Calcium signalling mediates self-incompatibility response in the Brassicaceae

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Self-incompatibility (SI) in the Brassicaceae is controlled by multiple haplotypes encoding pollen ligand (SP11/SCR) and its stigmatic receptor kinase (SRK). A haplotype-specific interaction between SP11/SCR and SRK triggers SI response that leads to self-pollen rejection, but the signalling pathway remains largely unknown. Here we show that Ca²⁺-influx into stigma papilla cell mediates the SI signalling. Using self-incompatible Arabidopsis thaliana expressing SP11/SCR and SRK, we found that self-pollination specifically induced an increase of cytoplasmic Ca²⁺ ([Ca²⁺]cyt) in papilla cells. Direct application of SP11/SCR to the papilla-cell protoplasts induced Ca²⁺-increase, which was inhibited by a glutamate receptor channel blocker AP-5. An artificial increase of [Ca²⁺]cyt in papilla cell arrested wild-type pollen hydration. Treatment of papilla cell with AP-5 interfered SI, and Ca²⁺-increase upon SI response
was reduced in the glutamate receptor-like channel (GLR) gene mutants. These results suggest that $\text{Ca}^{2+}$-influx mediated by GLR is the essential SI response leading to self-pollen rejection.
Flowering plants have developed SI as a genetic system to prevent inbreeding and thus promote outcrossing. In many species, SI is controlled by an \( S \) locus with multiple haplotypes\(^1\). Each \( S \)-haplotype encodes both male- and female-specificity determinants (\( S \)-determinants), and self/non-self discrimination is accomplished by the \( S \)-haplotype–specific interaction between these \( S \)-determinants.

In the Brassicaceae, the male and female \( S \)-determinants have been identified as \( S \)-locus protein 11 (SP11, also named \( S \)-locus cysteine-rich protein, SCR) and \( S \)-locus receptor kinase (SRK), respectively\(^1\). SP11/SCR is a polymorphic small peptide secreted from the anther tapetum that localizes to the pollen surface, whereas SRK is a polymorphic Ser/Thr receptor kinase that localizes to the plasma membrane of stigma papilla cells. SP11/SCR and SRK from each \( S \)-haplotype function respectively as a ligand and its cognate receptor. Upon self-pollination, the \( S \)-haplotype–specific interaction between SP11/SCR and SRK induces autophosphorylation of SRK, which is thought to trigger a signalling cascade in the papilla cell, resulting in the rejection of self-pollen\(^2\). Although self-pollination is known to evoke multiple physiological changes in the papilla cell including disruption of actin bundles, fragmentation of vacuolar structure, and modification of microtubules\(^3,4\), the signalling pathway downstream of SRK that leads to these processes remains largely unknown.

Thus far, two candidate molecules, \( M \)-locus protein kinase (MLPK) and Arm-repeat containing 1 (ARC1), have been identified as the direct downstream effectors of SRK. MLPK was identified as a gene responsible for a self-compatibility mutation in \textit{Brassica rapa}\(^5\), and encodes a membrane-anchored cytoplasmic protein kinase that interacts with SRK on the papilla cell membrane\(^6\). Recent studies have suggested that MLPK is also involved in intra-species unilateral incompatibility of \textit{B. rapa}\(^7\), but it remains unclear whether MLPK is required for SI throughout the Brassicaceae\(^8\).

ARC1 is known to interact with, and is phosphorylated by, the kinase domain of SRK in \textit{Brassica napus}\(^9,10\). ARC1 is a U-box protein with E3 ubiquitin ligase activity\(^11\), and interacts with Exo70A1\(^12\), a putative component of the exocyst complex, which generally functions in polarized secretion\(^13\). These results suggested a model in which activated SRK phosphorylates ARC1, leading to the preclusion of as-yet-unknown “compatibility factors” secretion to the stigmatic surface and inhibiting pollen entrance\(^14\). However, the suppression of \textit{ARC1} expression results in incomplete
breakdown of SI in both *B. napus* and *Arabidopsis lyrata*\textsuperscript{10,14}, and self-compatible *Arabidopsis thaliana* that lack ARC1 acquires SI phenotype by introducing SRK and *SP11/SCR* genes\textsuperscript{15}. Therefore, to what extent ARC1 is involved in the signalling pathway downstream of SRK remains controversial\textsuperscript{8,16,17}.

In this study, we focused to investigate the cytoplasmic Ca\textsuperscript{2+} dynamics in stigma papilla cells during the SI response. Previous study used injected dyes to monitor Ca\textsuperscript{2+} dynamics in the SI response\textsuperscript{18}. By combining the *in vivo* imaging using genetically encoded [Ca\textsuperscript{2+}]_cyt probes and pharmacological approaches, we found that cytoplasmic Ca\textsuperscript{2+} drastically increases in the papilla cell after self-pollination, which can be efficiently blocked by the inhibitors of glutamate receptor channels that mediates the influx of extracellular Ca\textsuperscript{2+}. Pre-treatment of papilla cells with glutamate receptor channel inhibitor compromised the SI response *in vivo*, whereas an artificial increase in [Ca\textsuperscript{2+}]_cyt in papilla cells induced an arrest of pollen hydration to compatible pollen. [Ca\textsuperscript{2+}]_cyt increase in papilla cells during the SI response of *GLR* mutants were significantly reduced. Our results overall strongly suggest that the Ca\textsuperscript{2+}-influx in papilla cells mediated by GLR is the key SI response that leads to self-pollen rejection.

**RESULTS**

**Dual pollination assay using self-incompatible *A. thaliana*.** To clarify the SI signalling pathway in Brassicaceae species, we utilized model plant *A. thaliana*. Although *A. thaliana* is self-compatible with disrupted *S*-locus and some other components required for SI response\textsuperscript{19-21}, some accessions including C24 retained SI signalling cascade and could confer stable SI by introducing *SP11/SCR* and SRK genes cloned from an SI relative, *A. lyrata*\textsuperscript{22,23}. We generated *A. thaliana* C24 accession lines expressing *SP11/SCR* or SRK gene from *A. lyrata* *S_b*-haplotype (*S_b*-SP11/SCR or *S_b*-SRK) and obtained the lines with strong and stable SI phenotype (Supplementary Fig. 1a,b), which is in consistent with the report by others\textsuperscript{24}. The SI response in the pistil was suggested to be dependent on kinase activity of SRK, because none of the transgenic lines expressing kinase-inactive form of SRK (*S_b*-SRK_K555E), in which the 555th lysine residue at an ATP-binding site was substituted to glutamic acid, exhibited SI response (Supplementary Fig. 1c,d)\textsuperscript{24,25}. 


Using these transformed lines of *A. thaliana*, we first re-examined the classical dual-pollination assay, which showed that a single *Brassica* papilla cell could simultaneously reject a self-pollen grain but accept a cross-pollen grain\(^{26}\). This observation was interpreted as indicating that SI signalling occurs locally at the site where pollen was attached. When we pollinated each single *Sbo*-SRK–expressing papilla cell almost simultaneously (within 1 min) with self (*Sbo*-SP11/SCR–expressing) pollen grain and cross (wild-type, WT) pollen grain using two micromanipulators, at distant attachment sites, in about 30% of tests, germination of the self-pollen grain was selectively inhibited as in the case of previous report (Fig. 1). While in about 70% of tests, germination of both self- and cross-pollen grains was inhibited. When we first pre-pollinated a papilla cell with a self-pollen grain and then pollinated with a cross-pollen grain after 5 min, germination of the cross-pollen grain was also completely inhibited. In the control experiment, papilla cell was first pre-pollinated by a cross-pollen grain instead of a self-pollen grain, which did not influence the germination rate of the re-pollinated cross-pollen grain. Our previous study showed that the SI response such as actin filament depolymerization and vacuole disorganization occurs throughout the whole papilla-cell\(^3\). These results together suggest that SI signalling is not restricted to the self-pollen attachment site, but is instead diffusible and can spread into the papilla cell within minutes.

**An increase in cytoplasmic Ca\(^{2+}\) in papilla cells after self-pollination.** As a candidate for the diffusible signalling molecule, we focused on cytosolic Ca\(^{2+}\). To examine cytosolic Ca\(^{2+}\) dynamics ([Ca\(^{2+}\)_cyt]) in the papilla cell during self- and cross-pollination, we generated transgenic *A. thaliana* co-expressing *Sbo*-SRK and the membrane-anchored form of Yellow Cameleon 3.60 (YC3.60\(_{pm}\)). Membrane anchoring of YC3.60 has been used to effectively monitor Ca\(^{2+}\) dynamics near the cytoplasmic faces of the plasma membrane in animal cells\(^{27}\). Based on the localization of YC3.60\(_{pm}\) in the transgenic papilla cells, as determined by immunoelectron microscopy (Supplementary Fig. 2), we predicted that this monitoring system would measure [Ca\(^{2+}\)_cyt] near the cytoplasmic faces of the plasma and endomembranes. Before pollination, the mean emission ratio of YFP to CFP at the tip region of papilla cells was 2.01 ± 0.39 (*n* = 20), whereas after self-pollination (*i.e.*, pollination with *Sbo*-SP11/SCR–expressing pollen), the values
increased and reached its maximum value of $2.87 \pm 0.61 \ (n = 20)$ at about 5 min (Fig. 2a,c; Supplementary Movie 1). Based on the [Ca$^{2+}$] calibration curve (Supplementary Fig. 3), these values correspond to an increase in [Ca$^{2+}$]$_{cyt}$ from ~ 0.1 µM (before pollination) to ~ 1 µM (after self-pollination). On the other hand, after cross-pollination (i.e., pollination with WT pollen), small and occasional [Ca$^{2+}$]$_{cyt}$ increase was observed and the value mostly stayed at around $2.01 \pm 0.48 \ (n = 20)$, ~ 0.1 µM (Fig. 1b,d; Supplementary Movie 2). Such [Ca$^{2+}$]$_{cyt}$ behavior in the cross-pollination was similar to our previous observations$^{28}$, and these local peaks were expected to correlate with pollen hydration, tube germination and penetration. Similar [Ca$^{2+}$]$_{cyt}$ dynamics were observed when we used the transgenic *A. thaliana* co-expressing *S*$_{b}$-SRK and the soluble form of YC3.60, although the maximum YFP/CFP ratio was slightly smaller compared with that in YC3.60$_{pm}$ transformants (Supplementary Fig. 4).

**An increase in cytoplasmic Ca$^{2+}$ in papilla-cell protoplasts triggered by the interaction between cognate SP11/SCR and SRK.** The specific increase of papilla [Ca$^{2+}$]$_{cyt}$ after self-pollination is probably attributable to *S*$_{b}$-SP11/SCR expressed in the transformed pollen grain. To confirm this, we chemically synthesized *S*$_{b}$-SP11/SCR protein and refolded it into the active form, according to the previously described procedures$^{29}$, and confirmed the biological activity as the male *S*-determinant (Supplementary Fig. 5).

However, the direct application of the synthesized *S*$_{b}$-SP11/SCR protein to papilla cell surface could not induce SI responses without pollination due to the cuticular layer and the wax coat covering over the surface of the papilla cells, preventing the soluble form of *S*$_{b}$-SP11/SCR protein from penetrating into the papilla-cell wall to interact with *S*$_{b}$-SRK on the plasma membrane. To solve this issue, we established a method for preparing protoplasts from papilla cells expressing both *S*$_{b}$-SRK and YC3.60, and then analyzed the effect of *S*$_{b}$-SP11/SCR. Before adding *S*$_{b}$-SP11/SCR, the steady-state [Ca$^{2+}$]$_{cyt}$ in papilla-cell protoplasts was ~ 0.51 µM (mean YFP/CFP ratio = 2.49 ± 0.20; $n = 13$), about five times higher than in intact papilla cells. However, when *S*$_{b}$-SP11/SCR was applied (at final concentration of 10 nM) to the papilla-cell protoplasts, [Ca$^{2+}$]$_{cyt}$ transiently increased after 6 min to ~ 2.85 µM (mean YFP/CFP ratio = 3.87 ± 0.65; $n = 13$), approximately six-fold higher than the initial concentration (Fig. 3a,e). On the other hand,
when synthesized $S_b$-SP11/SCR from *B. rapa* was added to $S_b$-SRK protoplasts (to 10 nM) (Fig. 3b,f) or when $S_b$-SP11/SCR was added to WT protoplasts expressing only YC3.60 (Fig. 3c,g), no significant $[\text{Ca}^{2+}]_{\text{cyt}}$ increase was observed. Furthermore, the protoplasts expressing kinase-inactive $S_b$-SRK ($S_b$-SRK$_{\text{K555E}}$) exhibited no $[\text{Ca}^{2+}]_{\text{cyt}}$ increase after the addition of $S_b$-SP11/SCR (Fig. 3d,h).

Further to confirm that the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase observed in $S_b$-SRK–expressing *A. thaliana* is not an artificial response incidentally induced by $S_b$-SP11/SCR, but is instead a bona fide universal SI response triggered by $S$-haplotype specific interaction between SP11/SCR and SRK, we conducted a reciprocal induction test using two $S$-haplotypes of *B. rapa*. We prepared papilla-cell protoplasts from the $S_8$- or $S_9$-haplotypes of *B. rapa* plants, loaded with the membrane-permeable Ca$^{2+}$ indicator Fluo4-AM$^{30}$. The $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the $S_8$- or $S_9$- haplotype papilla-cell protoplasts was induced by application of the synthesized cognate SP11/SCR (at final concentration of 10 nM) approximately after six minutes, but not by the non-cognate SP11/SCR, respectively (Supplementary Fig. 6). These results suggested that the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the papilla cell is a common response induced by the $S$-haplotype specific interaction between SP11/SCR and SRK in the Brassicaceae.

**Pharmacological analyses of the Ca$^{2+}$ channel involved in SI signalling.** The $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the papilla cell could be attributed to an influx from the extracellular space or an efflux from an intracellular organelle such as the vacuole and ER. When the protoplast medium was replaced with Ca$^{2+}$-free medium, chemically synthesized $S_b$-SP11/SCR induced no significant $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in $S_b$-SRK protoplasts (Fig. 3i). Protoplast viability was not affected during this experiment, because $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the protoplasts were completely restored by the addition of Ca$^{2+}$ in culture medium (Supplementary Fig. 7). In addition, when a broad Ca$^{2+}$ channel blocker such as GdCl$_3$ or LaCl$_3$ was added to the protoplast medium, the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase was significantly suppressed$^{31}$ (Fig. 3i, Supplementary Fig. 8). These results together suggested that $[\text{Ca}^{2+}]_{\text{cyt}}$ increase triggered by $S_b$-SP11/SCR was induced by an influx of extracellular Ca$^{2+}$, and that some Ca$^{2+}$ channel in the plasma membrane functions in this $[\text{Ca}^{2+}]_{\text{cyt}}$ increase.
To characterize the molecules mediating this Ca\(^{2+}\) influx, we examined the effect of various inhibitors for Ca\(^{2+}\) transporters; AP-5, NMDA receptor\(^{32}\); DNQX, and CNQX, AMPA/kainate receptor\(^{32}\); U-73122, phospholipase C\(^{33}\); Verapamil, L-type Ca\(^{2+}\) channel\(^{34}\); Mibefradil, T-type Ca\(^{2+}\) channel\(^{34}\); Carboxyoeosine, plasma-membrane Ca\(^{2+}\) pump\(^{35}\) (Fig. 3i). We found that all tested glutamate receptor channel blockers (AP-5, DNQX, and CNQX) significantly inhibited the Ca\(^{2+}\) increase (Fig. 3i, Supplementary Fig. 8). Especially AP-5, a selective NMDA receptor antagonist, completely blocked the SP11/SCR-induced [Ca\(^{2+}\)]\(_{cyt}\) increase at 0.5 mM. Moreover, co-treatment of known ligands of glutamate receptors such as D-glutamate, glycine and D-serine with AP-5 partially restored the SP11/SCR-induced [Ca\(^{2+}\)]\(_{cyt}\) increase (Supplementary Fig. 9). These results suggested that GLR is the candidate molecule involved in Ca\(^{2+}\)-influx by S\(_b\)-SP11/SCR treatment. In agreement with the protoplast assay, we found that maximum YFP/CFP ratio of the S\(_b\)-SRK–expressing stigmas pretreated with 50 mM AP-5 suppressed to 2.10 ± 0.50 (n = 24) (Supplementary Fig. 10), whereas in the control treated solely with tween 20 maximum YFP/CFP ratio recorded 2.69 ± 0.51 (n = 20).

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\text{[Ca}^{2+}\text{]}_{cyt} \text{ increase in papilla cell is required and sufficient to induce SI response.}
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Next, we pharmacologically investigated the involvement of GLR in the SI reaction using \textit{in vivo} pollination assay. When S\(_b\)-SRK–expressing stigmas were pretreated with AP-5 and then pollinated with self (S\(_b\)-SP11/SCR–expressing) pollen grains, significant compromise in SI reaction was observed (Fig. 4). Robust pollen tube growth was observed in the AP-5 treated stigmas, while such extent of tube growth was never observed in the untreated control stigma pollinated with self-pollen grains (Fig. 4a). Taken together, we concluded that preventing the increase of Ca\(^{2+}\) to the papilla cell by AP-5 treatment eventually leads to SI breakdown.

Furthermore, we investigated the causal relationship between pollen hydration and the increase of [Ca\(^{2+}\)]\(_{cyt}\). To examine whether the [Ca\(^{2+}\)]\(_{cyt}\) increase in papilla cells causes inhibition of pollen hydration, we microinjected aqueous Ca\(^{2+}\) solution into papilla cells co-expressing S\(_b\)-SRK and YC3.60pm, using a laser-assisted thermal-expansion microinjector\(^{36}\). Injection volume was held constant by adding Alexa Fluor 546 to the test solution and monitoring the fluorescence intensity. After each microinjection, the injected papilla cell was pollinated with a single WT-pollen grain, and then its hydration
was monitored under the microscope. We also measured the YFP/CFP ratio before and after microinjection to calculate the relative increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ in the injected papilla cell. We estimated that the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase after self-pollination (final to ~ 1 µM) corresponds to the microinjection of CaCl$_2$ solution at an intermediate concentration between 0.1 and 1 mM (Table 1). When we injected solution containing more than 0.1 mM CaCl$_2$, pollen hydration was significantly inhibited. By contrast, when we injected water or concentrated KCl solution, pollen hydration was not inhibited (Table 1). These results suggest that the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in papilla cells is required and sufficient to induce SI response that results in inhibition of pollen hydration.

**Nonsense mutants of the Arabidopsis GLR members exhibits reduced Ca$^{2+}$-increase upon SI response.** To further gain validation for the role of GLR in the SI response, we sought for GLR genes abundantly expressed in the *A. thaliana* papilla cell through the combination of laser-microdissection and microarray analysis. We found that GLR1.3, GLR3.3, GLR3.5 and GLR3.7 are the four most abundantly expressed genes in the papilla cells among 20 GLR members encoded in the *A. thaliana* genome (Supplementary Fig. 11). We obtained mutants that harbors premature stop codon in each of these four GLR genes using the TILLING platform (Supplementary Methods) and investigated whether if these nonsense mutations have an effect in the Ca$^{2+}$-increase response when introduced into the *Sb*-SRK/YC3.60 background (Supplementary Fig. 12). We found that relative Ca$^{2+}$-increase during the SI response in the line carrying homozygous nonsense mutations in GLR3.5 and GLR3.7 were significantly reduced compared to the wild-type (Fig. 5), suggesting that at least these two GLR genes are necessary for the $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in papilla during the SI response. We further confirmed the involvement of GLR3.7 by complementation test, in which *glr3.7/glr3.7* lines expressing GLR3.7 (Supplementary Fig. 13) restored the Ca$^{2+}$-increase response level comparable to the WT (Fig. 5).

Although the Ca$^{2+}$-increase was reduced in the mutants, we could not observe the breakdown of SI in the single mutants by the pollination assay, possibly attributable to functional redundancy of tandemly duplicated GLR3.5 and GLR3.7 (Supplementary Fig. 11).

**DISCUSSION**
Ca$^{2+}$ is an important secondary messenger molecule functioning during the entire fertilization process in animals and plants\textsuperscript{37}. The involvement of calcium signalling in Brassicaceae SI has been hypothesized based on the observation that callose (\(\beta\)-1,3-glucan) was deposited at the self-pollen-papilla cell interface\textsuperscript{38}, and that the activity of catalysing enzyme, \(\beta\)-1,3-glucan synthase, was calcium-dependent\textsuperscript{39}. However, nobody has succeeded in directly and non-disruptively monitoring calcium dynamics in living papilla cells, and the involvement of Ca$^{2+}$ in SI response remained ambiguous. Our present study strongly suggests that direct interaction between SP11/SCR and SRK results in drastic \([Ca^{2+}]_{cyt}\) increase in the papilla cell, possibly via the Ca$^{2+}$-influx mediated by GLR, although how SRK phosphorylation signaling activates GLR remains unknown. A previous study injecting calcium dyes into papilla cell concluded that both cross- and self- pollinations in Brassica napus produce only small localized peaks in \([Ca^{2+}]_{cyt}\) within the observed 10 minutes\textsuperscript{18}. This inconsistency with our present report is perhaps attributable to the difference in methods used in two studies to detect \([Ca^{2+}]_{cyt}\). The pollination responses occur much later in Brassica than in Arabidopsis (e.g. pollen hydration at 15-20 min after cross-pollination whereas 5-6 min in Arabidopsis). A longer experimental duration was most likely required for the previous study that used the Brassica species to observe the Ca$^{2+}$-increase in the SI response.

Ionotopic glutamate receptor channels in animal cells are known to be activated by membrane depolarization, ligand binding and phosphorylation to permeabilize sodium, potassium and calcium ions\textsuperscript{40}. We found that some amino acids, especially D-glutamate can compete with AP-5 to induce Ca$^{2+}$-increase during the SI response, in agreement with a previous report which suggested D-serine as the GLR ligand in pollen tube\textsuperscript{32}. However, sole application of D-glutamate did not induce the Ca$^{2+}$-increase (Supplementary Fig. 9b), indicating that other factors are required to induce the SI response. Possible role for SRK may not only be limited to direct phosphorylation of GLRs, but can also mediate GLR activation through papilla cell membrane depolarization via phosphorylation of another factor\textsuperscript{40}. Our current result showed that even using protoplasts and chemically synthesized SP11, approximately six minutes was required for the Ca$^{2+}$-increase SI response to occur. Although time required for the diffusion of applied SP11 is not negligible, necessity for multiple steps to
activate GLR may also be the reason for this delay. We found that \textit{GLR}3.5 and \textit{GLR}3.7 are at least required for the full $Ca^{2+}$-increase activity upon the SI response, and a recent study confirms the \textit{GLR}3.7 function to permeabilize $Ca^{2+}$ ions in the \textit{Xenopus} cells\textsuperscript{41}. From the series of data in this study, we consider that $Ca^{2+}$ ions are most likely imported from the extracellular space (Fig. 3i, Supplementary Fig. 7), although exact location of the source remains to be elucidated. Although our data suggested that the $Ca^{2+}$-influx is necessary for the SI response, $[Ca^{2+}]_{cyt}$ increase we observed in the papilla cells may include $Ca^{2+}$ ions released from other sources such as organelles possibly invoked by the $Ca^{2+}$-influx. A detailed study on papilla cell membrane channel activity including electrophysiology means is clearly necessary to further understand the underlying mechanism of $Ca^{2+}$-increase in SI response of the Brassicaceae. In contrast, our data showed that AP-5 does not inhibit compatible pollination (Fig. 4), suggesting the fundamental difference in the $Ca^{2+}$-dynamics between compatible and incompatible pollinations. Previous study showed the $Ca^{2+}$-supply mechanism to pollen in compatible pollination via $Ca^{2+}$-pump containing vesicles\textsuperscript{42}. SI downstream response such as disruption of actin bundles\textsuperscript{4} could be involved to prevent the $Ca^{2+}$-supply, although further investigations are necessary.

Relationship between the $[Ca^{2+}]_{cyt}$ increase pathway and other factors proposed to play a role in the SI signalling also needs to be clarified. It is possible that MLPK co-function with SRK to activate GLR. There has been a SI signalling pathway proposed involving ARC1 that do not involve $Ca^{2+}$-increase\textsuperscript{11,12}. It remains to be solved if this pathway runs in parallel with the $Ca^{2+}$-increase mediated SI response, or if two pathways interconnect with each other.

Recent molecular and genetic analyses have revealed that angiosperms possess diverse SI molecular systems\textsuperscript{1,43}. In the Papaveraceae, SI is controlled by an $S$-haplotype-specific interaction between the stigmatic ligand (PrsS) and the pollen receptor (PrpS). Although molecular natures of these $S$-determinants are completely different from those of the Brassicaceae, their $S$-haplotype specific interaction induces a $[Ca^{2+}]_{cyt}$ increase in the self-pollen tube that results in its growth arrest and programmed cell death\textsuperscript{44,45}. In the hermaphroditic solitary ascidian \textit{Ciona intestinalis}, SI is controlled by two loci (\textit{S} and \textit{Z})\textsuperscript{46}, both of which encode a tightly linked pair of proteins: a fibrinogen-like female ligand (v-Temis) and its transmembrane sperm receptor.
Upon self-fertilization their haplotype-specific interaction induces a $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the sperm, thereby inactivating fertilization \(^{47}\). Our current findings lead us to hypothesise that although the molecular systems of SI in angiosperms and hermaphroditic organisms differ from each other, an SI-induced $[\text{Ca}^{2+}]_{\text{cyt}}$ increase in the receptor side may be a common event triggered by self-recognition between male and female determinants.

**Methods**

**Plant growth.** Plants were grown in mixed soil in a growth chamber under controlled conditions (light intensity of 120–150 µmol m\(^{-2}\) s\(^{-1}\), 14-h light – 10-h dark cycle at 22 ± 2°C).

**Generation of genetic materials.** All transgenic plants were generated in *A. thaliana* accession C24 using the *Agrobacterium* infiltration procedure, as previously reported\(^{48}\). Details of the vector construction and transgenic line development procedures are described in Supplementary Methods.

**Preparation of papilla-cell protoplasts.** Stigmas from opening flowers were aligned on a glass slide, and papilla cells were separated under a dissecting microscope using surgical blades. Collected cells were incubated for 2 h at 23°C in enzyme solution (5 mM Mes-Tris, pH 5.5) containing 0.5 mM CaCl\(_2\), 0.5 mM MgCl\(_2\), 10 mM KH\(_2\)PO\(_4\), 0.5 mM ascorbic acid, 0.55 M sorbitol, 1.3% (w/v) Onozuka RS cellulase, 0.0075% (w/v) Pectolyase Y-23, and 0.25% (w/v) BSA. Released protoplasts were collected by centrifugation (50 x g for 5 min at 23°C). The protoplasts were then washed in protoplast medium (Murashige and Skoog medium containing 0.55 M sorbitol, pH 5.7 and 3 mM CaCl\(_2\)), and after re-collection by centrifugation, the protoplasts were resuspended in the protoplast medium. The dishes containing the protoplasts were maintained in dark at 23°C until use. For Ca\(^{2+}\)-free medium, 0.1 mM Na\(_2\)-EDTA was added in place of 3 mM CaCl\(_2\) to the protoplast medium. Viability of the isolated protoplasts after \(S_n\)-SP11/SCR addition, under different conditions (1 mM GdCl\(_3\), 1mM LaCl\(_3\), 1 mM AP-5 and in Ca\(^{2+}\)-free buffer) was confirmed by fluorescence diacetate (FDA) staining (Supplementary Fig. 14). FDA dissolved in DMSO (5 mg/ml) was
diluted to 0.25 mg/ml in distilled water before use. Ten minutes after \(S_b\)-SP11/SCR addition to the \(S_b\)-SRK expressing protoplasts in 50 \(\mu\)l medium, 1 \(\mu\)l of diluted FDA was applied. Protoplasts were placed at 23°C for 5 min and observed by epifluorescent microscopy.

**Ratiometric imaging.** For experiments in vivo, sample preparation was performed as previously reported\(^{28}\). For experiments in vitro, protoplasts suspended in protoplast medium were observed in a glass-bottom dish. Imaging was performed using a confocal laser-scanning microscope equipped with a 440-nm laser (LSM710; Carl Zeiss, Germany). Imaging of the YC3.60 emission ratio was performed using two emission ranges (465-495 for CFP and 515-555 for YFP) using a Zeiss 20×/0.8 fluorescence objective lens. Ratiometric images (YFP/CFP) were obtained using the ZEN image-processing software (Carl Zeiss). In each experiment, the ratio in a region with a diameter of 5 \(\mu\)m was measured, and these ratios are shown in figures as sequential line graphs. The mean maximum values were calculated using the Excel software (Microsoft). Exposure times were typically 1–1.5 sec, and images were collected every 15–30 sec.

**Calibration of YC3.60 and YC3.60pm ratiometric changes.** Calibration of \([Ca^{2+}]_{c_{yt}}\) was carried out as described previously\(^{28,48}\). Serial dilutions of purified YC3.60 were made in \(Ca^{2+}\) calibration buffer (Molecular Probes), in which the free \([Ca^{2+}]\) ranged from 0–1 mM. Each image for the titration was captured using the same confocal laser microscope (LSM710) under the same conditions used for imaging the in vivo and in vitro experiments. YC3.60 was diluted to yield signal intensities similar to those observed in YC3.60-expressing stigmatic papilla cells, and these dilutions were used to measure YFP/CFP ratios and to generate the calibration curve (Supplementary Fig. 3). The \([Ca^{2+}]_{c_{yt}}\) in the papilla cells and papilla-cell protoplasts was estimated from YFP/CFP values using this calibration curve.

**Synthesis of \(S_b\)-SP11/SCR.** \(S_b\)-SP11/SCR, which was synthesized by Bio-Synthesis Inc. (Texas), was reduced in the presence of dithiothreitol and purified by reversed-phase HPLC. The purified \(S_b\)-SP11/SCR was refolded in the presence of both the oxidized and
reduced forms of glutathione (1:5). The most major oxidized form of $S_b$-SP11/SCR was purified by reversed-phase HPLC. We verified the refolding (four disulphide-bonds formation) of the obtained $S_b$-SP11 by MALDI-TOF-MS, and confirmed its biological activity by pollination assay. In brief, stigmas were treated with 1.2 pmol of purified $S_b$-SP11 in 0.1 μl containing 0.05% Tween 20, and then dried in the air for 1 h. After cross-pollination, the stigmas were kept at 20°C for 6 h. Penetration of pollen tubes into each stigma was observed after aniline-blue staining as previously described.

**Microinjection.** Microinjection into papilla cells co-expressing YC3.60pm and $S_b$-SRK was performed using a wide-field microscope (Axio Observer; Carl Zeiss) equipped with micromanipulators (Narishige, Japan) and a laser-assisted microinjector (Nepa Gene, Japan). Stigma was harvested and aligned on a slide. Papilla cells were held fixed by Vacutip (Eppendorf). To keep the injection volume constant, Alexa Fluor 546 was added to sample solution (to 0.8 mM), and the injection site was monitored using the emission 641/75 filter. The YC3.60 fluorescence emission was measured using two emission filters (480/30 for CFP and 535/40 for YFP). A Zeiss 40×/0.75 objective lens was used during microinjection. The YFP/CFP ratio was determined using the MetaMorph software.
References


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Author contributions

Additional information
Supplementary information is available in the online version of the paper. Reprints and permissions information is available online at www.nature.com/reprints. Correspondence and requests for materials should be addressed to S.T.

Competing financial interests
The authors declare no competing financial interests.
FIGURE LEGENDS

Figure 1 | Dual pollination assay using self-incompatible *A. thaliana*. (a) Each *Sb*-SRK–expressing papilla cell was pollinated with both self (*Sb*-SP11/SCR–expressing) and cross (WT) pollen grains. (b) Germination of self pollen grain was selectively inhibited. Simultaneous pollination (within 1 min using two micromanipulators) of *Sb*-SRK–expressing papilla cell with self and cross pollen grains, germination of ca. 70% WT pollen grains were inhibited as well as *Sb*-SP11/SCR–expressing pollen grains. Dual-pollination by WT pollen grain 5 min after *Sb*-SP11/SCR–expressing pollen grain resulted in strong germination inhibition of WT pollen grain. Pollen germination was monitored 25 min after the last pollination.

Figure 2 | Dynamics of \([\text{Ca}^{2+}]_{\text{cyt}}\) in YC3.60pm expressing papilla cells following self- and cross-pollination. YFP/CFP ratio images of a single *Sb*-SRK–expressing papilla cell before and after pollination (a) with self (*Sb*-SP11/SCR–expressing) pollen and (b) with cross (WT) pollen. Scale bars, 5 µm. The time-course ratio change in the tip region below the pollen attachment site (c) after self pollination shown in (a), and (d) after cross pollination shown in (b). Arrows indicate pollination timing. Arrows labelled 1, 2, and 3 indicate the timings of pollen hydration, pollen tube germination, and pollen tube penetration into the papilla cell, respectively.

Figure 3 | \([\text{Ca}^{2+}]_{\text{cyt}}\) dynamics in papilla-cell protoplasts treated with SP11/SCR. (a-d) Typical YFP/CFP ratio images of protoplasts (bars, 20 µm) before (left) and 6 min after (right) the addition of SP11/SCR and (e-h) time-lapse of YFP/CFP ratio changes. Traces represent individual replicates (*n* = 3). *Sb*-SRK–expressing papilla-cell protoplast treated (a, e) with self *Sb*-SP11/SCR and (b, f) with cross *Sb*-SP11/SCR. (c, g) WT and (d, h) *Sb*-SRK_K555E–expressing papilla-cell protoplast treated with *Sb*-SP11/SCR. (i) Relative YFP/CFP ratio increase after SP11/SCR treatment in \(\text{Ca}^{2+}\)-free medium and in the presence of channel inhibitors or antagonists. Error bars indicate standard deviations.
**Figure 4 | A glutamate receptor antagonist AP-5 interferes with SI response.** (a) Typical images of manually pollinated open flower stigma stained with aniline-blue after AP-5 treatment. (b) Summary of the *in vivo* pollination test. Each stigma was treated with 0.1 µl of designated dosage of AP-5 in 0.025% Tween 20. Stigma was dried for one hour after the treatment, and then used for the pollination test. *p < 0.01 by Fisher’s exact probability test compared to the stigmas treated solely with 0.025% Tween 20 (0 mM AP-5).

**Figure 5 | Glutamate receptor-like channel nonsense mutants are compromised in the Ca²⁺-increase upon SI response.** Homozygous nonsense mutants (*glr*1.3, *glr*3.3, *glr*3.5, *glr*3.7) carrying both *S*₅-*SRK* and *YC*3.6 genes were obtained by TILLING and subsequent crossing. Relative YFP/CFP ratio increase in papilla-cell protoplasts (6 min after *S*₅-*SP11*/SCR treatment) in these mutant lines were compared to WT lines (*i.e.* *GLR1.3/GLR1.3, GLR3.3/GLR3.3, GLR3.5/GLR3.5* and *GLR3.7/GLR3.7*) that lost the *glr* mutations during crossing. Restoration of the YFP/CFP ratio increase was observed in two independent complementation lines (No. 2 and No. 8) carrying *GLR3.7*ₚｒｏ*:*GLR3.7 cDNA in the *glr3.7/glr3.7* genetic background. Error bars indicate standard deviations.
Table 1. Effects of injection of Ca\textsuperscript{2+} solution into papilla cell on pollen hydration

<table>
<thead>
<tr>
<th>Pollination</th>
<th>Injected solution</th>
<th>mM</th>
<th>Increase in [Ca\textsuperscript{2+}]\textsuperscript{a}</th>
<th>Hydrated pollen/Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(S_b)-SRK × WT</td>
<td>H\textsubscript{2}O</td>
<td>-</td>
<td>0.36 ± 0.30</td>
<td>18/20 (90)</td>
</tr>
<tr>
<td>(S_b)-SRK × WT</td>
<td>CaCl\textsubscript{2}</td>
<td>10</td>
<td>1.43 ± 0.52</td>
<td>1/10 (10)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>1.20 ± 0.54</td>
<td>2/20 (10)**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.1</td>
<td>0.74 ± 0.33</td>
<td>15/25 (60)*</td>
</tr>
<tr>
<td>(S_b)-SRK × WT</td>
<td>KCl</td>
<td>1000</td>
<td>0.42 ± 0.78</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>0.45 ± 0.17</td>
<td>5/6 (83)</td>
</tr>
<tr>
<td>(S_b)-SRK × (S_b)-SP11</td>
<td>-\textsuperscript{b}</td>
<td></td>
<td>1.00 ± 0.30</td>
<td>0/7 (0)</td>
</tr>
<tr>
<td>(S_b)-SRK × WT</td>
<td>-\textsuperscript{b}</td>
<td></td>
<td>0.09 ± 0.15</td>
<td>7/7 (100)</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Increase in [Ca\textsuperscript{2+}] was calculated by (“YFP/CFP ratio just after injections” – “YFP/CFP ratio before injections”) and normalized against the corresponding value for self-pollination (\(S_b\)-SRK × \(S_b\)-SP11). Data are expressed as means ± s.e.

\textsuperscript{b}- means no injection.

\(*P < 0.05, **P < 0.001 with Fisher’s exact probability test versus the H\textsubscript{2}O-injected control."
Figure 1 (Takayama)
Figure 2 (Takayama)
**Figure 3 (Takayama)**

- **Figure 3a:** Comparison of Sb-SRK and Sb-SP11 images with and without Sb-SP11.
- **Figure 3b:** Comparison of Sb-SRK images with and without Sb-SP11.
- **Figure 3c:** Comparison of WT images with and without Sb-SP11.
- **Figure 3d:** Comparison of Sb-SRK_K555E images with and without Sb-SP11.
- **Figure 3e:** Graph showing the ratio (YFP/CFP) over time for Sb-SRK and Sb-SP11.
- **Figure 3f:** Graph showing the ratio (YFP/CFP) over time for Sb-SRK and Sb-SP11.
- **Figure 3g:** Graph showing the ratio (YFP/CFP) over time for WT and Sb-SRK.
- **Figure 3h:** Graph showing the ratio (YFP/CFP) over time for Sb-SP11 and Sb-SRK_K555E.
- **Figure 3i:** Bar graph showing the relative ratio increase with different treatments.

**Graph Legend:**
- Sb-SRK, Sb-SP11, Sb-SRK_K555E
- Time (min): 0, 5, 10, 15, 20
- Ratio (YFP/CFP): 1.0, 2.0, 3.0, 4.0, 5.0
- Relative ratio increase
- Ca²⁺-free, GdCl₃, LaCl₃, AP-5, DMOX, CNOX, UT7122, Verapamil, Miltefradil, Carbamoylcholine

* indicates significant difference.
Figure 4 (Takayama)

**a**

♂ WT + 0 mM AP-5
♂ WT + 50 mM AP-5
♂ Sb-SP11/SCR + 0 mM AP-5
♂ Sb-SP11/SCR + 25 mM AP-5
♂ Sb-SP11/SCR + 50 mM AP-5

**b**

<table>
<thead>
<tr>
<th>AP-5 (mM)</th>
<th>♂ WT</th>
<th>♂ Sb-SP11/SCR</th>
</tr>
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<tr>
<td>0</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>50</td>
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<td>25</td>
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<tr>
<td>50</td>
<td>0</td>
<td>60</td>
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n = 6
n = 10
n = 27
n = 24
n = 24

* denotes statistically significant difference.
Figure 5 (Takayama)