Supporting Information

Naseer et al. 10.1073/pnas.1205726109

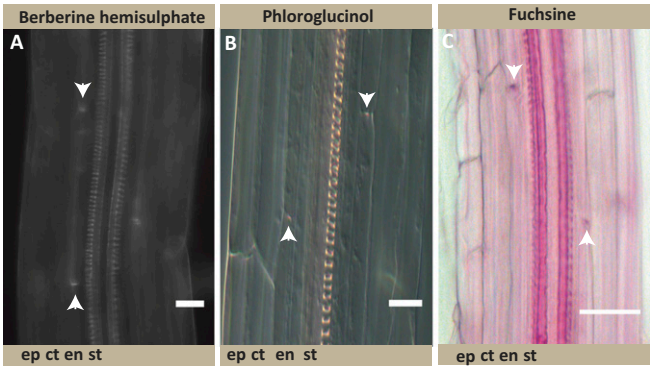

Fig. S1. Casparian strip detection with additional dyes. (*A*) Dot-like appearance of Casparian strips as visualized by Berberine hemisulfate staining. (*B*) Phloroglucinol staining and (*C*) Fuchsine staining. Stele (st), endodermis (en), cortex (ct), epidermis (ep). $n = 16$ (Scale bars: *A* and *B*, 20 μm; *C*, 50 μm.)

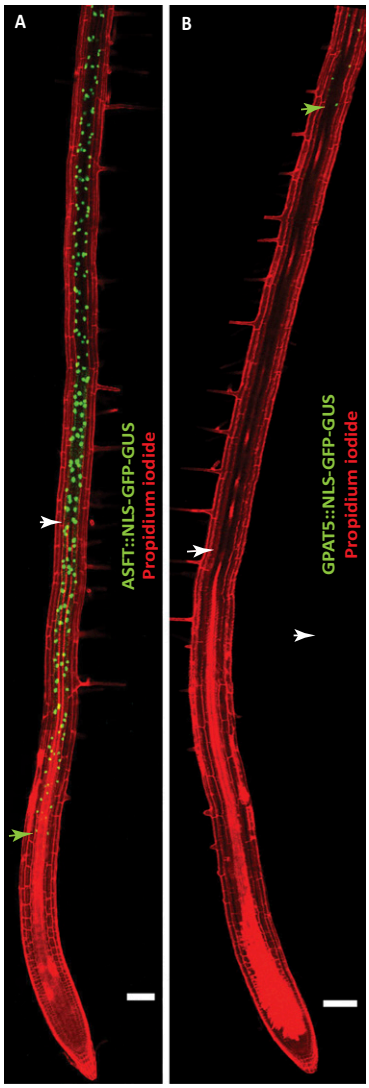

Fig. S2. Comparison of onset of ALIPHATIC SUBERIN FERULOYL TRANSFERASE (ASFT) and GPAT5 promoter activities. (*A*) *ASFT::NLS-GFP-GUS*: beginning of promoter activity in the elongation zone (green arrowhead) before establishment of a functional diffusion barrier, as visualized with propidium iodide (PI, white arrowhead). (*B*) *GPAT5::NLS-GFP-GUS*: beginning of activity late in the differentiated zone (green arrowhead), appears long after establishment of the diffusion barrier (white arrowhead). $n = 10$. (Scale bars, 100 μm.)

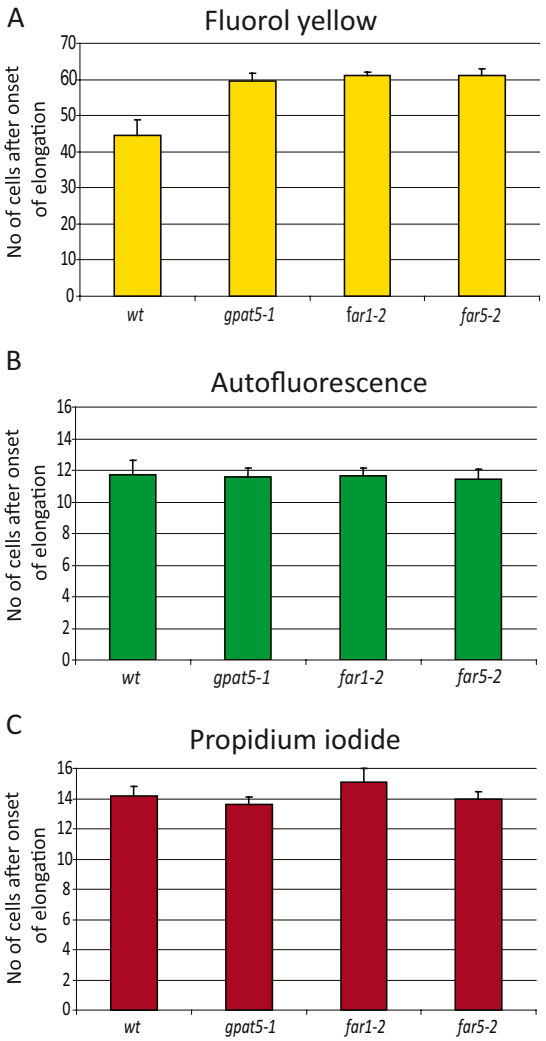

Fig. S3. Additional suberin biosynthetic mutants delay in suberin lamellae formation without affecting Casparian strips. (*A*) Fluorol yellow staining shows the significant delay in the formation of suberin lamellae; first signal was observed at around 44.5 endodermal cells after the onset of elongation in wild-type (*wt*), 59 endodermal cells in *gpat5-1*, 61 endodermal cells in *far1-2*, and 61 endodermal cells in the *far5-2* mutant. (*B*) Autofluorescence after clearing shows that both both insertion mutants do no significantly affect the appearance of Casparian strips, compared with wild-type. (*C*) PI staining shows no effect on the establishment of functional difussion barrier in both insertion mutants and *wt* (n = 16). "Onset of elongation" was defined as the zone where an endodermal cells was clearly more than twice its width.

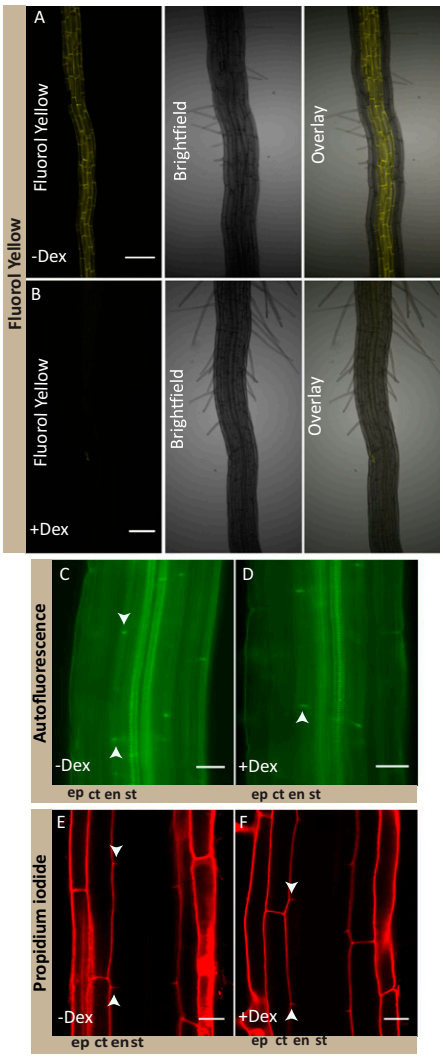

Fig. S4. Inducible suberin degradation does not affect formation of Casparian strips or diffusion barrier establishment. (*A* and *B*) Fluorol yellow staining reveals presence of suberin lamellae formation in the untreated seedling (−Dex), whereas no staining is observed in seedlings treated with 10 μM dexamethasone (+Dex). (*C* and *D*) Autofluorescence shows no effect on the formation of Casparian strips in both −Dex and +Dex seedlings. (*E* and *F*) PI shows that suberin degradation does not affect formation of a diffusion barrier in both −Dex and +Dex-treated seedlings. Stele (st), endodermis (en), cortex (ct), epidermis (ep). $n = 20$ (Scale bars: *A* and *B*, 100 μm; *C*–*F*, 20 μm.)

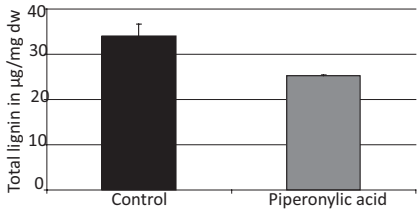

Fig. S5. Piperonylic acid (PA) significantly lowers overall lignin contents in young seedling roots. Quantitative analysis shows significant difference in the total amount of lignin extracted from 5-d-old seedling roots treated with 10 μM PA for 24 h compared with the control (untreated) samples.

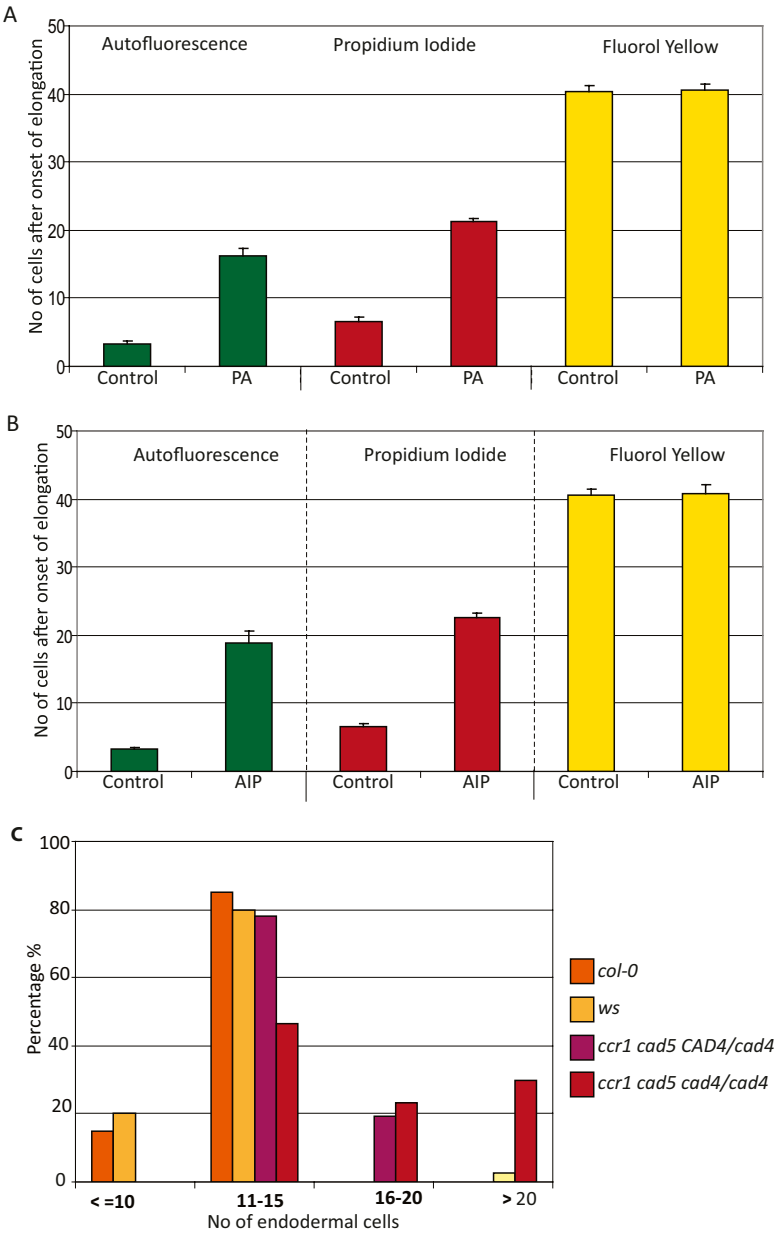

Fig. S6. Interference with lignin biosynthesis affects Casparian strip formation, but not suberin lamellae. (*A* and *B*) Quantification of seedling treated with two different lignin biosynthesis inhibitors (10 μM PA and 50 μM 2-aminoindan-2-phosphonic acid, AIP) blocks the appearance of green autofluorescent signal and of PI uptake in the newly formed cells, compared with control seedlings. However, suberin lamellae formation was not affected by the inhibitors. (*C*) Genetic interference using triple insertion mutants (*ccr1;cad4;cad5*) of lignin biosynthetic genes reveals a delay in the formation of the diffusion barrier, visualized by PI. In a population of double homozygote (*cad4;cad5*), segregating for *ccr1*, a delay in the formation of the diffusion barrier is observed in the double mutant, which is further increased in the triple mutant. Wild-type (*Col*): $n = 60$ and Wassilewskija (*Ws*), double mutant *(cad5;ccr1* with *CAD4* either *CAD4/CAD4* or *CAD4/cad4): n* = 82 and the triple mutant *(cad5;ccr1;cad4): n* = 30. Data of autofluorescence and PI in *A* the same as in Fig. 4 *D* and *H*.

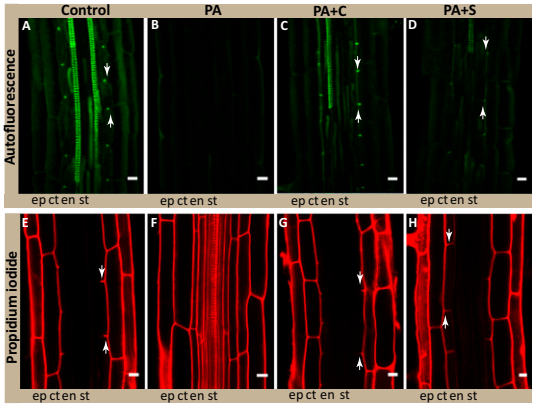

Fig. S7. Exogenous treatment with only coniferyl alcohol can lead to Casparian strip formation in lignin inhibitor-treated roots. (*A*) Autofluorescence after clearing shows the dot-like appearance of Casparian strips in control seedlings. (*B*) Block of Casparian strip formation in the PA-treated seedling. (*C*) Formation of functional Casparian strips by exogenous application of 20 μM coniferyl alcohol. (*D*) Formation of Casparian strips by exogenous application of 20 μM of sinapyl alcohol. (*E*) PI staining shows block of PI uptake in untreated (control) seedling. (*F*) Penetration of PI in the seedling treated with PA. (*G*) Establishment of functional diffusion barrier because of the complementation of the inhibitor-induced defect with coniferyl-alcohol. (*H*) Sinapyl-alcohol is less functional in the formation of a functional diffusion barrier than coniferyl alcohol. Stele (st), endodermis (en), cortex (ct), epidermis (ep). *n* = 20 roots counted. (Scale bars, 20 μm.)

Table S1. Sequences of primers used for genotyping

Primer name	Primer sequence
horst-1-LP	AAGAACCAGCTCAAGGCCACC
horst-1-RP	AGCAAAAAGCCTAAACCGGGA
horst-3-LP	AGGTAGCAACATCTGCTTCCC
horst-3-RP	ACCAGGATTTCAAATACGTCG
*gpat*5–1-LP	TTGGTTACTATATGCTCCTATTTTGG
*gpat*5–1-RP	TTCGGACAAATGGTGAATTTC
far 1–2 -LP	TTGTTGCAATAAATGAAATGAACAG
far 1–2 -RP	TACCTTGCACGACTATGTCCC
far 5–2-LP	TTCTTGCAACGTCCTTAGCTG
far 5–2-RP	AAAGGTGGTATATAAAATTTCTTGTAGC
ccr1- LP	CCGTAACAATACCAATTCTACAAAAC
ccr1-RP	TTTTATTGTTTTGATTGACAATTTGG
f5h1-LP	ATGTCGGATTCTTCAACTCGTCTGTCA
f5h1-RP	GGCTTCAGTTCGTGATGAAGTGGAC
f5h2-LP	TATGTGGGAGTCGTGAAATTTATATG
f5h2-RP	AACTCACCAAAGAGCTTAGAGAACTC
cad4-LP	GCTCAGAACTTGAGCAGTATTGTAAC
cad4-RP	TTAACAAATTTGAGTTCAAGTGGAAG
cad4-ccc -LP	GCCACCTTGAGTAGGTTTTCC
cad4-ccc -RP	CTGCAAGAGATCCTTCTGGTG
cad5-LP	AATACACACACATAAACAGCAAAAGC
cad5-RP	CTCTCTTCTTGTTTGATGAGCTTATG
cad5-ccc -LP	GATCTTGCAATGCCTCTTCTC
cad5-ccc -RP	GAAGTAGTGGAGGTGGGATCA
LBb1	ATTTTGCCGATTTCGGAAC
GABI-LB	ATATTGACCATCATACTCATTGC
Versailles-LB	CTACAAATTGCCTTTTCTTATCGAC

Table S2. Details of knock-out mutants associated with suberin biosynthesis

Gene number	Accession	Salk number	Mutant name	References
AT5G58860	Col	SALK_107454	*horst*-1	Hofer et al. (1)
AT5G58860	Col	SALK_050126.55.50.x	*horst*-3	Present work
AT3G11430	Col	SALK_018117	*gpat*5–1	Beisson et al. (2)
At5g22500	Col	SALK_149469	*far* 1–2	Domergue et al. (3)
At3g44550	Col	SALK_070363	*far* 5–2	Domergue et al. (3)
At1g15950	Col	GABI_622C01	*ccr1*	Ruel et al. (4)
At4g36220	Col	SALK_063792	*f5h-1*	Huang et al. (5)
At5g04330	Col	SALK_093419	*f5h-2*	Present work
At3g19450	Col	SAIL_1265_A06	*cad4-c*	Present work
At3g19450	Ws	Versailles collection	*cad4-c*	Sibout et al. (6)
At4g34230	Col	SALK_040062	*cad5-d*	Kim et al. (7)
At4g34230	Ws	Versailles collection	*cad5-d*	Sibout et al. (6)

1. Hofer R, et al. (2008) The Arabidopsis cytochrome P450 CYP86A1 encodes a fatty acid omega-hydroxylase involved in suberin monomer biosynthesis. *J Exp Bot* 59(9):2347–2360.
2. Beisson F, Li Y, Bonaventure G, Pollard M, Ohlrogge JB (2007) The acyltransferase GPAT5 is required for the synthesis of suberin in seed coat and root of Arabidopsis. *Plant Cell* 19(1): 351–368.
3. Domergue F, et al. (2010) Three Arabidopsis fatty acyl-coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. *Plant Physiol* 153(4):1539–1554.
4. Ruel K, et al. (2009) Impact of CCR1 silencing on the assembly of lignified secondary walls in Arabidopsis thaliana. *New Phytol* 184(1):99–113.
5. Huang J, et al. (2009) Pleiotropic changes in Arabidopsis f5h and sct mutants revealed by large-scale gene expression and metabolite analysis. *Planta* 230(5):1057–1069.
6. Sibout R, et al. (2003) Expression pattern of two paralogs encoding cinnamyl alcohol dehydrogenases in Arabidopsis. Isolation and characterization of the corresponding mutants. *Plant Physiol* 132(2):848–860.
7. Kim SJ, et al. (2004) Functional reclassification of the putative cinnamyl alcohol dehydrogenase multigene family in Arabidopsis. *Proc Natl Acad Sci USA* 101(6):1455–1460.

Table S3. Primer sequences used for construction of promoter::GUS fusions

At number	Gene name	Enzyme name	Bp before ATG	Primer name	Primer sequence
at1g04220	*DAISY*	3-ketoacyl CoA synthase	2,084 bp	prKCS2-attB4(12bp) Sense	ATAGAAAAGTTGCTCTCATGGTTG AGTAGTTGAATGTTG
				prKCS2-attB1r(12bp) AS	TTGTACAAACTTGCGGTAGGTTTT TTGGTTTTAAATGATA
at1g67730	*KCR1*	Ketoacyl CoA reductase	2,027 bp	prKCR1-attB4(12bp) Sense	ATAGAAAAGTTGCTCAAATGTGC AGGTTGCTCTATTAT
				prKCR1-attB1r(12bp) AS	TTGTACAAACTTGCTAGAGAAGAA AGGTTGAGACTTTGG
at5g58860	*HORST*	Fatty acid ω-hydroxylase	2,165 bp	prHORST-attB4(12bp) Sense	ATAGAAAAGTTGCTGAGTAGTACC CTCAGAGGAACTTGCA
				prHORST-attB1r(12bp) AS	TTGTACAAACTTGCTATCCCGGTTT AGGCTTTTTGCT
at5g23190	*CYP86B1*	Fatty acid ω-hydroxylase	1,734 bp	prCYP86B1-attB4(12bp) Sense	ATAGAAAAGTTGCTCACACCCAGTA AGAGATCAAACACA
				prCYP86B1-attB1r(12bp) AS	TTGTACAAACTTGCTGTGACAAAGA GAAGAGAGAGCGA
at4g15330	*CYP705A1*	Other CYP450 enzymes	1,917 bp	prCYP705A1-attB4(12bp) Sense	ATAGAAAAGTTGCTGTTCATCGTGCTG CCAAAGTAGTGA
				prCYP705A1-attB1r(12bp) AS	TTGTACAAACTTGCTGTTGCTGAAA AGCAAAGAAGAGGC
at3g11430	*GPAT5*	Glycerol-acyl-transferase	2,146 bp	prGPAT5-attB4(12bp) Sense	ATAGAAAAGTTGCTTGATCGCAA ACGTCAATGGTCTAT
				prGPAT5-attB1r(12bp) AS	TTGTACAAACTTGCTTCTTTTGTTT TTTGCTCGAATATTA
at5g41040	*ASFT*	Feruloyl-acyl-transferase	2,049 bp	prACT-attB4(12bp) Sense	ATAGAAAAGTTGCTGAAGATCAG CAGCAGAGTGCAGAG
				prACT-attB1r(12bp) AS	TTGTACAAACTTGCTTTGATCCAAA TGGAGAAAACAGC
at3g44540	*FAR4*	Alcohol-forming fatty acyl-CoA reductase	2,300 bp	prFAR4-attB4(12bp) Sense	ATAGAAAAGTTGCTGGAACCTAT GTCCGAACTCCG
				prFAR4-attB1r(12bp) AS	TTGTACAAACTTGCTGAAGAAA CTTATATCTATCCAATTAAT

For construction of transgenic line expressing CDEF1 (At4g30140), Gateway Directional TOPOentry vector(U15010) was used (Invitrogen). Fragments of entry clones were transferred into the destination vector pB7m34GW.