCGDKVEMGASWIHGIDGNP  
IYKIAKDINAFENDEPWPDLGGKMNKMAIT EDGHEVNSSIVNNISSFFKNLLDFASGEGDFGGGIGGKIVER  
LRIENGGGIDKVSIGSFLR KGLFEFYWGLR**KDQENVESFN****LDG****GSEK****DHENGEI****FNLD****NGGSEK****DQEHVKSFN**  
**LDDG****GSEK****DHENVESFN****LDG****GSEK****IQENVESFN****LDG****GSEK****DHENDESFN****LDY****GGSEK****DQENVEV****FENWS****SRK**  
**S****LE****E****G****V****F****T****M****F****E****N****I****H****R****H****Y****S****S****A****G****D****L****G****T****L****D****F****N****G****H****S****E****Y****I****D****F****P****G****D****D****I****T****I****A****K****G****F****S****S****V****I****E****S****L****G****S****V****L****P****S****G****L****I****Q****L****G****R****K****V****T****K****I**  
**E****W****Q****L****E****N****D****D****G****N****K****P****V****K****L****H****F****S****D****G****S****V****M****Y****A****D****H****V****I****V****T****I****S****L****G****V****L****K****Q****G****I****R****E****D****S****S****L****F****S****P****P****L****P****S****F****K****T****D****A****I****S****R****L****G****F****G****V****D****K****L****F****L**  
**Q****L****S****P****T****H****H****E****N****V****G****D****G****M****K****F****P****N****M****M****V****F****H****Q****P****D****A****K****L****N****N****P****K****I****P****F****W****I****R****R****T****T****L****V****H****P****V****H****P****E****S****R****V****V****M****L****F****F****A****G****E****E****A****L****K****V****E****S****L****D****D**  
**E****E****I****E****G****V****S****T****T****M****S****E****I****L****A****N****A****K****H****F****K****N****S****N****A****A****A****E****S****E****S****F****I****K****F****T****K****V****L****K****C****K****W****G****T****D****P****L****F****L****G****S****Y****T****H****I****A****V****G****S****S****G****D****D****L****D****S****M****A****E****P****L**  
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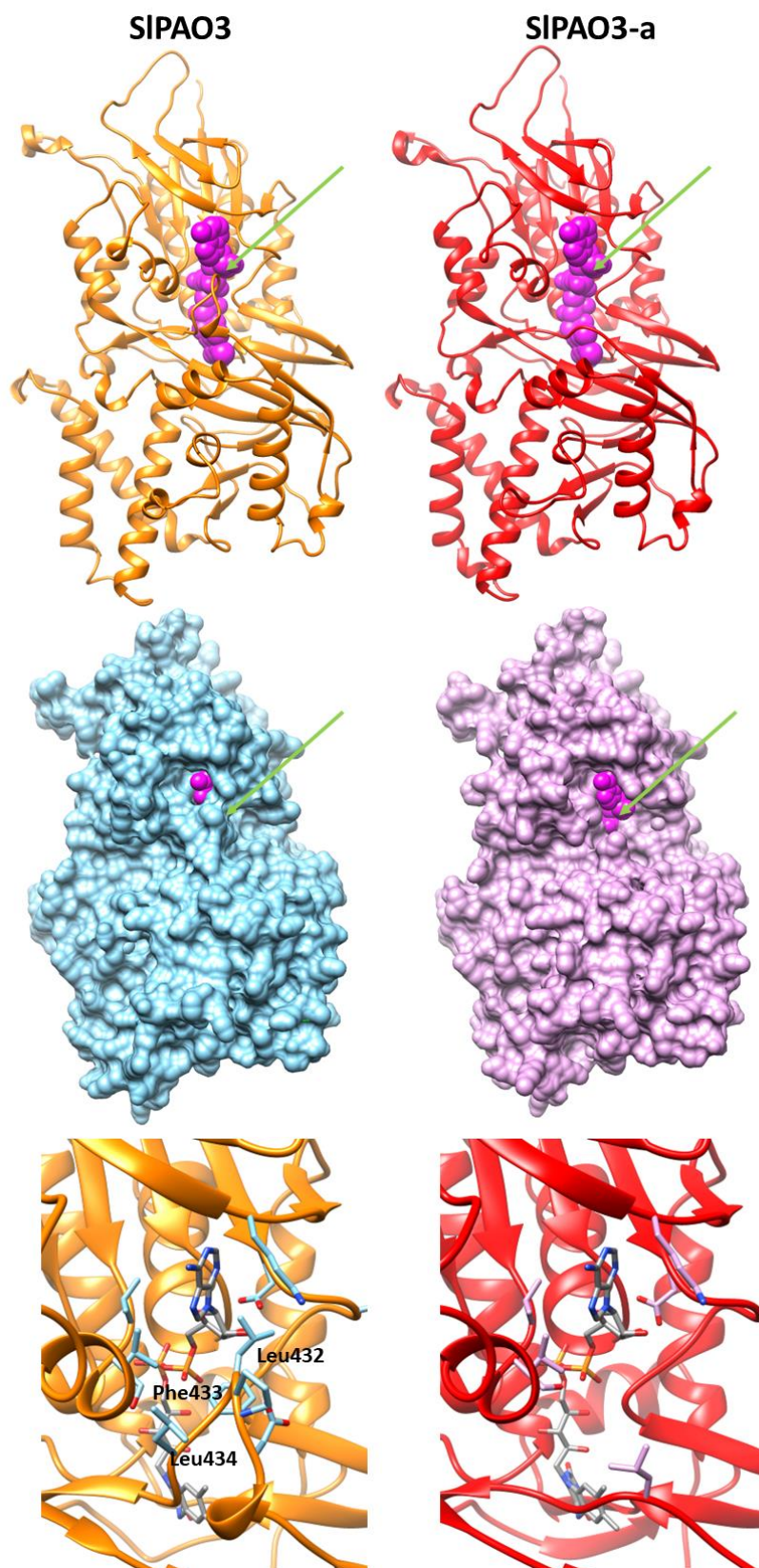


**Figure S2.** Schematic diagram of *SIPAO3* gene, vector construction, and CRSIPR/Cas9-mediated mutagenesis. (a) Schematic diagram of *SIPAO3* gene and position of target sites 1 and 2. (b) Scheme of the binary vector constructed for CRISPR/Cas9-mediated *SIPAO3* mutagenesis in which the relative position of the *NOSp::NPTII*, *2x35Sp::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; *2x35SP*: 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 coloured in magenta.



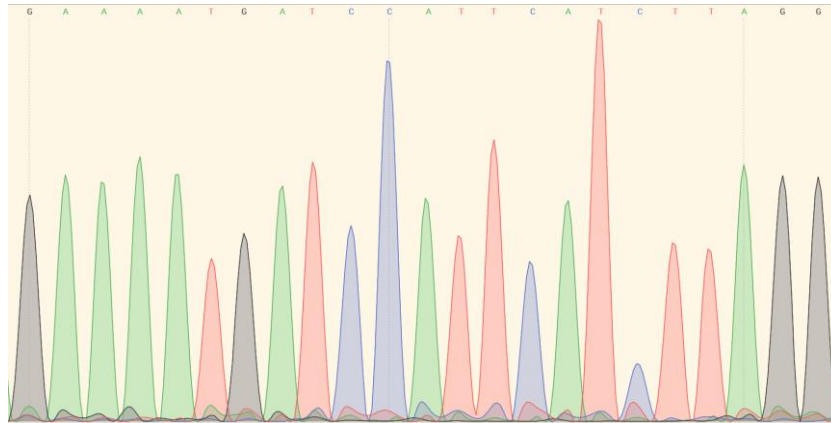


**Figure S4.** Comparison of SIPAO3 and SIPAO3-a structural models showing the changes affecting the FAD cofactor molecular environment. Top panels, ribbon representation; central panels, molecular surface representation; bottom panels, enlarged view showing the hydrophobic plug of the FAD binding pocket,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

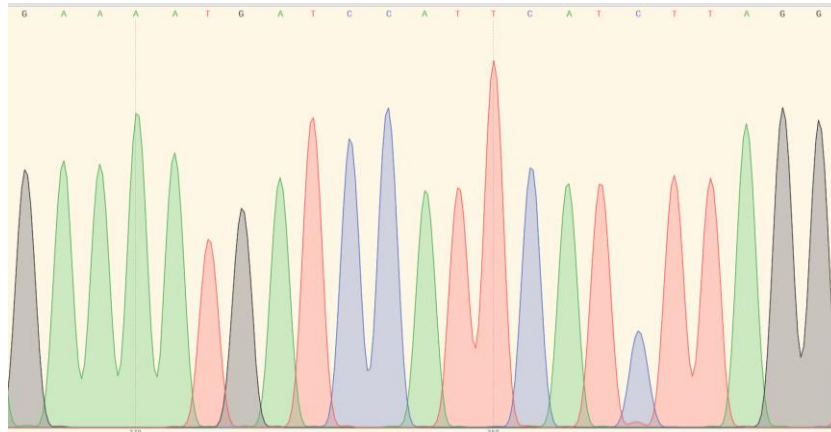
**Figure S5.** Sequence analysis of the top six potential genome-wide off-target sites in the *slpao3* mutants. The genomic regions flanking the selected putative off-target sites (OFF-A to OFF-F; Table S2) were amplified from *slpao3-a* and *slpao3-b* mutants, and wild-type plants (WT). Resulting amplicons were sequenced and chromatograms at the putative off-target sites are shown. Mismatch nucleotides in respect to the *SLPAO3 gRNA2* (GGAAACGATCCACTCTTCTTGGG) are marked in red, and PAM sites are shown in blue.

**OFF-A** (GAAAATGATCCATTCATCTTAGG)

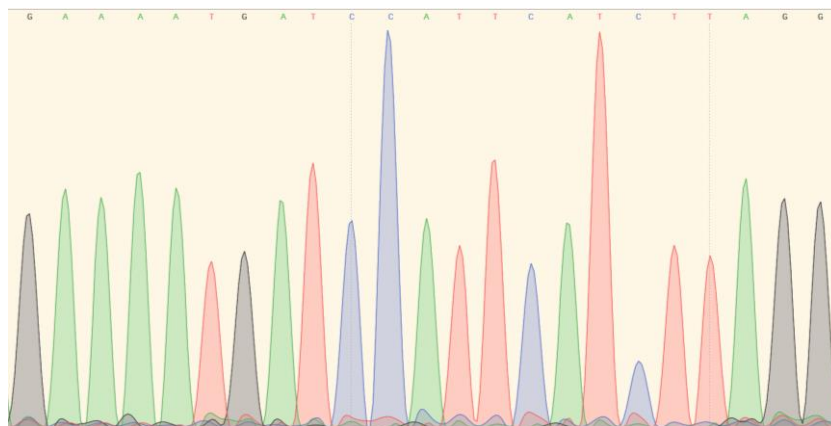
WT



*slpao3-a*

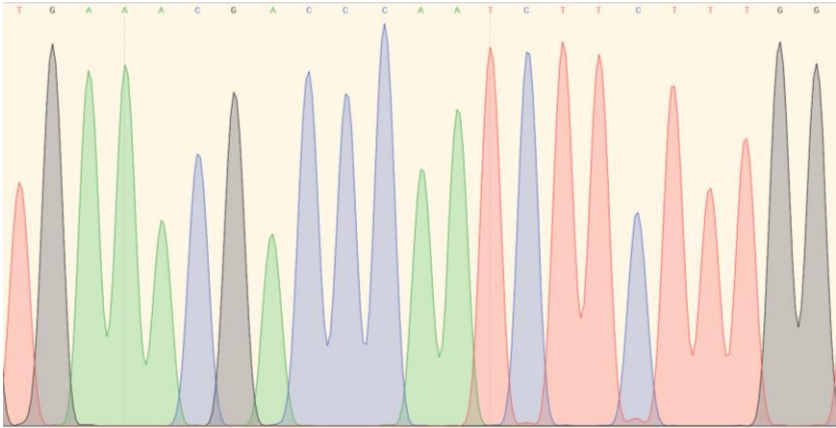


*slpao3-b*

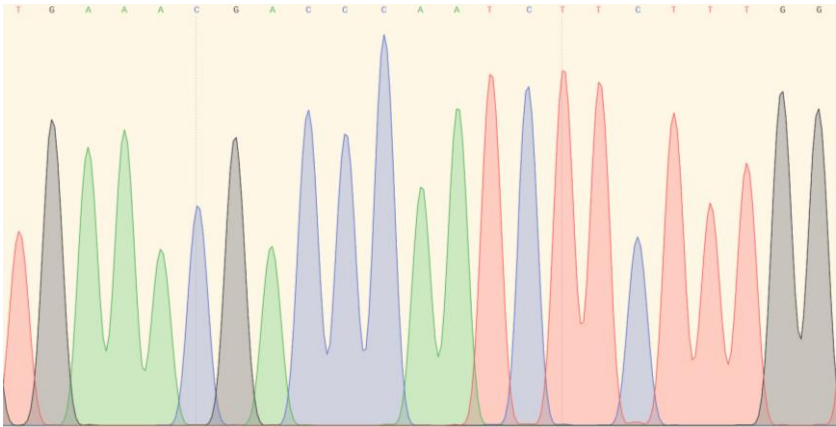


**OFF-B**(TGAAACGACCCAATCTTCTTTGG)

**WT**



*slpao3-a*



*slpao3-b*



**OFF-C** (ATAAATGATGCACTCTTCTTGG)

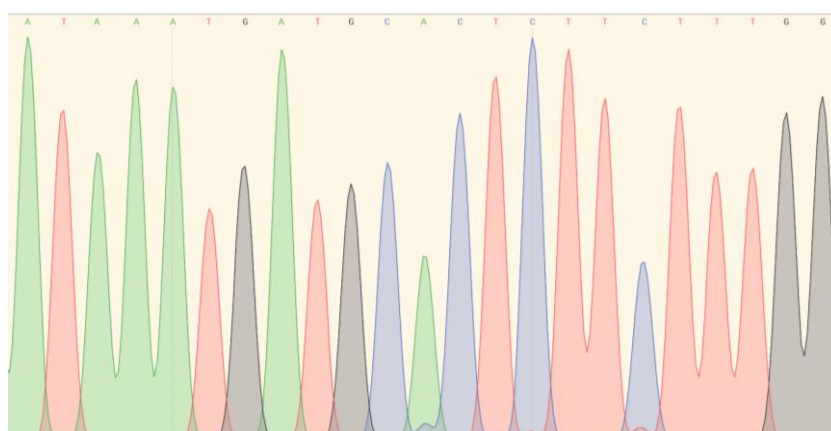
**WT**



*slpao3-a*



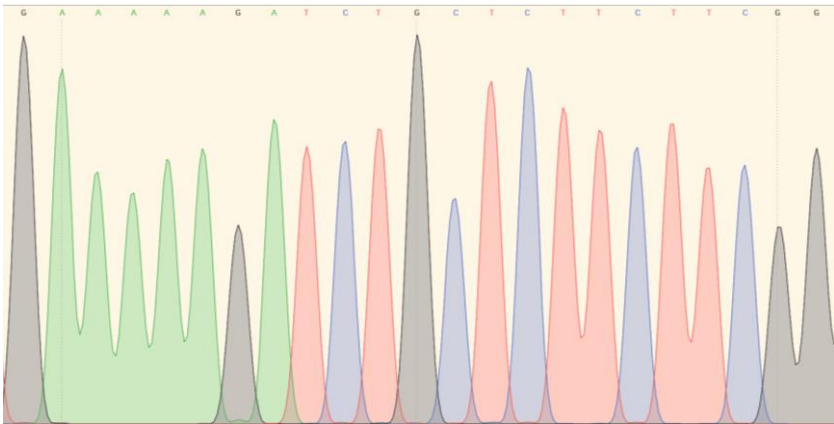
*slpao3-b*



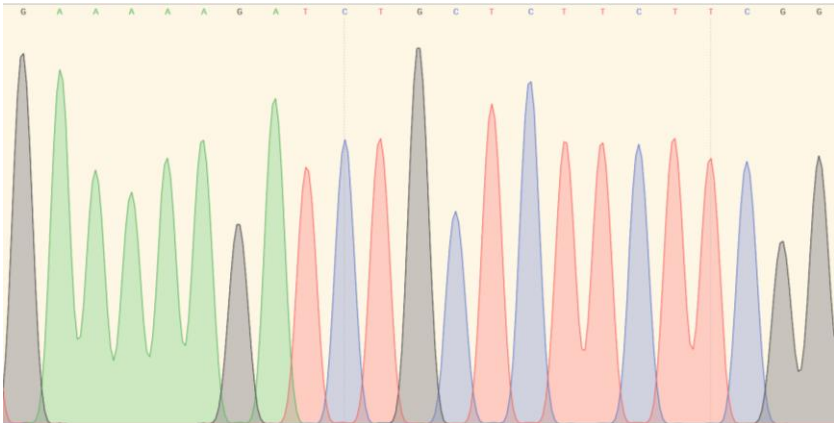


**OFF-D** (GAAAAAGATCTGCTCTTCTTCGG)

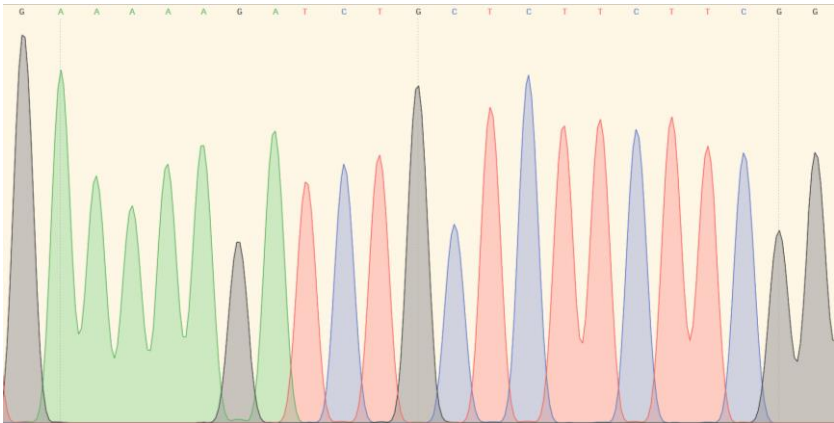
**WT**



*slpao3-a*

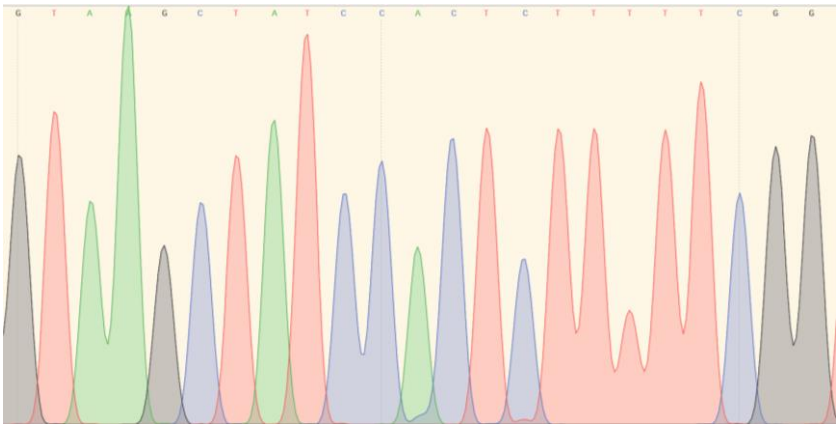


*slpao3-b*

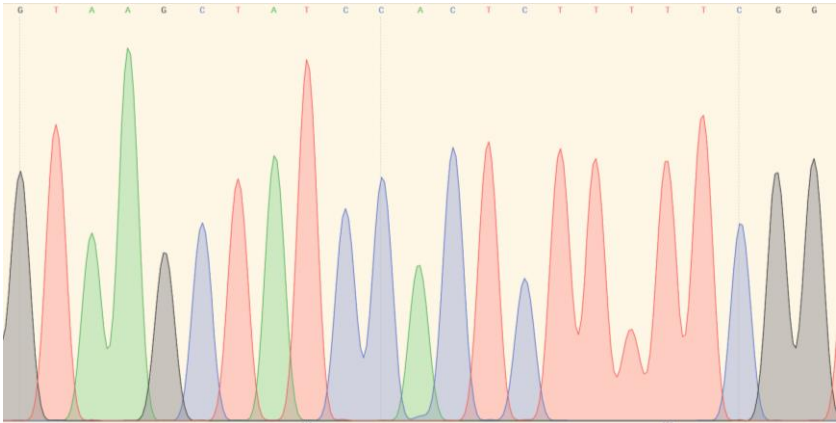


**OFF-E** (GTAAGCTATCCACTCTTTTTCGG)

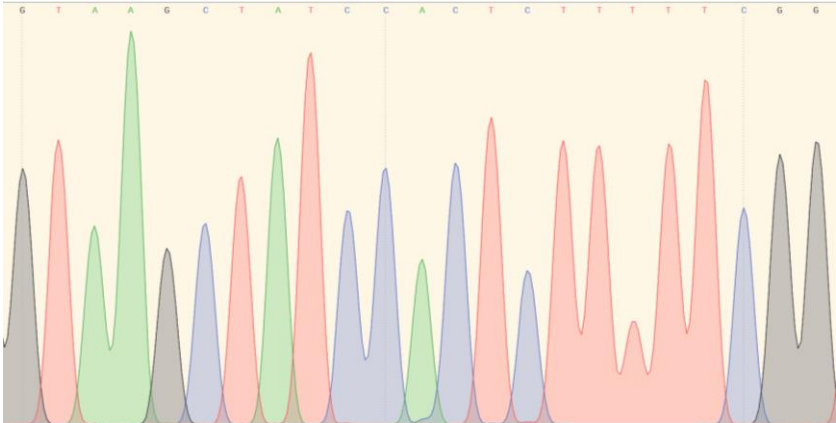
**WT**



*slpao3-a*

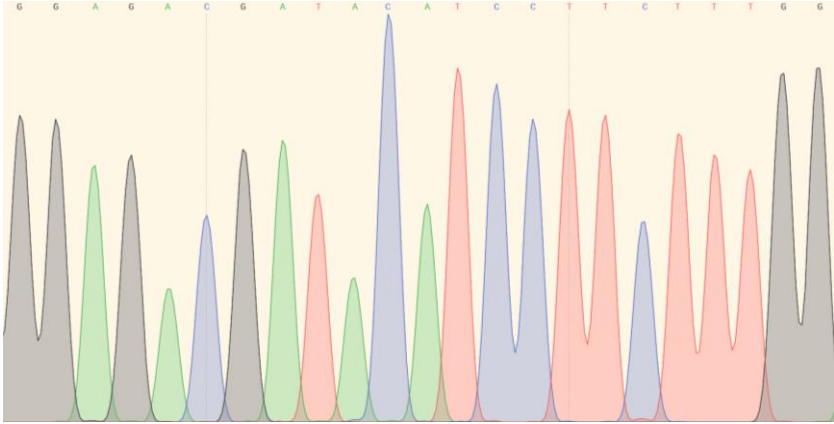


*slpao3-b*

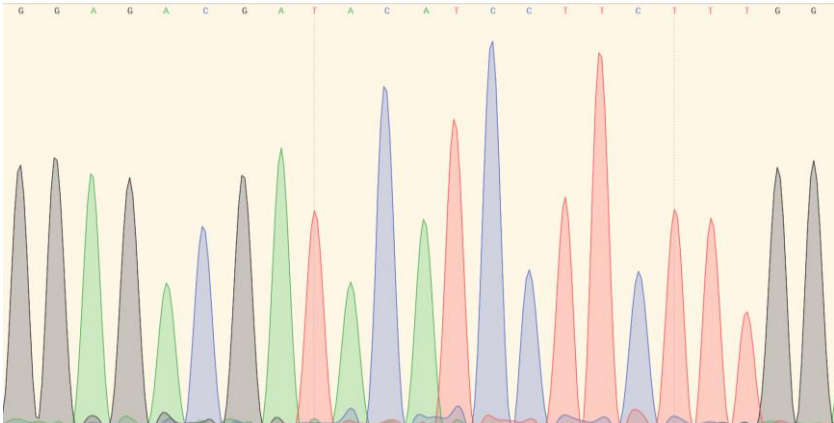


**OFF-F** (GGAGACGATACATCCTTCTTTGG)

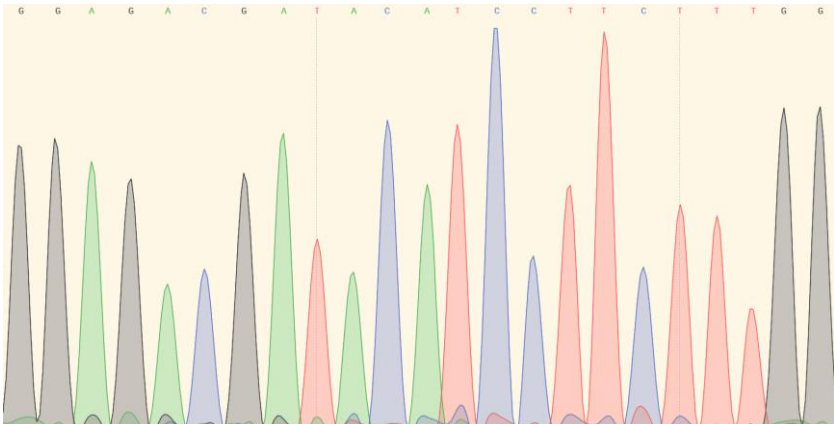
**WT**

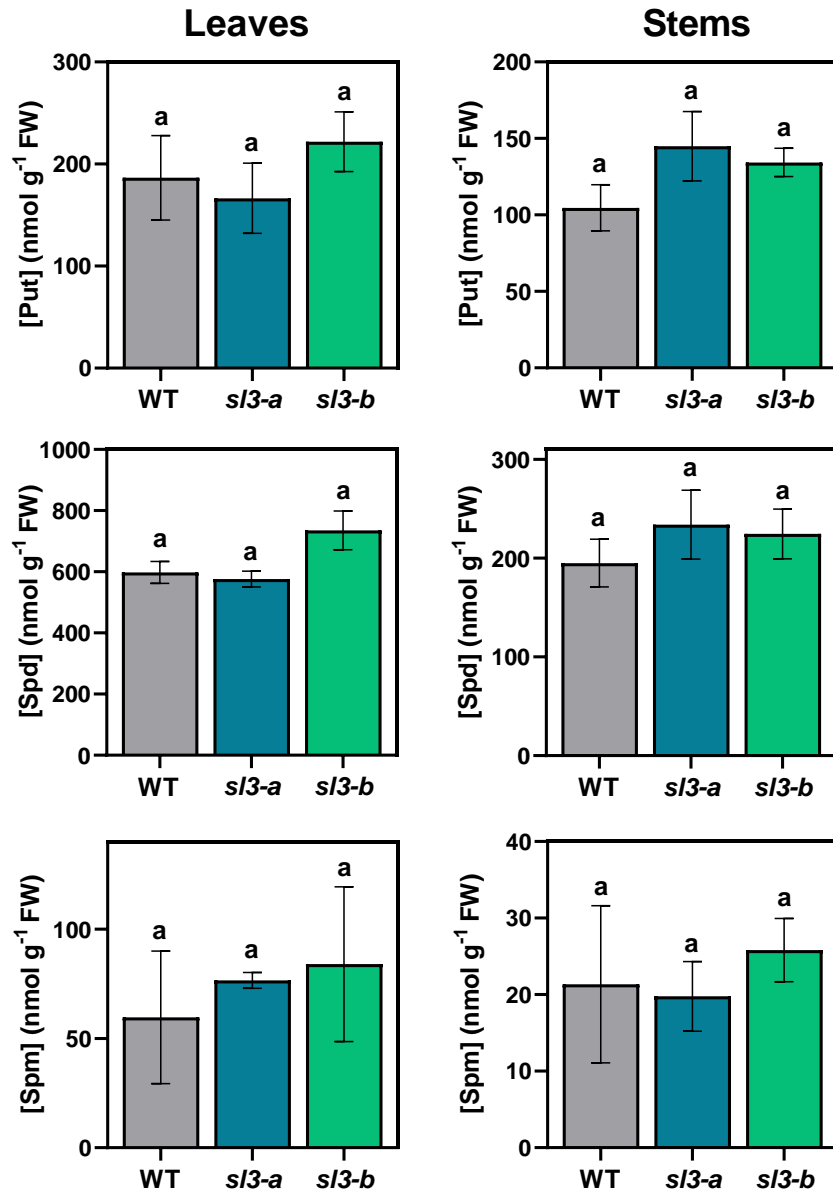


*slpao3-a*

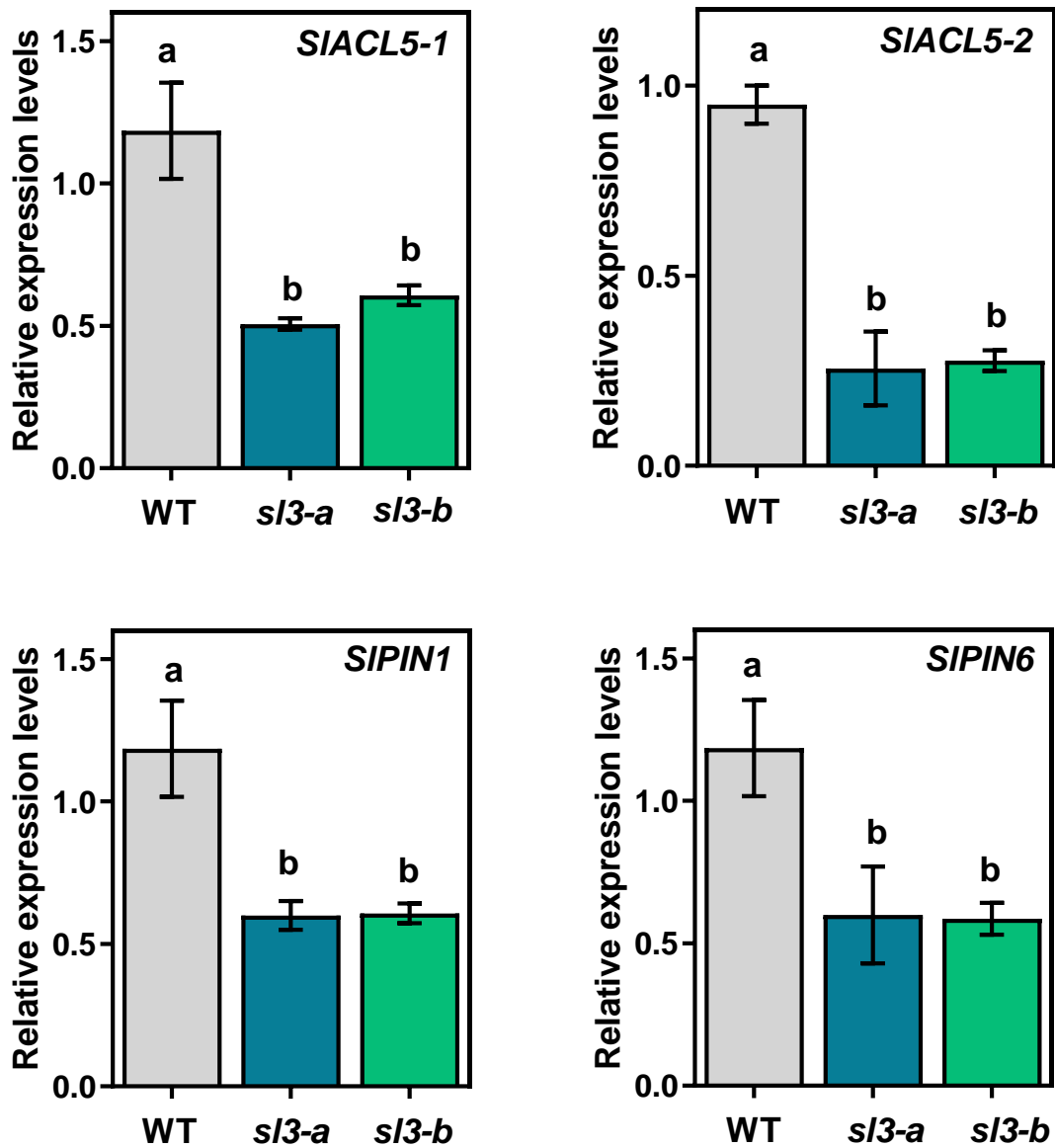


*slpao3-b*



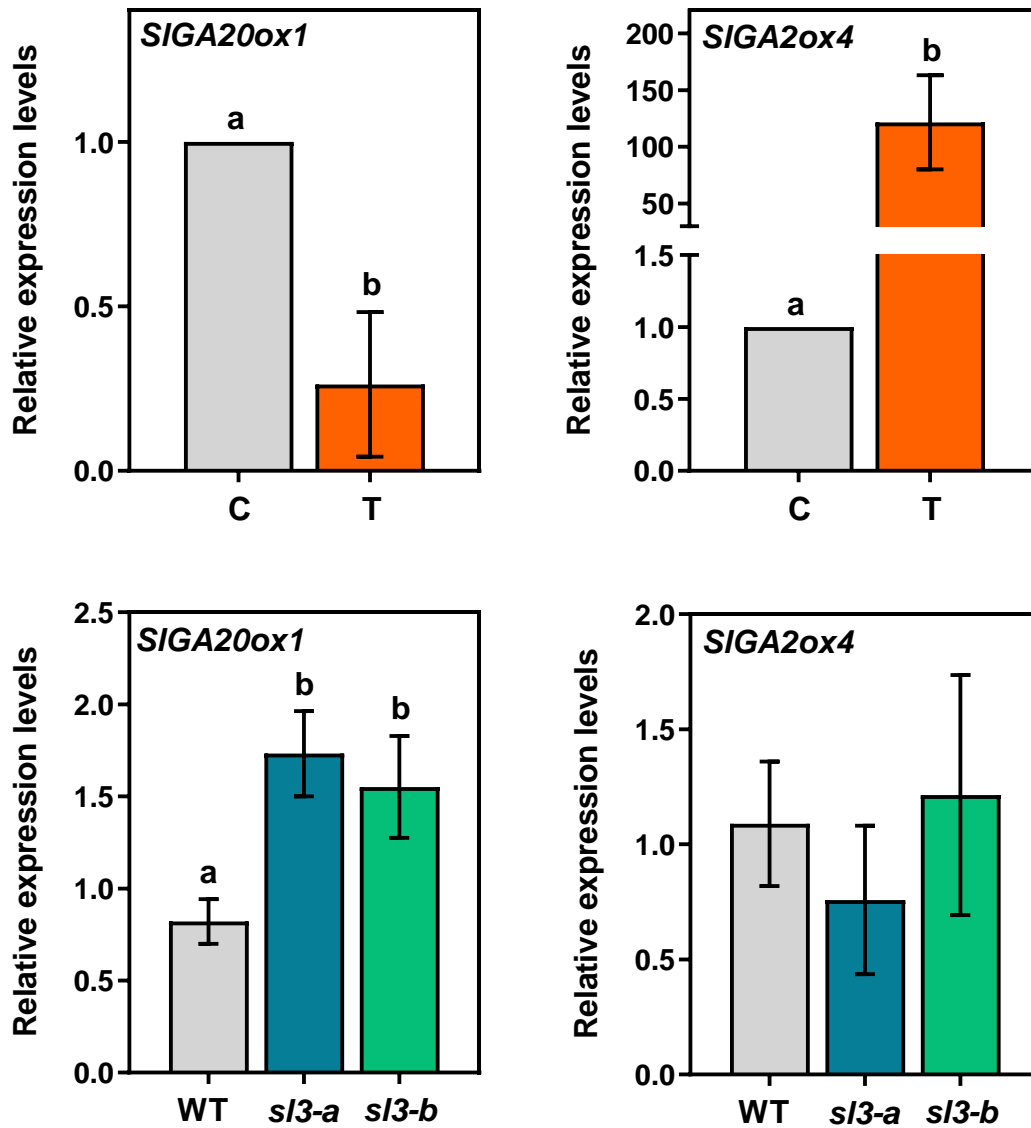


**Figure S6.** Putrescine, spermidine and spermine levels in *slpao3-a* and *slpao3-b* mutants. Polyamine levels were determined in leaves and stems of the third internode (from above) of 6-week-old *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, and wild-type (WT) tomato plants. Values are means 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.



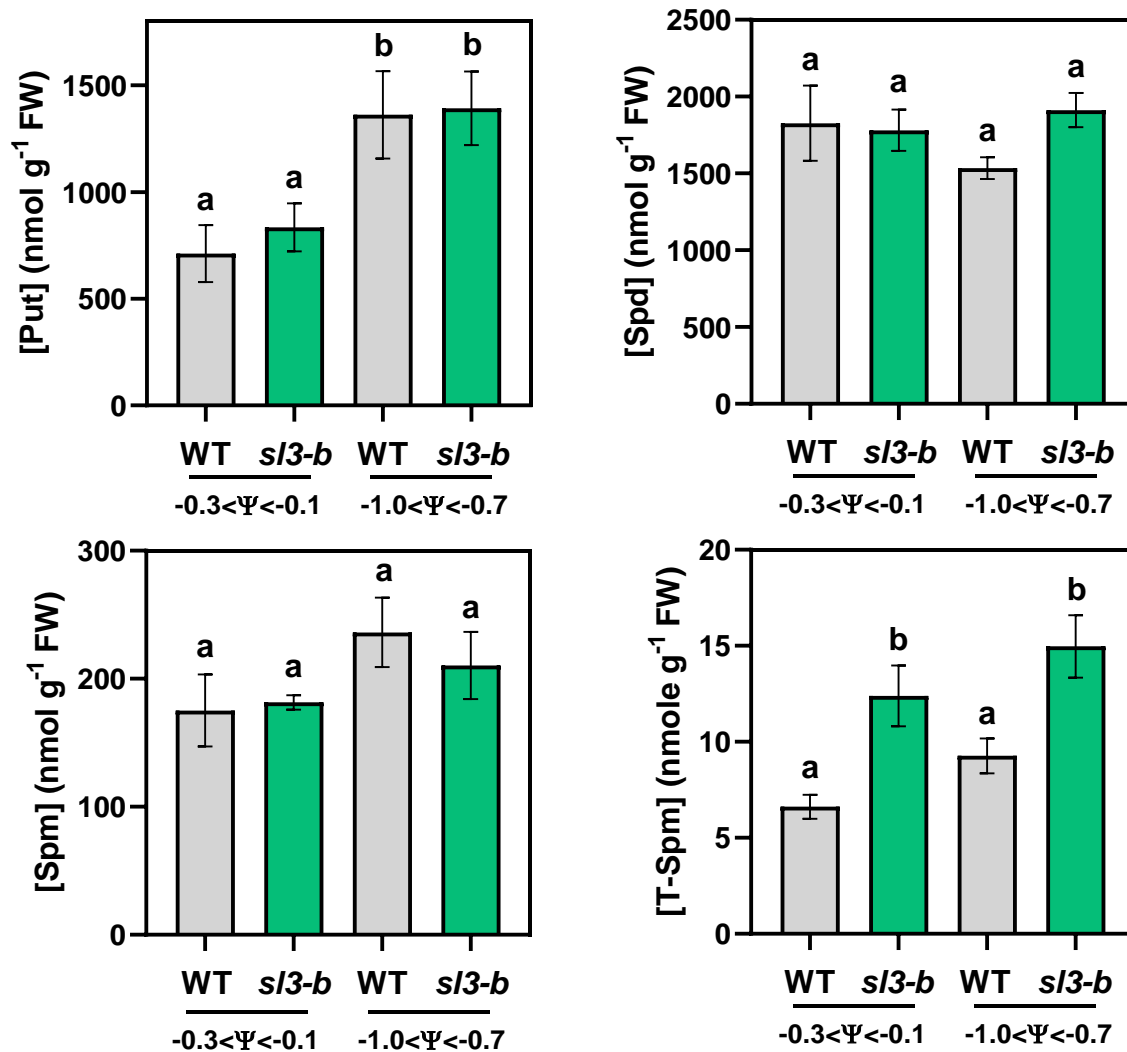
**Figure S7.** Expression levels of genes involved in T-Spm biosynthesis and auxin transport in the roots of *slpao3* mutants. Expression levels were analyzed by RT-qPCR in roots of 7-day-old *slpao3-a* (*sl3-a*), *slpao3-b* (*sl3-b*) and wild-type (WT) tomato genotypes. Relative expression levels are presented as fold-changes ( $2^{-\Delta\Delta C_t}$ ) from the control plants. Values are means of three independent repetitions  $\pm$  SEM. Different letters above the columns indicate statistically significant differences (one-way ANOVA;  $P < 0.05$ ).



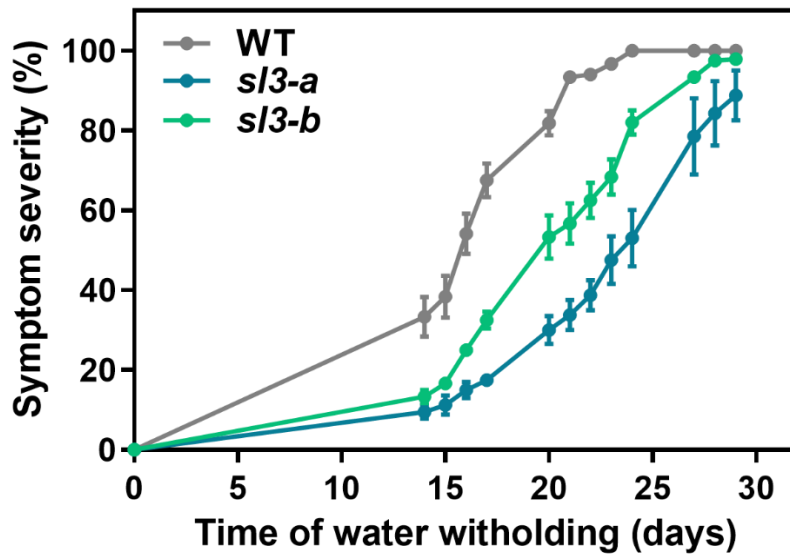


**Figure S8.** Effect of T-Spm on the expression levels of genes involved in gibberellin biosynthesis (*SIGA20ox1*) and deactivation (*SIGA2ox4*). Expression levels were analyzed by RT-qPCR in shoots of 7-day-old tomato wild-type seedlings treated (T) or not (C) with 100  $\mu$ M T-Spm for 24 h. Expression 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 ( $2^{-\Delta\Delta C_t}$ ) 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,  $P < 0.05$ ).

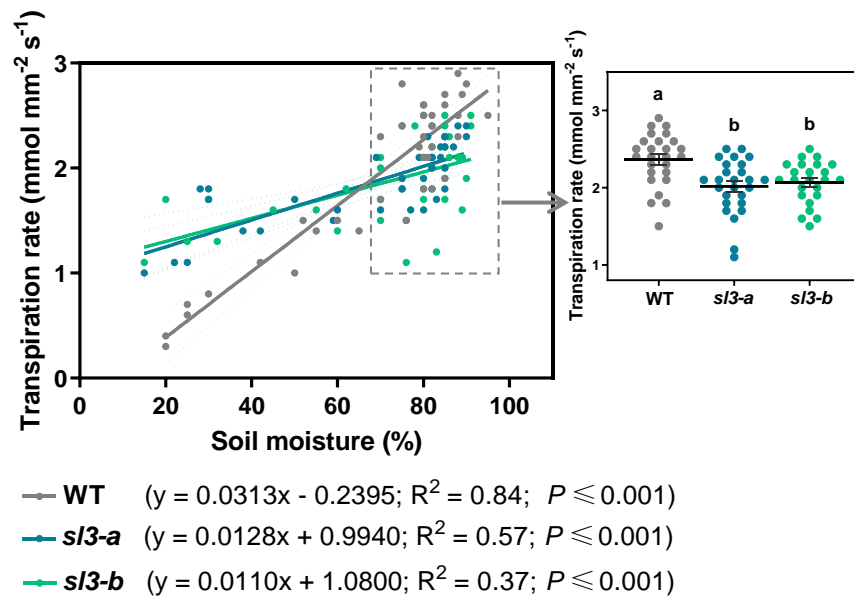




**Figure S10. Polyamine levels in *slpao3* mutants and wild-type plants under drought stress conditions.** Polyamine levels were determined in the second and third leaves from the apex of 4-week-old *slpao3-a* (*sl3-a*) and *slpao3-b* (*sl3-b*) mutants, and wild-type (WT) tomato plants. Samples were 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.

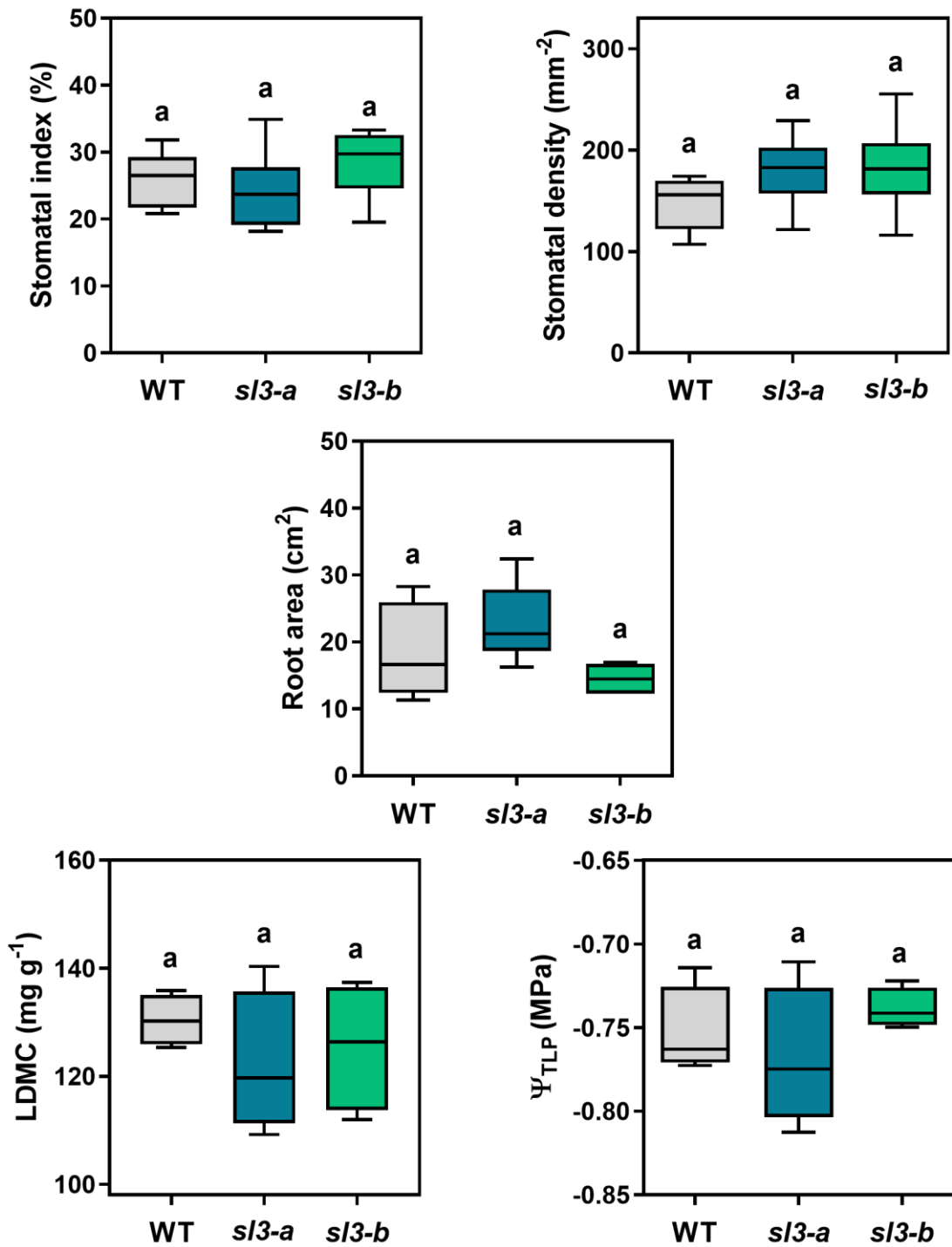


**Figure S11.** Levels of leaf wilting in *slpao3* mutants under drought stress conditions. For drought stress, irrigation of 6-week-old plants was completely interrupted and severity of wilting symptoms was monitored for 15-30 days after onset of drought. A scale of leaf wilting was arbitrarily set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

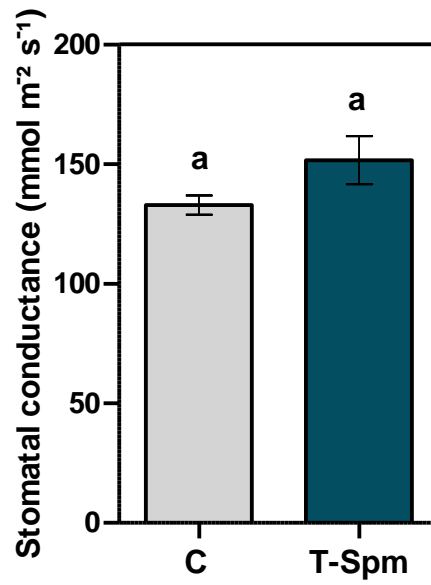


**Figure S12.** Transpiration rate of the different tomato genotypes at various soil humidity conditions. Transpiration rate was determined through measurement of  $\text{CO}_2/\text{H}_2\text{O}$  gas exchange in leaves of the different tomato genotypes. The slope of the correlation curve of wild-type 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 ( $\geq 70\%$ ).

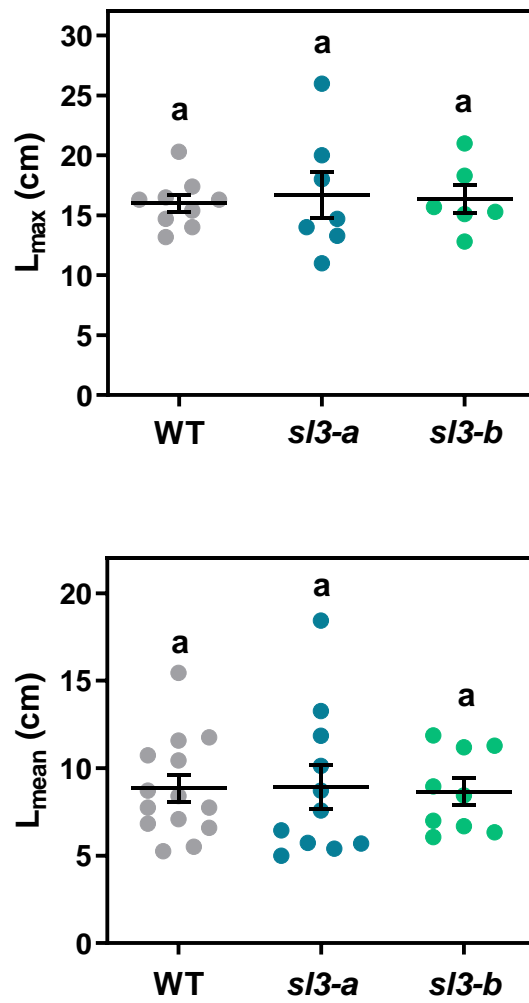




**Figure S13.** Stomatal index, stomatal density, root surface area, leaf dry matter content (LDMC) and leaf water potential at turgor loss point ( $\Psi_{tlp}$ ) in leaves of *slpao3* mutants and wild-type (WT) tomato plants. Letters above the box & whiskers plots indicate the statistical analysis (one-way 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 mM T-Spm and stomatal conductance was measured through measurement of CO<sub>2</sub>/H<sub>2</sub>O gas exchange. Values obtained after 1 h of treatment are shown. Values are means from three different plants ± 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.