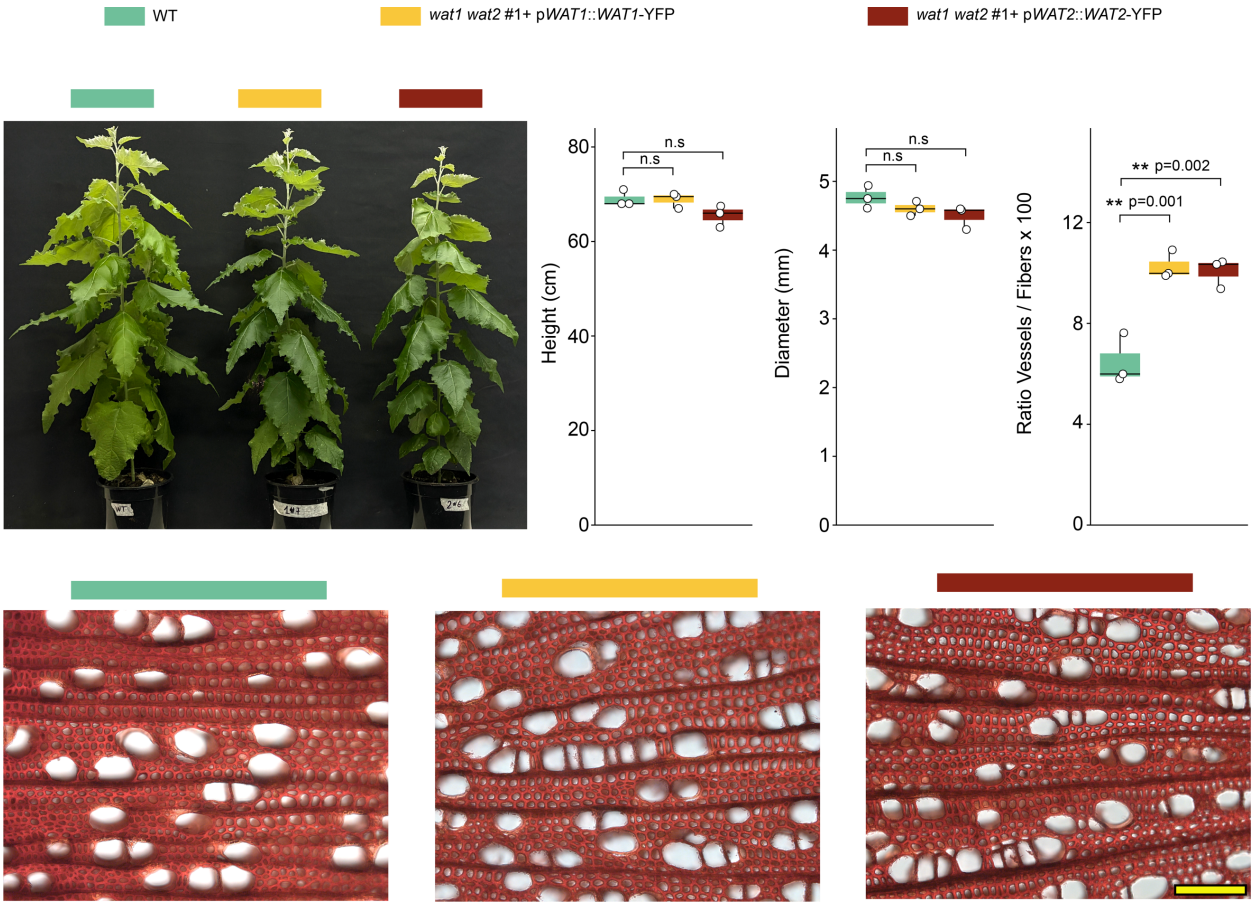
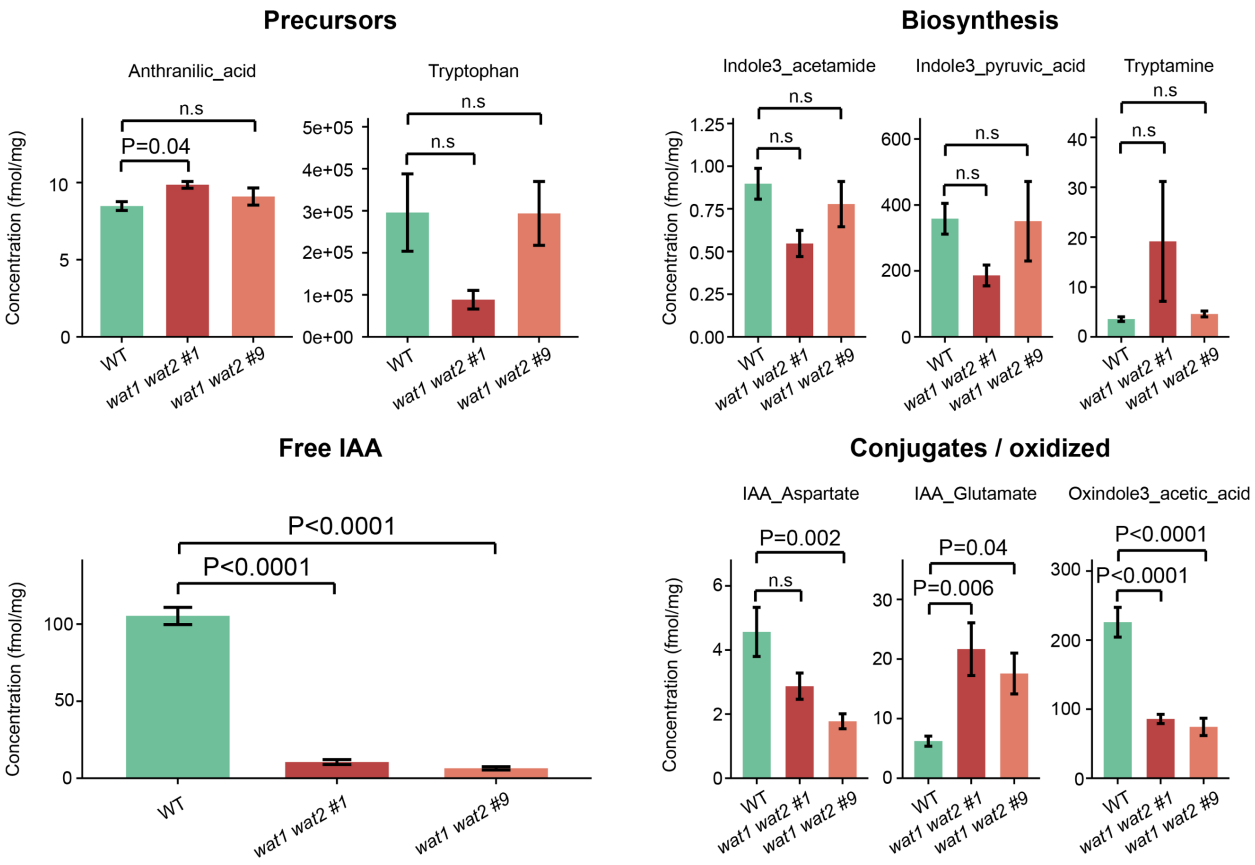


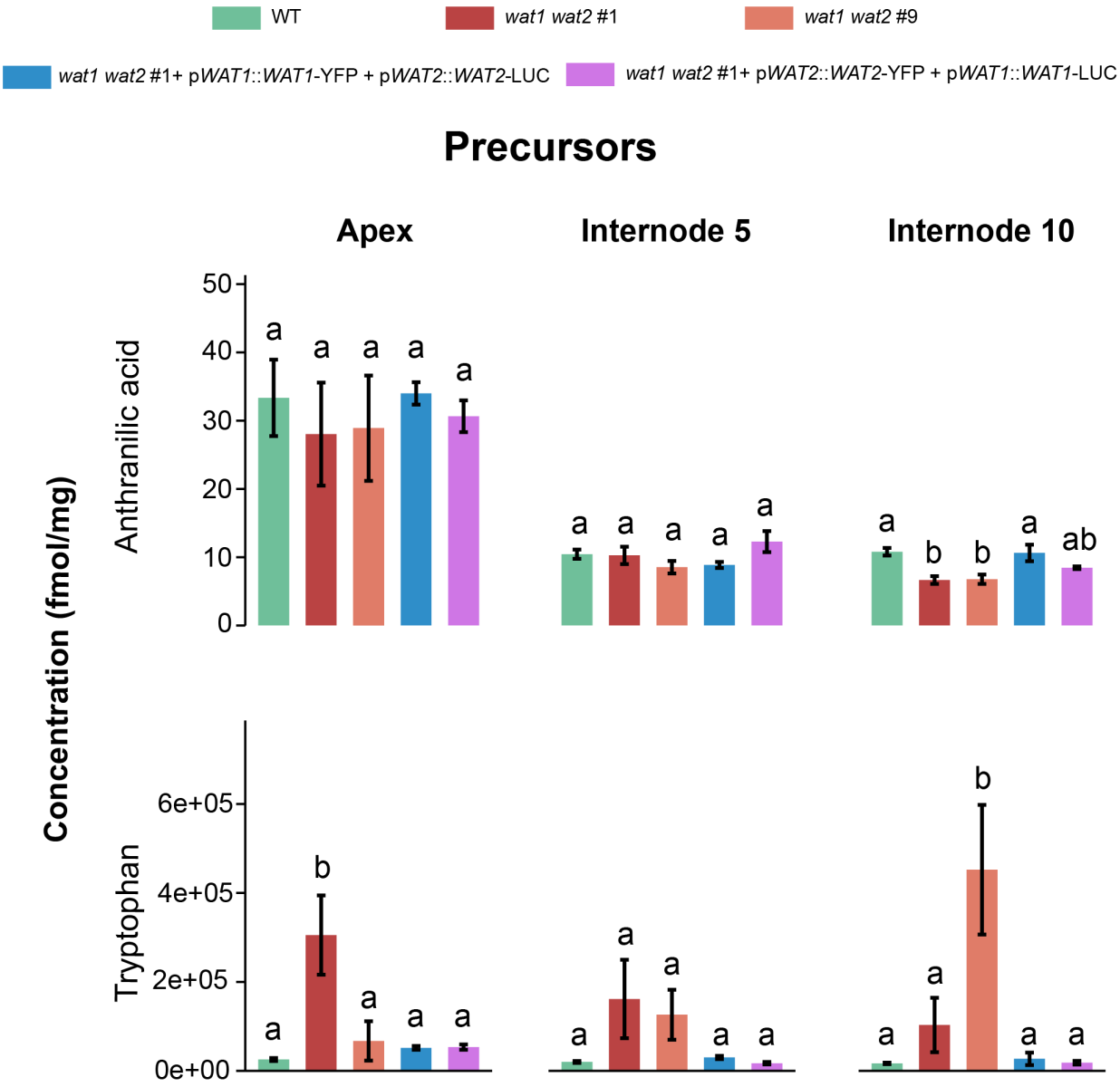
Supplementary Fig. 1. Either WAT1 or WAT2 is sufficient to restore normal vegetative growth in the *wat1 wat2* mutant.

Representative phenotype of WT, and *wat1 wat2* plants complemented with either *pWAT1*::*WAT1*-YFP or *pWAT2*::*WAT2*-YFP in the *wat1 wat2* background. Quantification of plant height and stem diameter is shown below. Expression of either *WAT1* or *WAT2* under its native promoter fully restored vegetative growth to WT levels, demonstrating that either paralogue is sufficient to sustain normal stem development. Data are presented as the mean ± SE with individual biological replicates shown as dots. Statistical significance was determined using one-way ANOVA followed by Dunnett's post hoc test to compare each genotype with WT (n.s., not significant; **P < 0.01; ***P < 0.001). Scale bars, 100 µm.



Supplementary Fig. 2. WAT1 and WAT2 are required to maintain free IAA homeostasis in poplar stems.

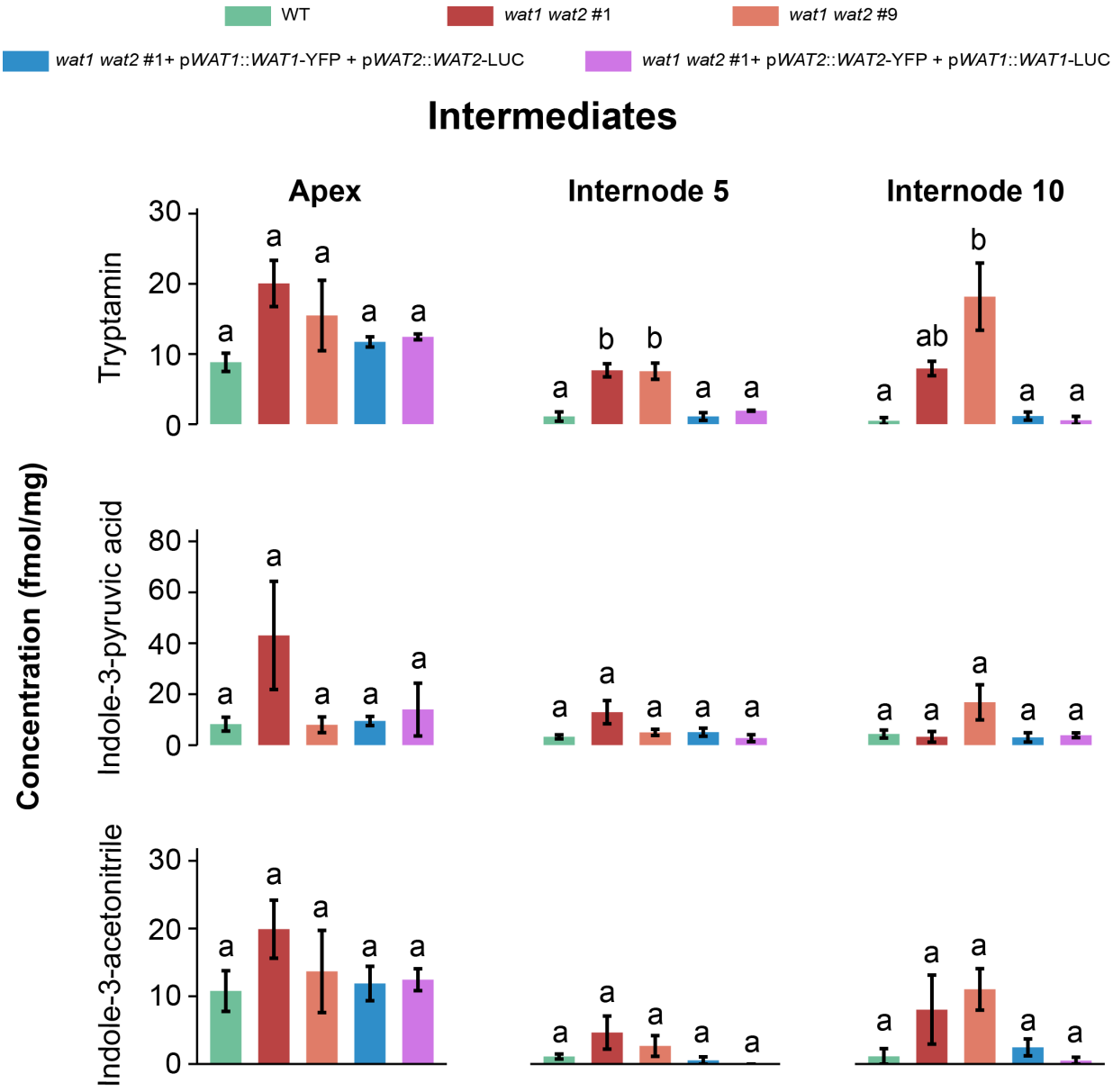
Quantification of auxin precursors, biosynthetic intermediates, free IAA, auxin conjugates, and catabolites in developing stems of WT and two independent *wat1 wat2* mutant lines (*wat1 wat2*#1 and *wat1 wat2*#9). Most auxin precursors and biosynthetic intermediates were not significantly altered in the mutants, whereas free IAA levels were dramatically reduced. By contrast, the abundance of several auxin conjugates and catabolites was altered in both mutant lines, indicating disrupted auxin homeostasis. Data are presented as mean ± SE (n = 8 biological replicates per genotype). Statistical significance was determined by one-way ANOVA followed by Dunnett's post hoc test. n.s., not significant. P values are shown when differences were significant.



Supplementary Fig. 3. WAT1 and WAT2 regulate free IAA homeostasis rather than auxin biosynthesis.

Quantification of auxin precursors in the shoot apex, internode 5, and internode 10 of WT, two independent *wat1 wat2* mutant lines (*wat1 wat2*#1 and *wat1 wat2*#9), and complemented lines expressing *pWAT1*::*WAT1*-YFP + *pWAT2*::*WAT2*-LUC or *pWAT2*::*WAT2*-YFP + *pWAT1*::*WAT1*-LUC in the *wat1 wat2* background. The early precursor anthranilic acid showed only modest reductions in specific mutant tissues; however, these changes were not accompanied by a corresponding decrease in tryptophan, which remained largely unchanged between WT and mutant plants and even accumulated in specific tissues, indicating that these changes do not explain the

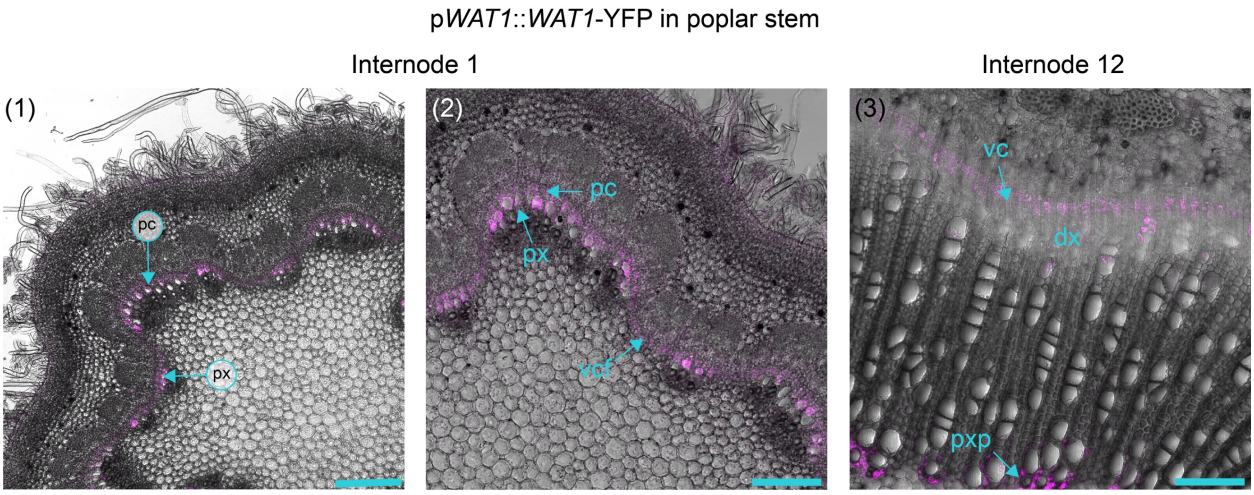
extreme reduction of free IAA in the stem of mutant trees. Data are presented as mean ± SE (WT and *wat1 wat2* mutant lines, n = 4 biological replicates; complemented lines, n = 3 biological replicates). Different letters indicate statistically significant differences (one-way ANOVA followed by Holm's post hoc test ($P < 0.05$).



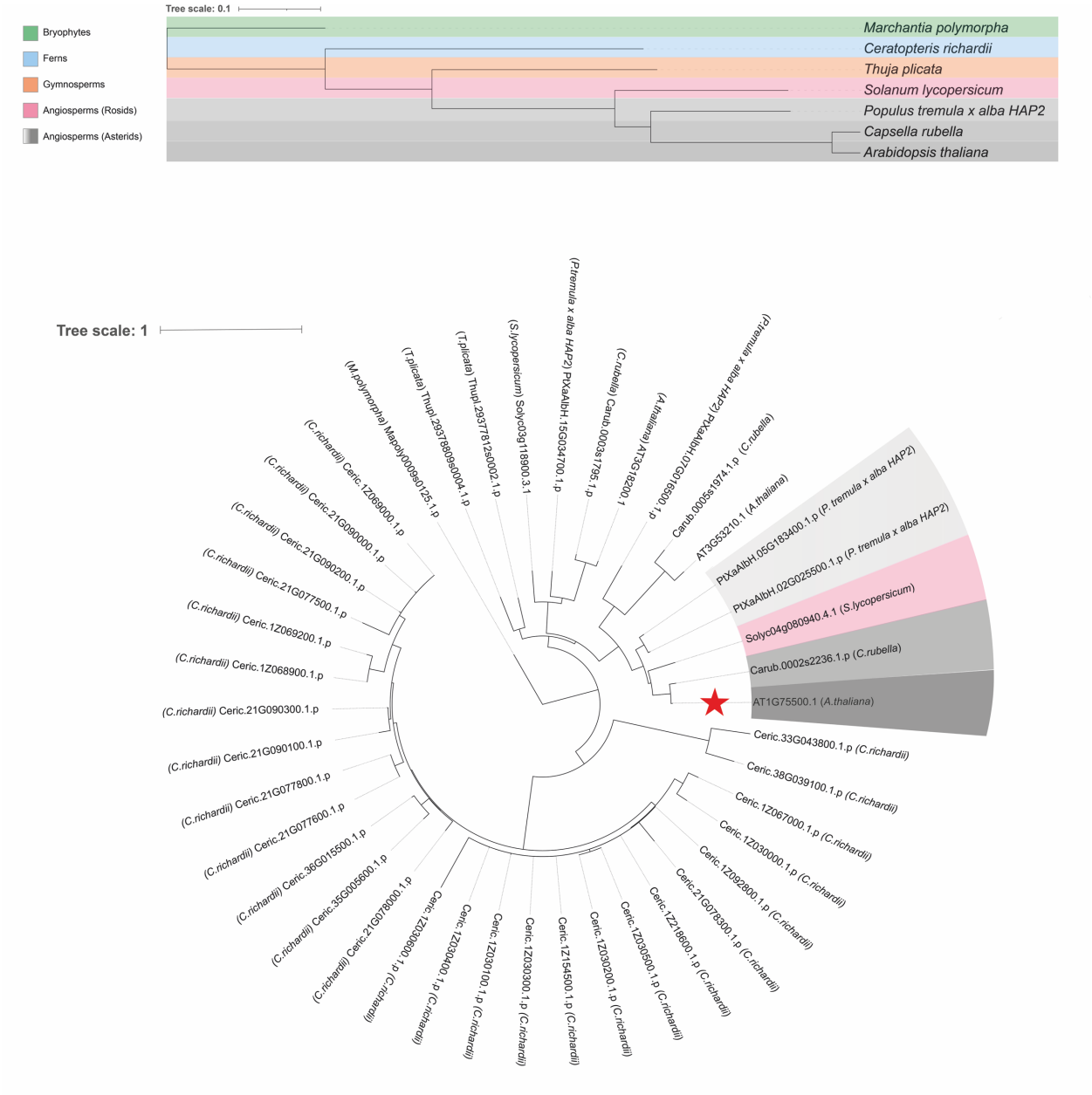
Supplementary Fig. 4. WAT1 and WAT2 regulate free IAA homeostasis rather than auxin biosynthesis.

Quantification of auxin biosynthetic intermediates in the shoot apex, internode 5, and internode 10 of WT, two independent *wat1 wat2* mutant lines (*wat1 wat2*#1 and *wat1 wat2*#9), and complemented lines expressing *pWAT1*::*WAT1*-YFP + *pWAT2*::*WAT2*-LUC or *pWAT2*::*WAT2*-YFP + *pWAT1*::*WAT1*-LUC in the *wat1 wat2* background. Tryptamine, indole-3-pyruvic acid, and indole-3-acetonitrile levels showed no consistent differences among genotypes across the three stem regions, indicating that WAT mutations have little effect on auxin biosynthesis. Data are presented as mean ± SE (WT and *wat1 wat2* mutant lines, n = 4 biological replicates;

complemented lines, n = 3 biological replicates). Different letters indicate statistically significant differences (one-way ANOVA followed by Holm's post hoc test ($P < 0.05$).



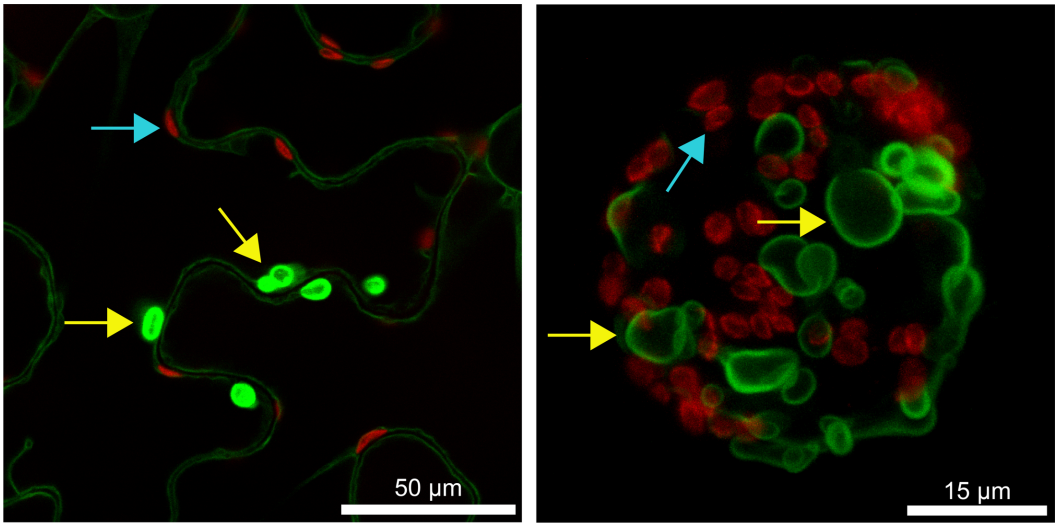
Supplementary Fig. 5. WAT1 is expressed throughout vascular development in poplar stems. Localization of p*WAT1*::*WAT1*-YFP in transgenic poplar stems. Representative confocal images of stem transverse sections at internode 1 (1-2) and internode 12 (3). YFP fluorescence (magenta) was detected in vascular tissues, including procambial cells, primary xylem, vascular cambium, and developing xylem. Abbreviations: dx, developing xylem; pc, procambium; px, primary xylem; vcf, vascular cambial founder cells; v, vessel; vc, vascular cambium; Scale bars, 190 μm (1), 140 μm (2), 150 μm (3).



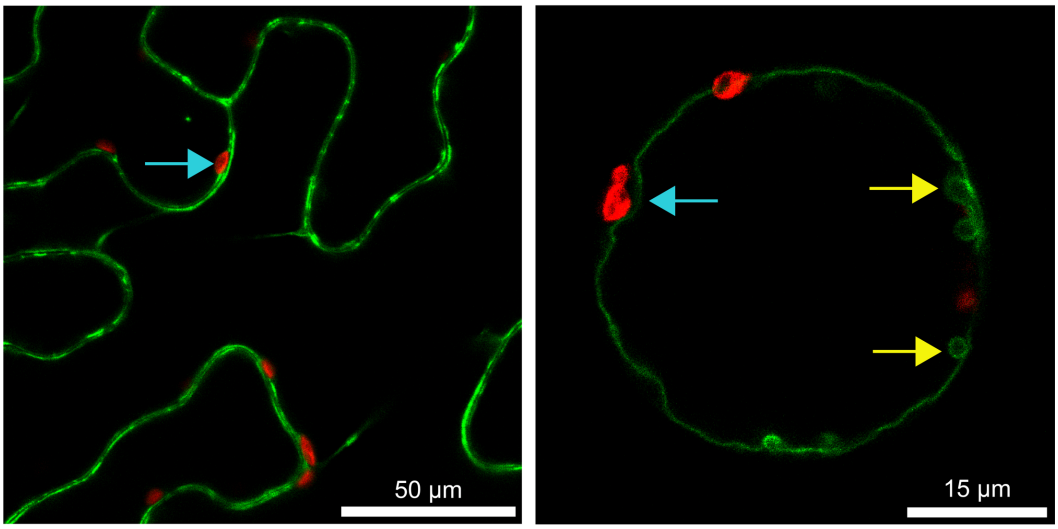
Supplementary Fig. 6. Phylogenetic analysis of the EamA/WAT protein family across representative land plants. A Species tree inferred by OrthoFinder from the complete proteomes of representative land plants, including the bryophyte *Marchantia polymorpha*, the fern *Ceratopteris richardii*, the gymnosperm *Thuja plicata*, and representative angiosperms (*Solanum lycopersicum*, *Populus tremula* × *alba*, *Capsella rubella*, and *Arabidopsis thaliana*). Colored backgrounds indicate the major taxonomic groups included in the analysis. **B** Gene tree of the WAT1-like protein family inferred by OrthoFinder. The shaded sector highlights the conserved angiosperm WAT1 clade, which includes the functionally characterized WAT1 proteins from

Arabidopsis thaliana and *Solanum lycopersicum*, along with the two *Populus tremula* × *alba* paralogues, WAT1 (PtXaAlbH.05G183400) and WAT2 (PtXaAlbH.02G025500). The red star denotes *Arabidopsis thaliana* WAT1 (AT1G75500). Scale bars indicate substitutions per site.

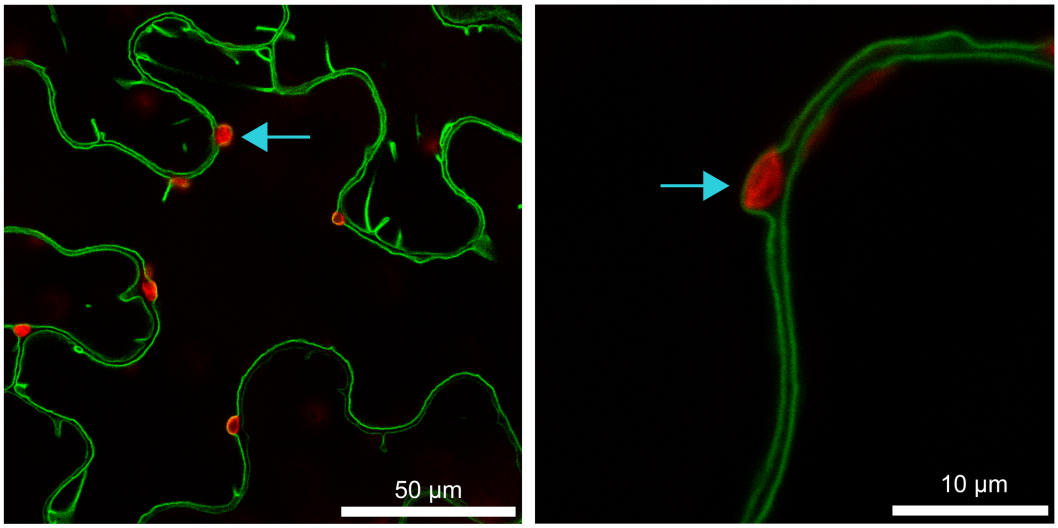
p*35S*::*WAT2*-eGFP



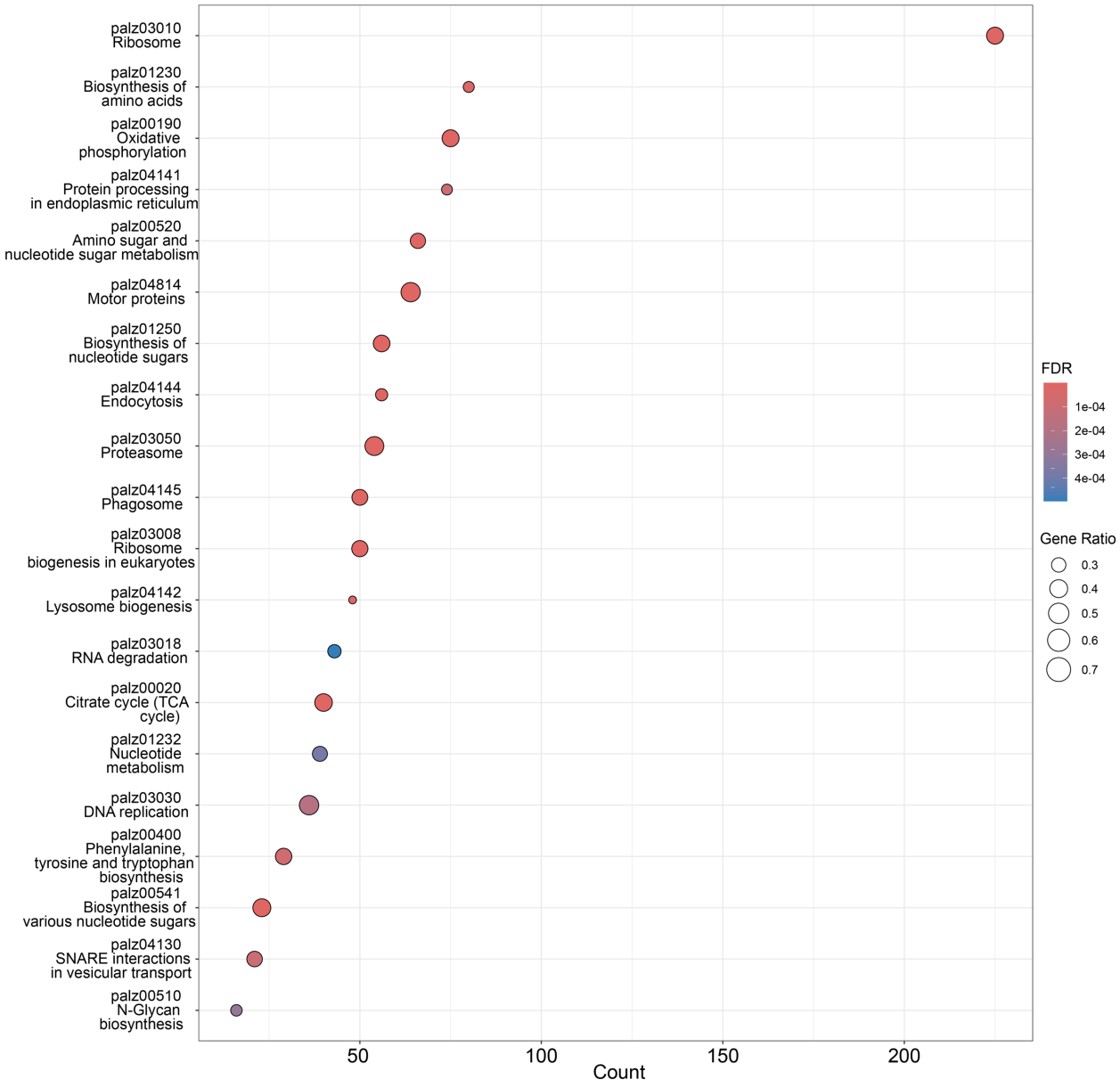
p*35S*::*WAT2*-**m**GFP



p*35S*::*WAT1*-**m**GFP



Supplementary Figure 7. WAT1 and WAT2 are localized at the tonoplast in *Nicotiana benthamiana* epidermal cells. Representative confocal images of *Nicotiana benthamiana* epidermal cells and isolated protoplasts transiently expressing 35S::*WAT2*-eGFP, 35S::WAT2-mGFP, or 35S::WAT1-mGFP. WAT2 fused to eGFP frequently induced prominent tonoplast bulbs (yellow arrows), whereas replacement of eGFP with the monomeric GFP variant (mGFP) markedly reduced bulb formation while preserving membrane-associated fluorescence. Chloroplast autofluorescence is indicated by cyan arrows and was used as a positional reference. Scale bars, 50 μm (left panels), 15 μm (upper and middle right panels), and 10 μm (lower right panel).



Supplementary Fig. 8. GSEA of repressed pathways in *wat1 wat2* stems. Dot plot showing the top 20 pathways repressed in *wat1 wat2* mutants, identified by one-tailed negative Gene Set Enrichment Analysis (GSEA) using differential expression results from the bulk RNA-seq dataset. Pathway names and their corresponding *Populus* KEGG identifiers are shown on the y-axis, whereas the x-axis indicates the number of genes from the enriched gene set assigned to each pathway (Count). Dot size represents the Gene Ratio, calculated as the proportion of enriched

genes relative to the total number of genes in each pathway, and dot color indicates the false discovery rate (FDR).

Gen	Primers qPCR (5’ - 3’)	
AFB2 (PtXaAlbH.05G121200)	fwd	GGAAATTAAATCTTGCAGGT
	rev	AGGGCTTCTGGATTGAATG
ARF3 (PtXaAlbH.04G039300)	fwd	TCTGCAACATCATCCCG
	rev	CTTTCACACAAGCTAATACG
ARF8 (PtXaAlbH.04G064100)	fwd	TGTGGCTTAAGACCCTGC
	rev	GAAGAATGCCATGCAGCTC
DAHPS2 (PtXaAlbH.05G123400)	fwd	AGCCAAGGTTAAGACTGC
	rev	AATGTCCGCCTCTTCCTT
QUASIMODO2 (PtXaAlbH.05G124100)	fwd	CCACAACTCTCAGAAGCC
	rev	CTTCTCATTTGCTCAGCACC
SK (PtXaAlbH.07G068300)	fwd	TGTATCCAGTATCACACCTG
	rev	GAAATAGAATCCCATTGCAGC
UBQ7 (PtXaAlbH.05G152100)	fwd	GGAACGGGTTGAGGAGAAAGAAG
	rev	GCAAGAACAAGATGAAGCACAGAGC

Supplementary Fig. 9. Primer sequences used in this study.

List of primer sequences used for RT-qPCR analyses performed in this study, including gene-specific primers used for transcript quantification.

Genotype	Stem diameter only with the secondary xylem (mm)
WT rep.1	7.17
WT rep.2	6.31
WT rep.3	6.40
WT rep.4	6.53
WT rep.5	6.76
wat1 wat2 #1 rep.1	7.76
wat1 wat2 #1 rep.2	6.47
wat1 wat2 #1 rep.3	7.13
wat1 wat2 #1 rep.4	6.74
wat1 wat2 #1 rep.5	5.86
wat1 wat2 #1 rep.6	6.39
wat1 wat2 #1 rep.7	6.03
wat1 wat2 #1 rep.8	6.42
wat1 wat2 #1 rep.9	5.92
wat1 wat2 #1 rep.10	5.86

Supplementary Fig. 10. Secondary xylem diameter of stems used for secondary cell wall analyses.

Diameter of the secondary xylem measured in the basal stems of WT and *wat1 wat2* plants used for secondary cell wall composition analyses. Plants were harvested after reaching a comparable degree of secondary xylem development to minimize developmental differences between genotypes. Each point represents one biological replicate.