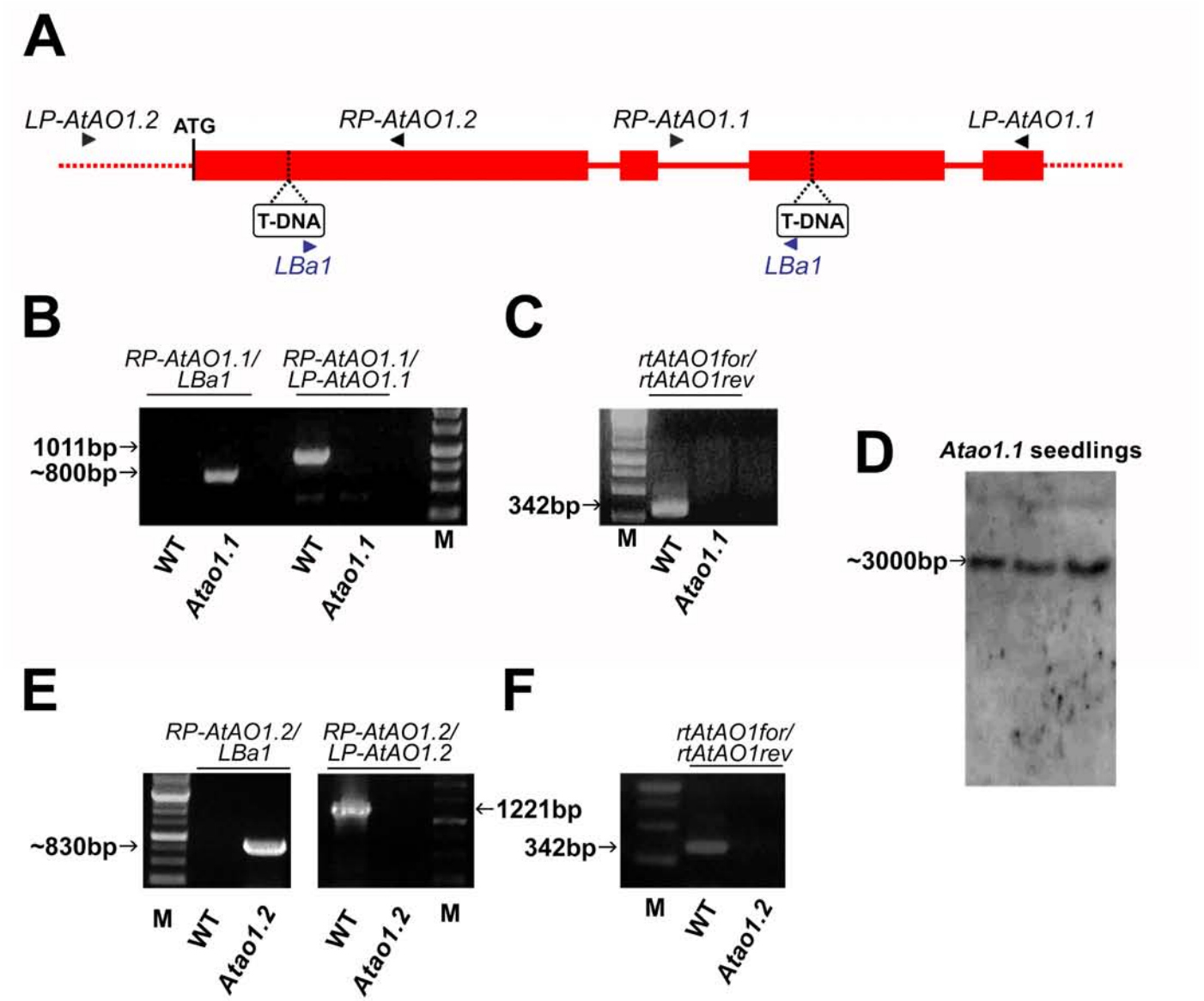
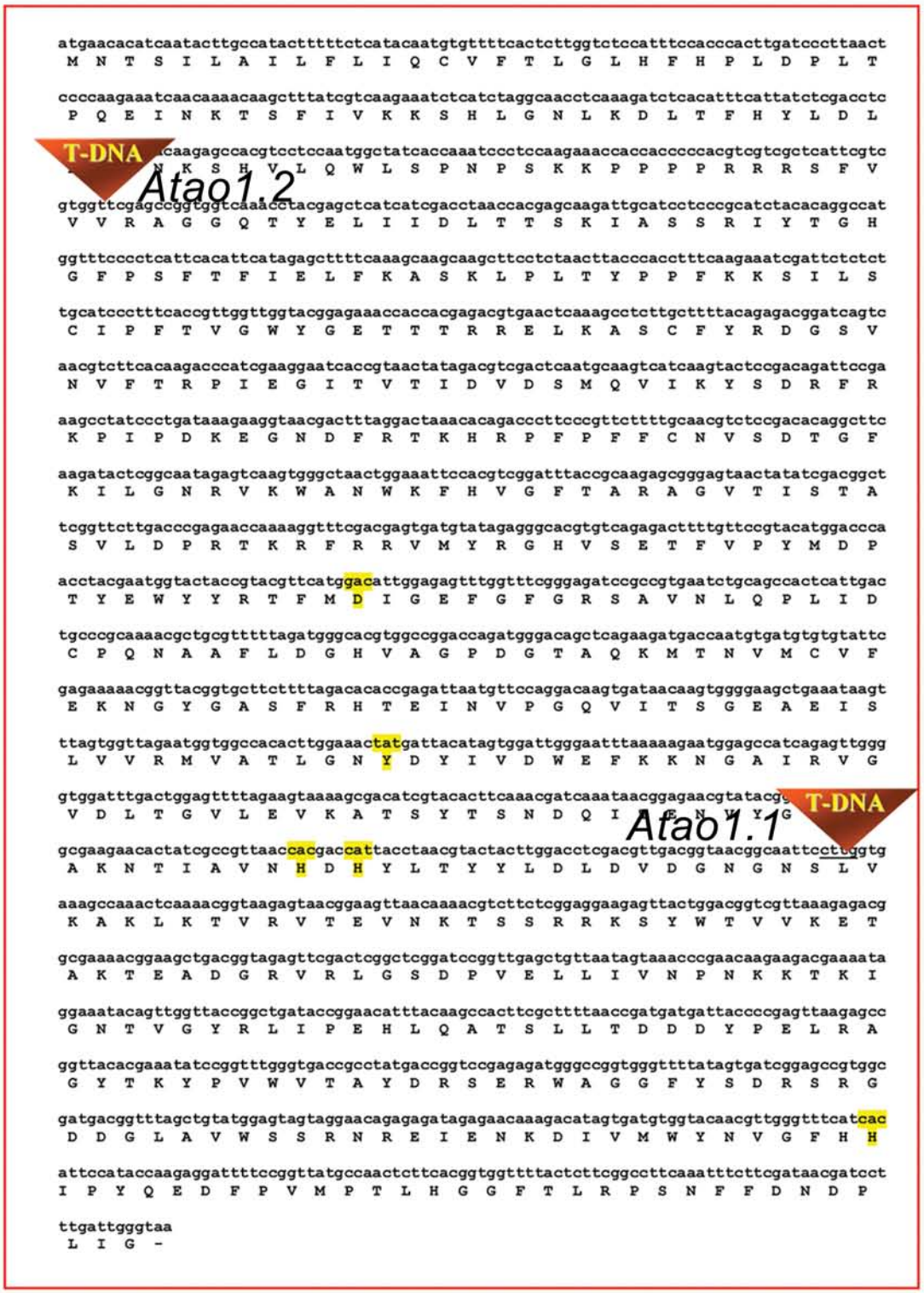
Supplemental Figure S1



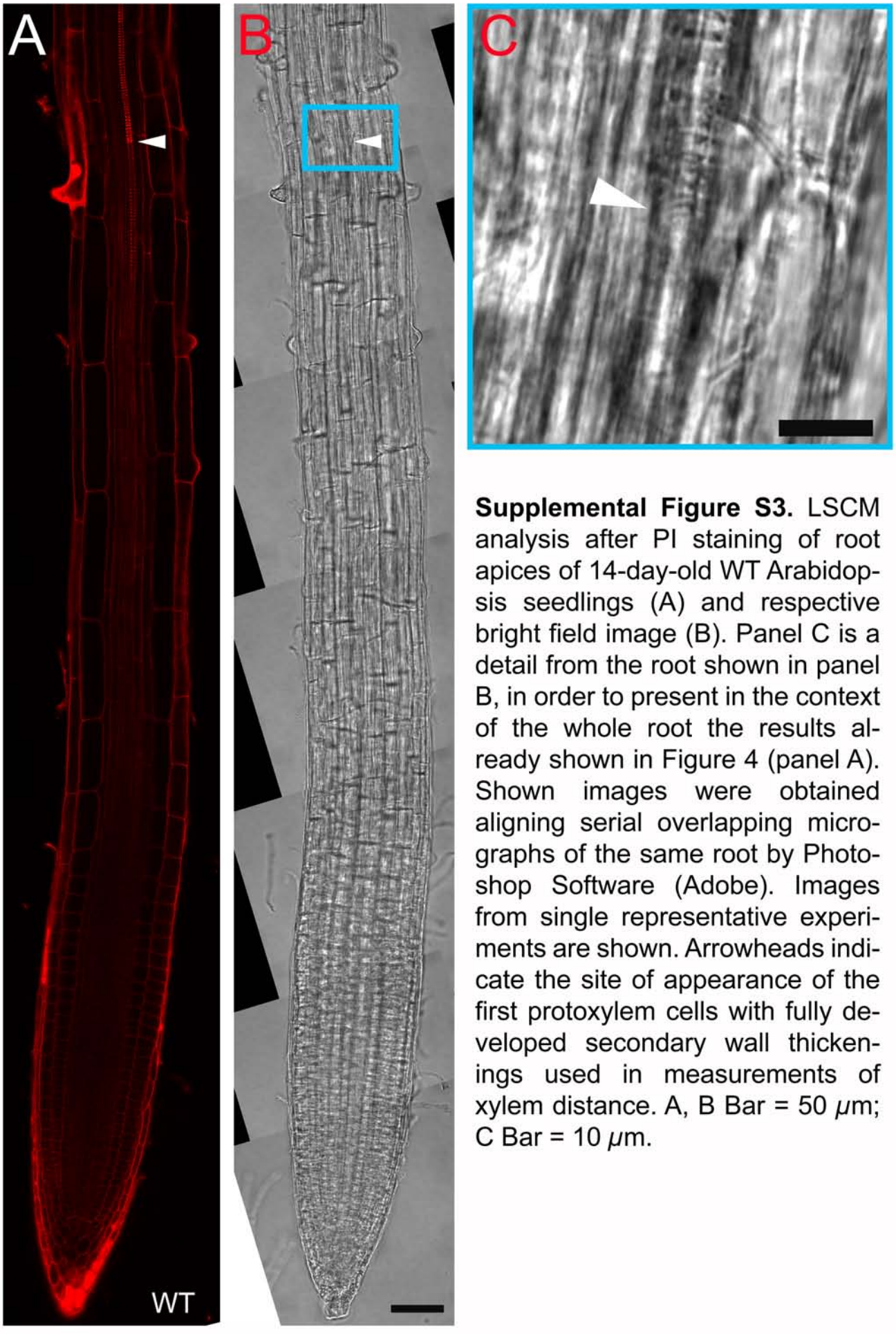
Supplemental Figure S1. Characterization of the two T-DNA insertional mutants for *AtAO1* (TAIR accession number: 2129519). A, Schematic representation of the T-DNA insertion sites in the *Atao1.1* (SALK_145639.55.25.X line; TAIR accession number: 1005841762) and *Atao1.2* (SALK_077391.40.85.X line; TAIR accession number: 4284859) mutants. *RP-AtAO1.1 / LP-AtAO1.1* and *RP-AtAO1.2 / LP-AtAO1.2* : gene specific primers; *LBa1*: T-DNA left border-specific primer for SALK T-DNA insertion lines. B and E, Genotyping of *Atao1.1* and *Atao1.2* mutants. In homozygous *Atao1* seedlings, T-DNA insertion in both alleles was demonstrated by the presence of the specific PCR fragment with *RP-AtAO1/LBa1* primers and the absence of amplification with *RP-AtAO1/LP-AtAO1* primers. Total DNA from WT was used as control. C and F, RT-PCR analysis of total RNA from *Atao1* seedlings. The absence of the full-length gene transcripts in homozygous *Atao1.1* and *Atao1.2* plants was determined using gene-specific primers (*rtAtAO1-for2/rtAtAO1-rev*). Total RNA from WT was used as control. D, Southern blot analysis of DNA extracted from three different *Atao1.1* seedlings after digestion with HindIII. M: DNA Marker.

Supplemental Figure S2



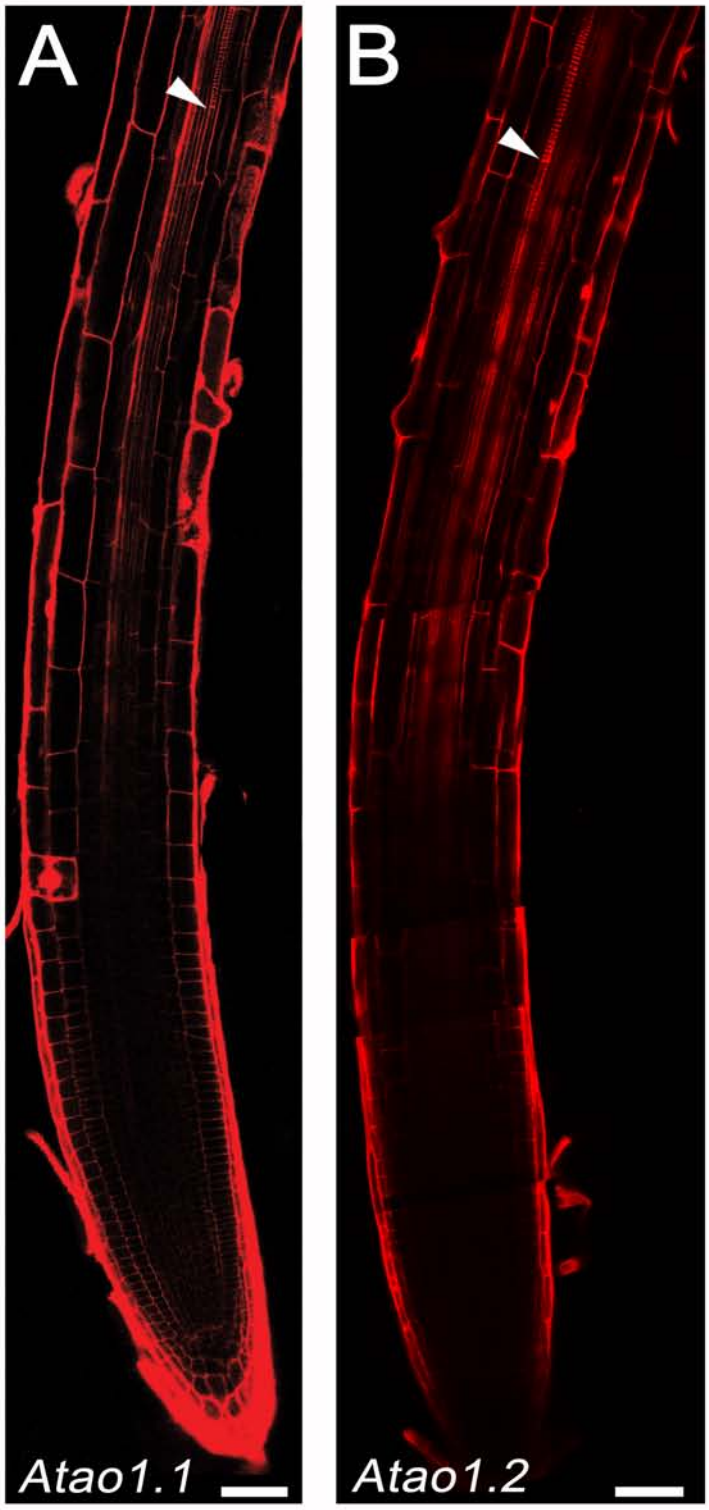
Supplemental Figure S2. Nucleotide and deduced amino acid sequences of *AtAO1* retrieved from TAIR database (TAIR accession number: 2129519). The red triangles represent the sites of T-DNA insertion in *Atao1.1* (SALK_145639.55.25.X line; TAIR accession number: 1005841762) and *Atao1.2* (SALK_077391.40.85.X line; TAIR accession number: 4284859) mutants according to TAIR database. The highlighted amino acids are the active site residues.

Supplemental Figure S3



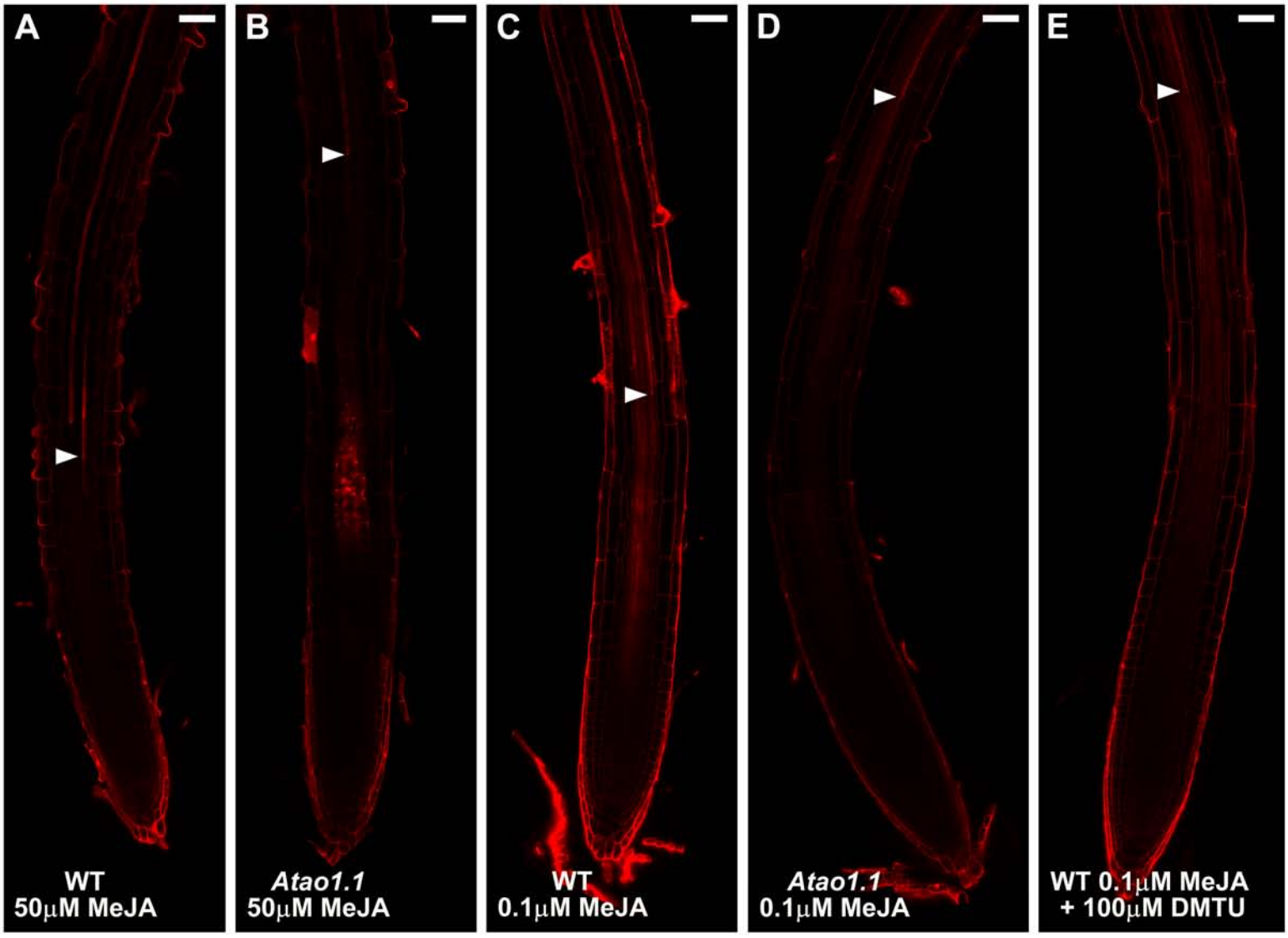
Supplemental Figure S3. LSCM analysis after PI staining of root apices of 14-day-old WT Arabidopsis seedlings (A) and respective bright field image (B). Panel C is a detail from the root shown in panel B, in order to present in the context of the whole root the results already shown in Figure 4 (panel A). Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. A, B Bar = 50 μm; C Bar = 10 μm.

Supplemental Figure S4



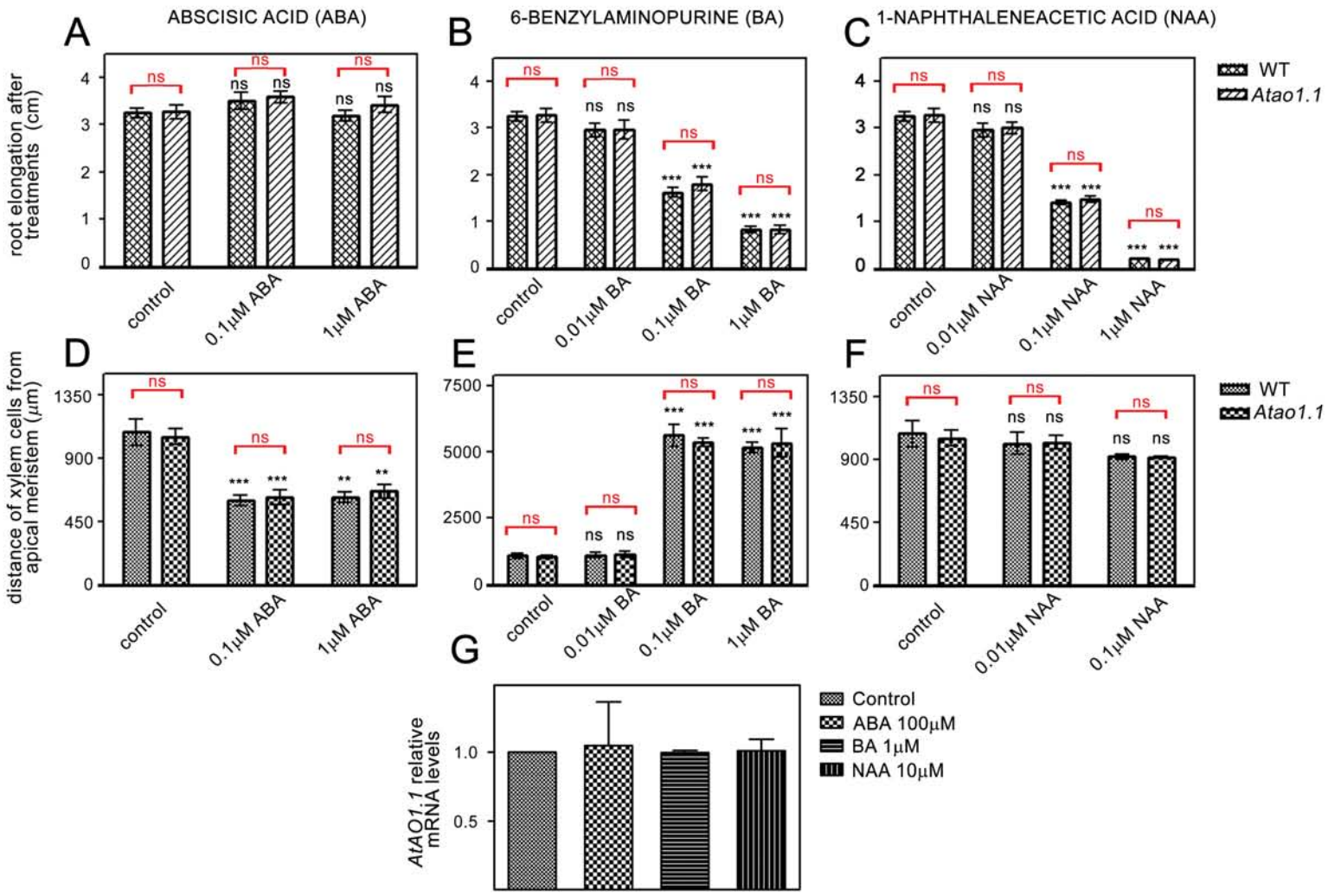
Supplemental Figure S4. LSCM analysis after PI staining of root apices of 14-day-old *Atao1.1* and *Atao1.2* Arabidopsis seedlings. Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. Bar = 50 μm.

Supplemental Figure S5



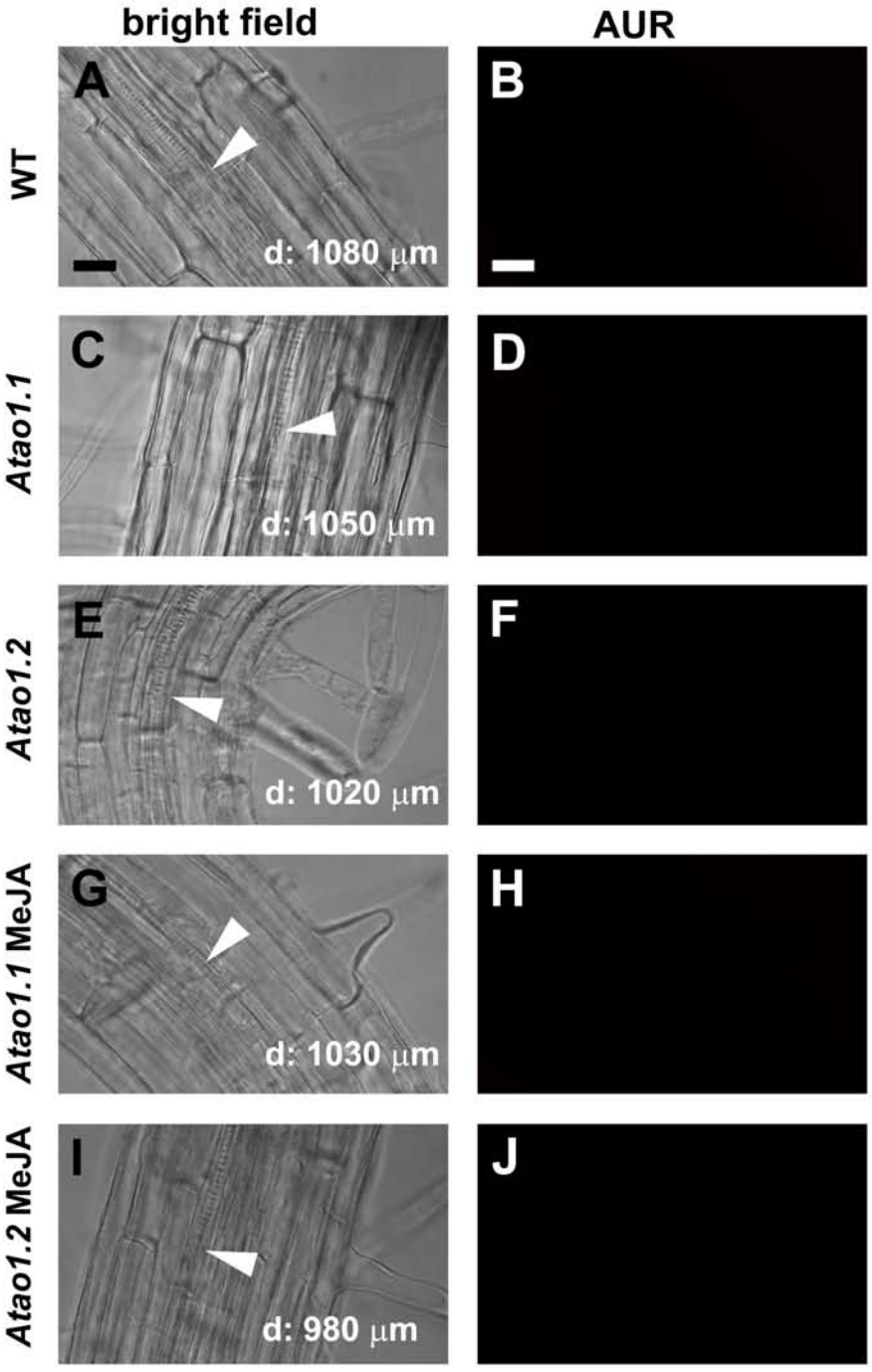
Supplemental Figure S5. Effect of MeJA and DMTU on root xylem differentiation in WT and *Atao1.1* Arabidopsis seedlings. A-E, LSCM analysis after PI staining of root apices of 14-day-old WT and *Atao1.1* Arabidopsis seedlings treated for the last 7 days with MeJA at 50 μM (A, B) and 0.1 μM (C, D) as well as WT plants treated with 0.1 μM MeJA and 100 μM DMTU (E). Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. Bar = 50 μm.

Supplemental Figure S6



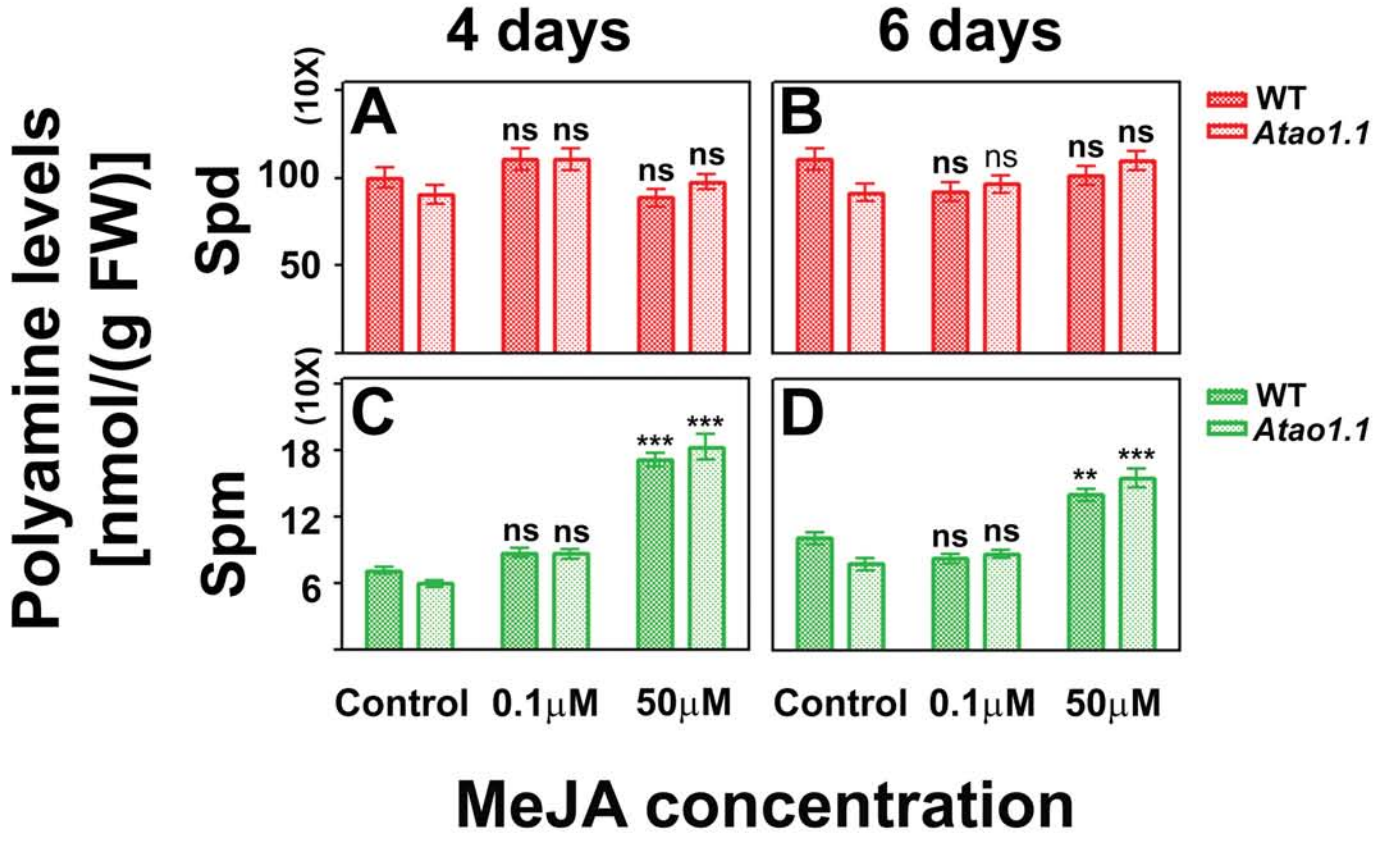
Supplemental Figure S6. Effect of abscisic acid (ABA), cytokinin (BA) or auxin (NAA) on root growth, xylem differentiation and *AtAO1* gene expression. A-C, The reported elongation in root length is the difference between the length measured in 14-day-old seedlings after 7 days treatment and that measured at the onset of the treatment (mean values ± SD; n = 5). D-F, The distance from the apical meristem of the first protoxylem cells with fully developed secondary wall thickenings was measured in 14-day-old seedlings after 7 days of treatment. Control: untreated plants. The significance levels between treated plants and the corresponding control plants are reported in black color. ns, Not significant, P value > 0.05; *, **, and ***, P values ≤ 0.05, 0.01, and 0.001, respectively. Differences between WT and *Atao1.1* plants supplied with the same concentration of hormone were not significant (ns: reported in red color). Similar results were obtained when *Atao1.2* mutants were analyzed (not shown). G, Analysis of *AtAO1* gene expression by RT-qPCR upon hormone treatment in WT plants. The expression of *AtAO1* gene was analyzed in 7-day-old WT seedlings untreated or treated with 100 μM abscisic acid (ABA) for 2h, 1 μM 6-benzylaminopurine (BA) for 1h or 10 μM 1-naphthaleneacetic acid (NAA) for 1h. C: control untreated seedlings after 1h or 2h respectively from the onset of the treatment.

Supplemental Figure S7



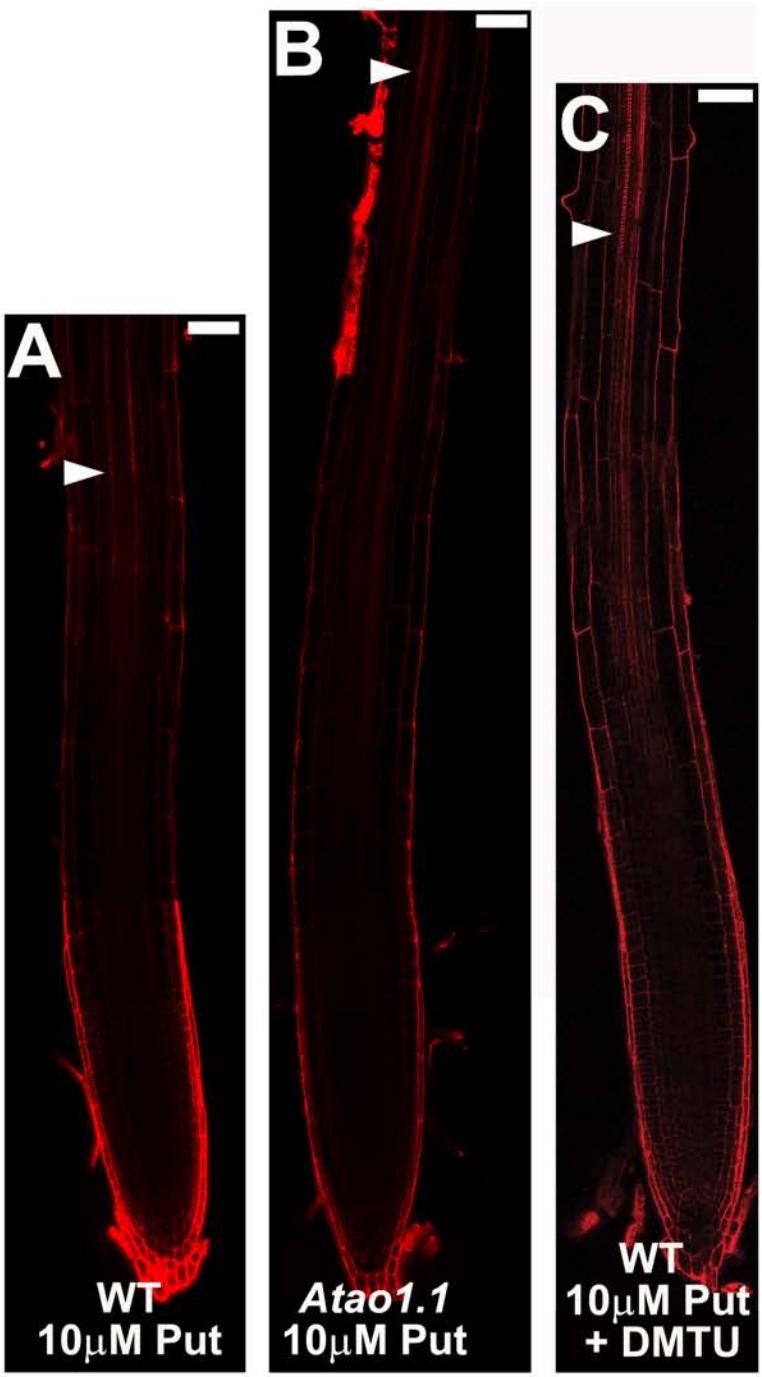
Supplemental Figure S7. *In situ* H_2O_2 detection by LSCM analysis after AUR staining and respective bright field images of roots from untreated WT, untreated *Atao1* and MeJA-treated *Atao1* mutants. Images show the root zone where the first protoxylem cells with fully developed secondary wall thickenings are found. A-F, Bright field and AUR staining images of roots from untreated (control) 14-day-old WT (A, B), *Atao1.1* (C, D) and *Atao1.2* (E, F) seedlings. G-J, Bright field and AUR staining images of roots from 0.1 μM MeJA-treated *Atao1.1* (G, H) and *Atao1.2* (I, J) seedlings. H_2O_2-dependent AUR fluorescence was not detectable at the site of differentiating protoxylem elements (B, D, F, H, J). Micrographs are relative to the confocal central section of the root and are representative of those obtained from 25 roots from 5 independent experiments. d: average distance from the apical meristem of the first protoxylem cells with fully developed secondary wall thickenings. Bar = 20 μm.

Supplemental Figure S8



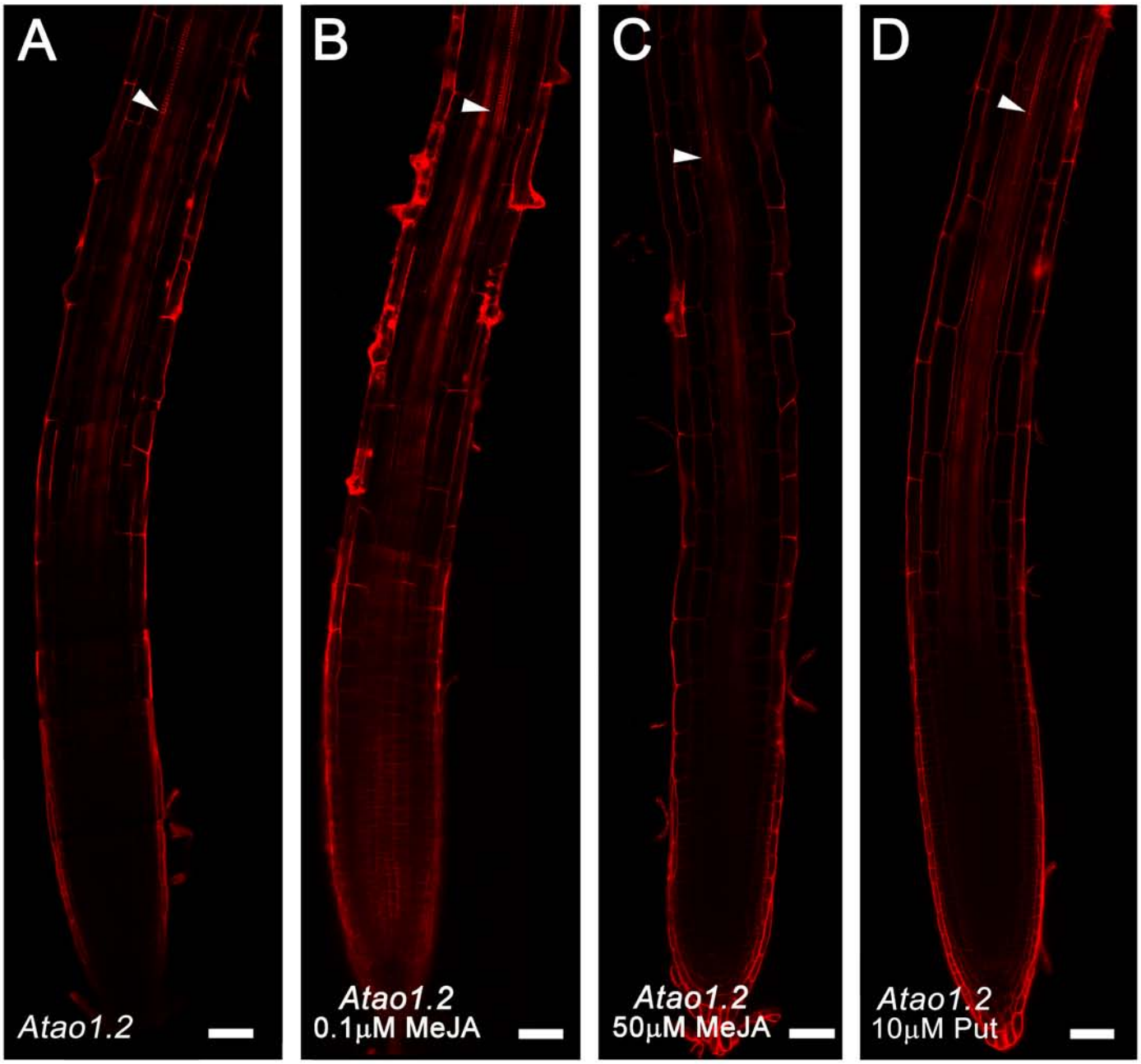
Supplemental Figure S8. Effect of MeJA treatment on Spd and Spm levels in roots of WT and *Atao1* mutants. Free soluble Spd and Spm levels expressed on a fresh weight (FW) basis in WT and *Atao1* roots upon 0.1 *μ*M or 50 *μ*M MeJA treatments. Analysis were carried out on 11-day-old seedlings untreated or treated with MeJA for 4 days (A, C) or 13-day-old seedlings untreated or treated with MeJA for 6 days (B, D). The levels of Spd (A, B) and Spm (C, D) are reported (mean values ± SD; n = 5). Control: untreated plants. ns, Not significant, *P* value > 0.05; *, **, and ***, *P* values ≤ 0.05, 0.01, and 0.001, respectively. Similar results were obtained when *Atao1.2* mutants were analyzed (not shown).

Supplemental Figure S9



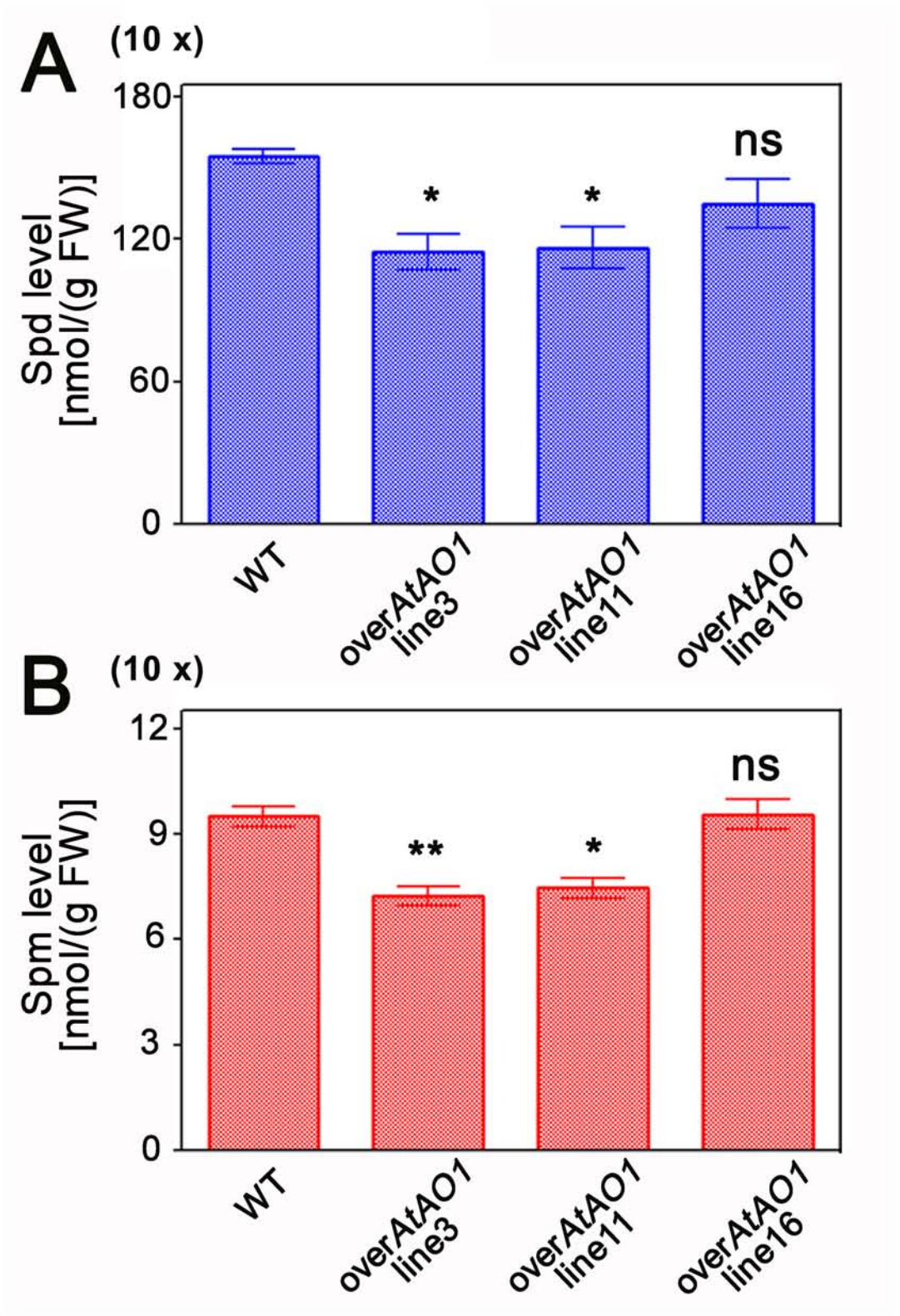
Supplemental Figure S9. Effect of exogenously supplied Put and/or DMTU on xylem differentiation in WT and *Atao1.1* Arabidopsis seedlings. LSCM analysis after PI staining of root apices of 14-day-old WT and *Atao1.1* Arabidopsis seedlings treated for 7 days with 10 μM Put (A, B) and WT plants treated with 10 μM Put and 100 μM DMTU (C). Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. Bar = 50 μm.

Supplemental Figure S10



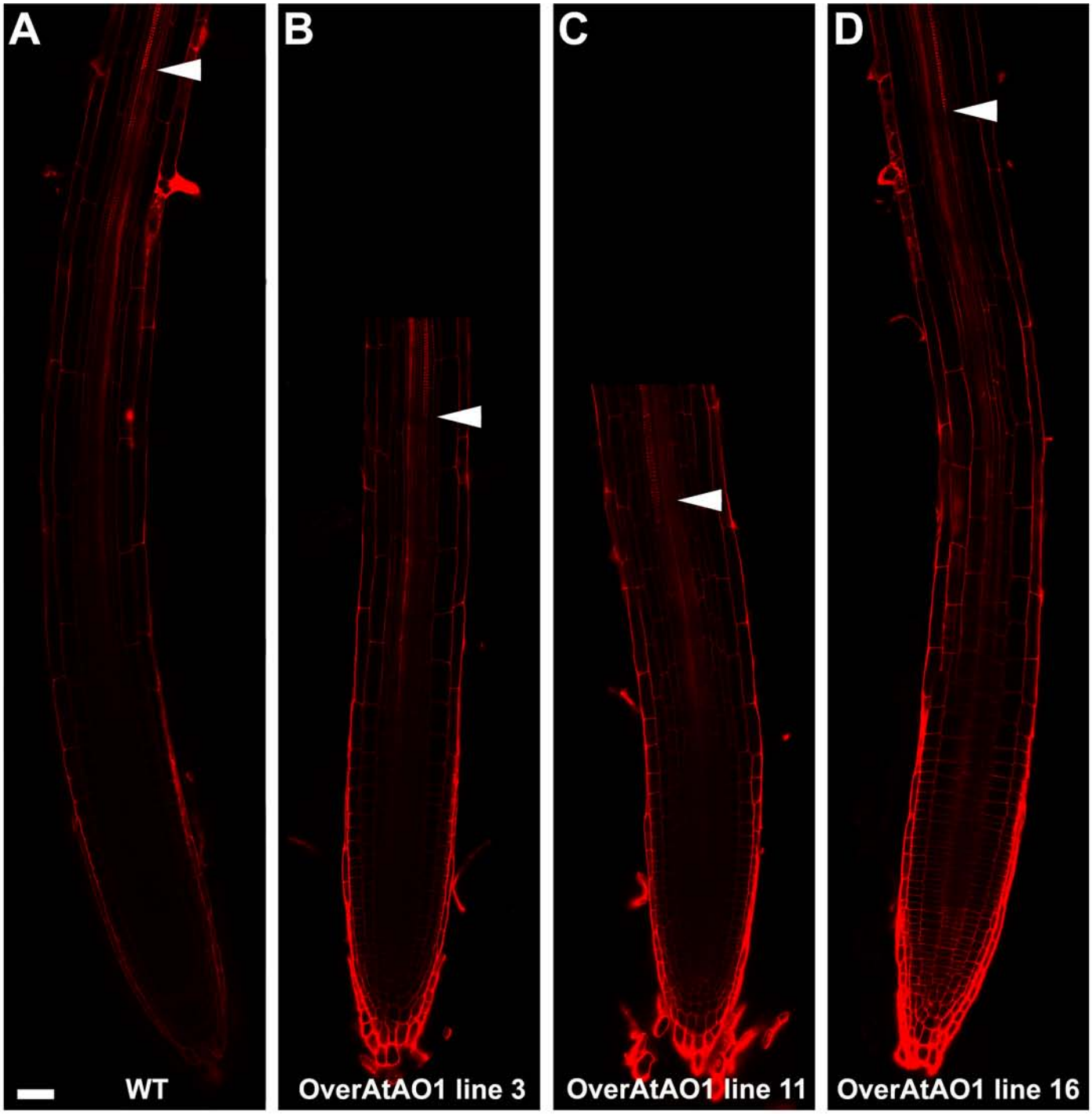
Supplemental Figure S10. Effect of MeJA and Put on root xylem differentiation in *Atao1.2* Arabidopsis seedlings. A-D, LSCM analysis after PI staining of root apices of 14-day-old mutants untreated (A) or treated for the last 7 days with MeJA at 0.1 μM (B), 50 μM (C) and Put at 10 μM (D). Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. Bar = 50 μm.

Supplemental Figure S11



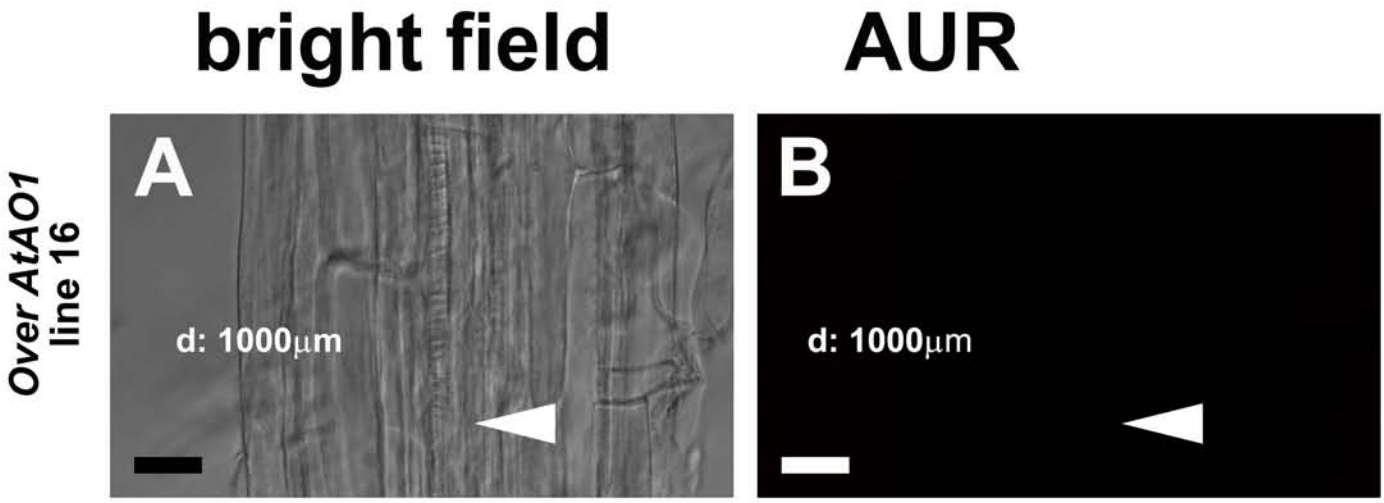
Supplemental Figure S11. Spd and Spm levels expressed on a fresh weight (FW) basis in 18-day-old WT and over*AtAO1* roots. A, Free soluble Spd levels. B, Free soluble Spm levels. ns, Not significant, *P* value > 0.05; *, **, and ***, *P* values ≤ 0.05, 0.01, and 0.001, respectively.

Supplemental Figure S12



Supplemental Figure S12. Effect of *AtAO1* over-expression on xylem differentiation. LSCM analysis after PI staining of root apices of 18-day-old WT (A) and over-expressing *AtAO1* (over*AtAO1* line 3, 11, 16; B-D) Arabidopsis seedlings. Shown images were obtained aligning serial overlapping micrographs of the same root by Photoshop Software (Adobe). Images from single representative experiments are shown. Arrowheads indicate the site of appearance of the first protoxylem cells with fully developed secondary wall thickenings used in measurements of xylem distance. Bar = 40 μm.

Supplemental Figure S13



Supplemental Figure S13. In situ H_2O_2 detection by LSCM analysis after AUR staining and respective bright field image of roots from *AtAO1* over-expressing seedlings, line 16. Images show the root zone where the first protoxylem cells with fully developed secondary wall thickenings are found. Bright field image (A) and AUR staining (B) of roots from 18-day-old seedlings. H_2O_2-dependent AUR fluorescence was not detectable at the site of differentiating protoxylem elements. Micrographs are relative to the confocal central section of the root and are representative of those obtained from 25 roots from 5 independent experiments. d: average distance from the apical meristem of the first protoxylem cells with fully developed secondary wall thickenings. Bar = 40 μm.

Supplemental Table 1. Primers used in the PCR procedures.

Reactions	Primer name	Primer sequence
Genomic amplification AtAO1 for Gateway cloning; forward	*overAtAO1-for*	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGCATATAAATGTAAATGTATTGTTG-3'
Genomic amplification AtAO1 for Gateway cloning; reverse	*overAtAO1-rev*	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTTAGTGGTGGTGGTGGTGGTGTCCTCCC CCAATCAAAGGATCGTTATCGAAG-3'
Genomic amplification AtAO1 promoter for Gateway Cloning; forward	*PromAtAO1-for*	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTGTGGAGGTACATGTGTAACTCTTAC-3'
Genomic amplification AtAO1 promoter for Gateway Cloning; reverse	*PromAtAO1-rev*	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTTGATGGAGTTGTTATTGAAGTAGAC-3'
Over-expression line analysis His tag; reverse	6-His tag specific primer	5'-GGGGACCACTTTGTACAAGAAAGC TGGGTCTAGTGGTGGTGGTGGTGGTGTCCTC-3'
Recombinant *AtAO1 RT-PCR*; forward	*rtAtAO1-for1*	5'-GGAGTAGTAGGAACAGAGAGATAG-3'
Atao1.1 T-DNA insertion analysis gene specific 1	*RP-AtAO1.1*	5′-TTTGAAATCCAGACCAACCTG-3′
Atao1.1 T-DNA insertion analysis gene specific 2	*LP-AtAO1.1*	5′-AGAAATTTGAAGGCCGAAGAG-3′
Atao1.2 T-DNA insertion analysis gene specific 1	*RP-AtAO1.2*	5′- CCGAGTATCTTGAAGCCTGTG -3′
Atao1.2 T-DNA insertion analysis gene specific 2	*LP-AtAO1.2*	5′- ATATCGAGTCCGCGACATATG -3′
T-DNA insertion analysis left border specific	*LBa1*	5′-GATGGTTCACGTAGTGGGCCATCGC-3′
Primer for Southern Probe 35S region; forward	*T-test-for*	5′-GAAGGGTCTTGCGAAGGATA-3′
Primer for Southern Probe 35S region; reverse	*T-test-rev*	5′-CTTCACAAACCAAGGCAAGTA-3′
AtAO1 gene specific primer for RT-PCR's; forward	*rtAtAO1-for2*	5′-CAAGTGGGGAAGCTGAAATAAGTTTAGTG-3′
AtAO1 gene specific primer for RT-PCR'; reverse	*rtAtAO1-rev*	5′-TCCTCCGAGAAGACGTTTTGTTAACTTC-3′
Control UBQ5 gene specific primer for RT-PCR; forward	*UBQ5-for*	5′-GGAAGAAGAAGACTTACACC-3′
Control UBQ5 gene specific primer for RT-PCR; reverse	*UBQ5-rev*	5′-AGTCCACACTTACCACAGTA-3′
Control Actin8 (At1g49240) gene specific primer for RT-qPCR; forward	*qActin8-for*	5'- AGTGGTCGTACAACCGGTATTGT -3'
Control Actin8 (At1g49240) gene specific primer for RT-qPCR; reverse	*qActin8-rev*	5'- GAGGATAGCATGTGGAAGTGAGAA -3'