Supporting Information (SI Appendix)

A Class II KNOX gene, *KNOX4*, controls seed physical dormancy

Maofeng Chai, Chuanen Zhou, Isabel Molina, Chunxiang Fu, Jin Nakashima, Guifen Li, Wenzheng Zhang, Jongjin Park, Yuhong Tang, Qingzhen Jiang and Zeng-Yu Wang

Supplementary Figures

Movie 1. Imbibition process of wild-type and *knox4* mutant seeds of *Medicago truncatula* in water.

Fig. S1. Seed vigor test and genetic segregation analysis for the non-dormant mutant in *M. truncatula.*

Fig. S2. Molecular cloning of *KNOX4* in *M. truncatula*.

Fig. S3. Surface feature of wild-type and *knox4* seed coat.

Fig. S4. Wax load in seed surfaces of wild type and *knox4-1* mutant.

Fig. S5. Volcano plot of 2-fold down-regulated and up-regulated genes in *knox4* knockout (KO) mutant.

Fig. S6*A*. Flow chart of GO enrichment with 2-fold down-regulated genes in *knox4* mutant.

Fig. S6*B*. MapMan general overview of differentially expressed genes related to cell wall pathways and lipid pathways.

Fig. S7. Phenotype observation of GFP:KNOX4 complementation lines and chromatin immunoprecipitation (ChIP) analysis.

Fig. S8. A proposed model for impermeable seed coat development regulated by *KNOX4* in *M. truncatula*.

Fig. S9. Toluidine blue staining of *knox4* mutant seeds in *M. truncatula*.

Supplementary Tables

Table S1. The most differentially expressed genes found in a microarray analysis of *knox4* and wild-type seed coat.

Table S2. Species used for the phylogenetic analysis.

Table S3. Primers used in this study
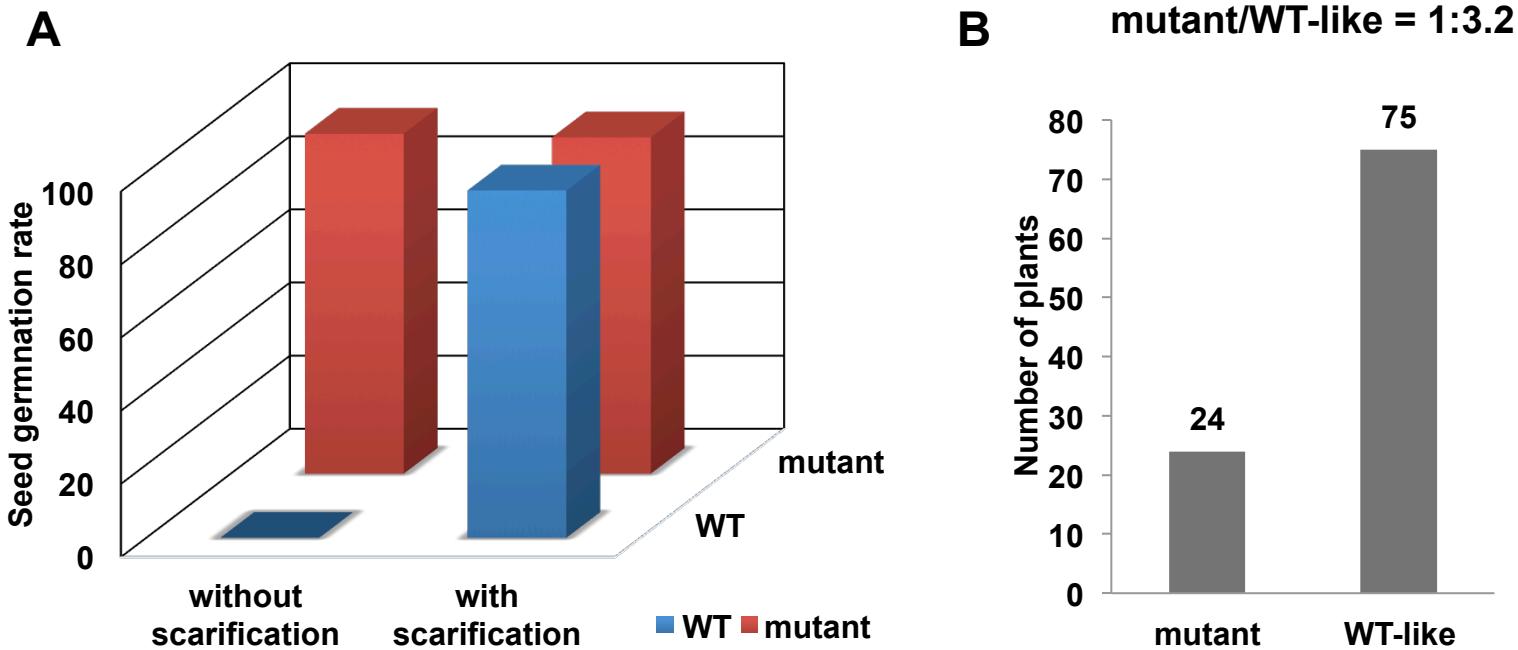
Fig. S1. Seed vigor test and genetic segregation analysis for the non-dormant mutant in *M. truncatula.*

(*A*) Seed germination frequency of wild type (WT) and mutant with and without scarification treatment in sterile water for 2 days.

(*B*) Number of wild-type-like and mutant plants obtained from F2 population.
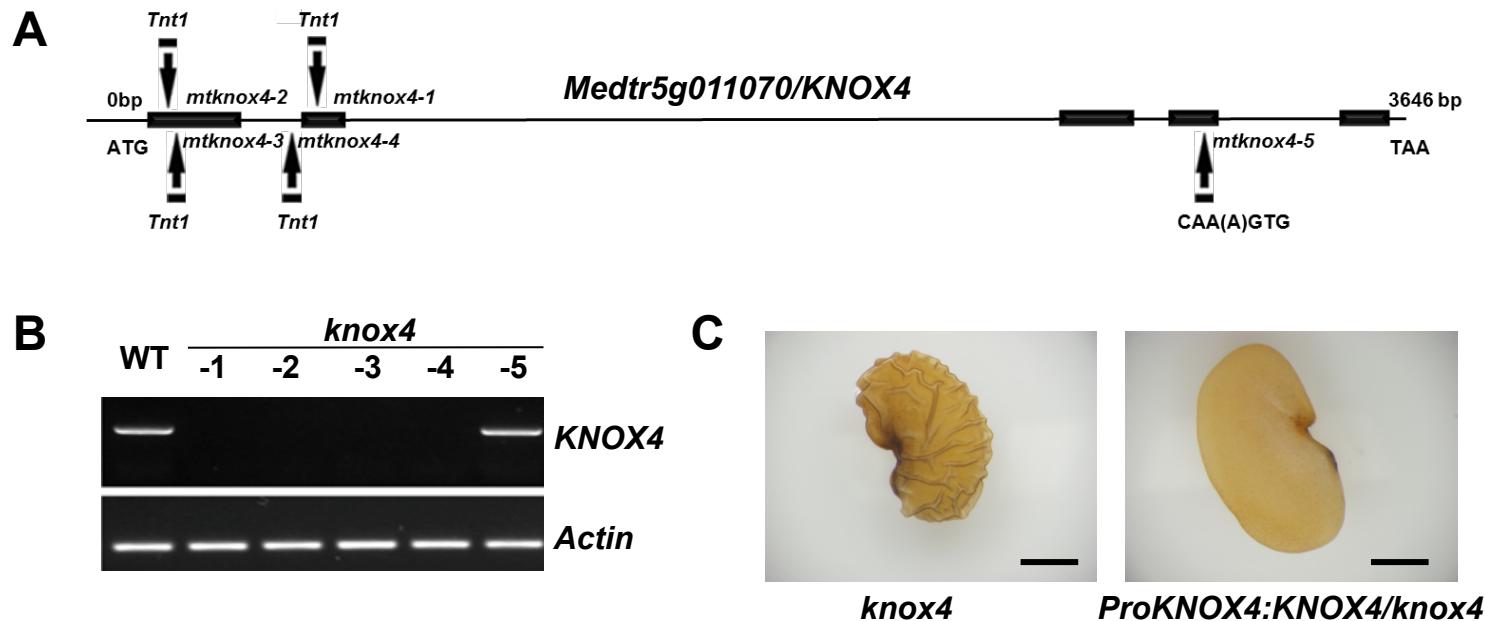
Fig. S2. Molecular cloning of *KNOX4* in *M. truncatula*.

(*A*) Schematic diagram of gene structure and five allele sites of *KNOX4*. Boxes represent exons and lines represent introns.

(*B*) RT-PCR analysis of *KNOX4* transcripts in the wild type and *knox4* mutants (*knox4-1* to *knox4-5*). Actin was used as the loading control.

(*C*) Phenotype observation of mutant and *KNOX4* complementation lines with water imbibition assay. Bar = 1mm.
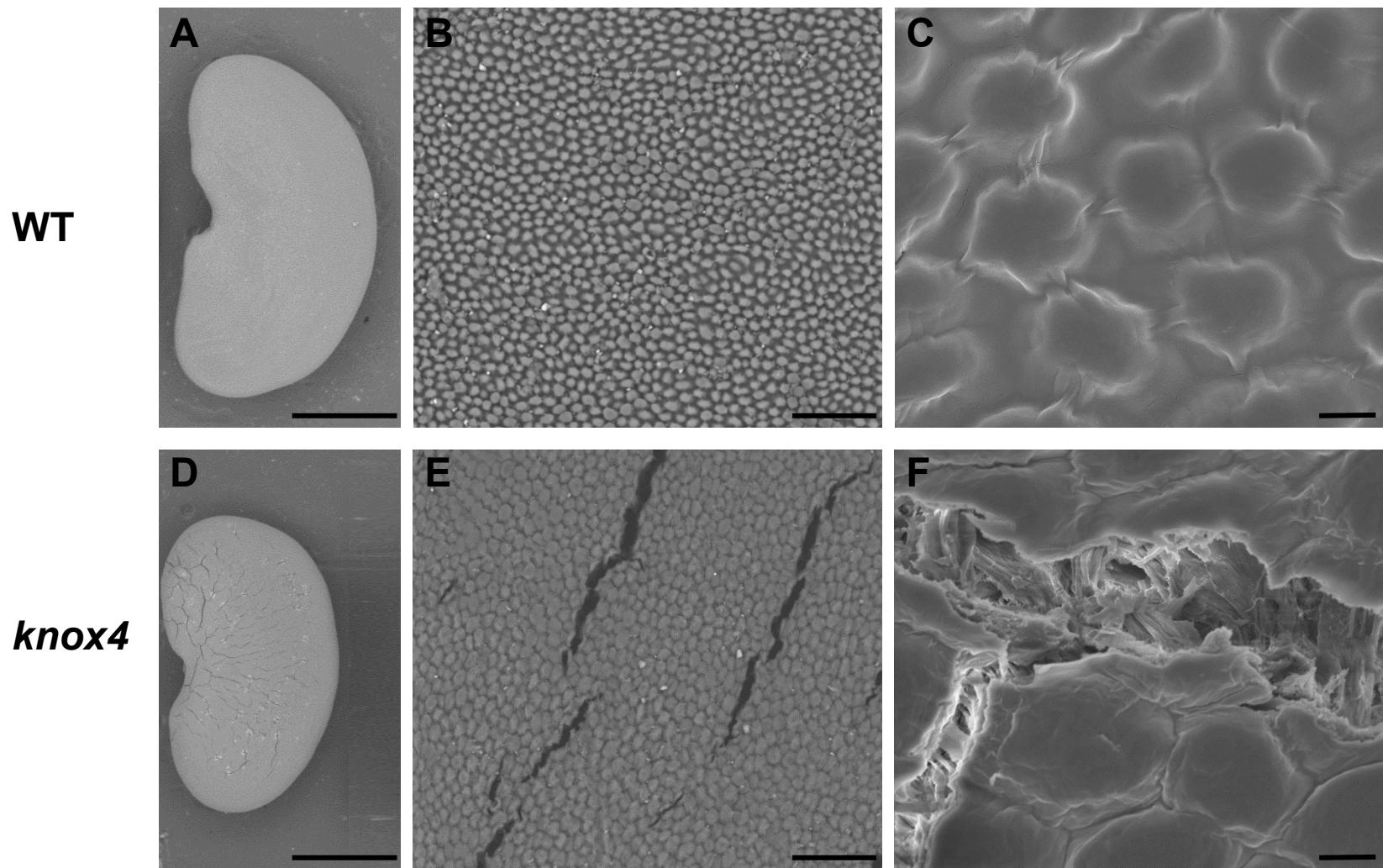
Fig. S3. Surface feature of wild-type and *knox4* seed coat.

(*A-F*) Scanning electron microscope images of seed coat from wild type (*A*, *B*, *C*) and *knox4* mutant (*D*, *E*, *F*). Minor cracks occurred under SEM with vacuum after 30 min. (*A*, *D*) Bar = 1cm. (*B*, *E*) Bar = 200µm. (*C*, *F*) Bar = 2µm.
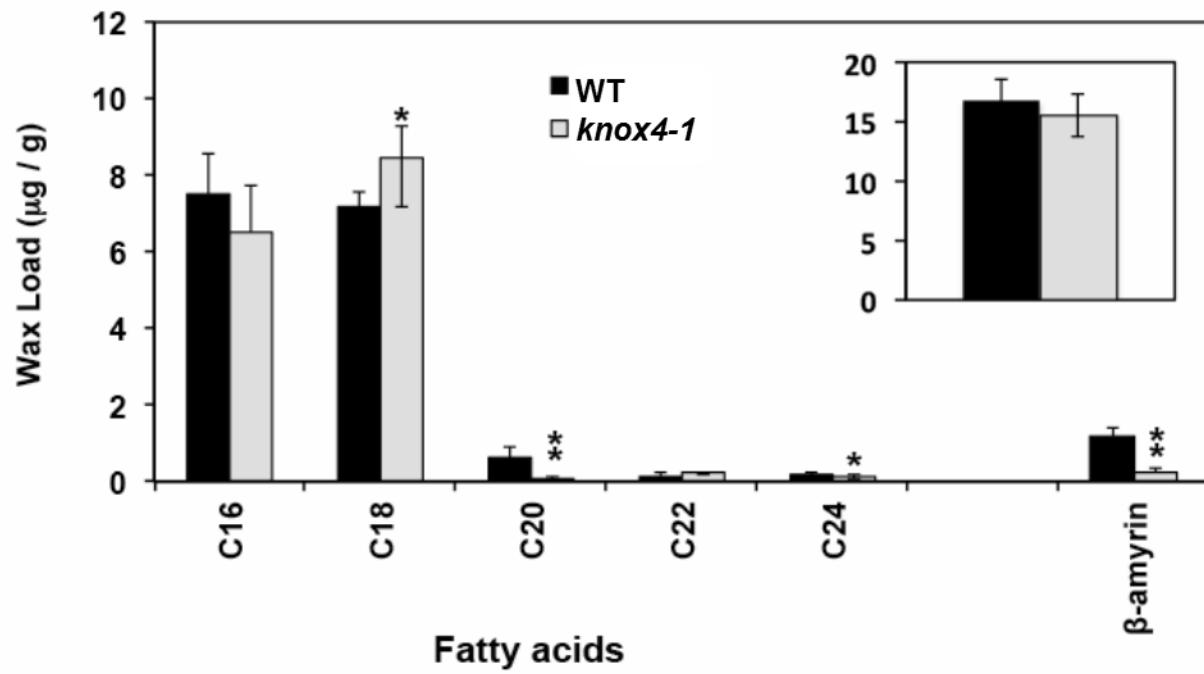
**Fig. S4.** Wax load in seed surfaces of wild type and *knox4-1* mutant. Inset: total seed wax loads.

Volcano plot
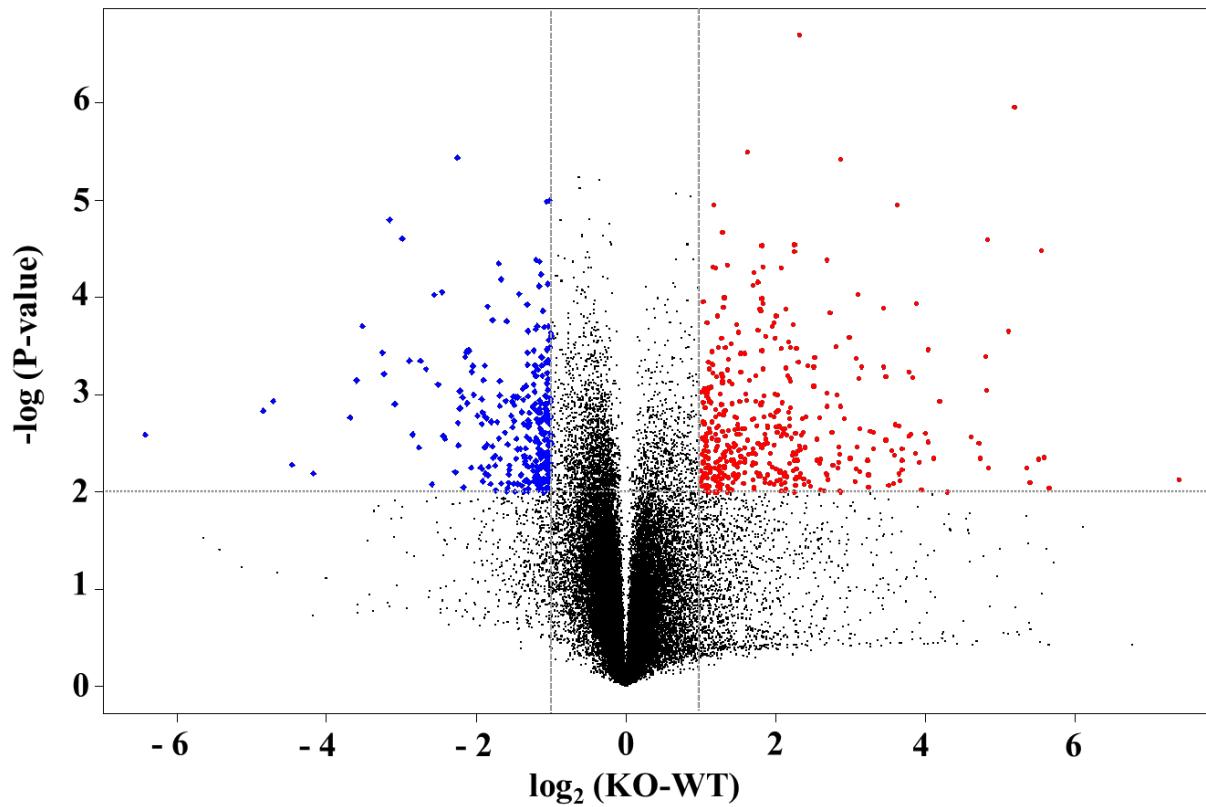
Blue dot: > 2 fold low expression with statistically highly significance (P- value < 0.01) in KO.
Red dot: > 2 fold high expression with statistically highly significance (P- value < 0.01) in KO.

Fig. S5. Volcano plot of 2-fold down-regulated and up-regulated genes in *knox4* knockout (KO) mutant.
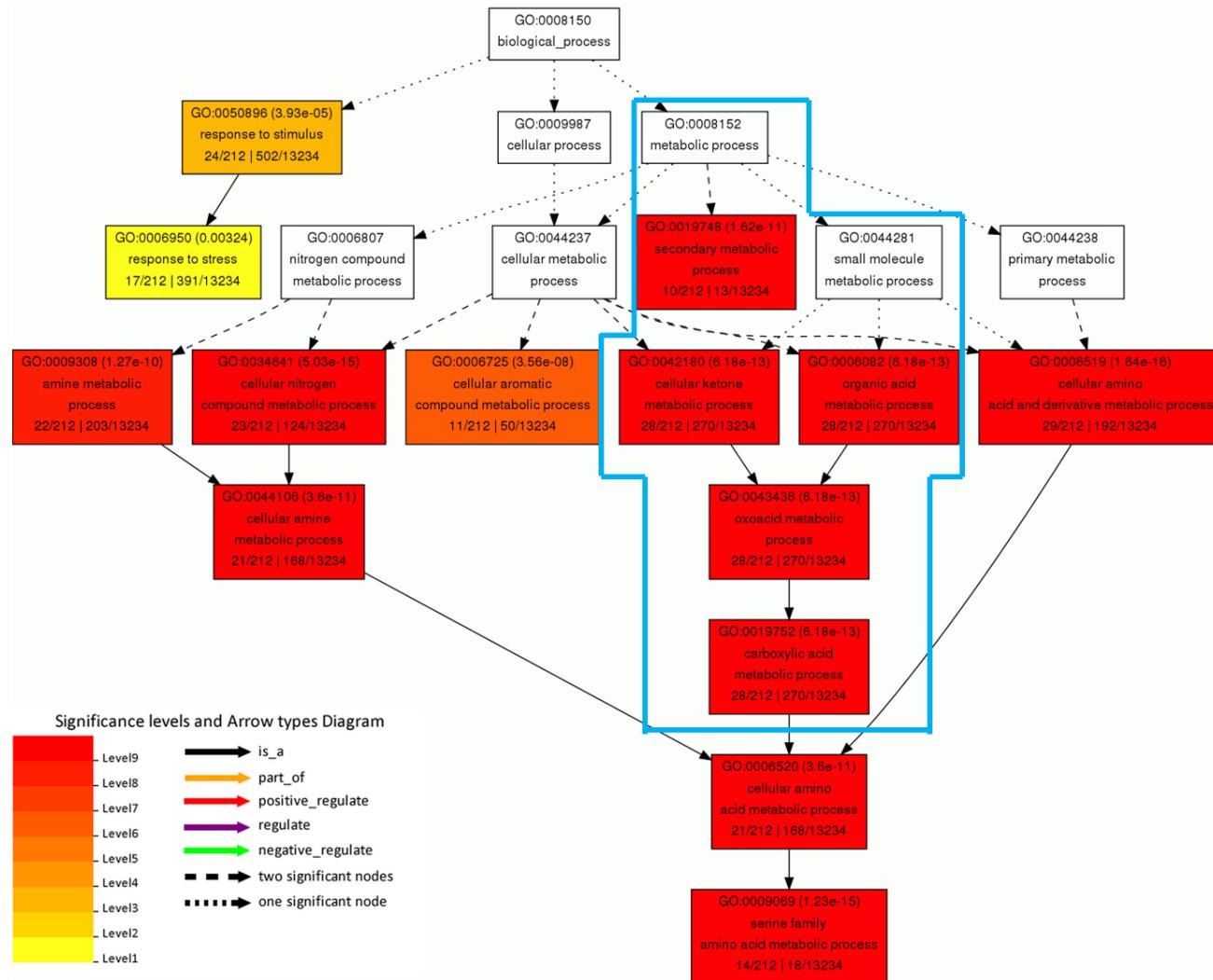
Fig. S6A. Flow chart of GO enrichment with 2-fold down-regulated genes in *knox4* mutant. Blue box: 2 fold lower expression (P- value < 0.01) in mutant.
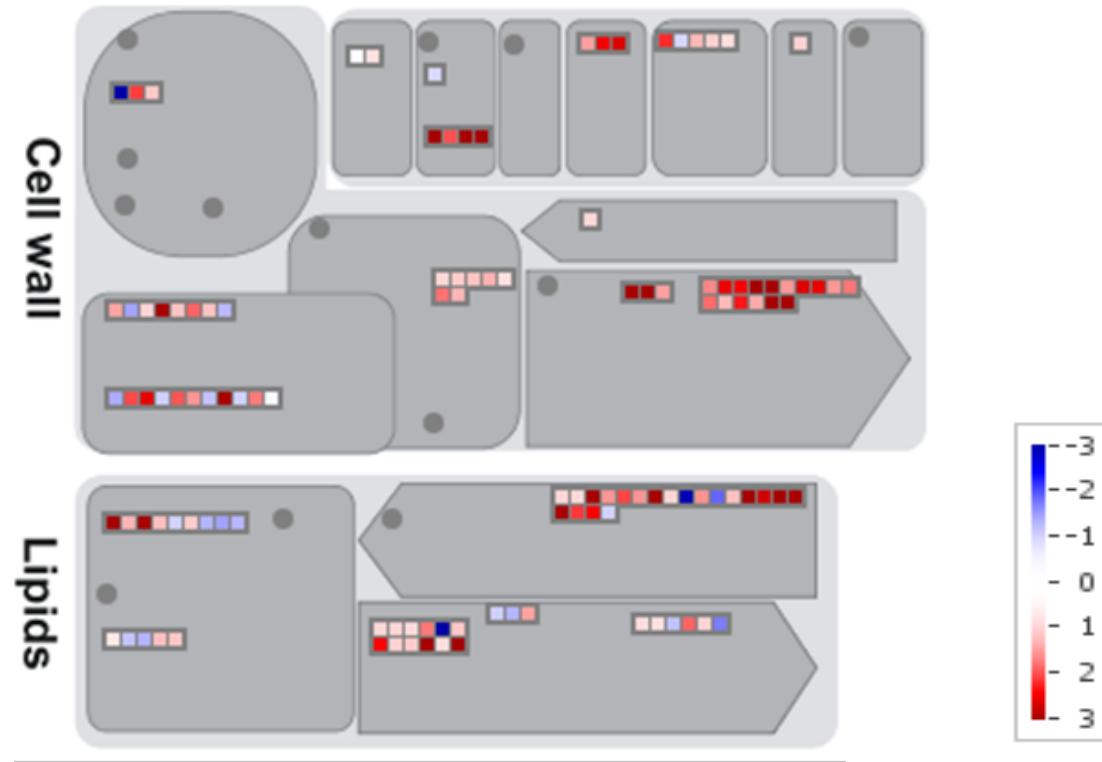
Fig. S6*B*. MapMan general overview of differentially expressed genes related to cell wall pathways and lipid pathways. Each square represents a gene. Red and blue indicate higher and lower expression in wild type compared to mutant. The figure is shown in log2-transformed ratios of wild-type divided by mutant.
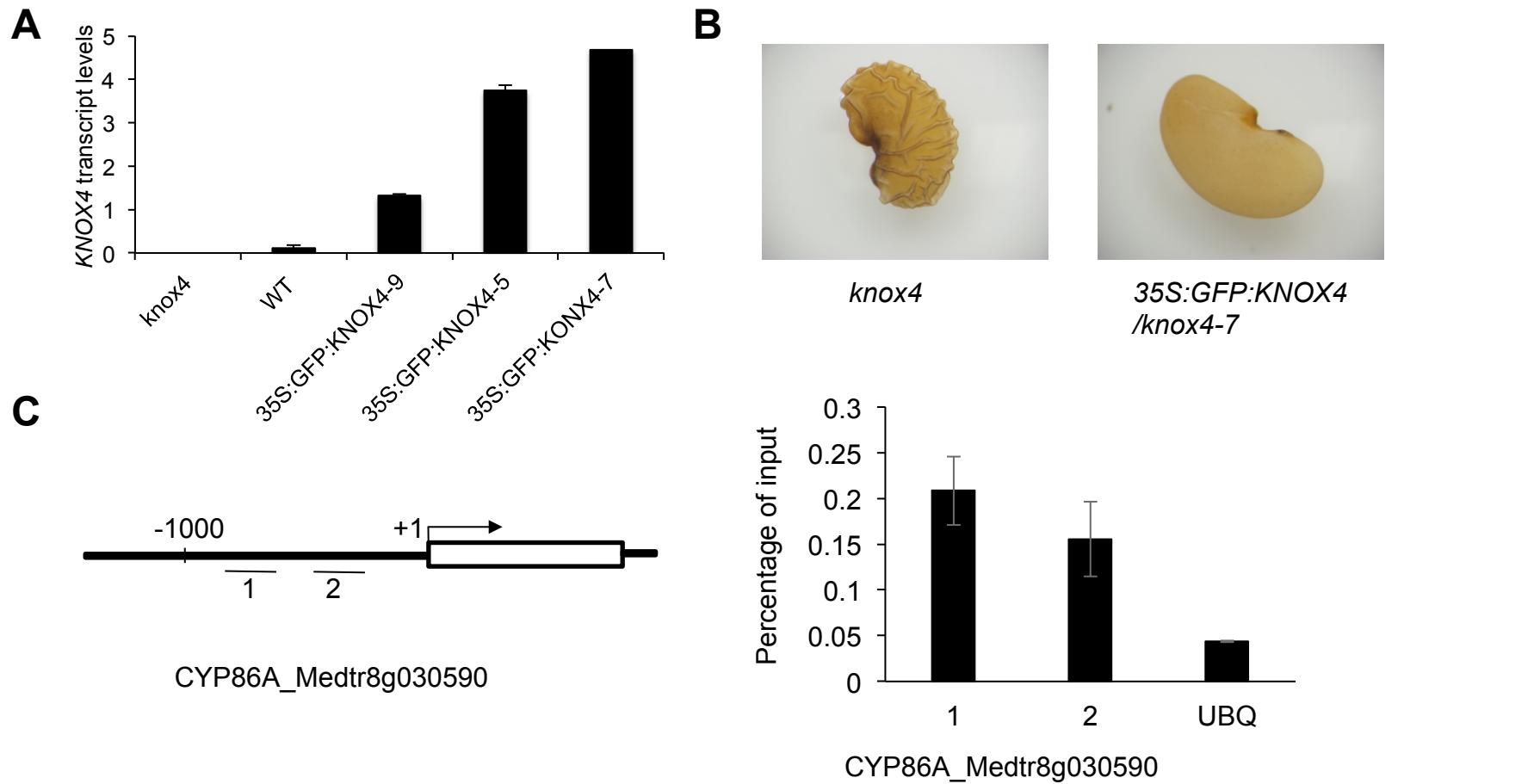
Fig. S7. Phenotype observation of GFP:KNOX4 complementation lines and chromatin immunoprecipitation (ChIP) analysis.

(*A*) Transcript levels of *KNOX4* in mutant, wild type and GFP:KNOX4 complementation lines, as determined by quantitative real-time PCR (qRT-PCR).

(*B*) Imbibition of seeds of wild-type and GFP:KNOX4 complementation lines in water.

(*C*) Chromatin Immunoprecipitation of GFP:KNOX4 protein with *CYP86A* gene chromatin regions. PCR amplification locations (bars with numbers) were diagrammed in the graph. The ChIP experiments were repeated three times using independent biological replicates with similar results.
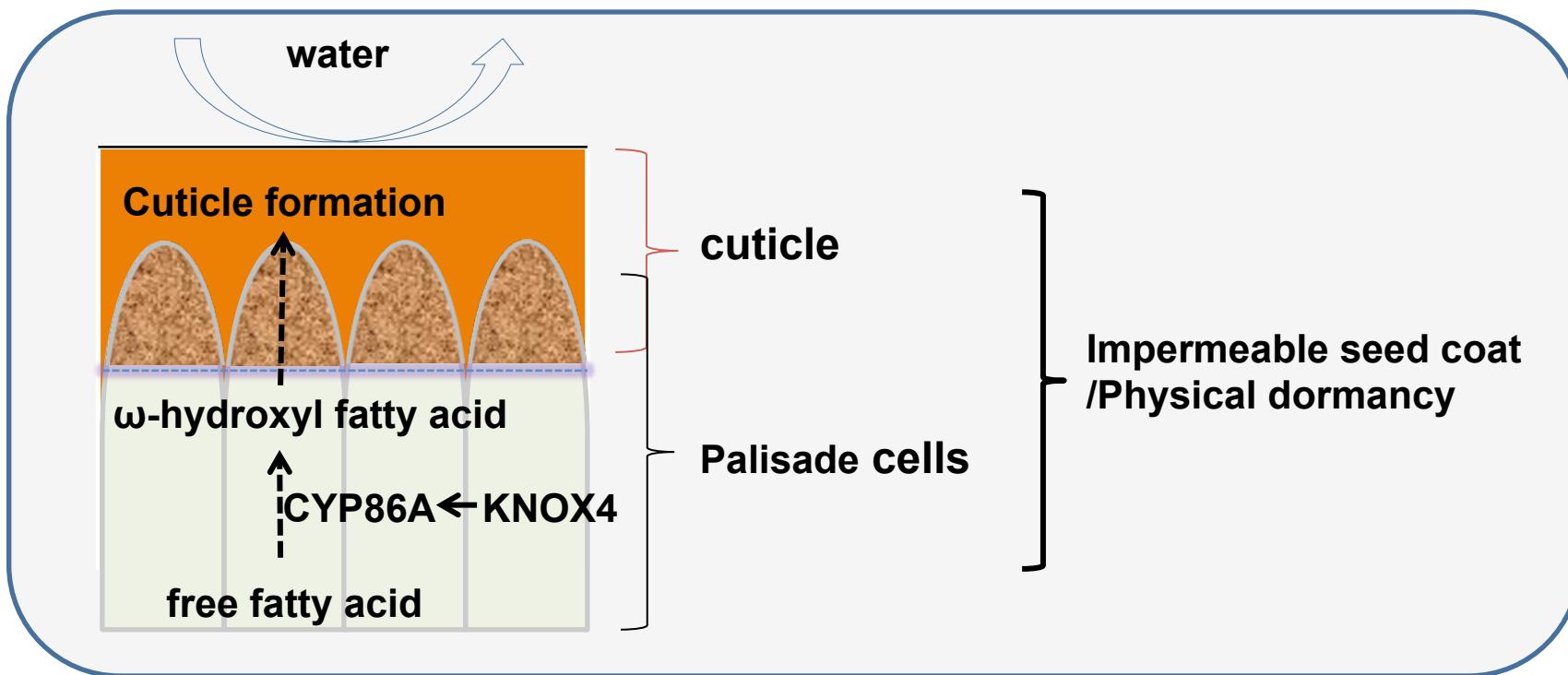
Fig. S8. A proposed model for impermeable seed coat development regulated by *KNOX4* in *M. truncatula*. In palisade cells, CYP86A is a crucial enzyme for cuticle formation. CYP86A gene is regulated by *KNOX4* in *M. truncatula*. Rigid palisade layer of cells covered with an intact cuticle layer constitute an impermeable seed coat.
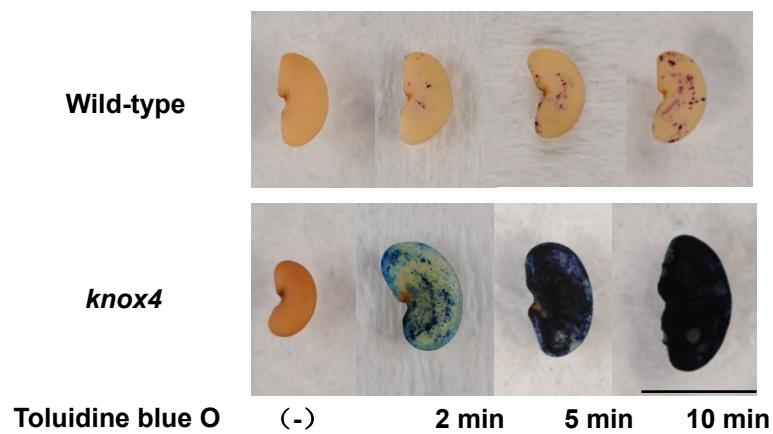
Fig. S9. Toluidine blue staining of *knox4* mutant seeds in *M. truncatula*. Seeds were immersed in 0.05% toluidine blue O for 2, 5 and 10 min and rinsed with water. Bar = 0.5 cm.

Table S1. The most differentially expressed genes found in a microarray analysis of *knox4* and wild-type seed coat.

| Probe sets	Gene name/annotation	Gene ID	Fold change (*knox4*/WT)	P Value
Mtr.15209.1.S1_at	Phosphatidylinositol transporter	Medtr4g096250.1	0.02	0.0296
Mtr.49936.1.S1_at	Cytochrome P450，ATT1	Medtr8g030590.1	0.1258	2E-05
Mtr.49305.1.S1_at	KCS12 | 3-ketoacyl-CoA synthase 12	Medtr2g114190.1	0.1313	0.042
Mtr.16532.1.S1_at	Glucan endo-1,3-beta-glucosidase-like protein	Medtr4g133690.1	0.0233	0.0393
Mtr.9377.1.S1_at	Cell wall pectin metabolic process	Medtr1g055085.1	0.0396	0.0669
Mtr.16432.1.S1_at	Represses phenylpropanoid biosynthesis	Medtr5g087140.1	0.055	0.0064
Mtr.8859.1.S1_at	cell wall protein	Medtr5g004980.1	0.0625	0.077
Mtr.34401.1.S1_s_at	Represses phenylpropanoid biosynthesis	Medtr5g087140.1	0.0776	0.0017
Mtr.49452.1.S1_at	D-arabinono-1,4-lactone oxidase	Medtr7g145950.1	0.0834	0.1756
Mtr.44985.1.S1_at	ANR，Negative regulator of flavonoid biosynthesis	Medtr4g092080.1	0.0868	0.0002
Mtr.12295.1.S1_at	Inorganic pyrophosphatase	Medtr8g077180.1	0.0912	0.0319
Mtr.49572.1.S1_s_at	CHS WD40 same pattern	Medtr5g007810.1	0.1175	0.029
Mtr.20185.1.S1_x_at	CHS WD40 same pattern	Medtr5g007840.1	0.1726	0.0207

Table S2. Species used for the phylogenetic analysis.

All information used for phylogenetic analyses comes from Phytozome v11.

Organism	Version	Common Name	Source
Aquilegia coerulea Goldsmith	v1.1	Colorado blue columbine	JGI annotation of assembly v1
Amborella trichopoda	v1	Amborella	The Amborella Genome Project
Arabidopsis lyrata	v1.0	Lyre-leaved rock cress	JGI release v1.0
Arabidopsis thaliana columbia	TAIR10	Thale cress	The Arabidopsis Information Resource
Boechera stricta	v1.2	Drummond's rock cress	JGI annotation of assembly v1
Brachypodium distachyon	v3.1	Purple false brome	JGI annotation of assembly v3.0 (strain Bd21)
Brachypodium stacei	v1.1	*B. stacei*	JGI annotation of assembly v1.0 (strain ABR114)
Brassica rapa FPsc	v1.3	Turnip mustard - FasPlant self-compatible	JGI annotation of assembly v1
Capsella grandiflora	v1.1	-	JGI annotation of assembly v1
Capsella rubella	v1.0	Red shepherd's purse	JGI annotation of assembly v1
Carica papaya	ASGPBv0.4	Papaya	ASGPB release of 2007
Chlamydomonas reinhardtii	v5.5	Green Algae	JGI v5.0 assembly, JGI annotation based on Augustus u11.6

Citrus clementina	v1.0	Clementine	JGI annotation of assembly v1
Citrus sinensis	v1.1	Sweet orange	JGI annotation of assembly v1
Cucumis sativus	v1.0	Cucumber	from Roche 454 assembly and JGI 2009 annotation pipeline
Eucalyptus grandis	v2.0	Eucalyptus	JGI annotation v2.0 (mapping of v1.1 models forward) on assembly v2.0
Eutrema salsugineum	v1.0	Salt cress	JGI annotation of assembly v1
Fragaria vesca	v1.1	Woodland strawberry	Fragaria vesca Genome v1.1 from Sulaev et al with annotations from v1.0
Glycine max	Wm82.a2.v1	Soybean	JGI annotation of assembly v2.0
Gossypium raimondii	v2.1	Cotton	JGI annotation of assembly v2.0
Kalanchoe marnieriana	v1.1	Marnier's kalanchoe	JGI annotation v1.1 on assembly v1.0
Linum usitatissimum	v1.0	Flax	BGI v1.0 on assembly v1.0, provided by Mike Deyholos at the University of Alberta
Malus domestica	v1.0	Apple	Genome Database for Rosaceae
Manihot esculenta	v6.1	Cassava	JGI annotation v6.1 of assembly v6
Medicago truncatula	Mt4.0v1	Barrel medic	JCVI Medicago Truncatula Genome Project
Mimulus guttatus	v2.0	Monkey flower	JGI annotation of assembly v2.0
Musa acuminata	v1	Banana	Release v1 from The Banana Genome Hub
Oryza sativa	v7.0	Rice	MSU Release 7 on assembly Os-Nipponbare-Reference-IRGSP-1.0, TE inactivated/corrected

Panicum hallii	v2.0	Hall's panicgrass	JGI v1.1 annotation mapped forward to assembly v2.0
Panicum virgatum	v1.1	Switchgrass	JGI annotation of assembly v1.0
Phaseolus vulgaris	v1.0	Common bean	JGI annotation of assembly v1.0
Physcomitrella patens	v3.3	Moss	COSMOSS annotation of JGI assembly v3
Populus trichocarpa	v3.0	Poplar	JGI annotation of assembly v3
Prunus persica	v2.1	Peach	JGI annotation v2.1 of assembly v2.0
Ricinus communis	v0.1	Castor bean	TIGR release 0.1
Salix purpurea	v1.0 (ER)	Willow	JGI annotation v1.0 (in collaboration with JCVI) of assembly v1.0
Selaginella moellendorffii	v1.0	Spikemoss	from original JGI FilteredModels3 database Selmo1, with only one haplotypic variant
Setaria italica	v2.2	Foxtail millet	JGI annotation of assembly version 2.0
Setaria viridis	v.1	Green foxtail	JGI annotation of assembly version 1.0
Solanum lycopersicum	iTAGv2.3	Tomato	SGN Tomato Genome Project ITAG2.3
Solanum tuberosum	v3.4	Potato	SGN Solanum tuberosum Group Phureja DM1-3 516R44 (CIP801092) Genome Annotation v3.4 mapped to pseudomolecule sequence
Sorghum bicolor	v3.1	Cereal grass	JGI annotation of assembly v3.0
Spirodela polyrhiza	v2	Greater Duckweed	MIPS/IBIS annotation
Sphagnum fallax	v0.5	Bog moss	JGI annotation of assembly v0.5
Theobroma cacao	v1.1	Cacao	International Wheat Genome Sequencing Consortium

Vitis vinifera	Genoscope.12X	Grape	Genoscope
Zea mays	6a	Maize	Zea mays gene annotation v6a on B73 assembly AGPv3 from the Maize Genome Sequencing Project

Table S3. Primers used in this study

Prime name	Primer sequence	Application
MtKNOX4-F2	ATAGAGTGGTCCTCAACCTCATC	For confirmation of the Tnt1 insertion of knox4-1,knox4-2,knox4-3
MtKNOX4-R3	CATACCTGTGAGGGCTTGTAAGGT	For confirmation of the Tnt1 insertion of knox4-1,knox4-2,knox4-3
Tnt1-Reverse-F2	GGCTACCAAACCAAAGAAAACTC	For confirmation of the Tnt1 insertion of knox4-1,knox4-2,knox4-3
Tnt1-6R	GCTACCAACCAAACCAAGTCAA	For confirmation of the Tnt1 insertion of knox4-1,knox4-2,knox4-3
Tnt1-4F	TACCGTATCTCGGTGCTACA	For confirmation of the Tnt1 insertion of knox4-1,knox4-2,knox4-3
M4like LP	TCAGGAGTGAGTTTGGGAGAAGG	For confirmation of the Tnt1 insertion of knox4-4 and knox4-5
M4like RP	TTTGTAAATCAGTGTCGGGCTCA	For confirmation of the Tnt1 insertion of knox4-4 and knox4-5
MK4 F	CACCATGCAAGAACCAAGCTTAGGGATG	For cloning of the MtKNOX4 full length CDS
MK4 R	TTACTACCTCTTGCGTTTCGACTTC	For cloning of the MtKNOX4 full length CDS
K4-F	ACTCGACAACTTCCTCGCACAA	For cloning of the KNOX4 cDNA as probe for in situ hybridization
K4-R	TTTGCTTCAGCTGCAGTCCTGT	For cloning of the KNOX4 cDNA as probe for in situ hybridization
MK4P F	CACCTTTAGTGCTCATCAAGTTTGTTCTCGCC	For making the complementation construct
MK4 R	TTACTACCTCTTGCGTTTCGACTTC	For making the complementation construct
MK4P F-2	CACCTATTCAAAGCAATCTACCTCTGCCACTA	For cloning of the KNOX4 promoter
MK4P R-3	TTCTGAAGAAAATGAAAGCTTGTTG	For cloning of the KNOX4 promoter
MtATT1/NF6718 F1	ATTACTTTTAACGGCCATAACAGCT	For confirmation of the Tnt1 insertion of CYP86A_Medtr8g030590
MtATT1/NF6718 R1	TGAATAACCAACCAAAAGAACCAG	For confirmation of the Tnt1 insertion of CYP86A_Medtr8g030590
SQ34 CHIP F1	GGTAGGTTGCTCACCAAAACG	For ChIP-PCR analysis of CYP86A_Medtr8g030590
SQ34 CHIP R1	AGCAAGATGAGGGTGCTGTTT	For ChIP-PCR analysis of CYP86A_Medtr8g030590
SQ34 CHIP F2	ACAGGTGGGGTGATTTCAACT	For ChIP-PCR analysis of CYP86A_Medtr8g030590
SQ34 CHIP R2	AGGCCAGACTGGTTTTCTTCA	For ChIP-PCR analysis of CYP86A Medtr8g030590
MtUBC Q F-2	CTGACAGCCCACTGAATTGTGA	For ChIP-PCR analysis of UBQ control
MtUBC Q R-2	TTTTGGCATTGCTGCAAGC	For ChIP-PCR analysis of UBQ control
Mt-ACTIN11-F	ACGAGCGTTTCAGATG	RT-PCR control
Mt-ACTIN11-R	ACCTCCGATCCAGACA	RT-PCR control