A two-way molecular dialogue between embryo and endosperm is required for seed development

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The plant embryonic cuticle is a hydrophobic barrier deposited de novo by the embryo during seed development. At germination, it protects the seedling from water loss and is, thus, critical for survival. Embryonic cuticle formation is controlled by a signaling pathway involving the ABNORMAL LEAF SHAPE1 subtilase and the two GASSHO receptor-like kinases. We show that a sulfated peptide, TWISTED SEED1 (TWS1), acts as a GASSHO ligand. Cuticle surveillance depends on the action of the subtilase, which, unlike the TWS1 precursor and the GASSHO receptors, is not produced in the embryo but in the neighboring endosperm. Subtilase-mediated processing of the embryo-derived TWS1 precursor releases the active peptide, triggering GASSHO-dependent cuticle reinforcement in the embryo. Thus, a bidirectional molecular dialogue between embryo and endosperm safeguards cuticle integrity before germination.

CASPARIAN STRIP INTEGRITY FACTORS (CIFs), a family of small sulfated signaling peptides, are ligands for GSO1 and GSO2 (10–12). CIF1 and CIF2 are involved in Casparian strip formation in the root endodermis (10, 11). The function of CIF3 and CIF4 is still unknown. To assess the role of CIF peptides in cuticle development, the quadruple mutant (cif1 cif2 cif3 cif4) was generated (fig. S1A). Neither cuticle permeability nor seed twisting phenotypes were observed in this quadruple mutant (fig. S1, B to E). However, reduction [in the leaky sgn2-1 allele (10)] or loss [in the tsp1-1 mutant (15)] of tyrosyl-protein sulfotransferase (TPST) activity results in seed-twisting and cuticle-permeability phenotypes resembling those observed in ale1 mutants (Fig. 1, A to D, and fig. S2, A to D). These data suggest that a sulfated peptide may act as the ligand of GSO1/2 during seed development.

Consistent with the hypothesis that TPST acts in the same pathway as GSO1 and GSO2, no difference was observed between the phenotype of tsp1-1 gso1-1 gso2-1 triple and gso1-1 gso2-1 double mutants (fig. S2E). In contrast, TPST and AELE1 appear to act synergistically, as a phenotype resembling that of gso1 gso2 double mutants was observed in tsp1-1 ale1-4 double mutants (Fig. 1, E to I, and fig. S2F). This result supports the hypothesis that TPST and AELE1 act in parallel regarding their roles in embryonic cuticle formation, possibly through independent posttranslational modifications that contribute to the maturation of the hypothetical peptide signal.

Identification of the peptide signal was facilitated by a study of TWISTED SEED1 (TWS1) (14), which reported a loss-of-function phenotype that was notably similar to that of gso1 gso2 double mutants. Because existing alleles of TWS1 are in the Wassilewskija (WS) background, we generated new CRISPR alleles (tws1-3 to tws1-10) in the Col-0 background and confirmed the phenotype of resulting mutants (Fig. 1 and fig. S3). No additivity was observed when loss-of-function alleles of TWS1 and of other pathway components (GSO1, GSO2, TPST, and ALE1) were combined, providing genetic evidence for TWS1 acting in the GSO signaling pathway (fig. S4). Furthermore, gaps in the cuticle of embryos and cotyledons, similar to those observed in ale1 and gso1 gso2 mutants (2), were detected in both the tws1 mutants and tsp1 mutants (Fig. 1, J to N, and fig. S5). Inspection of the TWS1 protein sequence revealed a region with limited similarity to CIF peptides, including a DY motif that marks the N terminus of the CIFs (Fig. 1O) and is the minimal motif required for tyrosine sulfation by TPST (15). Corroborating the functional importance of the putative peptide domain, the tws1-6 allele (deletion of six codons in the putative peptide-encoding region) and the tws1-5 allele (substitution of eight amino acids, including theDY motif) both showed total loss of function of the TWS1 protein (fig. S3).

We tested whether TWS1 is a substrate of ALE1 by coexpression of ALE1:(His)6 and TWS1::GFP:(His)6 fusion proteins in tobacco (Nicotiana benthamiana) leaves. A specific TWS1 cleavage product was observed upon coexpression of ALE1 but not in the empty-vector control, suggesting that TWS1 is processed by ALE1 in planta (Fig. 1P). Likewise, recombinant TWS1 expressed as GST-fusion in Escherichia coli was cleaved by purified ALE1 in vitro, (Fig. 1Q). Mass spectroscopy analysis of the TWS1 cleavage product purified from tobacco leaves showed that ALE1 cleaves TWS1 between His24 and Gly35 (fig. S6). These residues are important for cleavage site selection, as ALE1-dependent processing was not observed when either His24 or Gly35 was substituted by site-directed mutagenesis (Fig. 1Q). His34 corresponds to the C-terminal His or Asn of CIF peptides (Fig. 1O). Thus, the data suggest that ALE1-mediated processing of the TWS1 precursor marks the C terminus of the TWS1 peptide. Because the CIF1 and CIF2 peptides are located at the very end of their respective precursors, C-terminal processing could represent a mechanism of peptide activation operating in the developing seed but not in the root. A summary of TWS1 modifications is provided in Fig. 1R.

To test the biological activity of TWS1, the predicted peptide encompassing the conserved N-terminal DY motif and the C terminus defined by the ALE1 cleavage site was custom-synthesized in tyrosine-sulfated form. As synthetic TWS1 cannot easily be applied to developing embryos, a root bioassay for CIF activity was used. In wild-type roots, TWS1 induced ectopic endodermal lignification, as previously observed for the CIF1 and CIF2 peptides (12). TWS1 activity was GSO1-dependent, suggesting that processed TWS1 peptide can replace...
CIF1 and CIF2 as a ligand for GSO1 during Casparian strip formation (Fig. 2A and fig. S7). Supporting this, TWS1 application complemented the cif1 cif2 mutant, albeit with reduced activity compared with CIF2 (Fig. 2B and fig. S8). TWS1 activity in this assay was reduced when sulfation on the DY motif was missing (Fig. 2B). Versions of TWS1 in which Y33 was mutated to either F or T only partially complemented the mutant phenotype of tws1-4 (fig S9), consistent with a residual but weak activity for nonsulfated TWS1 in vivo and with the weak loss-of-function phenotype of the tpst-1 mutant.

To confirm TWS1 as a ligand of GSO1 and GSO2, the interaction of the synthetic peptide with the leucine-rich repeat (LRR) ectodomains of the receptors was analyzed in
Grating-coupled interferometry binding assays. GSO1 bound sulfated TWS1 with a $K_D$ (dissociation constant) of $\sim 30$ nM (Fig. 2C). The observed binding affinity is $\sim 1/10$ that of the CIF2 peptide ($K_D = 2.5$ nM) (fig. S10), which is consistent with the reduced ability of TWS1 to complement the root phenotype of the cif1 cif2 double mutant (Fig. 2B). Sulfated TWS1 also bound to the LRR domain of GSO2, albeit with slightly reduced affinity ($K_D \sim 100$ nM) (Fig. 2D). As previously shown for other CIF peptides (11), tyrosine sulfation was critical for the interaction of TWS1 with GSO1 and GSO2 in vitro (Fig. 2, E and F). Technical issues at high peptide concentrations may explain discrepancies between in vitro binding assays and the in vivo activity of nonsulfated TWS1. In vivo activities for nonsulfated versions of other normally sulfated peptides, including CIF2, have been reported (11, 16–18). Adding a 3AA C-terminal extension to the sulfated TWS1 peptide reduced binding affinity to both GSO1 and GSO2 (fig. S10), consistent with the need for ALE1-mediated C-terminal processing for efficient signaling.

Taken together, our results suggest the sulfated TWS peptide as the missing link in the

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**Fig. 2. The TWS1 peptide is a functional GSO1/GSO2 ligand.** (A) Root over lignification following treatment with the active CIF2 or TWS peptide in Col-0 and in the gso1 (sgn3-3) background. Lignin is stained in purple and CASP-GFP fusion protein, marking the Casparian strip domain, in green. Scale bar, 5 μm. (B) Complementation of cif1-2 cif2-2 Casparian strip integrity phenotype by peptide treatments. Number of gaps in CASP1-GFP signal counted after treatment with CIF2 sulfated peptide, TWS1 sulfated peptide, TWS1 nonsulfated peptide. N = 10. a, b, and c correspond to classes statistically supported by one-way ANOVA analysis, followed by Tukey tests ($P < 0.05$). (C to F) Grating-coupled interferometry (GCI)–derived binding kinetics. Shown are sensorgrams with raw data in red and their respective fits in black. $k_a$, association rate constant; $k_d$, dissociation rate constant; $K_D$, dissociation constant. (C) Data for the GSO1 extracellular domain in the presence of the sulfated TWS1 peptide. (D) Data for the GSO2 extracellular domain in the presence of the sulfated TWS1 peptide. (E) Data for the GSO1 extracellular domain in the presence of the nonsulfated TWS1 peptide. (F) Data for the GSO2 extracellular domain in the presence of the nonsulfated TWS1 peptide. n.d., not determined.
intercompartmental signaling pathway for embryonic cuticle formation. The activities of ALE1 and TPST both contribute to the formation of the bioactive peptide (Fig. 1R), which is perceived by GSO1 and GSO2 to ensure appropriate cuticle deposition.

To understand how the elements of the signaling pathway cooperate to ensure the formation of a functional cuticle, we analyzed their spatial organization. In silico data indicate that the TPST gene is expressed in all seed tissues (fig. S11) (19, 20). To investigate in

Fig. 3. Spatial separation of ALE1 and TWS1 expression is critical for pathway function. (A to C) F1 seedlings from reciprocal crosses stained with Toluidine blue. (D and N) Toluidine blue quantification as in Fig. 1. a to d indicate statistical differences with one-way ANOVA followed by a post hoc Scheffé multiple comparison test (P < 0.01). (D) Complementation of tpst-1 mutant with endosperm-specific expression of TPST (pRGP3::TPST), embryo-specific expression of TPST (pPIN1::TPST), and ubiquitous expression of TPST (pRPS5a::TPST) compared with tpst-1 and Col-0. Three independent lines were analyzed. (E) Confocal images of pTWS1::mCitrine::NLS-mCitrine reporter lines, signal in yellow, autofluorescence in red. Scale bars, 50 μm. (F and G) Dry seeds (scale bars, 400 μm) and chloral hydrate cleared seeds (9 DAP) (scale bars, 100 μm), respectively, from a line expressing ALE1 in the embryo in the tws1-4 background (pTWS1::ALE1 line#7). (H and I) Seeds from crosses of Col-0 pollen onto line#7. (J and K) Self-fertilized tws1-4 seeds as a control. (L and M) Seeds from a cross of Col-0 pollen on a tws1-4 pistil as a control. Results for three further independent transgenic lines are shown in figs. S18 and S19. (N) Complementation of tws1-4 mutants by expression of TWS1 in the endosperm. Four independent lines were analyzed. (O) Model for embryonic cuticle integrity monitoring. Left shows the wild-type situation before gap-filling (nascent cuticle), illustrating the diffusion and processing of TWS1 across the embryo-endosperm interface. Right shows the wild-type situation when the cuticle is intact, spatially separating signaling components and, thus, attenuating signaling.
which compartment TPST [which acts cell autonomously (33)] is required for TWS1 maturation, reciprocal crosses and complementation assays using tissue-specific promoters were performed. No cuticle permeability defects were observed when homozygous mutants were pollinated with wild-type pollen, confirming their zygotic origin. (Fig. 3, A to C). Expressing TPST under the ubiquitously active RPS5A promoter (21) or the PIN1 promoter [which is embryo-specific in seed (fig. S12)] complements tpst-1 cuticle defects. In contrast, no complementation was observed using the endosperm-specific RGP3 promoter (22), indicating that TPST activity is required for TWS1 sulfation specifically in the embryo to ensure cuticle integrity (Fig. 3D and fig. S13). Consistent with this observation and with a previous report (16), the TWS1 promoter was found to drive expression specifically in the developing embryo from the early globular stage onwards (Fig. 3E and fig S14). The TPST promoter (10) drove expression throughout the embryo proper at the onset of embryo cuticle establishment (the globular stage) before becoming restricted to the root tip (fig. S11). We conclude that the TWS1 peptide is both sulfated and secreted specifically in the embryo.

However, production of mature TWS1 requires a C-terminal cleavage event that we have shown to be mediated by ALE1. ALE1 is expressed only in the endosperm (4, 23), on the opposite side of the nascent cuticle to the GSO1 and GSO2 receptors, which are localized on the membranes of the epidermal cells that produce the cuticle (figs. S15 to S17) (2). Our data therefore support a model in which activation of the GSO signaling pathway depends on the diffusion of the TWS1 peptide precursor to the endosperm, where it is cleaved and activated by ALE1 before diffusing back to the embryo to trigger GSO1/2-dependent cuticle deposition. An intact cuticle would separate the embryo from the endosperm, as suggested by the weak phenotype of ale1 mutants.

The proposed bidirectional signaling model allows efficient embryo cuticle integrity monitoring. The sulfated TWS1 precursor is produced by the embryo and secreted (probably after N-terminal cleavage of the pro-peptide) to the embryo apoplast. In the absence of an intact cuticular barrier, it can diffuse to the endosperm and undergo activation by ALE1 and potentially other subtilases. Activated TWS1 peptide then leaks back through cuticle gaps to bind the GSO1 and GSO2 receptors and activate local gap repair (Fig. 3O). When the cuticle is intact, proTWS1 peptides are confined to the embryo where they remain inactive.

Our results demonstrate a role for a subtilase in providing spatial specificity to a bidirectional peptide signaling pathway. In contrast, the related CFI1-, CFI2-, and GSO1-dependent signaling pathway controlling Caspian strip integrity is unidirectional, negating the need for C-terminal cleavage-mediated peptide activation (10, 12). Both pathway components and their spatial organization differ between the two systems, suggesting an independent recruitment of the GSO receptors to different integrity monitoring functions within the plant.

REFERENCES AND NOTES
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SUPPLEMENTARY MATERIALS
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Materials and Methods. Figs. S1 to S23
References (24–39)

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Filling in the gaps
In a plant seed, the embryo lies dormant surrounded by nutritive endosperm while awaiting suitable conditions to germinate. A hydrophobic cuticle around the embryo protects it from catastrophic water loss during the early days of growth. Doll et al. identified a back-and-forth signaling pathway that ensures an intact cuticle. The precursor of a signaling peptide is made in the embryo and transferred to the endosperm, where it is processed into an active form. The activated peptide diffuses back into the embryo to activate receptor-like kinases that drive cuticle development. Serve and return continues until all leaks in the cuticle are filled in and the peptide can no longer cross the barrier. Science, this issue p. 431