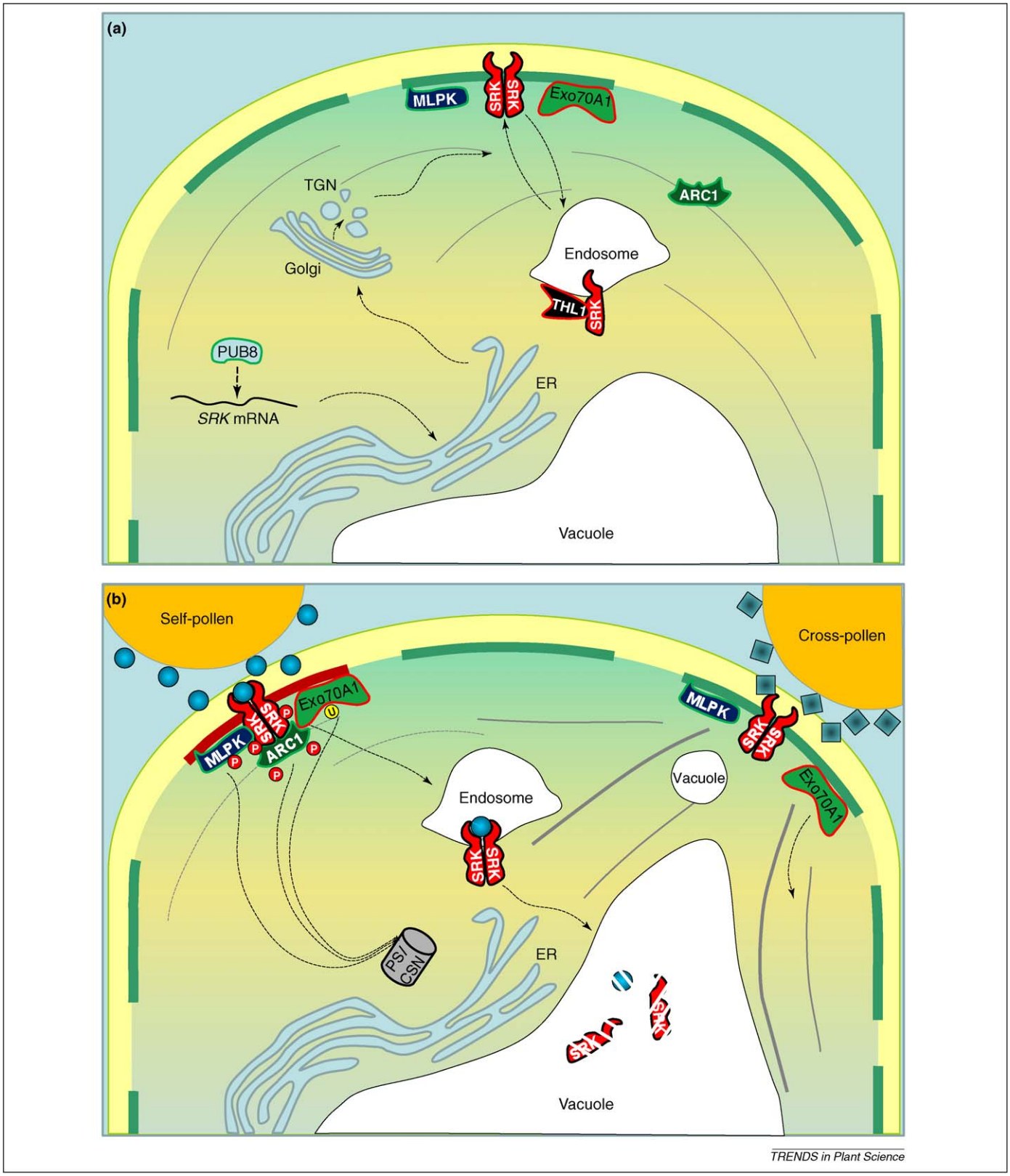

Figure 1. Molecular events in the papilla cell underlying Brassicaceae SI: a putative model. **(a)** Events in an unpollinated stigma papilla cell. *SRK* gene encoding the *S*-receptor kinase is transcribed at the onset of pollination [23,24]. High levels of *SRK* mRNA are required in these early stages and are, at least in part, ensured by the U-box protein PUB8 [27]. SRK protein passes through the secretory system to reach the endosomes and the PM. In the endosomes, it interacts with the thioredoxin THL1, which prevents the nonspecific autoactivation of the overaccumulated SRK. At the PM, SRK is distributed in zones, designated 'SI domains' (in green), and exists as both monomer (not depicted) and homodimer, the homodimer being a prerequisite for ligand binding [34]. The protein kinase MLPK is N-terminally anchored at the PM but probably does not interact with SRK. The E3 ubiquitin ligase ARC1 is located in the cytosol and is inactive [38]. Exo70A1 travels through the Golgi apparatus to the PM, where it is distributed in zones, similarly to SRK [39]. **(b)** Events in the stigma papilla cell following pollination (a simultaneous self- and cross-pollination event is depicted). Upon self-pollination, SRK recognizes its cognate ligand (blue circles) that has passed through the cell wall, and binds it. This causes SRK phosphorylation [14] and the recruitment of MLPK and ARC1 to the complex. MLPK is phosphorylated by SRK [43] and, together with SRK, phosphorylates ARC1 [45]. Thus activated, the complex is able to spread the signal to neighboring SRK molecules; however, this chain reaction is contained within the same SI domain (in red) leaving the rest of the PM-located SRK

The expression of *SRK* initiates in young flower buds, peaking when they reach maturity [22–24], and is specific to stigmas [25]. *SRK* promoters can mediate a similar expression pattern even when introduced in tobacco (*Nicotiana tabacum*) [23]. Part of the *SRK* gene is transcribed also in the antisense direction, although the significance of these antisense transcripts remains unknown [26]. The use of a transgenic self-incompatible *A. thaliana* line revealed that *SRK* expression is regulated by the *PLANT U-BOX8* (*PUB8*), which is required for maintenance of sufficient *SRK* mRNA levels and manifestation of SI response [27]. *PUB8* is strongly expressed in stigmas, but is also present in other tissues, suggesting a more general function. PUB8 contains ARMADILLO(ARM)-repeats and a U-box, but the way it regulates *SRK* mRNA availability is not clear. Additionally, inactivation of the RNA-dependent RNA polymerase RDR6, which functions in the production of *trans*-acting short interfering RNA (ta-siRNA), causes slight inhibition of *SRK* expression. This effect is probably indirect because it is accompanied by enhanced SI response [28] and might be connected to the epigenetic silencing of *SRK* in tetraploid *Brassica* plants [29].

SRK mRNA undergoes alternative splicing resulting in a number of mostly uncharacterized transcripts [1,25]. One of these, comprising the first exon, encodes the full extracellular domain of SRK, named eSRK (a glycosylated protein comprising the extracellular domain of SRK. The abbreviation is also used to designate the extracellular domain of the full-length SRK in certain studies). Existence of eSRK has been demonstrated experimentally [30,31]. It exists in different glycosylation forms and appears even when the full-length *A. lyrata SRK* gene is expressed in *A. thaliana* [32]. A membrane-bound form of eSRK, named tSRK, has also been found [30] and more recently been shown to have high affinity towards the ligand SCR [5,21,31]. tSRK contains the extracellular portion of the receptor, the transmembrane domain and heterogeneous C-terminus, which mostly terminates before the end of the juxtamembrane domain. Presence of tSRK was detected even if SRK cDNA was expressed in tobacco BY2 cells, which led to the conclusion that it is a product of a post-translational modification, rather than alternative splicing [31].

SRK is predicted to be synthesized in the endoplasmic reticulum (ER), because the protein contains a signal peptide [1], is glycosylated [30], and shows manifestation at the PM [23,33–36]. Immunohistochemical studies have indeed demonstrated ER localization, as well as presence in the Golgi and trans-Golgi network? [34], which mark the most probable way of SRK trafficking in the cell. Ultimately, SRK reaches the endosomes and the PM [23,33–36]. Electron microscopy immunolocalization shows that SRK is also present in small vesicles in close proximity to the PM. This suggests a continuous SRK traffic between the PM and the endosomes. In the endosomes, the receptor is very abundant and colocalizes with its inhibitor THIOREDOXIN H-LIKE 1 (THL1) [34]. Overexpression of SRK leads to nonspecific autoactivation [37], which explains why the highly concentrated endosomal SRK needs to be inhibited.

Only small amounts of SRK are present at the PM which are distributed in zones, or 'SI Domains' [36], resulting in areas of the membrane lacking the receptor [34]. This phenomenon was also observed when expressing SRK in a heterologous system [37]. Contrary to expectations, no THL1 was detected at the PM [34], implying that here the receptor is in an uninhibited ready-to-be-activated state. However this speculation has to be treated with caution because other factors might inhibit SRK at the PM instead.

The *M*-locus Protein Kinase (MLPK) is essential for the manifestation of the SI response. Plants lacking MLPK are incapable of rejecting self-pollen [12]. It is expressed as two types of transcripts, *Mlpkf1* and *Mlpkf2*, differing in the transcription initiation site and their tissue specificity. None of the two forms is strictly stigma-specific, suggesting additional roles unrelated to SI. The difference in mRNA is reflected in the N-termini of the translated proteins. In spite of this difference, both isoforms complement MPLK-deficient plants. Both isoforms are targeted and anchored to the cytoplasmic face of the PM by their N-termini, despite using different membrane association mechanisms. MLPKf1 undergoes myristoylation at Gly2, a Gly2Ala mutation completely abolishing myristoylation and PM localization in BY2 protoplasts. By contrast, Gly2 of MLPKf2 is not myristoylated and the protein associates with PM using an N-terminal hydrophobic region [35].

ARM-REPEAT CONTAINING 1 (ARC1) is an E3 ubiquitin ligase, which was identified as a SRK interactor in a yeast two-hybrid screen. The interaction is mediated by its C-terminal ARM-repeats and is specific to the phosphorylated kinase domain of SRK. Expression of *ARC1* is stigma-specific and is induced together with *SRK* [10]. Down-regulation of *ARC1* impairs SI response and therefore ARC1 is considered a positive regulator [11]. Its ubiquitination activity during self-pollen rejection has been demonstrated [38] but so far it is unclear whether SRK is a substrate of ARC1. Localization experiments in tobacco BY2 cells have shown that in the absence of active SRK, ARC1 is predominantly cytoplasmic although part of ARC1 migrates to the nucleus. This dual localization, whose significance is so far poorly understood, is a result of active nuclear import and export [38].

Recently, Exo70A1, a member of the exocyst complex, was identified as an ARC1 interactor, and is necessary in the stigma for accepting compatible pollen [39]. Similarly to the yeast and animal homologues, the plant Exo70A1

inactive (domains in green) and free for further decision events. Exo70A1 is probably removed from the SI domain by ARC1-mediated ubiquitination and proteasome relocalization. The phosphorylated ARC1 and MLPK dissociate from the complex. ARC1 travels to the proteasome/COP9 signalosome (marked 'PS/CSN', in gray) [38,39]. MLPK can also be found in the PS/CSN [39]. SRK is endocytosed to the endosomes, where it may remain active before being sent for degradation. New receptors are synthesized from the available mRNA pool (not depicted) [34]. The actin filaments (gray lines) are partially depolymerized [48]. All this ensures targeted pollen rejection allowing a compatible pollen grain to be accepted by the same papilla cell at the same time. Upon compatible pollination, SRK is not activated by the non-cognate SCR (blue squares). Exo70A1 targets so far unidentified components to the PM for post-pollination events such as pollen hydration (not depicted). This is followed by disappearance of the protein from the PM [39]. Prominent actin filaments are formed (gray lines) focusing on the contact site, many vesicular structures are transported in this direction and the central vacuole is elongated towards the accepted pollen [48]. With the exception of SRK and SCR and proteasome structures, proteins that are known to positively regulate SI response are depicted with green borders, whereas known negative regulators have red borders.

whose expression is not stigma-specific, may be involved in the polarized secretion of vesicles to the PM [40]. RFP-fusions of the protein show Golgi localization in immature stigmas and later traffic to the PM at anthesis [39].

The papilla PM at the onset of pollination: the SRK complex

At the time of flower opening, the papilla cell has a full set of correctly distributed factors enabling it to respond adequately to pollination attempts.

A subset of the full-length SRK molecules form homodimers (Figure 1a). The process happens spontaneously and is not caused by the ligand [31,37]. On the contrary, it seems that the dimerization is a prerequisite to ligand-binding, as the extracellular domain of SRK can bind its cognate SCR only as a dimer but not in monomeric form [31]. Receptor dimerization occurs through the extracellular part of SRK and involves the PAN_APPLE domain and to a lesser extent the epidermal growth factor (EGF)-like domain [41] (see Figure 2). Because this interaction analysis was done using the yeast two-hybrid system, it can be noted that SRK dimerization is dependent only on the protein backbone and there is no requirement for glycosylation. Importantly, heterodimerization of SRK molecules from different *S*-haplotypes seems possible, but interactions are weak and the dimers would be unstable [41]. It is possible that these interactions contribute to the known phenomena of dominance, codominance and mutual weakening,because in nature plants are usually heterozygous for the *S*-locus [42].

MLPK was shown to interact with SRK in BY2 protoplasts in the absence of SCR suggesting that it is a component of the inactive receptor complex [35]. However, when overexpressed in a heterologous system, SRK tends to be autoactivated [37,38] and experimental data suggests that the SRK–MLPK interaction is only transient [35,43]. Therefore, the question of whether MLPK binds activated or inactive SRK remains open.

While present at the PM, there is no evidence at this time that Exo70A1 interacts with any of the other components.

Rendezvous: SRK–SCR interaction at the plasma membrane

Upon pollination, SCR travels from the pollen coat through the papilla cell wall but only with the help of as yet unidentified pollen coat proteins [21].

The receptor–ligand interaction happens at the PM of the papilla cell. In an *in vitro* study, the binding of SCR was mapped to the two hypervariable regions within the Lectin-like 2 domain of SRK [44] (Figure 2). These regions were additionally shown to be responsible for the ligand-selective activation of SI response *in vivo*, and were suggested to form a three-dimensional SCR-binding pocket [32]. Surprisingly, in a recent study, interaction was found between non-cognate receptor–ligand couples [44], confirming the earlier demonstration that ligand-binding does not necessarily cause the activation of SRK [41]. Thus, activation of the SI response is based on two phenomena: physical interaction between SRK and SCR, and activation of the SRK kinase domain. Interestingly, SRK activation can be induced by a monoclonal antibody designed against the N-terminus of SRK_3, which can functionally substitute the natural ligand [14,34].

The outcome of the encounter between the receptor and the ligand is crucial for the pollen grain. If no signal is transmitted, the pollen will hydrate, germinate and the pollen tube will penetrate the papilla cell (see Box 1). If signaling is initiated, the pollen will be rejected.

A negative decision: activation and fate of the SRK complex

Following SCR recognition, the signal is relayed across the PM and after a series of phosphorylation events, an activated receptor complex is formed that includes SRK, MLPK and ARC1 (Figure 1b). SRK and MLPK can autophosphorylate *in vitro* [12,37], and SRK is phosphorylated specifically after self-pollination in papilla cells [14]. Thus, within the complex both proteins are probably in a phosphorylated state, creating a scaffold for recruitment of ARC1, which specifically binds the phosphorylated kinase domain of SRK [10]. An interaction with MLPK is also suggested by *in vitro* experiments. Both kinases can phosphorylate ARC1 *in vitro* and, surprisingly, MLPK shows much higher activity than SRK [45]. This fact shows a molecular basis for the observed loss of SI response after inactivation of the MLPK kinase domain [12].

The molecular role of ARC1 in SI is still unclear. It possesses an E3 ubiquitin ligase activity and upon incompatible pollination ubiquitinates multiple targets [38]. A recent study on the flagellin receptor FLAGELLIN SENSING2 receptor kinase (FLS2) demonstrated that PRKs, similarly to their animal counterparts, can be targets of ubiquitination upon signaling [46]. Although it is tempting

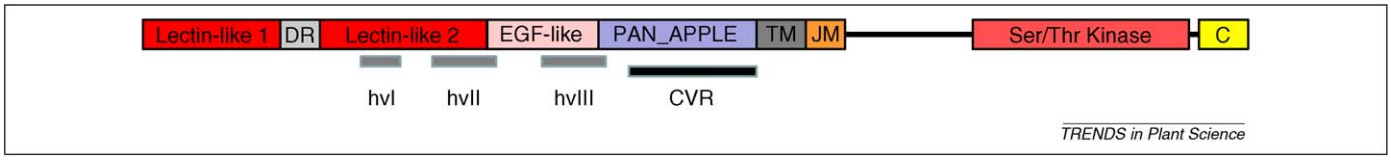

Figure 2. Structure of SRK. For the domain structure of the extracellular portion of SRK, we used the classification from the study of Naithani *et al.* [41]. From left to right the domains and their functions are as follows: The Lectin-like domain 1 – might have implications in the activation of SRK. A monoclonal antibody raised against the first several amino acids in the N-terminus of this domain is able to activate SRK *in vivo* [14,34]. DR – 'deletable region'. A linker sequence of variable size [41]. Lectin-like domain 2 – together with the EGF-like domain, responsible for binding of SCR [44]. Contains two hypervariable regions that define haplotype specificity [32,58]. EGF-like domain – has a supporting role in receptor dimerization [41]. PAN_APPLE domain – responsible for homo and heterodimerization of SRK molecules. The extracellular domain of SRK contains three hypervariable regions (hvI–III) in the Lectin-like 2 and EGF-like domains. The first two define SCR binding [44] and the haplotype specificity of the receptor [32,58]. The C-terminal variable region (CVR), found within the PAN_APPLE domain determines SRK dimerization affinity [41]. TM – transmembrane domain. JM – juxtamembrane domain. Its function is not well studied in SRK, but PRKs lacking juxtamembrane domain lose their kinase activity [59]. Kinase domain – has Ser-Thr kinase activity [22,37]. When phosphorylated, it is responsible for the interaction with ARC1 [10]. C-terminal stretch – no data is available on the function of this region.

Box 1. Pollen acceptance after compatible pollination

Papilla cells take an active role in pollen acceptance (Figure 1b). SRK–SCR interaction has no role in compatible pollination, because plants such as *A. thaliana*, which lack one or both components, can accept pollen. The earliest response to cross-pollination is the disappearance of Exo70A1 from the PM. Mutants lacking this protein are incapable of pollen acceptance, which suggests that it carries out its function prior to its removal from the PM [40]. Exo70A1 is a member of the exocyst complex involved in vesicle tethering during polarized secretion [40,62]. Brassicaceae plants possess a dry stigmatic surface and the function of the exocyst in pollen acceptance may be to facilitate targeted exocytosis and delivery of water to support pollen hydration and germination. Additionally, enzymes need to be transported to the cell wall in order to support pollen tube penetration.

Investigation on the actin dynamics showed that cross-pollination induces bundle formation directed towards the site of pollen contact [48]. This is followed by the reorganization of the vacuolar structure [48] and targeted exocytosis [63]. By analogy to other systems [52], Ca^{2+} concentration is proposed to regulate actin formation through the action of actin-binding proteins [48]. As for the Brassicaceae, a calmodulin was isolated as an interactor of SRK, but its role has not been investigated in detail.

Ca^{2+} is essential to pollen germination and growth [64,65] and, along with water for hydration [66], the papilla cell needs to provide Ca^{2+} to the pollen grain. In support of this, Ca^{2+} accumulation was observed at the site of compatible pollen contact [67].

to speculate that SRK might also be ubiquitinated, this has not been experimentally demonstrated. Instead, ARC1 was proposed to target and negatively regulate factors that promote the process opposite to SI – compatible pollen acceptance [38].

Such a factor may be Exo70A1. It is ubiquitinated by ARC1 *in vitro* and sent to the proteasome. Its overexpression leads to partial loss of SI, possibly due to providing excessive substrate for ARC1. Exo70A1 has also a critical role for pollen acceptance during compatible pollination [39] (see Box 1). In this context, initiation of SI would require removal of Exo70A1 at the PM, thus preventing vesicle secretion and pollen acceptance. However, additional experiments are needed to specify the role of Exo70A1 in the SI response; in particular its trafficking dynamics in a SI plant background. As *Brassica* papilla cells are not suitable for live imaging [39], an SI-restored *A. thaliana* plant [8] will be more suitable to address such questions.

A particularity of Brassicaceae SI response is the ability of the papilla cell to remain receptive to cross-pollen, even if it is simultaneously self-pollinated [47]. This phenomenon provides an advantage for the cross-pollen, which competes with the self-pollen for cell surface, rather than a limited number of cells. At the same time, it demands a strictly localized SI response. This is likely to be ensured by the unequal distribution of the SI components on the PM. SRK is known to transphosphorylate rapidly [37] and activation of the receptor would spread the signal laterally across the membrane. This chain reaction will continue until the border of the 'SI domain' is reached. Thus, activation will be contained within the domain underlying the contact site, ensuring proper SI response, while leaving the rest of the PM intact for further independent decisions [36] (Figure 1b).

Following this activation step, the receptor complex dissociates and its components are internalized following different pathways. SRK, perhaps together with SCR, is endocytosed and enters the endosomes where it colocalizes with THL1. This ligand-induced internalization is an intermediate step before the degradation of the receptor, which probably occurs in the vacuole [34]. Studies in tobacco protoplasts suggest that after leaving the active complex, ARC1 relocalizes to ER-associated proteasome/COP9 signalosome (PS/CSN) and was proposed to carry ubiquitinated substrates there [38]. Indeed, in the presence of active SRK, Exo70A1 colocalizes with ARC1 in proteasomal compartments [39]. Surprisingly, when coexpressed, MLPK can also be found colocalizing with ARC1 in the perinuclear region, suggesting a stronger connection between it and ARC1 during SI response than previously expected [45]. So far, no data are available on the stability of ARC1, MLPK or Exo70A1 but their relocalization pattern suggests that following successful signaling, all the known members of the complex, together with SRK, are destroyed to be replaced by newly synthesized ones.

A result of the incompatible pollination is the partial depolymerization of the actin cytoskeleton in the region of pollen contact, which affects the vesicle secretion and the disruption of the vacuolar system [48].

The 'No' factor: SRK and PRK signaling

Receptor signaling has become a central topic in plant biology. Advances in understanding signaling of the brassinosteroid receptor BRASSINOSTEROID INSENSITIVE1 receptor kinase (BRI1) and the flagellin receptor FLS2 have underlined the importance of partner proteins and cellular compartmentalization [49,50]. In both cases, receptor–ligand interaction happens at the PM and is followed by the formation of an active complex with the coreceptor BAK1 (Figure 3). This initiates additional phosphorylation events allowing the complex to transmit the signal to downstream components [50–52]. In the case of SI, the complex formation at the PM seems to follow a similar principle, even if utilizing different factors. In order to initiate SI, the SRK complex requires the presence of MLPK (Figure 3). Despite being structurally different to BAK1, MLPK is also not involved in ligand perception but rather plays a role in complex activation. Even though it has no extracellular or TM domains, the PM localization of MLPK is crucial to its function. Mutated proteins with no PM anchoring sequences are not able to complement the *m*-locus mutation in *Brassica* [35]. In addition to its function in phosphorylation, BAK1 has been shown to mediate the internalization of BRI1 and FLS2 [53,54]. Endocytosis has emerged in recent years as a major factor in regulation of plant receptor kinase (PRK) signal transduction. Trafficking of active receptors to the endosomes has traditionally been regarded as a silencing step. However, the concept of endosomes as signaling compartments in plant cells has gained support with studies on BRI1 [49,55].

SRK also undergoes internalization after binding SCR. So far, an implication of MLPK in this process has not been addressed. Once in the endosomes, SRK colocalizes with its inhibitor THL1, which is the basis of the suggestion that any signaling initiated by SRK is terminated in endosomes. This idea is supported by the finding that the receptor is later degraded [34], and that most of the known

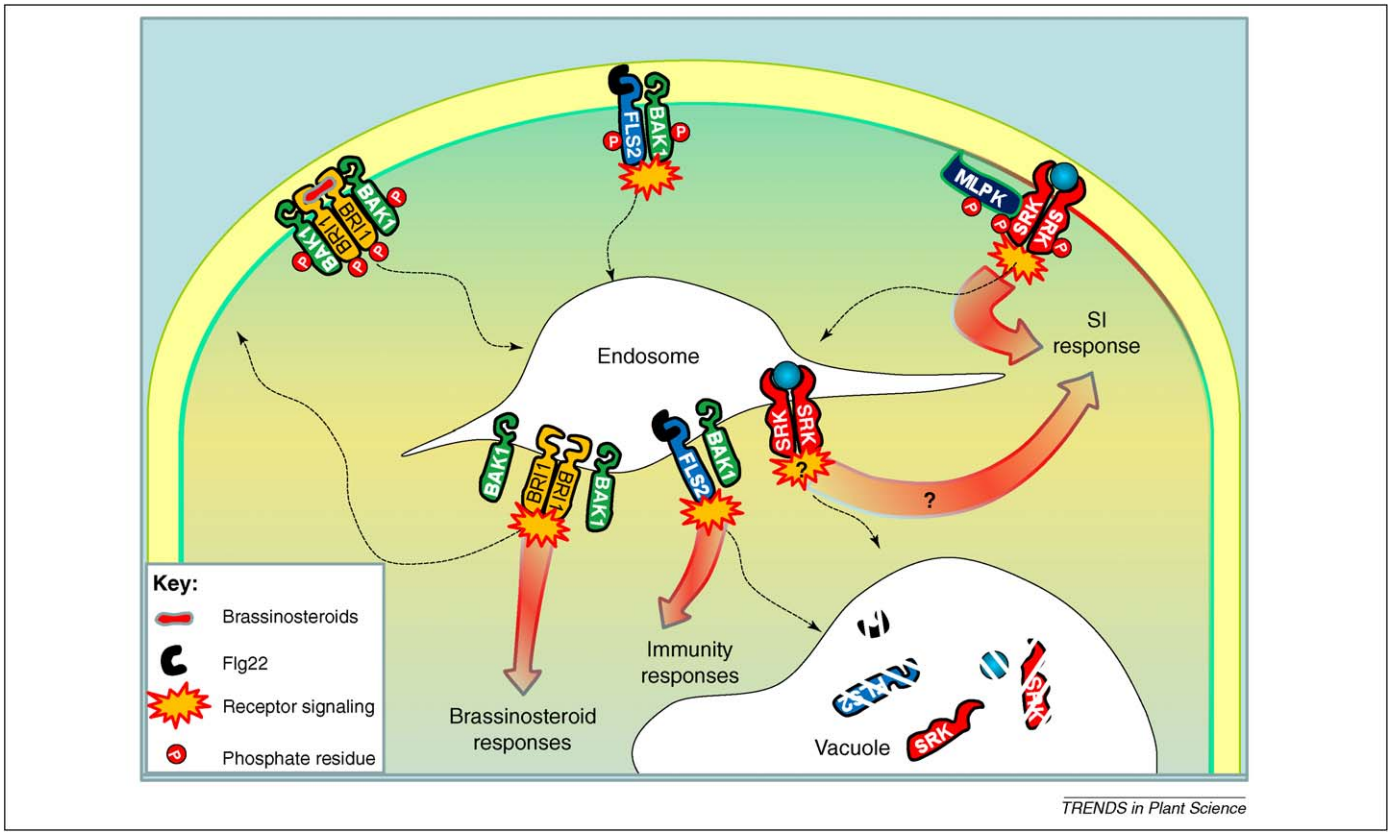

Figure 3. Endocytosis in plant receptor kinase signaling. To achieve active status, plant receptor kinases require additional kinase activity. For BRI1 and FLS2, this is provided by the coreceptor BAK1. BAK1 probably does not participate in ligand recognition [54,60]. Following the same principle, SRK activation requires the activity of the membrane-anchored kinase MLPK [12]. It has been proposed that signaling for FLS2 and SRK initiates at the PM [34,51,61] and such a possibility exists for BRI1 as well (not depicted) [49]. Activation of PRKs is followed by internalization to endosomes. BRI1 signals from these compartments to initiate the brassinosteroid signal transduction [55]; the same may be true for FLS2, though in this case the hypothesis is based on indirect evidence. Currently available data for SRK suggests signaling from the PM. However, the observation that active SRK is accumulated in the endosomes, rather than being immediately sent for degradation indicates that additional signaling steps might occur in this compartment. In this case output is probably targeted back to the part of the PM where the receptor was initially activated, and which corresponds to the site of self-pollen rejection. The hypothetical SRK endosomal signaling is marked with a question mark.

positive SI regulators, perhaps also ARC1 during SI, are localized at the PM. However, we cannot exclude that SRK may continue to signal from the endosomes before being ultimately silenced. Following this concept, SI signaling would consist of a two-step process: (i) labeling of the PM underneath the pollen grain followed by endocytosis of the activated SRK (possibly associated with other components); and (ii) recruitment of additional factors at the endosomes, which are then targeted to the labeled PM.

Shaping the future: perspectives for SI research

Since 2003, many new components involved in SI response have been identified. Because of the lack of accessible genetic resources many of them, such as calmodulin, KAPP and SNX1, despite showing great promise for SRK regulation [16,53,56,57], have remained poorly characterized in terms of SI. Factors such as PUB8 and Exo70A1 need to be further investigated to clarify their role. In this respect, one of the major aims for the near future will be to widen the use of *A. thaliana* as a model system to study SI. The recent discovery that despite the loss of SI, at least four accessions have retained functional SRK [9] will have impact not only on the understanding of SI evolution within Brassicaceae, but will also provide a good tool to enhance our understanding of SRK and receptor kinase signaling in plants.

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