Supplementary Materials and Methods

Quantification of epidermal cell types

Epidermal cell types were quantified in bright field images of cleared cotyledons of 10-d-old seedlings or leaves of 21-d-old plants using ImageJ 1.43u software (Wayne Rasband, NIH, Bethesda, USA). Depending on cotyledon or leaf size, up to three areas in the abaxial epidermis were selected, avoiding the margin and the area close to the petiole, and cell types in these areas were counted. Area size was 250 µm x 250 µm in cotyledons of light-grown seedlings and 100 µm x 100 µm in cotyledons of dark-grown seedlings. Epidermal cells were classified as stomata (i.e. pairs of guard cells), stomatal precursors (i.e. meristemoids and GMCs) or other epidermal cells according to morphological differences described in Nadeau and Sack (2002). Percentage of stomata was calculated as stomata / (stomata + stomatal precursors + other epidermal cells) and percentage of stomatal precursors was calculated as stomatal precursors / (stomata + stomatal precursors + other epidermal cells).

For quantification of total cell numbers, cells were quantified in confocal images of whole, PI-stained cotyledons of 2- or 10-d-old seedlings using ImageJ 1.43 software. For light-grown seedlings, cells in one half of each cotyledon were counted and the total cell numbers were extrapolated.

Eight to ten cotyledons or leaves of individual seedlings and plants, respectively, were analysed per genotype and condition. Percentages of stomata and stomatal precursors were calculated for each cotyledon or leaf individually before mean and s.e.m. were calculated from these data.

Statistical analysis

Statistical analyses were performed in R (http://www.r-project.org). Data were checked for deviations from normality using the Shapiro-Wilk test. Variances of normally distributed data were then analysed using Levene's test for homogeneity of variance. Comparisons of two normally distributed samples with equal variances were carried out using a two-tailed *t*-test; in case of unequal variances a Welch-corrected *t*-test was applied. For multiple comparisons of normally distributed data, an ANOVA (or Welch-ANOVA for samples with unequal variances) was performed. If significant differences were detected by ANOVA, a post-hoc

analysis was carried out using Tukey's HSD test for samples with equal variances or Dunnett's modified Tukey-Kramer test for samples with unequal variances.

In rare cases when data showed strong deviations from a normal distribution, non-parametric tests were performed. For pairwise comparisons, a Wilcoxon rank sum test was used while for multiple comparisons, a Kruskal-Wallis one-way analysis of variance was carried out, which was followed by multiple pairwise Wilcoxon rank sum tests if significant differences were detected.

Plasmid construction

To generate *promoter::axr3-1-YFP* constructs the Venus-YFP coding sequence was PCR-amplified from the pDEST-Venus-GW-Ter vector (R. Hänsch, unpublished) with primers introducing XhoI and SpeI restriction sites, the purified PCR product was digested with XhoI and SpeI and ligated into pJIC30 (Corbesier et al., 2007), thereby generating pJIC30-YFP. The *axr3-1* ORF was PCR-amplified from the pBS-axr3-1 vector (Ouellet et al., 2001) with primers introducing HindIII and XhoI restriction sites, the purified PCR product was digested with HindIII and XhoI and ligated into pJIC30-YFP, thereby generating pJIC30-axr3-1-YFP.

Promoter fragments 2421 bp upstream of the *AXR3* ORF, 2581 bp upstream of the *ICE1* ORF and 2574 bp upstream of the *SPCH* ORF were amplified from Col-0 genomic DNA and the purified PCR products were cloned into pDONR^{TM}207 using GATEWAY® BP technology, thereby generating the promoter entry clones pDONR^{TM}207-AXR3pro, pDONR^{TM}207-ICE1pro and pDONR^{TM}207-SPCHpro. Entry clones containing the *ML1* and *CAB3* promoters were described previously (An et al., 2004; Ranjan et al., 2011). *Promoter::axr3-1-YFP* constructs were generated by GATEWAY® LR reactions between these promoter entry clones and the pJIC30-axr3-1-YFP destination vector. Primers used for cloning are listed in Table S3.

Supplementary References

An, H., Roussot, C., Suárez-López, P., Corbesier, L., Vincent, C., Piñeiro, M., Hepworth, S., Mouradov, A., Justin, S., Turnbull, C., et al. (2004). CONSTANS acts in the phloem to regulate a systemic signal that induces photoperiodic flowering of Arabidopsis. *Development* **131**, 3615–3626.

Corbesier, L., Vincent, C., Jang, S., Fornara, F., Fan, Q., Searle, I., Giakountis, A., Farrona, S., Gissot, L., Turnbull, C., et al. (2007). FT Protein Movement

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Nadeau, J. A. and Sack, F. D. (2002). Stomatal Development in Arabidopsis. *Arabidopsis Book* **1**, e0066.

Ouellet, F., Overvoorde, P. J. and Theologis, A. (2001). IAA17/AXR3: Biochemical Insight into an Auxin Mutant Phenotype. *Plant Cell* **13**, 829–841.

Ranjan, A., Fiene, G., Fackendahl, P. and Hoecker, U. (2011). The Arabidopsis repressor of light signaling SPA1 acts in the phloem to regulate seedling de-etiolation, leaf expansion and flowering time. *Development* **138**, 1851–1862.

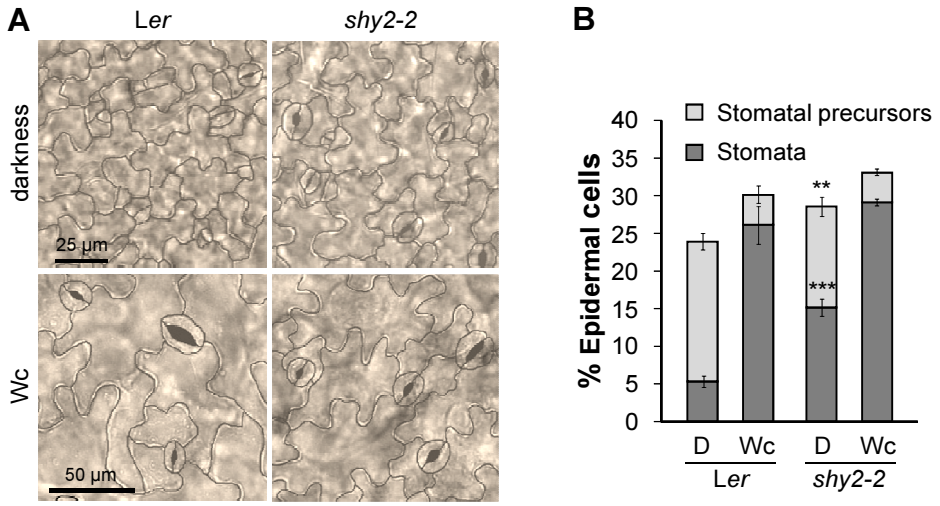

Fig. S1. The *shy2-2* mutant shows increased stomata formation in darkness. (**A**) Brightfield images of the cotyledon epidermis of dark- and Wc-grown seedlings of the indicated genotypes. Cell outlines were traced in dark grey. (**B**) Quantification of stomata and stomata precursors of the genotypes shown in A. Error bars represent the s.e.m. Asterisks indicate indicate significant differences in the proportion of stomatal precursors or stomata, respectively, when compared to the L*er* wild type (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

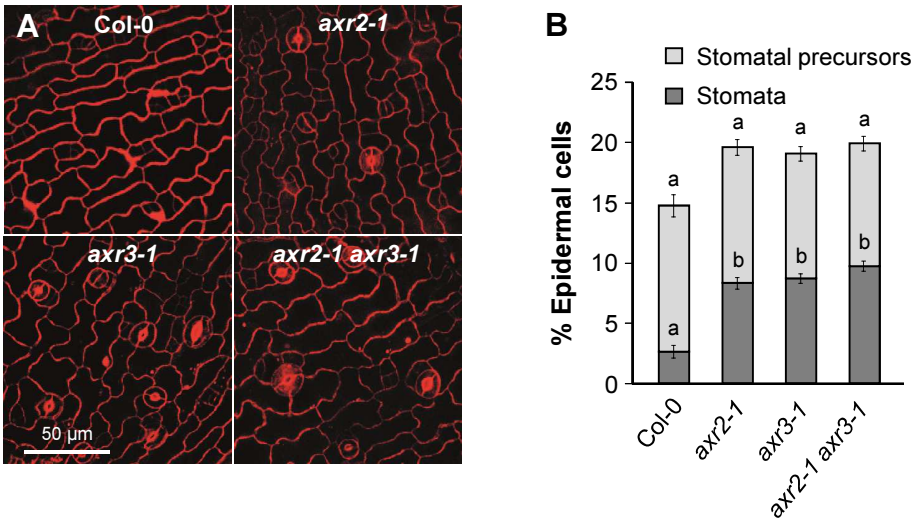

Fig. S2. The *axr2-1 axr3-1* double mutant shows no increased stomata formation in comparison to the respective single mutants. (**A**) Confocal images of the cotyledon epidermis of dark-grown seedlings of the indicated genotypes. Cell outlines were visualised by PI staining. (**B**) Quantification of stomata and stomatal precursors of the genotypes shown in A. Error bars represent the s.e.m. Letters indicate significance groups for each cell type; samples with the same letters are not significantly different ($p < 0.05$).

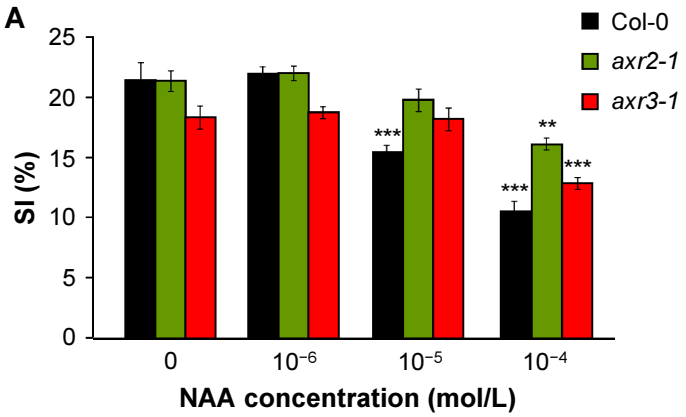

Fig. S3. NAA treatment affects stomatal development. SI of the cotyledon epidermis of Wc-grown Col-0, *axr2-1* and *axr3-1* seedlings treated with different concentrations of NAA. Error bars represent the s.e.m. Asterisks indicate significant differences to the wild type (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

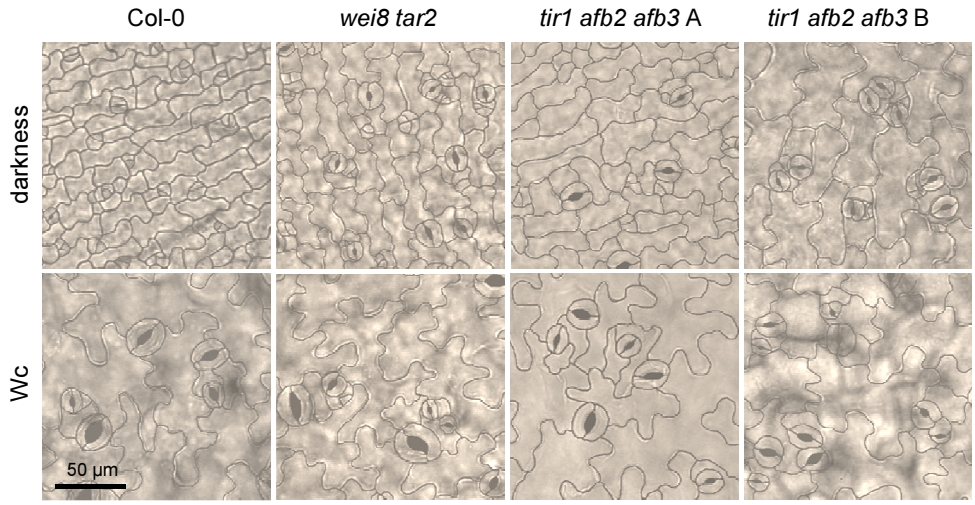

Fig. S4. Mutants deficient in auxin biosynthesis or auxin perception are defective in stomatal development. Brightfield images of the cotyledon epidermis of dark- and Wc-grown seedlings of the indicated genotypes. *tir1 afb2 afb3* seedlings were grouped into classes A and B, which refer to mild and strong phenotypes, respectively. Cell outlines were traced in dark grey.

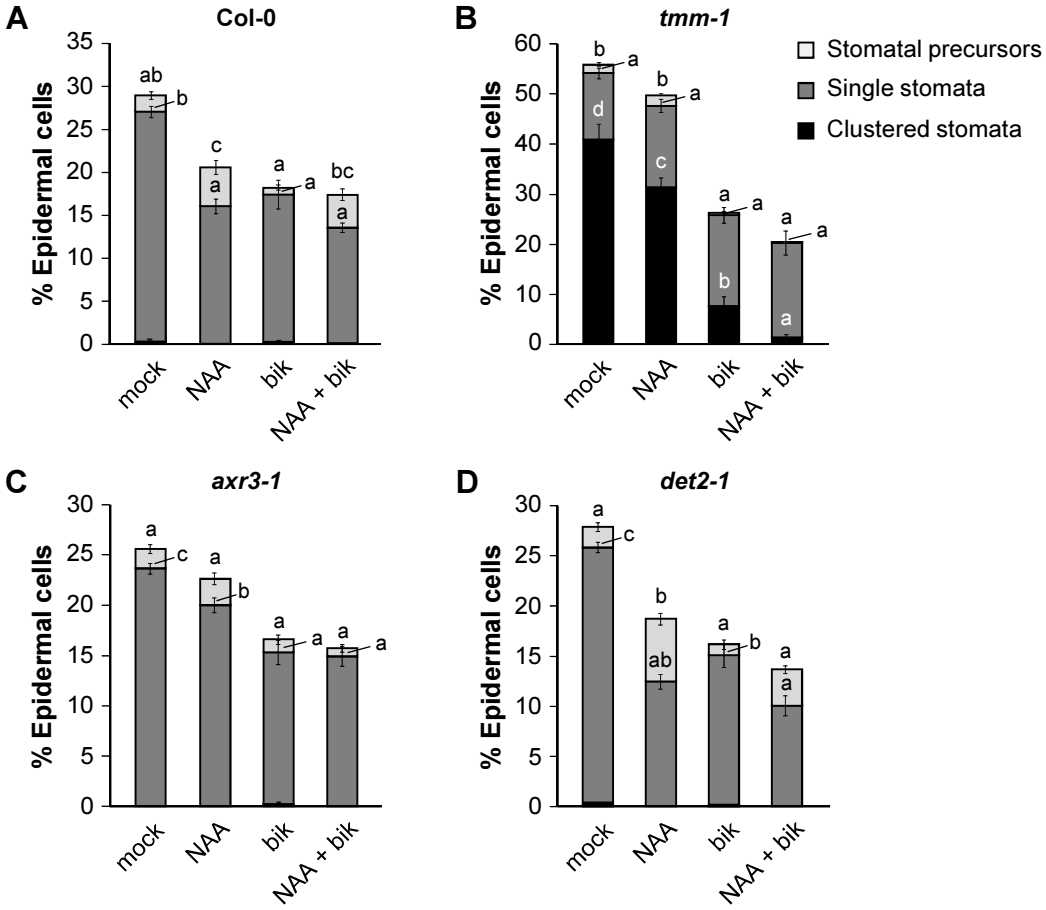

Fig. S5. NAA and bikinin have additive effects on stomata formation. Quantification of stomata and stomatal precursors in the abaxial cotyledon epidermis of Wc-grown Col-0 (A), *tmm-1* (B), *axr3-1* (C) and *det2-1* (D) seedlings treated with 10 µM NAA, 30 µM bikinin, a combination of both or mock-treated. Error bars represent the s.e.m. Letters indicate significance groups for each cell type; samples with the same letters are not significantly different ($p < 0.05$).

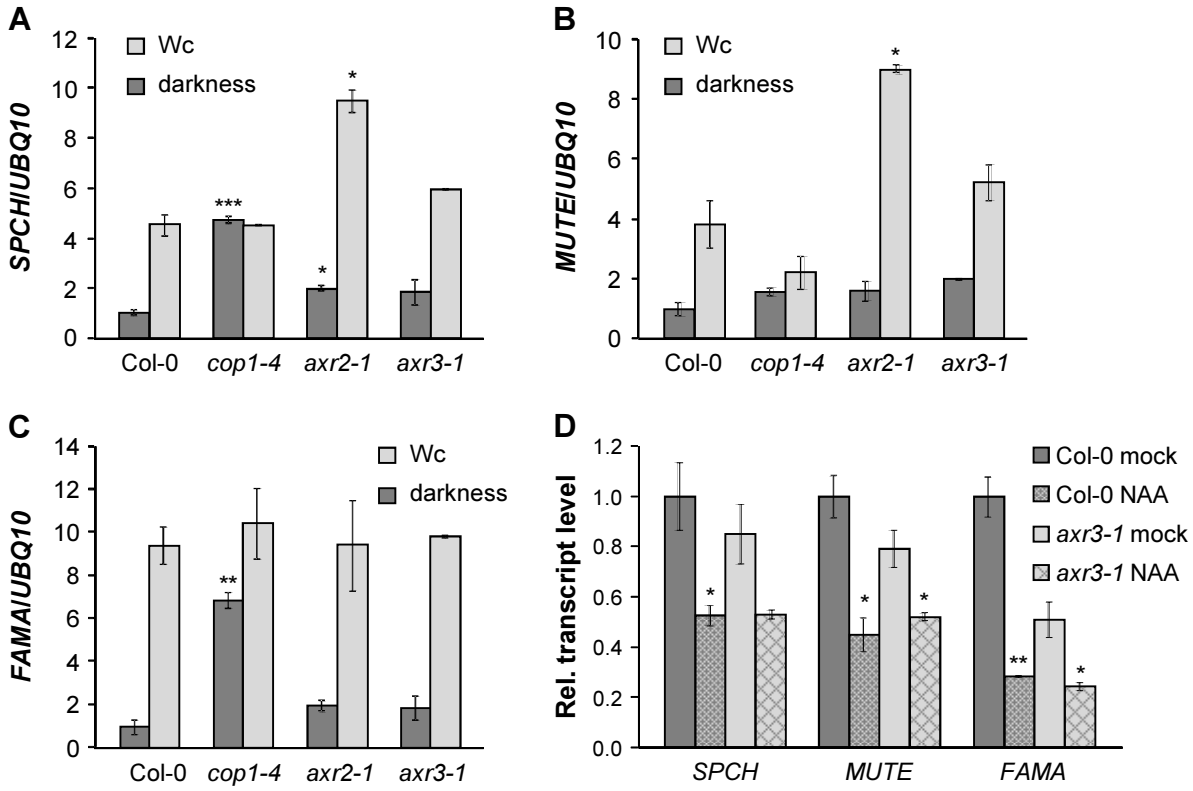

Fig. S6. Analysis of *SPCH*, *MUTE* and *FAMA* transcripts. (**A-C**) Transcript levels of *SPCH* (A), *MUTE* (B) and *FAMA* (C) in 5-d-old dark- or Wc-grown seedlings of the indicated genotypes. (**D**) *SPCH*, *MUTE* and *FAMA* transcript levels in 5-d-old Wc-grown Col-0 and *axr3-1* seedlings treated with 10 µM NAA or mock-treated. Transcript levels were normalised to *UBQ10* and are shown relative to the levels of dark-grown Col-0 seedlings (A-C) or the mock-treated Col-0 control (D). Error bars represent the s.e.m. Asterisks indicate significant differences to dark-grown wild type (A-C) or the mock-treated control (D) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

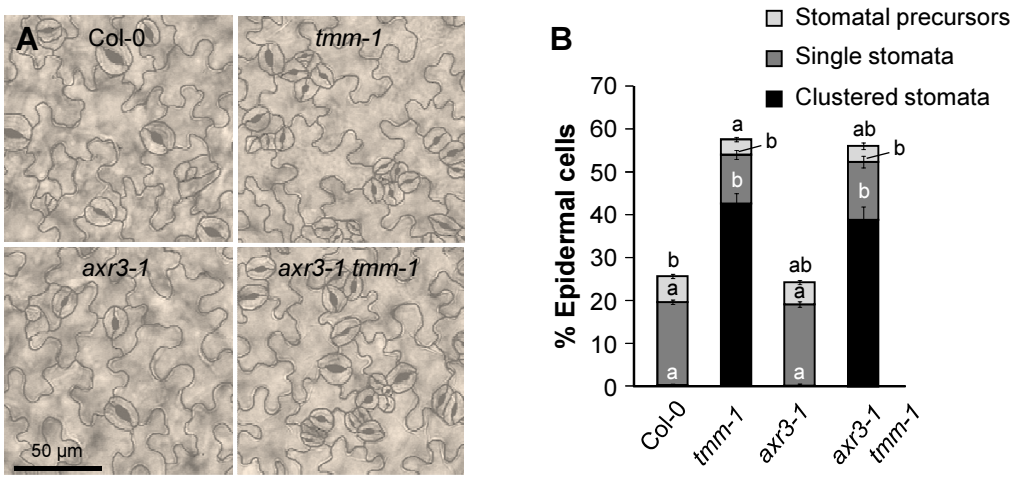

Fig. S7: The *axr3-1* mutation has no effect on the phenotype of Wc-grown *tmm-1* mutant seedlings. (**A**) Brightfield images of the cotyledon epidermis of Wc-grown seedlings of the indicated genotypes. Cell outlines were traced in dark grey. (**B**) Quantification of stomata and stomatal precursors of the genotypes shown in A. Error bars represent the s.e.m. Letters indicate significance groups for each cell type; samples with the same letters are not significantly different ($p < 0.05$).

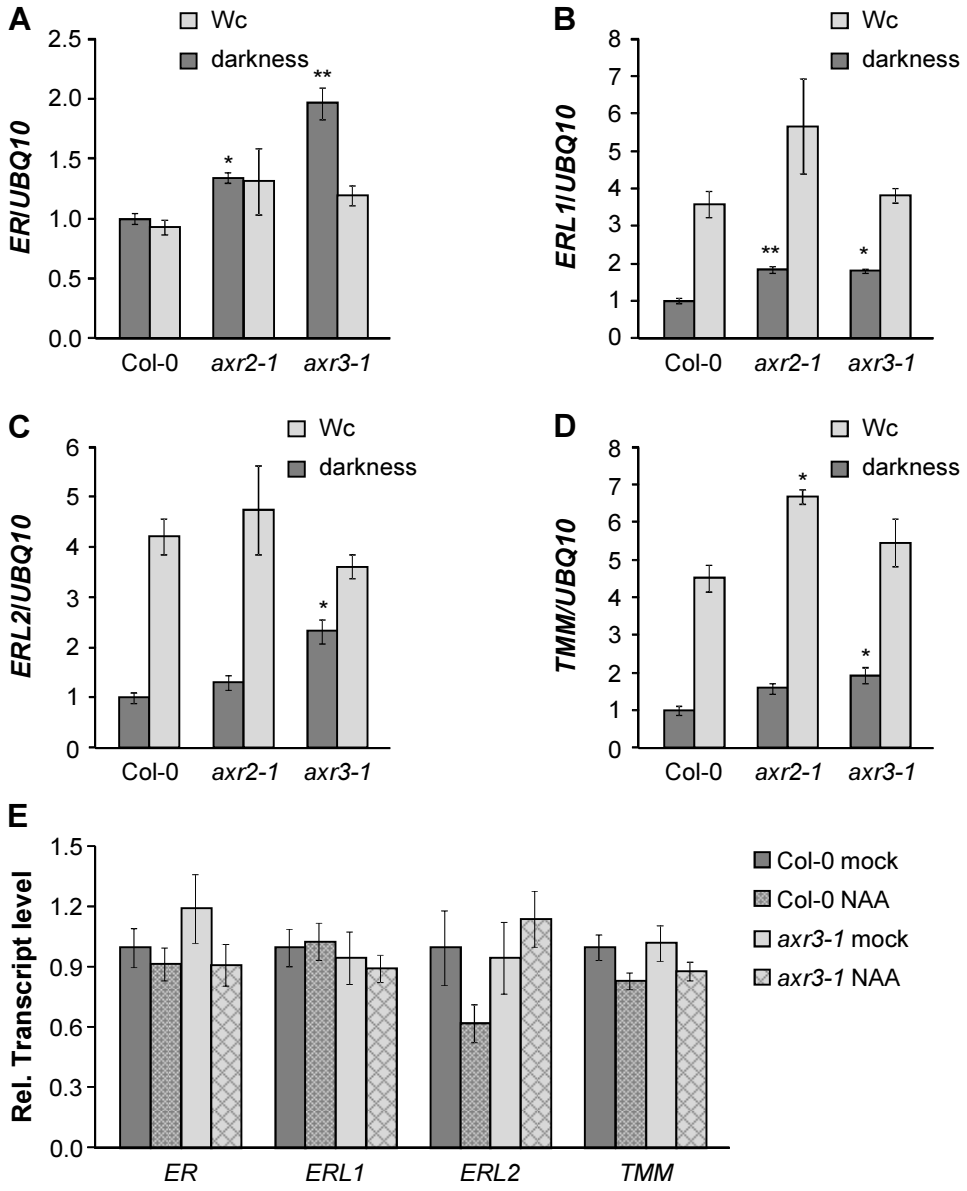

Fig. S8. Analysis of *ER*(*L*) *and TMM* transcripts. (**A-D**) Transcript levels of *ER* (A), *ERL1* (B), *ERL2* (C) and *TMM* (D) in 5-d-old dark- or Wc-grown seedlings of the indicated genotypes. (**E**) *ER*(*L*) and *TMM* transcript levels in 5-d-old Wc-grown Col-0 and *axr3-1* seedlings treated with 10 µM NAA or mock-treated. Transcript levels were normalised to *UBQ10* and are shown relative to the levels of dark-grown Col-0 seedlings (A-D) or the mock-treated Col-0 control (E). Error bars represent the s.e.m. Asterisks indicate significant differences to dark-grown wild type (A-D) or the mock-treated control (E) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

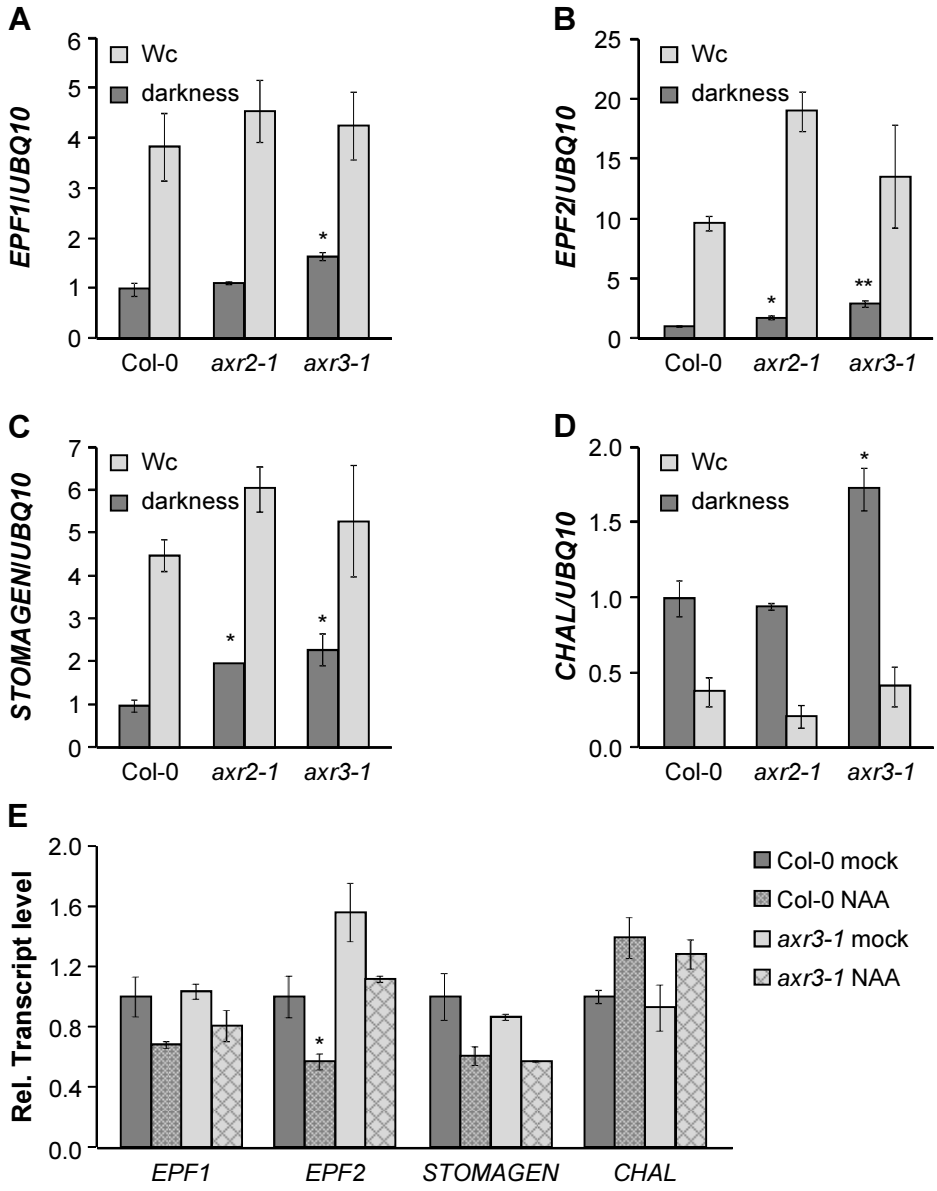

Fig. S9. Analysis of *EPFL* transcripts. (**A-D**) Transcript levels of *EPF1* (A), *EPF2* (B), *STOMAGEN* (C) and *CHAL* (D) in 5-d-old dark- or Wc-grown seedlings of the indicated genotypes. (**E**) *EPFL* transcript levels in 5-d-old Wc-grown Col-0 and *axr3-1* seedlings treated with 10 µM NAA or mock-treated. Transcript levels were normalised to *UBQ10* and are shown relative to the levels of dark-grown Col-0 seedlings (A-D) or the mock-treated Col-0 control (E). Error bars represent the s.e.m. Asterisks indicate significant differences to dark-grown wild type (A-D) or the mock-treated control (E) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

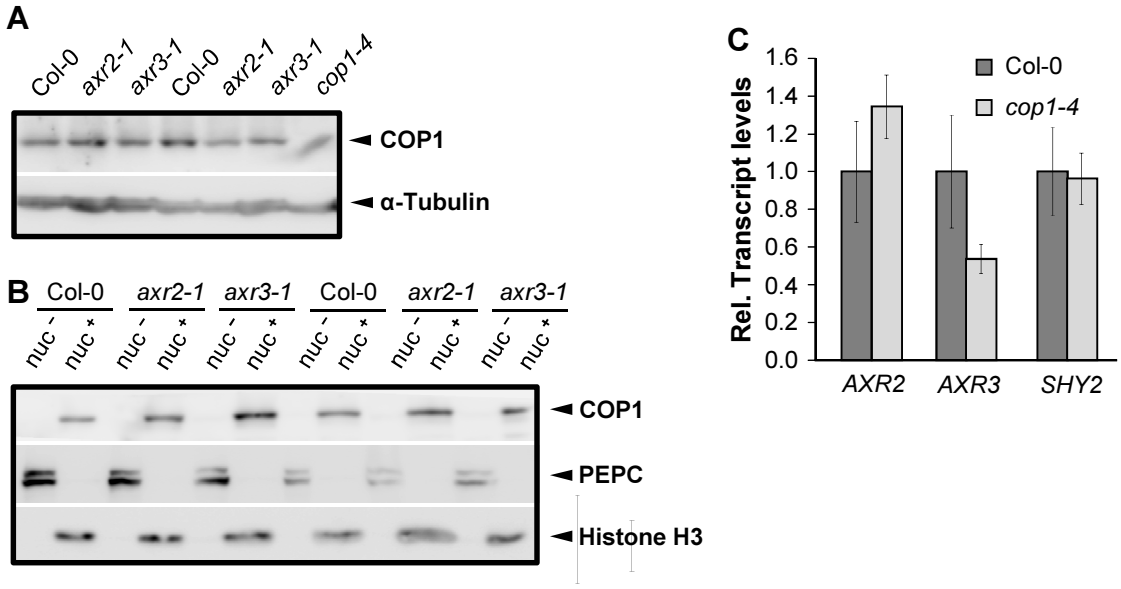

Fig. S10. Aux/IAA proteins and COP1 seem to act independently in light-dependent stomata development. (**A, B**) Immunodetection of COP1 protein in total protein extracts (A), nuclei-depleted (nuc^-) and nuclei-enrichted (nuc^+) protein fractions (B) of 5-d-old dark-grown Col-0, *axr2-1* and *axr3-1* seedlings. Tubulin is shown as loading control, PEPC is shown as cytosolic marker and histone H3 is shown as nuclear marker. The nuclei-enriched fractions were 15 x enriched compared to the nuclei-depleted fractions. Two independent biological replicates are shown. (**C**) Transcript levels of *AXR2*, *AXR3* and *SHY2* in 5-d-old dark-grown Col-0 and *cop1-4* seedlings. Transcript levels were normalised to *UBQ10* and are shown to relative to the levels of dark-grown Col-0 seedlings. Error bars represent the s.e.m.

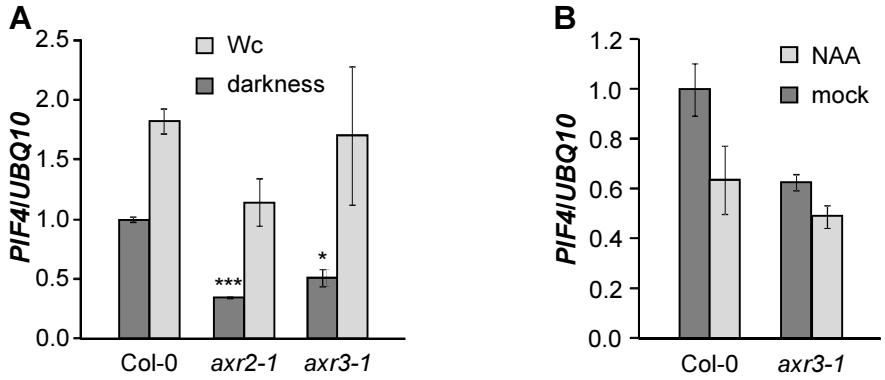

Fig. S11. Analysis of *PIF4* transcript. (**A**) Transcript levels of *PIF4* in 5-d-old dark- or Wc-grown seedlings of the indicated genotypes. (**B**) *PIF4* transcript levels in 5-d-old Wc-grown Col-0 and *axr3-1* seedlings treated with 10 µM NAA or mock-treated. Transcript levels were normalised to *UBQ10* and are shown relative to the levels of dark-grown Col-0 seedlings (A) or the mock-treated Col-0 control (B). Error bars represent the s.e.m. Asterisks indicate significant differences to dark-grown wild type (A) or the mock-treated control (B) (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

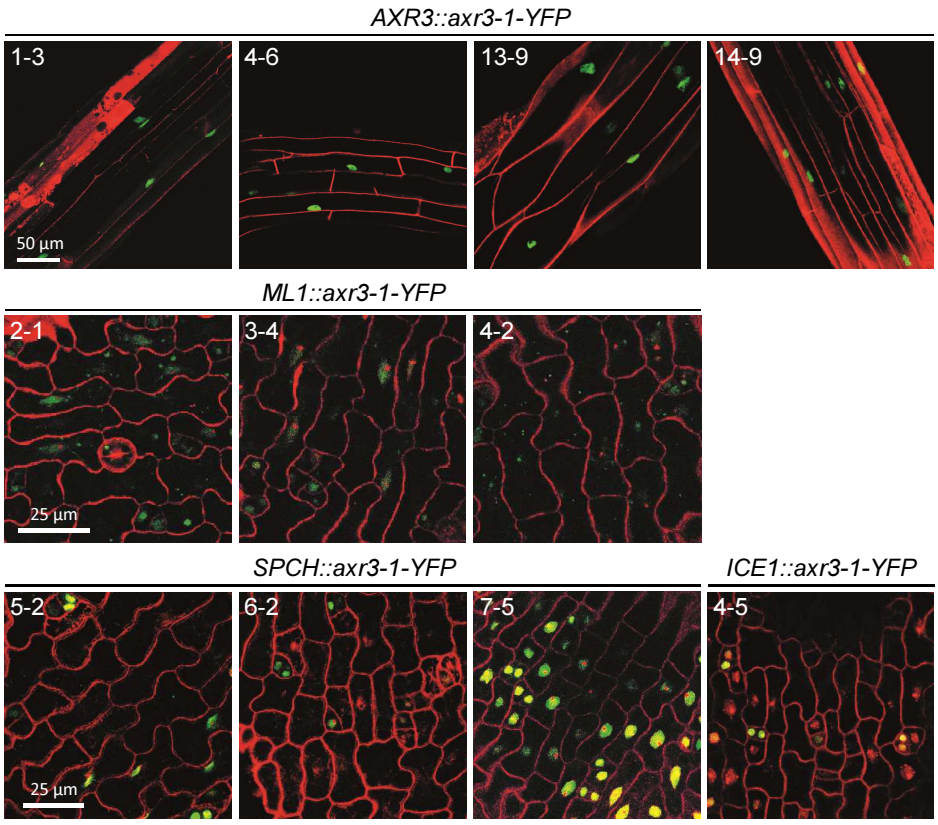

Fig. S12. Accumulation of axr3-1-YFP protein in dark-grown seedlings of additional *promoter::axr3-1-YFP* lines. Confocal images of the hypocotyl of Col-0 seedlings expressing axr3-1-YFP from the *AXR3* promoter (upper panel) and of the cotyledon epidermis of Col-0 seedlings expressing axr3-1-YFP from the *ML1* (middle panel), *SPCH* and *ICE1* (lower panel) promoters. Cell outlines were visualised by PI staining.

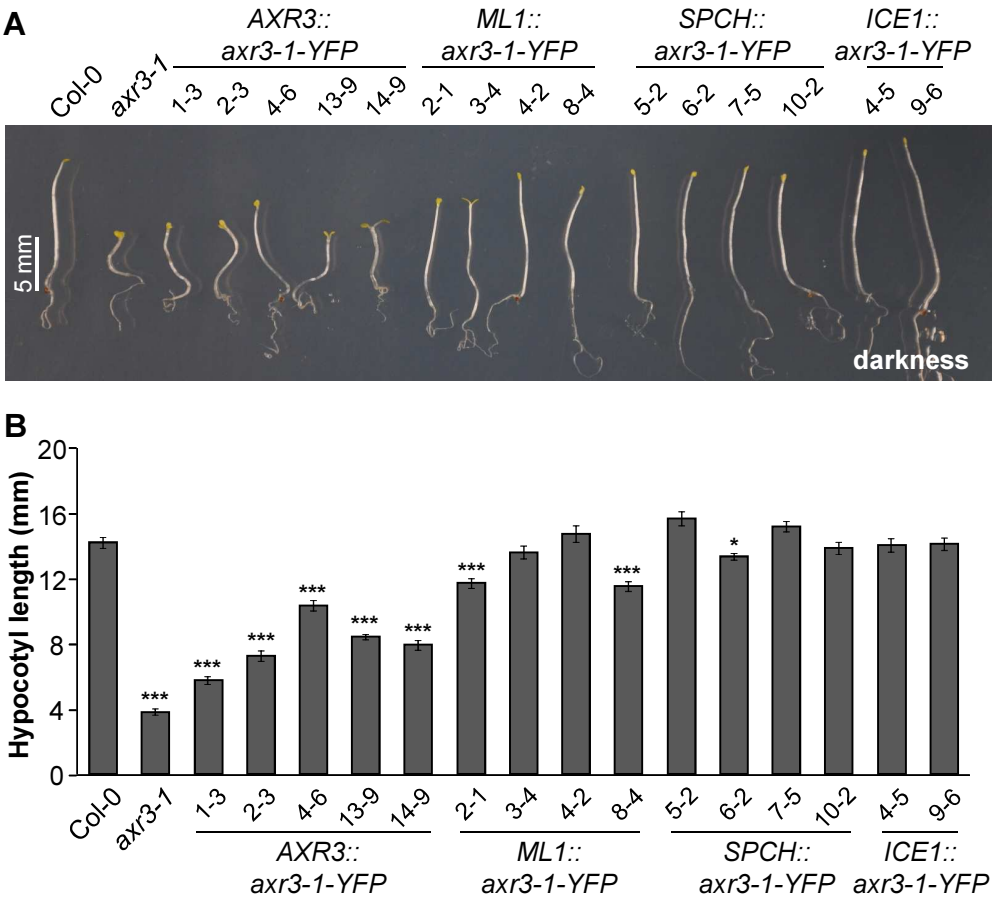

Fig. S13. Plants expressing axr3-1-YFP in the epidermis partially mimic the phenotype of dark-grown *axr3-1* seedlings. (**A**) Visual phenotype of 10-d-old dark-grown seedlings of the indicated *promoter::axr3-1-YFP* lines. Numbers denote independent transgenic lines. Dark-grown Col-0 and *axr3-1* seedlings are shown as controls. (**B**) Quantification of hypocotyl length of the lines shown in A. Error bars represent the s.e.m. Asterisks indicate significant differences in hypocotyl length compared to the wild type (*** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$).

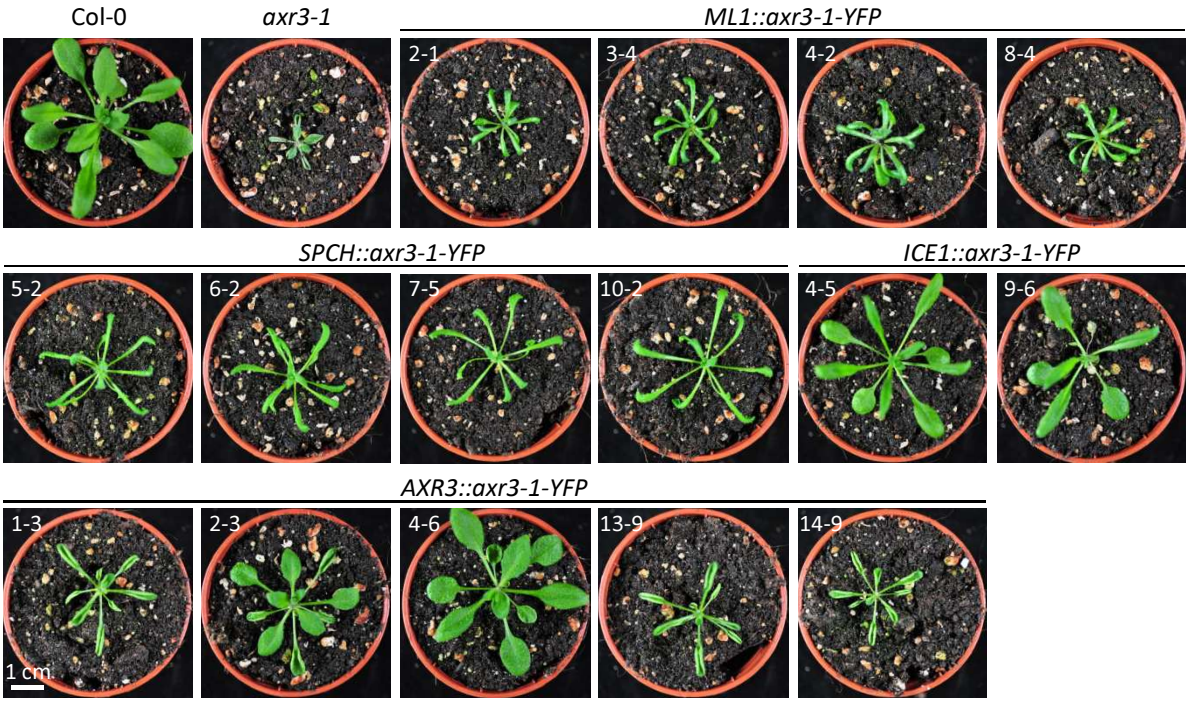

Fig. S14. The *axr3-1* adult plant phenotype is not completely mimicked by any *promoter::axr3-1-YFP* line. Visual phenotype of 24-d-old adult plants of the indicated *promoter::axr3-1-YFP* lines grown in long day conditions. Col-0 and *axr3-1* plants are shown as controls.

Supplementary Tables

Table S1. Primers and restriction enzymes used for genotyping.

Mutant	Primer name	Primer sequence	Restr. enzyme
axr2-1	AXR2-geno_F2	CAATACATACATGCGTACAAGC	MslI
	AXR2-geno_R2	ATGACTCTAACTCGGTAAGGTTC	
axr3-1	AXR3-geno_F1	TTTTCCACTCTTCTCTACTGCTC	BshTI
	AXR3-geno_R1	CTCCGTCCATTGATACCTTC	
cop1-4	COP1-geno_F1	GATGCGCTGAGTGGGCCA	HpyF10VI
	COP1-geno_R1	TGCCATTGTCCTTTTACCATTTCAGC	
er-105	ERg2248	AAGAAGTCATCTAAAGATGTGA	–
	Erg3016rc	AGAATTTTCAGGTTTGGAATCTGT	
	er-105	AGCTGACTATACCCGATACTGA	
erl1-2	ERL1-geno_F2	AGGGAAAAATACCAGTTGAGC	–
	ERL1-geno_R2	CGGAGAGATTGTTGAAGGAG	
	JL-202	CATTTTATAATAACGCTGCGGACA TCTAC	
erl2-1	ERL2-geno_F3	GAACTATCCCAGAGAGCATT	–
	ERL2-geno_R3	TGATTCAAGGCAGCACAG	
	JL-202	CATTTTATAATAACGCTGCGGACA TCTAC	
fama-1	FAMA-geno_F1	TGGTCTTGCTCGTTCTAGCTC	–
	FAMA-geno_R1	CTATCTTGCATGTCTTGCGTC	
	SALK-LBb1.3	ATTTTGCCGATTTCGGAAC	
mute-1	mute-dCAPS_F1	TTCGTTCTTTGACTCCTTGTTTCT ACCTCAAAAG	BslI
	mute-dCAPS_R1	CTTCGAGAAAATAATTAGGATTGT GAATTGAG	
spch-3	SPCH-geno_F1	GAAAAACCTAGATCCTCCCCC	–
	SPCH-geno_R3	AACCTGAAGAATCTCAAGAGCC	
	SAIL-LB1	GCCTTTTCAGAAATGGATAAATAG CCTTGCTTCC	
tmm-1	tmm-1-dCAPS_F1	AACGCGTTCAAAGGGCTCAAGAACGT	AclI
	tmm-1-dCAPS_R2	AGACTGTTATCGTTGAGCC	

Table S2. Primers used for qPCR.

Gene	Primer name	Primer sequence
AXR2	AXR2-RT_F2	AGAGTCCTGCCAAATCGG
	AXR2-RT_R2	TGAGATCAACGGTTTCGG
AXR3	AXR3-RT_F1	CTCTTTTACCATGGGCAAACATGGA
	AXR3-RT_R1	AGGGAACATAGTCCCAGCTATTCA
CHAL	CHAL-RT_F2	CATTACAACCATCAGAAAACACGAG
	CHAL-RT_R2	TGACACAGAGGACGAAGAAGAGTAG
EPF1	EPF1-RT_F2	GGCATTTACCAACATCCTCC
	EPF1-RT_R2	CCTCCTCCTCTGACGCTTC
EPF2	EPF2-RT_F2	CACTAAAAAACACGGTCAATGG
	EPF2-RT_R2	TGCTTATTTCCTTCTTGTGGTG
ER	ER-RT_F2	GACTCTTGATTGGGACACACG
	ER-RT_R2	AGAATGTTGGACGACTTCACG
ERL1	ERL1-RT_F1	GCAGCAAGAGAATGAAGTTAGG
	ERL1-RT_R1	AACCATCCTTTCCTTCCAATC
ERL2	ERL2-RT_F2	AAGTGATTTAGGGCTAGTATGAGGG
	ERL2-RT_R2	GAACTCGTGAAGATGTCCATTG
FAMA	FAMA-RT_F2	CTGCTTTGGAGGATCTTCATCTCT
	FAMA-RT_R2	CTTCTGCCGTAAACCTCGTTTC
MUTE	MUTE-RT_F2	GACGATCACTTCATCAGACACAAAG
	MUTE-RT_R2	CCTCAATATTAGTAGCATGGAGGAGACT
PIF4	PIF4-RT_F1	GCTTCGGCTCCGATGAT
	PIF4-RT_R1	CGCGGCCTGCATGTGTG
SHY2	SHY2-RT_F1	CTTAAAGCTTTAGAAGTGATGTTCAA
	SHY2-RT_R1	CACGTACATATGAACATCTCCCA
SPCH	SPCH-RT_F2	TTCTGCACTTAGTTGGCACTCAAT
	SPCH-RT_R2	GCTGCTCTTGAAGATTTGGCTCT
STOMAGEN	STOM-RT_F2	AATACGGTCTCCCTTCTCCC
	STOM-RT_R2	CTGGAACTTGCTCTGCTCTG
TMM	TMM-RT_F3	AACAGTCTTCGGGTCCTTCAC
	TMM-RT_R3	GCTCGCTTAGATGCTTCACG
UBQ10	UBQ10-RT_F	CACACTCCACTTGGTCTTGCGT
	UBQ10-RT_R	TGGTCTTTCCGGTGAGAGTCTTCA

Table S3. Primers used for cloning.

Primer name	Primer sequence
AXR3pro-attB-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTTGTGGTAGAATGTTGAGAGTTGTGGC
AXR3pro-attB-R	GGGGACCACTTTGTACAAGAAAGCTGGGTTATTAACCTTTCTTCTTCTTTGGTGTTC
AXR3-XhoI-R	CATGCTCGAGAGCTCTGCTCTTGCACTTCTCC
HindIII-AXR3-F	CTTGAAGCTTATGATGGGCAGTGTCGAGCTG
ICE1pro-attB-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTACCGGACCACCGTCAATAACATCG
ICE1pro-attB-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGCCAAAGTTGACACCTTTACCC
SPCHpro-attB-F	GGGGACAAGTTTGTACAAAAAAGCAGGCTCAAGATCATCACTGCGATAAGGAG
SPCHpro-attB-R	GGGGACCACTTTGTACAAGAAAGCTGGGTCGTGATTAGAGATATATCCTTCTC
XhoI-YFP-F	GCATCTCGAGATGGTGAGCAAGGGCGAGGAG
YFP-SpeI-R	TCACACTAGTCTACTTGTACAGCTCGTCCATGC