A peptide-mediated, multilateral molecular dialogue for the coordination of pollen wall formation

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The surface of pollen grains is reinforced by pollen wall components produced noncell autonomously by tapetum cells that surround developing pollen within the male floral organ, the anther. Here, we show that tapetum activity is regulated by the GASSHO (GSO) receptor-like kinase pathway, controlled by two sulfated peptides, CASPARIAN STRIP INTEGRITY FACTOR 3 (CIF3) and CIF4, the precursors of which are expressed in the tapetum itself. Coordination of tapetum activity with pollen grain development depends on the action of subtilases, including AtSBT5.4, which are produced stage specifically by developing pollen grains. Tapetum-derived CIF precursors are processed by subtilases, triggering GSO-dependent tapetum activation. We show that the GSO receptors act from the middle layer, a tissue surrounding the tapetum and developing pollen. Three concentrically organized cell types, therefore, cooperate to coordinate pollen wall deposition through a multilateral molecular dialogue.

Significance

Pollen viability depends on a tough external barrier called the pollen wall. Pollen wall components are produced by tapetum cells, which surround developing pollen grains within the anther. Precise coordination of tapetum activity with pollen grain development is required to ensure effective pollen wall formation. Here, we reveal that this is achieved through a multilateral dialogue involving three distinct cell types. We show that peptide precursors from the tapetum are activated by proteases produced stage specifically in developing pollen grains. Unexpectedly, we found that activated peptides are perceived not in the tapetum, but in the middle layer, which encloses the developing tapetum and pollen grains, revealing an unsuspected role for this enigmatic cell layer in the control of tapetum development.

Pollen grains, housing dispersible male gametophytes, form a critical element in plant sexual reproduction. The pollen from a single plant can be carried by wind or pollinators toward multiple other individuals, allowing efficient exchange of genetic material. However, the release of pollen grains into the terrestrial environment requires protection from dehydration, sunlight, and other environmental stresses. Protection is provided by a multilayered pollen wall with sporopollenin, one of the most resistant biological polymers known, as the main constituent of the outermost layer (the exine) (1).

Pollen is produced in the anthers from diploid precursor cells, the pollen mother cells (PMCs). Each PMC undergoes meiosis, forming four haploid cells. These are temporarily held together as tetrads before they are released as individual immature microspores (2, 3). The subsequent assembly of the pollen wall is a highly dynamic, multistep process involving the sporophytic tissues that surround developing pollen grains. In Arabidopsis, these comprise four concentric layers of cells: the tapetum (most internal cell layer), middle layer, endothecium, and epidermis (outer cell layer) (Fig. 1A). The tapetum contributes the most to pollen wall formation by producing the biochemical precursors of sporopollenin and of other wall components (1, 3, 4). However, the developing pollen grains are not in direct contact with the tapetum but are suspended in a matrix of largely unknown composition (often called the locular fluid), which is presumably also produced by the tapetum (5). The tapetum cells export pollen wall components, which must then traverse this matrix for deposition on the pollen surface to form an intact and functional pollen wall (Fig. 1B).

The export of pollen wall components from the tapetum is under developmental control. It terminates with programmed cell death of the degenerating tapetum, liberating the final components of the pollen wall. Although the critical contribution of the tapetum to pollen wall development is uncontested, the question of whether—and, if so, how—it is coordinated with pollen grain development, as suggested in the literature (6, 7), remains unanswered.

Two extracellular structural barriers have recently been shown to be developmentally monitored to ensure physical integrity prior to their functional deployment. These are the embryonic cuticle (necessary to prevent water loss from the seedling surface at germination) and the Casparian strip (necessary for the regulation of water and ion homeostasis in roots). The integrity of both the nascent Casparian strip and the nascent embryonic cuticle depends on intercellular signaling pathways involving GASSHO (GSO) receptor-like kinases (RLKs) and CASPARIAN STRIP INTEGRITY FACTOR (CIF)-related sulfated peptide ligands (8–10). In both cases, the diffusion of posttranslationally processed ligands across the barrier into tissues containing functional receptor complexes signals defects in barrier integrity and triggers gap-filling responses.

Here, we show that the proper tapetum function necessary for the formation of a third extracellular structure, the pollen wall, is controlled through a molecular dialogue...
among the middle layer, the tapetum, and the developing pollen grains. This dialogue involves two previously functionally uncharacterized CIF peptides (CIF3 and CIF4) and their cognate receptors GSO1 and GSO2.

**Results**

The Receptors GSO1 and GSO2 and the Sulfated Peptides CIF3 and CIF4 Are Necessary for Normal Tapetum Development and Pollen Wall Formation. We noticed in previous crossing experiments that pollen from gso1 gso2 double-mutant flowers forms large clumps. Scanning electron microscopy (SEM) confirmed that in contrast to wild-type mutant pollen, which is released as individual grains (Fig. 1 C and D), gso1-1 gso2-1 double-mutant pollen tends to form large, fused masses of misshapen grains (Fig. 1 E and F) with some free nonfused pollen grains remaining. This phenotype was confirmed in the independent gso1-2 gso2-2 double mutant carrying alternative transfer DNA insertions and was rescued by complementation with the GSO1 wild-type sequence (SI Appendix, Fig. S1 A and B). The single gso1 and gso2 mutants produced normal pollen (SI Appendix, Fig. S1 C and D). Five ligands of GSO1 and/or GSO1 and GSO2 have already been identified; TWISTED SEED1 (TWS1), involved in embryonic cuticle formation (9); CIF1 and CIF2, involved in Casparian strip formation (8, 10); and CIF3 and CIF4 (11). While pollen of tws1 and cif1 cif2 double mutants was unaffected, simultaneous loss of CIF3 and CIF4 function

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**Fig. 1.** GSO1 and GSO2 RLKs are required for pollen wall formation and tapetum development. (A) Inside the anther, the haploid (n) pollen are surrounded by four diploid (2n) sporophytic cell layers: tapetum, middle layer, endothecium, and epidermis. (B) At the end of the tetrad stage (stage 7), the tapetum cells start to synthesize and release sporopollenin precursors into the locular matrix, where they ultimately attach to the surface of the pollen grains. When pollen wall construction is finished (stage 11), the tapetum cells degrade, and the resulting cellular debris also adheres to the pollen surface. Pollen wall structural components are indicated. The exine is composed of nexine, bacula, and tectum. (C–F) SEM images of the wild type (C and D) and the gso1-1 gso2-1 (E and F) mature pollen. (G and H) Pollen and anther development in wild-type anthers (G) and gso1-1 gso2-1 anthers (H) from the tetrad stage (stage 7) to pollen release (stage 13). The anther cross-sections are stained with acriflavine. I–L) Pollen wall formation in wild-type (I and J) and the gso1-1 gso2-1 mutant (K and L) anthers shown by toluidine blue staining at stages 8 (I and K) and 10 (J and L). Black arrow indicates reddish-purple staining (toluidine blue) on the surface of the pollen grains in the gso1-1 gso2-1 mutant; red arrow indicates ectopic yellow color detected between tapetum cells in the gso1-1 gso2-1 mutant. (M–P) TEM of the pollen wall in wild-type (M and N) and gso1-1 gso2-1 (O and P) pollen. Arrow indicates fused pollen wall in the mutant. (Q and R) Cryo-SEM of the pollen wall in wild-type (Q) and gso1-1 gso2-1 (R) pollen. Arrows indicate the pollen walls. (S and T) Pollen wall material stained with Auramine-O in wild-type (S) and gso1-1 gso2-1 (T) anthers. Arrows indicate ectopic staining in the mutant. (U and V) TEM showing ectopic deposition of sporopollenin-like material around the tapetum cells in the gso1-1 gso2-1 mutant (V) (arrow) compared to the wild type (U). Scale bars: C and E, 50 μm; G and H, 20 μm; D, F, I–L, S, and T, 10 μm; M–R, U, and V, 1 μm; P, pollen; T, tapetum; ML, middle layer.
phenocopied the glo1 glo2 pollen phenotype (*SI Appendix*, Fig. S2 A and B). Occasional pollen adhesion was also observed for single cif4 but not for the cif3 single mutant (*SI Appendix*, Fig. S1 E, F, M, and N), suggesting that these genes do not act fully redundantly. Because CIF3 and CIF4 belong to a family of sulfo-peptides that rely on posttranslational tyrosine sulfation (TPST). Similar pollen defects were, indeed, observed in the *tpst* mutant, although, as reported in the seed, the phenotype tended to be less severe than that caused by loss of ligand or receptor function (*SI Appendix*, Fig. S2 C and D). Despite their dramatic phenotype, the glo1-1 glo2-1, cif3 cif4, and *tpst* mutants were not sterile, as viable pollen was detected using Alexander staining (*SI Appendix*, Fig. S3 A–H), and pollen located at the periphery of the clusters was still able to germinate (*SI Appendix*, Fig. S3 I–L). Furthermore, plants left to self-fertilize produced viable seeds, although seed number per silique was reduced compared to wild type (*SI Appendix*, Fig. S3A).

We traced the origin of the pollen defects by histological analyses. Anther development appeared normal in all backgrounds until stage 8 (staging according to ref. 12), the point of microspore release (see Fig. 1B). From this stage onward, pollen grains were uniformly distributed within the locular matrix of wild-type anthers. Tapetal cells were well defined and thinned progressively until they underwent cell death, prior to the release of pollen grains between stages 12 and 13 (Fig. 1G) (13). In contrast, in *glo1 glo2*, cif3 cif4, and *tpst* mutants, the apparent volume of locular matrix surrounding the developing pollen grains was dramatically reduced. The swollen and hypertrophied tapetum occupied a much larger volume, resulting in an apparent “crowding” of the developing pollen grains and a lack of locular fluid (Fig. 1H and *SI Appendix*, Fig. S2). Further aspects of this phenotype were related to the deposition of pollen wall components. In wild-type stage 8 anthers, the pollen wall stain reddish purple with toluidine blue (Fig. 1I) (14, 15). The reddish-purple staining is masked in subsequent stages by wall components onto the surface of developing pollen grains. In *glo1 glo2*, cif3 cif4, and *tpst* mutants’ anthers, the pollen wall components are distributed in a predominantly between tapetal cells, indicating a lack of cell polarization (*SI Appendix*, Fig. S5 D–M). No change in expression was observed for the sporopollenin biosynthesis genes (*SI Appendix*, Fig. S6), suggesting that the accumulation of pollen wall material in the mutants is likely not due to enhanced sporopollenin biosynthesis, but may be due to polarity defects.

In summary, GSO1/2, CIF3/4, and TPST all appear to be necessary for normal tapetum function. Tapetum function, in turn, is necessary for normal pollen grain spacing within the locular fluid and for the targeted and regular deposition of pollen wall components onto the surface of developing pollen grains, suggesting that GSO-mediated signaling is necessary for the coordination of these processes.

**GSO Receptors Are Produced in the Middle Layer, while CIF3 and CIF4 Are Expressed and Sulfated in the Tapetum Cells.** To understand the organization of the GSO signaling pathway in developing anthers, we studied the expression of pathway components. Transcriptional reporters revealed expression of *GSO1* and *GSO2* in the middle layer (Fig. 2 A and B and *SI Appendix*, Fig. S7). Despite the difficulty of counterstaining the inner cell layers of the anther wall, middle-layer nuclear signals can be clearly distinguished due to their flattened form (Fig. 2 A and B). Expression was initiated prior to meiosis of the PMC, at around stage 5 (*SI Appendix*, Fig. S7), significantly earlier than pollen wall deposition, and was maintained until the degradation of the middle layer at stage 11 (13). Translational reporter constructs revealed the presence of GSO1 and GSO2 proteins at the periphery of middle-layer cells, consistent with plasma membrane localization (Fig. 2 D and E and *SI Appendix*, Fig. S7). The GSO1 translational reporter fully complemented the pollen and tapetum phenotypes of *glo1 glo2* mutants (*SI Appendix*, Fig. S1B), confirming that this expression pattern accurately reflects native GSO1 expression in the anther. Although both transcriptional reporters and fusion proteins were occasionally seen in the endothecium, neither gene expression nor protein accumulation was ever detected in the tapetum. Protein accumulation appeared uniform within the membrane of middle-layer cells, similar to the situation previously reported in the embryo epidermis (9).
In contrast, transcriptional reporters pCIF3-NLS-3xmVenus and pCIF4-NLS-3xmVenus revealed that the expression of CIF3 and CIF4 is restricted to the tapetum and initiates around the onset of PMC meiosis (Fig. 2C and SI Appendix, Fig. S8). Tapetal expression is distinguished from that in other cell layers by the presence of characteristic double nuclei. While CIF3 expression diminishes shortly after microspore release, CIF4 expression continues until tapetum degradation at stage 11 (SI Appendix, Fig. S8). Expression of the CIF4 Open Reading Frame (ORF)—either under its own promoter or under the tapetum-specific AMS (17) (SI Appendix, Fig. S9) and SHT (18) (SI Appendix, Fig. S9) promoters—fully complemented the cif3 cif4 phenotype (SI Appendix, Fig. S1 G, H, J, O, and Q), confirming that these expression patterns likely reflect the native expression of CIF3 and CIF4.

To explore the spatial requirement for TPST, which acts cell autonomously in the Golgi apparatus during peptide secretion (19), we expressed the TPST ORF under the tapetum-specific AMS promoter in the tpst-1 mutant. All transformed lines showed a complete or nearly complete complementation of the pollen and tapetum phenotypes of tpst-1, consistent with TPST activity being required for the production of sulfated peptide ligands in the tapetum (SI Appendix, Fig. S1 L and R).

In summary, our results show that normal pollen wall deposition and tapetum function depend on the activity of CIF3 and CIF4 peptides sulfated and secreted from the tapetum and their cognate receptors, GSO1 and GSO2, in the anther middle layer.

**A Pollen-Specific Subtilisin Serine Protease, SBT5.4, Can Cleave the Extended C Terminus of the CIF4 Precursor.** Analysis of the embryonic cuticle integrity signaling in seeds revealed a bidirectional signaling pathway in which an inactive, sulfated precursor of the TWS1 peptide requires C-terminal subtilase (SBT)-mediated processing to release the mature and bioactive TWS1 peptide as a ligand for GSO1/2 (9). Like TWS1, both CIF3 and CIF4 possess C-terminal extensions (Fig. 3A), suggesting that they may also require C-terminal processing for activation. We found that both the full length CIF4 ORF and a truncated version lacking the sequence encoding the part of the precursor C-terminal to the predicted active peptide could complement the cif3 cif4 mutant phenotype when expressed under the CIF4 promoter or under the tapetum-specific SHT promoter in developing anthers (SI Appendix, Fig. S1 G, I-K, O, and P). The C-terminal extension of CIF4 is, thus, not required for activity. Our finding that C-terminal extensions in
SBT5.4 is, thus, a good candidate for CIF activation.

SBT5.4 can process the CIF4 precursor, and ectopic SBT5.4 expression leads to deregulated pollen wall formation. (A) Sequence alignment of the C terminus of CIF family precursors, with mature peptide sequences in boldface. Fully, strongly, and weakly conserved residues (Clustal W; Gonnet PAM250 matrix) are highlighted in red, blue, and green, respectively. Arrowheads indicate processing sites. (B) Coomassie-stained SDS-PAGE showing digests of recombinant GST-CIF4 (the full-length and a truncated version of the precursor are marked by blue and black arrowheads, respectively) with SBT5.4 purified from tobacco leaves. The molar SBT:substrate ratio is indicated for each lane; cleavage products are highlighted by asterisks. (C) Recombinant GST-CIF4 (the full-length and a truncated version of the precursor are marked by blue and black arrowheads, respectively) with SBT5.4 purified from tobacco leaves. The molar SBT:substrate ratio is indicated for each lane; cleavage products are highlighted by asterisks. (D) Cleavage of a synthetic CIF4 precursor peptide by SBT5.4. Bar graphs show relative abundance of the substrate peptide (Top) and fragments thereof for the control (C) and SBT5.4 (D) digests as the percentage of all peptides detected by MS/MS analysis. Arrowheads mark N- and C-terminal processing sites flanking the mature CIF4 peptide. The cleavage product generated by SBT5.4-specific processing of the C-terminal extension is highlighted in dark blue. (E–H) SEM images of pAMS:SBT5.4 pollen from two independent lines in E and F and in G and H. Similar phenotypes were observed in four independent lines. (I–P) TEM of the pollen wall formation in the wild-type (I, K, M, O) and the pAMS:SBT5.4 (J, L, N, P) anthers at the tetrad stage (stage 7) and the free microspore stage (stage 9). Black arrows indicate putative detached primexine in the pAMS:SBT5.4 tetrads. (Q) Pollen and anther development in the pAMS:SBT5.4 line at different developmental stages. The anther cross-sections are stained with acriflavine. Scale bars: E, G, and Q, 20 μm; F, H, J, M, and N, 5 μm; K, L, O, and P, 1 μm.

CIF-class ligands impair receptor binding (9), and the apparent contribution of the free C terminus of CIF peptides to receptor/coreceptor complex formation (11), suggests that the C-terminal extension needs to be removed for bioactivity. We, therefore, tested the hypothesis that as in the seed, SBTs could contribute to activating CIF peptides in the anther. We found that SBT5.4, one of numerous SBT-encoding genes expressed in pollen grains from stage 8 onward, is, thus, able to remove the C-terminal extension for CIF4.

CIF4 (encoded by SBT5.4) is, thus, a good candidate for activating CIF4. We therefore tested directly whether SBT5.4 is able to cleave the CIF4 precursor. N-terminally glutathione S-transferase (GST)-tagged proCIF4 expressed in E. coli was coincubated with SBT5.4(His)6 transiently expressed in tobacco (N. benthamiana) leaves and purified from cell wall extracts. Several cleavage products were generated upon coincubation with SBT5.4 but not with extracts from untransformed plants, indicating that proCIF4 is processed by SBT5.4 (Fig. 3B). To confirm cleavage at sites relevant for CIF4 maturation, a synthetic CIF4 peptide extended by three amino acids of the precursor at either end was used as substrate for recombinant SBT5.4. Mass spectrometry analysis of cleavage products revealed specific cleavage only at the C-terminal processing site between His89 and Gly90 (Fig. 3E–H). SBT5.4, a SBT specifically expressed in pollen grains from stage 8 onward, is, thus, able to remove the C-terminal extension for CIF4.

To further confirm the potential role of SBT5.4, we expressed it under the AMS promoter, which drives expression specifically in the tapetum, from the point of PMC meiosis onward (SI Appendix, Fig. S9) (and, thus, earlier than the initiation of endogenous SBT5.4 expression in pollen grains). This led to strong pollen phenotypes (Fig. 3). Pollen in these lines tended to fuse together in a mass (Fig. 3 E–H). However, defects in these lines were very distinct from those observed in...
gs1 gs2 or cif3 cif4 mutants. Firstly, tapetum hypertrophy was either weaker or absent (Fig. 3Q). Secondly—and, again, unlike the situation in loss-of-function gs1 gs2 or cif3 cif4 mutants, where the reticulate patterning of the pollen wall is still visible in most peripheral pollen grains (Fig. 1 C–F)—the patterning of the pollen wall was severely compromised in lines expressing the pAMS:SBT5.4 construct (Fig. 3 F and H). Defects varied from loss/fusion of tecta, giving rise to a “broken” appearance and to an apparent massive deregulation of pollen wall deposition, giving pollen grains a “lumpy” appearance (Fig. 3 F and H). Deregulation of pollen wall deposition could be observed as early as the tetrad stage in the pAMS-SBT5.4 lines. At this stage, the organization of the primexine, which serves as a scaffold for the pollen wall formation, was abnormal (Fig. 3 I–L). At later stages, the bacula appeared to form very closely to each other, resulting in abnormally dense exine (Fig. 3 N and P). Bacula also appeared to be poorly attached to the pollen surface (Fig. 3P). Thus, the ectopic expression of the SBT5.4 leads to strong deregulation of the pollen wall formation.

Our results strongly support the hypothesis that the strict spatial and temporal regulation of CIF-cleaving SBT activity in developing pollen grains is a critical factor in ensuring the organized deposition of the pollen wall.

**GSO1 Expression in the Tapetum Interferes with Pathway Function.** Our results suggest a model in which tapetum-derived peptides, processed by pollen-derived SBTs, must diffuse to the middle layer to activate GSO-mediated signaling (Fig. 4G). To test this model further, we expressed full-length GSO1 and a truncated version lacking the cytoplasmic kinase domain (GSO1 ΔKinase) under the tapetum-specific AMS promoter. ΔKinase versions of Leucine Rich Repeat-RLKs have previously been shown to provoke dominant negative phenotypes, possibly through ligand sequestration (20). Consistent with this, wild-type plants transformed with pAMS:GSO1-ΔKinase showed a strong loss-of-function phenotype identical to the gs1 gs2 mutant (tapetum hypertrophy with both ectopic and defective pollen wall deposition) (Fig. 4 A–C). This could be explained by the sequestration of activated CIF

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**Fig. 4.** The complex spatial organization of GSO pathway components is necessary for controlled tapetum development. (A–C) SEM images and histological cross-section of anthers of the pAMS:GSO1-ΔKinase line. Phenotype confirmed in seven independent lines. (D–F) SEM images and histological cross-section of anthers of the pAMS:GS01 line. Phenotype confirmed in five independent lines. (G) Taken together, our data support the following model for the coordination of pollen wall formation with tapetum development. The CIF precursors produced in the tapetum are processed by the pollen-derived SBTs. The activated peptides diffuse between tapetum cells to bind GSO receptors in the middle layer, which induces downstream signaling, leading to polarized sporopollenin secretion toward the pollen grains. The completion of the pollen wall on the pollen surface prevents the interaction of SBTs with their substrates thus attenuating signaling. The anther cross-sections are stained with acriflavine. Scale bars: 10 μm.
peptides, which, according to our model, need to diffuse around tapetum cells to reach their receptors in the middle layer. In contrast, wild-type plants transformed with pAMS:GSO1 presented a different phenotype with disrupted pollen wall patterning, frequent pollen death, and pollen grain adhesions, but no tapetum hypertrophy (Fig. 4D-F). These phenotypes are reminiscent of those observed upon SBT5.4 expression under the AMS promoter, suggesting that ectopic GSO1 expression in the tapetum may cause a deregulation of pathway activation.

Discussion

The coordination of tapetum activity (polar secretion of nutrients, enzymes, pollen wall components, and the locular matrix) with pollen development (growth and initial patterning of the pollen wall) is likely a key factor in ensuring the generation of viable pollen grains. Taken together, our data indicate that this coordination involves a molecular dialogue among the tapetum, the developing pollen grains, and the middle layer. The localization of GSO1 and GSO2 receptors on the membrane of middle-layer cells and the expression of CIF3 and CIF4 precursor-encoding genes in the tapetum initiate significantly before microspore release from tetrads and the onset of pollen wall deposition (Fig. 2G and SI Appendix, Fig. S7 and S8). However, our phenotypic data show that this early production of signaling pathway components, which is also observed in the seed system (9), may "poise" the signaling system for action but is not necessary for early stages of tapetum or pollen grain development, since loss-of-function phenotypes in both ligand and receptor mutant backgrounds appear only after microspore release.

In contrast, our results suggest that the trigger in the pathway is the production of pollen-derived CIF-activating pro-peptides including SBT5.4, the microspore-specific expression of which is synchronized perfectly with the onset of pollen wall deposition (Fig. 2G and SI Appendix, Fig. S12). The importance of accurately timing pathway activation is underlined by the dramatic effects of expressing SBT5.4 early in the tapetum, which would be predicted to trigger a precocious activation of GSO-mediated signaling through constitutive ligand activation. The highly disrupted deposition of pollen wall components observed in these lines, including the difficulty of wall components in anchoring to the pollen surface, could be a consequence of a lack of coordination of pollen wall component production by the tapetum with microspore release from tetrads.

Our model predicts that SBTs produced in immature pollen grains mediate the timely processing and liberation of activated CIF3/4 peptides in the pollen wall or locular matrix. Activated peptides must then diffuse between tapetum cells to the middle layer, where they can bind to GSO1 and GSO2, ultimately leading to the activation of the tapetum, and the organized secretion of pollen wall components into the anther locule. Either pollen wall completion or specific changes in the tapetum presumably subsequently attenuate signaling activity by separating SBTs from their substrates, thus adjusting tapetum activity to the needs of the pollen grains (Fig. 4G).

This model raises the intriguing question of why GSO1 and GSO2 are expressed in the middle layer rather than in the tapetum. One possible explanation is that separating prepeptide and receptor production prevents untimely receptor-ligand interaction in the secretory pathway. However, our published data suggest that C-terminally extended CIF peptides show impaired receptor binding (9), and, consistent with this, coexpression of the TWS1 precursor and the GSO receptors in the embryo epidermis does not lead to receptor activation. A second possibility is that GSO signaling could interfere with other signaling pathways if it occurs in the tapetum (e.g., through competition for Somatic Embryogenesis Receptor Kinase coreceptors, which are known to interact with GSO1 and GSO2, but also with other receptors involved in another development) (11, 21). A final possibility is that the directional output of GSO signaling from the middle layer helps to polarize secretion from the tapetum, as suggested by the ectopic deposition of sporopollenin in pathway mutants.

In conclusion, our work has revealed that a complex peptide-mediated dialogue spanning three distinct cell types is necessary to synchronize secretion and deposition of pollen wall components within the anther locule. The middle layer, an understudied and poorly understood anther tissue, plays an important role in this dialogue, apparently acting as an organizing hub for the activation and associated polarization of the neighboring tapetum.

Materials and Methods

Plant Material and Growth Conditions. Seeds were sown on half-strength MS (Murashige Skoog) medium with 1% sucrose and 1% agar, stratified for 2 d at 4 °C and grown for 7 d in short-day conditions (8 h light/16 h dark). Seedlings were then transferred to soil and grown for 3 more weeks in short-day conditions (8 h light/16 h dark) and subsequently transferred to long-day conditions (16 h light/8 h dark) to promote flowering. Some of the transgenic lines described were examined in the T1 generation; these were first selected on half-strength MS medium with 1% sucrose and 1% agar supplemented with either 50 μg/mL kanamycin or 10 μg/mL glucose ammonium (Basta).

For promoter::GUS(uidA) reporter lines, seeds were incubated overnight at −75 °C; resuspended in 0.1% agar; stratified for 48 h at 4 °C in the dark; and sown on either half-strength MS, 1% sucrose, 0.6% agar plates, or a mix of potting compost with 3.6% (vol/vol) sand and 7.2% (vol/vol) perlite. Plants were grown under short-day conditions (12-h photoperiod) at 22 °C and 100 to 120 μE white light.

Mutant alleles used were gao1-1 (SALK_064029), gao2-1 (SALK_130637) (9), gao1-2 (SALK_103965), gao2-2 (SALK_143123) (10), cif3-2 (GABI_516E10), cif4-1 (CRISPR mutant line) (11), ipt1-1 (SALK_009847) (19), sbt5.4 (At5g59810, SALK_025087), sbt5.5 (At5g54560, SALK_107233), and sbt6.5 (At5g54560, CRISPR mutant generated as described below) (SI Appendix, Fig. S13); genotyping primers used are listed in SI Appendix, Table S1. The pGSO1:GSO1-mVenus and pGSO2:GSO2-mVenus lines are described in Doll et al. (9).

Generation of Transgenic Plant Lines. Gateway technology (Invitrogen) was used for the production of genetic constructs. For the NLS-3xmVenus transcriptional reporter lines, the following promoter fragments were amplified by PCR from Arabidopsis (Col-0) genomic DNA: pSBT5.4 −2,639 to −1 bp, pSBT5.5 −1,903 to −1 bp, pSBT5.6 −4,144 to −1 bp, pAMS −2,618 to −1 bp, pSHF −859 to −1 bp, pGSO1 −5,583 to −1 bp, pGSO2 −3,895 to −1 bp, pCIF3 −2,092 to −1 bp, and pCIF4 −2,201 to −1 bp. The fragments were inserted into pDONR P4-P1R and recombined with NLS-3xmVenus-N7 and pDONR211, OCS terminator pDONR 2P2-R3 (containing STOP codon followed by the OCTOPINE SYNTHASE terminator), and pKm34GW destination vector (with kanamycin in planta resistance); this yielded plasmids containing pBSBT5.4-NLS-3xmVenus, pBSBT5.5-NLS-3xmVenus, pBSBT6.6-NLS-3xmVenus, pAMS-NLS-3xmVenus, and pSHF-NLS-3xmVenus. To construct pGSO1-NLS-3xmVenus, pGSO2-NLS-3xmVenus, pCIF3-NLS-3xmVenus, and pCIF4-NLS-3xmVenus, promoter regions cloned into pDONR P4-P1R vectors and NLS-3xmVenus in pDONR2 were integrated into the pB7m24GW3 destination vector (with Basta in planta resistance and with the 3SS terminator) using a Gateway LR reaction. These constructs were transformed into the Col-0 background.

The promoter::GUS(uidA) reporter lines were generated by amplifying promoter fragments of SBT5.15 (2,634 bp) and SBT5.4 (4,159 bp) from Arabidopsis (Col-0) genomic DNA. Promoter fragments were digested with NotI/XhoI and NotI/SalI, respectively, and cloned into pGreen 0029 (23) containing a...
promoterless β-glucuronidase (GUS)/green fluorescent protein fusion reporter gene from pCAMBIA 1303 (CAMBIA GPO Box 3200, Canberra, ACT 2601, Australia) followed by the 282-bp OCS terminator.

To produce GSO1 misexpression constructs, the GSO1 genomic fragment lacking the cytoplasmic kinase-encoding domain (from the ATG [+1 bp] to +2,850 bp) was inserted into pDONR211 and recombined with pAM5 pDONR P4-P1R (see above), OCS terminator pDONR P2R-P3 (see above), and pk7m34GW destination vector to yield the pAM5 GSO1 ΔKinase construct. Similarly, the entire GSO1 genomic fragment from ATG [+1 bp] to the STOP codon (+3,826 bp) was inserted into pDONR211 and recombined with pAM5 pDONR P4-P1R (see above), OCS terminator pDONR P2R-P3, and pk7m34GW destination vectors to yield the pAM5 GSO1 vector. These constructs were transformed into Col-0 background.

The CIF4 complementation construct was produced by amplifying the CIF4 genomic fragment, including the promoter region and the entire coding sequence from −4,242 to +309 bp (including the STOP codon), and introducing it into pDONR211. Alternatively, a truncated version of the CIF4 genomic fragment, from −4,242 to +267 bp, with an artificial STOP codon before the C-terminal extension, was introduced into pDONR211. The CIF4 terminator fragment from +310 to +2,541 bp was inserted into pDONR P2R-P3. Genomic fragments were recombined into the pk7m34GW destination vector to yield pCIF4-CIF4 or pCIF4-CIF4 truncated constructs, which were then transformed into cif3-2 cif4-1 double mutant.

To produce CIF4 expression constructs, CIF4 coding sequence from +1 (ATG) to +309 bp (STOP codon) was introduced into pDONR211. The construct was recombined with either pAM5 pDONR P4-P1R or pSH7 pDONR P4-P1R and with OCS terminator pDONR P2R-P3 and pk7m34GW destination vectors to yield the pAM5-CIF4 and pSH7-CIF4 constructs. Alternatively, a truncated version of the CIF4 coding sequence and including N-terminal part from +1 (ATG) to +267 bp (and, therefore, lacking the C terminus that is removed in the mature peptide) was introduced into pDONR211. The construct was recombined with pSH7 pDONR P4-P1R and with OCS terminator pDONR P2R-P3 and pk7m34GW destination vectors to yield the pSH7-CIF4 truncated construct. These constructs were transformed into the cif3-2 cif4-1 double mutant.

To produce the TPS7 complementation construct, a plasmid containing the TPS7 ORF in pDONR211 (9) was recombined with the pAM5 pDONR P4-P1R, the OCS terminator pDONR P2R-P3, and the pB7m34GW destination vector to yield the pAM5 TPS7 construct. This construct was introduced into the tps7-1 background.

For ectopic expression of SBT5.4 in the tapetum, the pAMS:GSO1 was recombined with the pENTR 5′-TOPO vector. The pAMS:GSO1 vector was transformed into the double-mutant background. The trans-
ace and lead citrate. Sections were examined under JEOL 1400 TEM at 120 kV and imaged with the Gatan Rion 16 camera.

Confocal Microscopy. For the mVenusexpressing transcriptional and transla-
tional reporter lines, anthers were stained with 20 μg/ml propidium iodide (PI) solution and examined using TCS SP5 (Leica) or ZEISS 710 confocal microscopes with excitation at 514 nm and emission at 526 to 560 nm for mVenuse and 605 to 745 nm for PI.

For the pABC26/ABCG26-mQ2 reporter lines, the anthers were stained with 20 μg/ml PI solution and examined using ZEISS 710 confocal microscopes with sequential excitation at 458 nm for mQ2 and emission peak at 490 nm, as well as with excitation at 514 nm and emission peak at 642 nm for PI. In total, 40 anthers per genotype from the same developmental stage were imaged.

Peptide Cleavage Assay and Cleavage Product Analysis. The SBTS4.4 ORF was amplified by PCR from a RIKEN Institute of Physical and Chemical Research full-length complementary DNA (cDNA) clone (RFL119-27-C18). Primers included KpnI and XbaI restriction sites and six terminal His codons in the reverse primer. The PCR product was cloned between the CaMV 35S promoter and terminator in pART7 (28). The expression cassette was then transferred into the Nott site of the binary vector pART27 and introduced into Agrobacterium tumefaciens (CS851C). C terminally His-tagged SBST4.4 was transiently expressed in E. coli (DH5α). Cells were grown in 250 mL Lysogeny Broth (LB) medium at 37 °C and 200 rpm, until OD600 reached 0.6 to 0.8. Subsequently, the culture was cooled down to 30 °C, and after 20 min at 20 rpm, 1 mM Isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression. Cells were harvested by centrifugation after 2 h and lysed by sonication in 50 mM Na2HPO4/NaH2PO4 pH 7.0, 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine hydrochloride, and a spatula tip DNaseI. Then, 4 mM imidazole was added to the cleared lysate, and the recombinant protein was purified on Ni-Ni agarose following the manufacturer’s instructions (Qiagen; Hilden, Germany).

The C4-4 ORF, without the sequence coding for the signal peptide, was ampli-
fied by PCR from cDNA using primers that included a BamHI site at the 5′ end and six His codons and an EcoRI site at the 3′ end. The PCR product was cloned downstream and in frame with the GST ORF in pGEX-3x (GE Healthcare). The expression construct was transferred into E. coli BL21 CodonPlus(DE3) RIL (Agil-
ent Technologies). Cells were grown in 250 mL Lysogeny Broth (LB) medium at 37 °C and 200 rpm, until OD600 reached 0.6 to 0.8. Subsequently, the culture was cooled down to 30 °C, and after 20 min at 20 rpm, 1 mM Isopropyl β-D-1-thiogalactopyranoside was added to induce protein expression. Cells were harvested by centrifugation after 2 h and lysed by sonication in 50 mM Na2HPO4/NaH2PO4 pH 7.0, 150 mM NaCl, 1 mM PMSF, 5 mM benzamidine hydrochloride, and a spatula tip DNaseI. Then, 4 mM imidazole was added to the cleared lysate, and the recombinant protein was purified on Ni-Ni agarose following the manufacturer’s instructions (Qiagen). The eluate (3× 500 μl) was concen-
trated by ultratrrifugation (Vivaspin concentrators, 10-kDa molecular weight cutoff; Sartorius; Göttingen, Germany) and dialyzed against 50 mM Na2HPO4/NaH2PO4 pH 6.0 and 300 mM NaCl.

Then, 2.5 μg GST-CIF4-His in a total volume of 10 μl 50 mM Na2HPO4/NaH2PO4 pH 6.0, 300 mM NaCl were incubated at 25 °C with recombinant SBTS4.5 at the indicated concentrations. As a negative control, apoplastic washes from N. benthamiana plants that had been agro-infiltrated with the empty pART27 vector were subjected to a mock purification, and an equal volume of the final fraction was used in the assay. The reaction was stopped after 2 h by addition of 4× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE) sample buffer. The digest was analyzed by SDS-PAGE (15%) followed by Coomassie-Brilliant Blue (Instant Blue; Abcam, Cambridge, U.K.) staining.

For mass spectrometric analysis of the cleavage site, 6 μg of a synthetic CIF4 peptide extended at both the N and the C termini by three precursor-derived amino acid residues were digested with 50 ng recombinant SBTS4.5 and a mock-
purified fraction as a control. The reaction was stopped after 1 h at 25 °C by addition of 1% trifluoroacetic acid. Reaction products were purified on C18 stage tips equilibrated in 0.5% (vol/vol) acetonitrile as described (30). Peptides were eluted in 0.5% (vol/vol) acetic acid and 50% (vol/vol) acetonitrile, evaporated to dryness in a SpeedVac concentrator (Savant Instruments; Holbrook, NY), and stored at −20 °C until further analysis. Digests were analyzed by Liquid Chromatography-Tandem Mass Spectrometry using an UltiMate 3000 RSLCnano system (Dionex, Thermo Fisher Scientific) coupled to an Orbitrap mass spectrometer (Explotis 480, Thermo Fisher Scientific). The Explotis 480 was operated under XCalibur software (version 4.4, Thermo Fisher Scientific) and calibrated internally (31). Peptides were trapped on a precolumn (C18 PepMap100, 5 μm × 5 mm, Thermo Fisher Scientific) and then eluted from a 75 μm × 250-mm analytical C18 column (NanoEase, Waters) by a linear gradient from 2 to 55% acetonitrile over 30 min. MS spectra (m/z = 200 to 2,000) were acquired at a resolution of 60,000 at m/z = 200. Data-dependent MS/MS mass spectra were generated for the 30 most abundant peptide precursors using high-energy collision dissociation fragmentation at a resolution of 15,000 and a normalized collision energy of 30. Peptides were identified by Mascot 2.6 search (Matrix Science, U.K.) and quantified using peak areas calculated from extracted ion chromatograms in XCalibur 4.4 (Thermo Fisher Scientific).

Pollen Viability and Germination Assays, Seed Count. Pollen viability was tested by staining mature anthers and pollen with Alexander solution (32). For the pollen germination assays, mature pollen grains were transferred onto medium containing 0.5% low melting agarose, 18% sucrose, 0.01% boric acid, 1 mM CaCl2, 1 mM Ca(NO3)2, 1 mM KCl, 0.03% casein enzymatic hydrolysate, 0.01% ferric ammonium citrate, 0.01% myo-inositol, and 0.25 mM spermidine, pH 8.0 for 8 h at 25 °C. The images were taken with Zeiss Axio Imager M2 microscope.

For the seed number analysis, seeds were counted in the fourth-eighth silique of the respective plants. In total, 50 siliques per genotype were counted. Unpaired two-tailed t tests were used for statistical analysis.

Data Availability. All study data are included in the article and/or SI Appendix. All lines used in the study will be provided upon signature of an appropriate Material Transfer Agreement.

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