Investigations into a putative role for the novel BRASSIKIN pseudokinases in compatible pollen-stigma interactions in Arabidopsis thaliana

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Abstract

Background: In the Brassicaceae, the early stages of compatible pollen-stigma interactions are tightly controlled with early checkpoints regulating pollen adhesion, hydration and germination, and pollen tube entry into the stigmatic surface. However, the early signalling events in the stigma which trigger these compatible interactions remain unknown.

Results: A set of stigma-expressed pseudokinase genes, termed BRASSIKINs (BKNs), were identified and found to be present in only core Brassicaceae genomes. In Arabidopsis thaliana Col-0, BKN1 displayed stigma-specific expression while the BKN2 gene was expressed in other tissues as well. CRISPR deletion mutations were generated for the two tandemly linked BKNs, and very mild hydration defects were observed for wild-type Col-0 pollen when placed on the bkn1/2 mutant stigmas. In further analyses, the predominant transcript for the stigma-specific BKN1 was found to have a premature stop codon in the Col-0 ecotype, but a survey of the 1001 Arabidopsis genomes uncovered three ecotypes that encoded a full-length BKN1 protein. Furthermore, phylogenetic analyses identified intact BKN1 orthologues in the closely related outcrossing Arabidopsis species, A. lyrata and A. halleri. Finally, the BKN pseudokinases were found to be plasma-membrane localized through the dual lipid modification of myristoylation and palmitoylation, and this localization would be consistent with a role in signaling complexes.

Conclusion: In this study, we have characterized the novel Brassicaceae-specific family of BKN pseudokinase genes, and examined the function of BKN1 and BKN2 in the context of pollen-stigma interactions in A. thaliana Col-0. Additionally, premature stop codons were identified in the predicted stigma specific BKN1 gene in a number of the 1001 A. thaliana ecotype genomes, and this was in contrast to the out-crossing Arabidopsis species which carried intact copies of BKN1. Thus, understanding the function of BKN1 in other Brassicaceae species will be a key direction for future studies.

Keywords: Compatible pollen, Stigma, Signaling, Pseudokinase, Receptor-like cytoplasmic kinase, Brassicaceae
Background
In the Brassicaceae, the early post-pollination stages of pollen adhesion and hydration, and pollen tube entry into the stigma are highly-regulated and represent the first of several stages leading to the release of the sperm cells at the ovule for fertilization (reviewed in [1–5]). The characteristic Brassicaceae “dry stigmas” lack surface secretions to facilitate pollen hydration and germination; thus, pollen recognition is required for the stigma to be receptive [6, 7]. The Brassicaceae stigma surface is covered with unicellular stigmatic papillae, and the process of pollen capture is very rapid, occurring in as little as 30 s following a compatible pollination in Arabidopsis thaliana [8]. Following this, the pollen coat and stigma surface components mix to form a “pollen foot” at the location of the pollen-papillar contact, and this contributes to the process of pollen adhesion [9]. The next checkpoint of pollen acceptance is pollen hydration, where the desiccated pollen grain takes up water released by the stigmatic papilla to become metabolically active [6, 10–12].

Despite being a critical step leading to successful fertilization, the cell-cell communication events that facilitate early pollen-stigma interactions are poorly understood. There are proteins in the pollen coat that are required for pollen hydration such as the A. thaliana GRP17 oleosin-domain protein, the EXL4 extracellular lipase, and the Pollen Coat Protein-B family (PCP-B) [13–15]. The PCP-Bs are particularly interesting as they are small cysteine-rich proteins that represent promising compatible pollen recognition factors for unknown stigma receptors. A. thaliana pcp-bc/b/γ triple mutants displayed impaired pollen hydration and delayed pollen tube growth on wild-type stigmas [15]. Perception of peptide ligands by receptor kinases plays a prominent role in the regulation of downstream compatible pollen-pistil interactions and pollen tube guidance, as well as the rejection of self-pollen in self-incompatible Brassicaceae species (reviewed in [1, 2, 4, 5, 16]).

Other factors identified on the pollen side for these early post-pollination stages are connected to the production of reactive oxygen species (ROS). Pollen NADPH oxidases were shown to be important for Ca^{2+}-dependent ROS production in the apoplast for A. thaliana pollen tube elongation into the stigmatic papillar cell wall [17, 18]. ROS production was again implicated in A. thaliana T-DNA insertion mutants disrupting the β and γ subunits of the SNF1-related protein kinase 1 complex. Mutant kinfy pollen grains displayed reduced ROS levels as a result of mitochondrial and peroxisomal defects, and this was associated with reduced hydration and germination on wild-type stigmas [19]. Finally, the SHAKER POLLEN INWARD K⁺ channel (SPIK) gene was found to be downregulated in kinfy mutant pollen, and spik mutant pollen grains also displayed reduced hydration on wild-type stigmas [20].

On the stigmatic papillar side, ultrastructural studies of the pollen-papillar interface previously implicated both secretory activity and vacuolar expansion in the stigmatic papillae of Brassica and Arabidopsis species [21–25]. This exocyst complex, a vesicle-tethering complex composed of eight different subunits (SEC3, SEC5, SEC6, SEC8, SEC10, SEC15, EXO70 and EXO84), was implicated in mediating this secretory activity in the stigma [26–28]. Through the use of knockout mutants and stigma-specific RNA silencing constructs, all eight subunits were found to be required in the stigma for the compatible pollen acceptance. Wild-type pollen applied to stigmas from the exocyst subunit knockdown/knockout mutants displayed reduced pollen hydration and germination, and showed signs of disrupted secretion [22, 26, 27, 29, 30]. Other cellular responses in Brassica and Arabidopsis stigmatic papillae have also been connected to vesicle trafficking (reviewed in [31]). For example, Brassica compatible pollinations were associated with actin reorganization in the stigmatic papilla towards the pollen attachment site and microtubule depolymerization [25, 32]. Recently, another vesicle trafficking-related component, Brassica phospholipase Da1, has been shown to be required in the stigma for compatible pollinations [33]. As well, changes in Ca^{2+} dynamics were observed, with small Ca^{2+} increases at the site of pollen attachment in A. thaliana stigmatic papillae [34]. Through transcriptome analyses of A. thaliana stigmas pre- and post-pollination, the ACA13 Ca^{2+} ATPase was identified as a stigmatic component and proposed to secrete Ca^{2+} for the developing pollen tube [35]. Finally, we have recently identified the secreted Arabidopsis E6-like 1 protein as a potential structural component of the stigmatic papillae required for these early post-pollination stages [36].

While the PCP-Bs represent potential pollen ligands for compatible pollen recognition, the corresponding recognition system in the stigma is unknown. The process of pollen acceptance by the stigma is thought to be conserved in the Brassicaceae since pollen from several Brassicaceae species were able to hydrate and germinate on Arabidopsis stigmas whereas pollen from non-Brassicaceae species failed to hydrate [37]. Moreover, when pollen from various species were applied to A. thaliana or B. oleracea stigmas, there was some specificity at the pollen adhesion stage [8, 38]. Thus in this reverse-genetics study, we utilized publicly available transcriptome datasets to search for potential signalling genes that display stigma-enriched expression and were conserved within the Brassicaceae. Through this approach, we identified a novel group of Brassicaceae-specific pseudokinase genes which we termed the BRASSIKINs (BKNs).
Results

BKNs are stigma-expressed receptor-like cytoplasmic kinases

To identifying candidate stigma signalling genes, we used the expression angler tool from the Bio-Analytic Resource for Plant Biology [39]. For this search, we used the stigma-specific SLR1 gene [40] as a bait to identify other genes with similar expression patterns across the A. thaliana developmental series microarray datasets [36, 41, 42]. The gene, At5g11400, was a top hit (Additional file 2: Table S1) and displayed stigma-specific expression in the transcriptome datasets (Additional file 1: Figure S1). This gene is predicted to encode a novel receptor-like cytoplasmic kinase (RLCK) which we named BRASSIKIN 1 (BKN1). Interestingly, adjacent to the BKN1 gene is a tandemly linked parologue, At5g11410, named BKN2 in the A. thaliana genome (Fig. 1a). BKN2 was ranked 64th in the expression angler dataset (Additional file 2: Table S1), with expression in a wider range of tissues (Additional file 1: Figure S1). Both BKN genes are also predicted to encode pseudokinases (discussed below) [46, 47].

The expression patterns for BKN1 and BKN2 were examined by RT-PCR on RNA extracted from A. thaliana stigmas (top ½ pistil), ovaries (bottom ½ pistil), leaves and roots. Both BKN1 and BKN2 were expressed in the stigma samples, with some expression for BKN2 in ovary samples (Fig. 1b). The BKN1 and BKN2 expression patterns were also examined in promoter-GUS transgenic plants (promoter regions are indicated by arrows, Fig. 1a). For the transgenic BKN1p:GUS A. thaliana lines, stained inflorescences showed high levels of GUS activity in stigmas from flowers across developmental stages, but not in the other tissues in the inflorescences or in seedlings (Fig. 1c–e). The transgenic BKN2p:GUS inflorescences displayed GUS activity primarily in the flower abscission zones and in the stems (Fig. 1f–g). There was also GUS activity in some BKN2p:GUS seedlings at the leaf edges and petiole (Fig. 1h). GUS activity was not observed in BKN2p:GUS stigma tissues; however, this may be due to BKN2 having much lower expression in the stigmas that may be undetectable by GUS staining (Additional file 1: Figure S1). Alternatively, the adjacent stigma specific BKN1 promoter or other unknown regulatory regions may be responsible for the BKN2 expression detected in the stigma tissues (Fig. 1a,b).

Analysis of compatible pollen responses for BKN1 and BKN2 single and double knockout mutants in A. thaliana

Given BKN1’s stigma-specific expression, we investigated whether BKN1 was required for compatible pollinations by examining loss-of function mutants. A knockout line with a T-DNA inserted in the fifth exon of BKN1 was assessed for post-pollination responses (bkn1–1; Additional file 1: Figure S2a, b). The bkn1–1 mutant plants did not display any discernible developmental defects and appeared fully fertile with wild-type siliques. Furthermore, pollinated bkn1–1 pistils stained with aniline blue were similar to wild-type for adhered pollen grains and pollen tube growth (Additional file 1: Figure S2c, d). Given that this mutant displayed some expression upstream of the T-DNA insertion, additional BKN1 mutants were generated using a CRISPR/Cas9 genome editing system [48]. Furthermore, a similar approach was taken for BKN2 since it could potentially function redundantly to BKN1. Single deletion mutants were generated resulting in two new independent homozygous mutants for each BKN1 and BKN2: bkn1–2 and bkn1–3, and bkn2–1 and bkn2–2 (Fig. 2a).

Similar to bkn1–1, all four CRISPR deletion mutants bkn1–2, bkn1–3, bkn2–1 and bkn2–2 displayed wild-type pollen tube growth in aniline blue stained pistils that had been manually pollinated with wild-type Col-0 pollen (Fig. 3a–c). There were no discernable phenotypes at this stage for individual bkn1 and bkn2 loss-of-function mutants. These mutants also did not show any observable defects in the number of pollen grains adhered to the stigma or seed set, relative to Col-0 (Fig. 3f–g). We then examined one of the earliest post-compatible pollination stages, pollen hydration, which is dependent on water release from the stigma [6, 26, 27]. Col-0 pollen was applied to all stigmas and pollen hydration was assessed by measuring the diameter of pollen grains which become rounder in shape with water uptake. Col-0 pollen on Col-0 stigmas had a mean pollen grain diameter of 21.9 μm at 10 min post-pollination compared to 12.6 μm at 0 min. The bkn1–2 and bkn1–3 mutant stigmas supported similar levels of Col-0 pollen hydration when compared to Col-0 stigmas (Fig. 2b). In contrast, Col-0 pollen place on the bkn2–1 and bkn2–2 mutant stigmas showed a small but significant decrease in diameter at 10 min post-pollination. This suggested that there was a mild Col-0 pollen hydration defect on the bkn2–1 and bkn2–2 mutant stigmas (Fig. 2b).

To test for potential functional redundancy, double bkn1-bkn2 mutants were generated. Since the BKN1 and BKN2 genes are tandemly arrayed, the strategy taken was to transform a transgene-free bkn1–3 mutant with a BKN2 CRISPR construct to knock out both genes. From this screen, three new bkn2 mutants, bkn2–3, bkn2–4 and bkn2–5, were identified in the bkn1–3 background (Fig. 2a). Pollen hydration assays were conducted on these double bkn1-bkn2 homozygous mutants, and again mild, but significant reductions were observed for the Col-0 pollen placed on these mutant stigmas (Fig. 2b). Similar to the single mutants, pollinated double bkn1-bkn2 mutant pistils stained with aniline blue displayed wild-type levels of adhered pollen.
grains and pollen tube growth (Fig. 4). Thus, there did not appear to be any additive effect by knocking out both BKN1 and BKN2, and suggests that the highly expressed, stigma-specific BKN1 did not display a noticeable function in pollen-stigma interactions.

Variations in predicted protein translation products for BKN1 in different A. thaliana ecotypes

As the bkn1 mutants displayed wild-type post-pollination phenotypes, the predicted protein sequences encoded by the BKN genes were examined more closely. BKN
cDNAs were cloned from the *A. thaliana* Col-0 ecotype and compared to the TAIR/Araport gene annotations [45, 49]. While the BKN2 cDNA sequence and predicted amino acid sequence matched the gene annotation, the full-length BKN1 cDNA showed some differences (Fig. 5a, Additional file 1: Figure S3). Importantly, the second exon in the cDNA included an additional 17 bp at the 5′ end resulting in a frameshift and a premature stop codon (Fig. 5a, asterisk). As a result, the predicted *A. thaliana* Col-0 BKN1 protein would only be 42 aa in length, in comparison to the predicted 304 aa (Additional file 1: Figure S4). The cloned BKN1 cDNA matched the carpel RNA-Seq mapping data displayed on Araport (Fig. 5a); nevertheless, there also appeared to be potential alternative splice sites at the beginning of the second exon that could restore the BKN1 reading frame and encode a larger protein (i.e. the BKN1 gene annotations; yellow arrow in Fig. 5a; Additional file 1: Figure S3, S4). While two BKN1 RT-PCR bands were observed in Fig. 1b, the larger band was identified by sequencing to include the third intron, rather than an alternatively spliced transcript. Signs of the unspliced third intron were also present in the carpel RNA-Seq mapping displayed on Araport (orange arrow in Fig. 5a). Despite several attempts, we were unable to clone BKN1 cDNAs that corresponded to the TAIR/Araport gene annotations.

A search for BKN orthologues in the genomes of two outcrossing *Arabidopsis* species, *A. lyrata*, and *A. halleri*, uncovered BKN1 coding regions that were predicted to be fully intact. This was confirmed by cloning the corresponding cDNAs from *A. lyrata* (Additional file 1: Figure S3, S4 and S5). When the BKN1 sequences were aligned, two indels were identified in the *A. thaliana* Col-0 BKN1 cDNA sequence...
that would disrupt the reading frame (Fig. 5, asterisks), the first being a 1 bp deletion (ΔT128) and the second being a 1 bp insertion (A597; Additional file 1: Figure S3). We then searched through the 1135 genotypes dataset to determine how widespread these BKN1 indels were across the different A. thaliana ecotypes [50]. Most ecotypes carried ΔT128 causing the premature stop codon in Col-0 BKN1 (Additional file 3: Table S2). Interestingly, three A. thaliana ecotypes were predicted to have fully intact BKN1 coding regions. The first ecotype identified was Hh-0, and the corresponding cDNA was cloned and confirmed by sequencing to encode a full-length BKN1 protein, similar to Al-BKN1 (Fig. 5, Additional...
file 1: Figure S3, S4 and S5). Subsequent searches identified two other ecotypes, Dju-1 and Västervik, that were also confirmed to carry the same two indels as Hh-0 to encode a full-length BKN1 protein (Fig. 5b, Additional file 1: Figure S6, Additional file 3: Table S2). However, in a number of other ecotypes, the presence of the two ORF-restoring indels (^T128, ΔA597) were associated with new SNPs that would again knock out the BKN1 coding region. This included the loss of the start methionine (ATG → ACG) and a new stop codon (TAA) downstream of ΔA597 (Fig. 5, Additional file 1: Figure S6, Additional file 3: Table S2). With Hh-0 expressing an intact At-BKN1 gene, pollen hydration assays were conducted on Hh-0 flowers to see if there was any variation at this early post-pollination stage and then compared to Col-0 in reciprocal pollinations, but no obvious differences were observed (Additional file 1: Figure S7).

BKNs are conserved within the Brassicaceae but are absent in species outside this family

Given the BKN1 polymorphisms found in the Arabidopsis species genomes, we also investigated related BKN genes in other plant species. The BKNs are part of the group VII RLCKs (Additional file 1: Figure S8; as defined by [51]) which include a number of important signalling proteins such as the BOTRYTIS-INDUCED KINASE1 (BIK1 [52]) and the various PBS1-Like (PBL) proteins associated with plant immune signalling [53, 54]. The BKNs are most closely related to CASTAWAY (CST [55]) and PBL31 [53] (Additional file 1: Figure S8). RLCKs are related to plant receptor kinases [51, 56], except that they lack extracellular domains and typically function in complexes with receptor kinases [57–59]. Alignments between BKN1 and BKN2 with CST (an active kinase involved in floral abscission [55]) clearly show that the BKNs are missing several key residues for ATP binding and catalytic activity, including the glycine-rich loop and the VAIK, HRD and DFG motifs, and as a result, are defined as being pseudokinases [46] (Additional file 1: Figure S9). Although some pseudokinases may exhibit partial kinase activity, BKNs are predicted to be inactive due to the number of missing residues, particularly the glycine-rich loop and the VAIK motif, which are required for catalytic activity [46, 60].

To investigate the distribution of BKN genes in plant genomes, BLAST searches were conducted using the A. thaliana BKN amino acid sequences along with three closely related RLCK amino acid sequences: At5g25440, PBL31 (At1g76360) and CST (At4g35600). These three predicted proteins were selected as they cluster with the BKNs in the RLCK-VII tree (Additional file 1: Figure S8). In these searches, a third A. thaliana BKN parologue was located nearby on chromosome 5, At5g11360.
BKN3; Additional file 1: Figure S4). At-BKN3 is predicted to have a large internal deletion of ~140 amino acids, while the corresponding orthologues in A. lyrata and A. halleri encode a full length BKN3s (Additional file 1: Figure S4 and S5). We identified BKN homologues for all Brassicaceae genomes searched, including A. lyrata, A. halleri, Arabis alpina, Boechera stricta, Capsella rubella, C. grandiflora, Brassica cretica, B. oleracea, Eutrema salsugineum (formerly Thellungiella halophila) and Schrenkiella parvula (formerly T. parvula). The number of homologues ranged from one in S. parvula to nine in B. oleracea. Interestingly, the BKNs were all predicted to be the pseudokinases (Additional file 1: Figure S9) and only found in core Brassicaceae genomes, not in the genome of the basal Brassicaceae species, Aethionema arabicum [61].

Despite lacking kinase activity, pseudokinases do play a variety of roles in biological systems and typically are in association with other signalling proteins at the cell membrane [66–74]. While the BKNs, as typical RLCKs, lack extracellular and transmembrane domains, they have conserved residues for N-terminal myristoylation and/or palmitoylation. These N-terminal lipid anchors can target proteins to the cell membrane where they would be proximal to other signalling proteins and receptors [75–77]. The presence of a glycine at position two is essential for myristoylation, while the cysteine at position 4 is required for palmitoylation [78]. Interestingly, all the Brassicaceae BKN homologues have a predicted myristoylation site (G2; Additional file 1: Figure S9) and only found in core Brassicaceae genomes, not in the genome of the basal Brassicaceae species, Aethionema arabicum [61].

**Plasma membrane localization of BKNs by predicted N-terminal myristoylation and palmitoylation sites**

Despite lacking kinase activity, pseudokinases do play a variety of roles in biological systems and typically are in association with other signalling proteins at the cell membrane [66–74]. While the BKNs, as typical RLCKs, lack extracellular and transmembrane domains, they have conserved residues for N-terminal myristoylation and/or palmitoylation. These N-terminal lipid anchors can target proteins to the cell membrane where they would be proximal to other signalling proteins and receptors [75–77]. The presence of a glycine at position two is essential for myristoylation, while the cysteine at position 4 is required for palmitoylation [78]. Interestingly, all the Brassicaceae BKN homologues have a predicted myristoylation site (G2; Additional file 1: Figure S9) while several also have a predicted palmitoylation site at the N-terminus (C4; Additional file 1: Figure S9). Specifically, A. thaliana BKN1, A. thaliana BKN2 and A. lyrata BKN2 have both the G2 and C4 sites while A. lyrata BKN1 only has the G2 myristoylation site (Additional file 1: Figure S5 and S9). Using a transient expression system in Nicotiana benthamiana leaf epidermal cells, C-terminal YFP fusions [79] of the four
proteins were then tested for potential plasma membrane localization. Full-length At-BKN1 from Hh-0 was tested along with At-BKN2 from Col-0, Al-BKN1 and Al-BKN2 (Fig. 7). All four BKN:YFP proteins appeared to be predominantly localized to the plasma membrane (Fig. 7a-d), with At-BKN1:YFP and Al-BKN1:YFP also showing some localization to the nucleus (Fig. 7a-b). It was unclear whether the unexpected partial nuclear localization is related to the protein function or an artifact caused by cleavage and mis-localization of the YFP. As well, Al-BKN1:YFP’s pattern of localization to the plasma membrane did not appear to be as tight as the other BKNs, but this may be related to Al-BKN1 only having a myristoylation site \[81\]. Myristoylation allows for transient associations with the membrane while the combination of palmitoylation and myristoylation more effectively...
anchors proteins to the plasma membrane, though these protein modifications remain reversible to facilitate transient membrane associations [81].

With Al-BKN2:YFP and At-BKN2:YFP containing both the G2 and C4 sites and showing strong plasma membrane localization, we also tested if these proteins...
could be palmitoylated when expressed in yeast cells. The BKN2 proteins were isolated from transformed yeast cells (Fig. 8a), and then tested for S-palmitoylation using the in vitro acyl-RAC (resin-assisted capture) assay [82]. After free thiols were blocked, the proteins were either treated with hydroxylamine (+) to remove palmitoylate and exposing free thiols at the palmitoylation sites or left untreated (−). Both Al-BKN2 and At-BKN2 were detected in the (+) lanes indicating that these proteins had been S-palmitoylated and were captured on the thiol-reactive resin following hydroxylamine treatment (Fig. 8b). Finally, since Al-BKN2:YFP displayed particularly high fluorescent levels as well as strong localization to the plasma membrane (Fig. 7d), we tested the effects of disrupting the myristoylation and palmitoylation sites on its localization pattern. Amino acid substitutions of the myristoylation (G2A) site, the palmitoylation (C4A) site or both (G2A, C4A) in Al-BKN2:YFP disrupted its plasma membrane localization, resulting in mis-localization to the nucleus and the cytoplasm of N. benthamiana leaf epidermal cells (Fig. 7e-g), similar to the CAM4:YFP control (Fig. 7h). The cells infiltrated with Al-BKN2:YFP were also plasmolysed by treating with 0.8 M mannitol to cause cell shrinkage and plasma membrane dissociation from the cell wall. BKN2:YFP localization was observed at the plasma membrane, at the sites where the plasma membrane has detached from the cell wall, and with Hechtian strands in the apoplastic space (Fig. 7i). Similar results were observed for the other BKNs following plasmolysis (Additional file 1: Figure S10). With the Al-BKN2 versions mutated at the myristoylation (G2A) and/or palmitoylation (C4A) sites, disrupted plasma membrane localization was again observed in the plasmolysed cells with the YFP signal becoming more diffuse and some localization occurring in the nucleus (Additional file 1: Figure S10). Thus, this data strongly supports that the BKNs have N-terminal lipid anchors to localize to the plasma membrane, and disruption of the fatty acid modification sites (G2, C4) causes a mis-localization to other subcellular compartments.

**Discussion**

In this study, we have investigated a novel family of pseudokinase genes, the BRASSIKINs (BKNs) that are only found in core-Brassicaceae species. The Brassicaceae genomes examined typically carried two to three different BKN genes, except for the Brassica genomes which tended to have higher numbers, up to nine predicted BKN genes for the diploid species. BKNs belong to the receptor-like cytoplasmic kinase (RLCK) subfamily, and so it is conceivable that BKNs function in a receptor kinase complexes for signaling pathways. For instance, A. thaliana CASTAWAY (CST) is a plasma membrane localized RLCK that has been shown to interact with two receptor kinases, HAESA and EVERSHEDE, and function as an inhibitor of floral organ abscission [55]. CST also localized to the plasma membrane through N-terminal myristoylation and palmitoylation sites, and this localization pattern was shifted towards the cytoplasm when the N-terminal lipid anchor sites were mutated [55]. Here, we have shown that BKN1 and BKN2 have predicted N-terminal palmitoylation and/or myristoylation sites and localized to the plasma membrane in N. benthamiana epidermal cells. As well, yeast-expressed BKN2 was confirmed to

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**Fig. 8** Palmitoylation of Al-BKN2 and At-BKN2. (a) Protein expression and (b) Palmitoylation assay for Al-BKN2 and At-BKN2. BKN2 proteins were purified from yeast cells and tested for S-palmitoylation using the in vitro acyl-RAC assay [82]. The BKN2 proteins were detected via C-terminal V5 epitope tags and western blotting with an anti-V5 antibody. The presence of a band in the + NH₂OH bound lanes indicates that both BKN2 proteins were palmitoylated when expressed in yeast cells.
be palmitoylated using the in vitro acyl-RAC assay. Finally, we observed that mutations of the myristoylation and palmitoylation sites for Al-BKN2 disrupted membrane localization in *N. benthamiana* epidermal cells. Thus, the plasma membrane localization of BKN1 and BKN2 could position these RLCKs for interactions with receptor complexes.

While many RLCKs, such as CST, are functional kinases, the BKNs are predicted to be pseudokinases meaning that they lack the catalytic motifs required for phosphotransfer [46, 60]. There are examples of pseudokinases with very low levels of autophosphorylation activity and this typically requires the catalytic lysine of the VAIK motif, and usually the aspartates of the HRD and DFG motifs as well [46]. In addition, while most pseudokinases are devoid of catalytic activity, it is estimated that close to 40% of all pseudokinases are capable of nucleotide binding [46]. However, the BKNs lack all of the key motifs required for ATP binding and catalytic activity, including VAIK with the key catalytic lysine as well as the HRD and DFG motifs [46, 60]. In a recent study, 168 of the 1005 *Arabidopsis* predicted kinases were classified as pseudokinases [47]. A number of these *Arabidopsis* pseudokinases (also referred to as atypical kinases [83]) have been shown to have biological functions related to signalling [66–74, 84, 85]. One example is the Brassinosteroid Signalling Kinases (BSKs), which are a group of 12 closely-related, functionally redundant cytoplasmic pseudokinases involved in brassinosteroid signalling through interactions with the receptor BR1 [66, 67, 86]. The BSKs are phosphorylated by the BRI1 receptor kinase during BR hormone perception and are proposed to function as scaffolds in the signaling complex [67, 87]. Another example is the stem cell signalling protein CORYNE (CRN) which interacts with the receptor CLAVATA 2 to promote localization to the plasma membrane [88] and functions with receptor kinases to regulate stem cell fate in the shoot and root apical meristems [68, 69, 89]. Finally, roles for pseudokinases have been defined in immune complexes that regulate plant immunity responses [70, 84, 85, 90]. Interestingly, Liu et al. [91] recently discovered a role for BKN2 in plant immunity (SUPPRESSOR OF ZED1-D2; SZE2) and found that it was localized to the plasma membrane as part of an immune complex. Thus, given the tendency of pseudokinases to function in protein complexes, potential interactors will need to be identified in the stigma to further understand the functions of BKNs.

Our interest in the BKN family started with a search for stigma-enriched signalling proteins that may function in compatible pollen responses and, this search led to the identification of the stigma-specific BKN1 in *A. thaliana* Col-0. However, the bkn1 mutants in the Col-0 ecotype did not display any detectable changes in compatible pollen responses and only a mild-hydration defect was observed when wild-type Col-0 pollen was placed on mutant bkn2 stigmas (a tandemly linked paralogue). In addition, the level of impairment did not increase for the bkn1-bkn2 double mutant stigmas. This was rather puzzling since BKN2 was only expressed at a low level in the stigma, particularly in comparison to BKN1. Further investigations uncovered that the BKN1 gene in most *A. thaliana* ecotypes carried two indels, ΔT128 and ΔA597, that would cause frameshifts in the BKN1 coding region resulting a loss of full-length protein. Interestingly, three ecotypes, Hh-0, Dju-1 and Västervik, were found to carry two ORF-restoring SNPs (^T128 and ^A597) and predicted to encode a full-length BKN1 protein. Furthermore, two outcrossing *Arabidopsis* species, *A. lyrata* and *A. halleri*, also carry BKN1 orthologues that are predicted to encode full-length BKN1 proteins. These combined results raise a few questions for further investigation. Does the mild hydration phenotype associated with bkn2 mutants point to a related function for BKN1 that was lost during the transition to selfing for *A. thaliana*? For example, is BKN1’s stigma function related to pollen-stigma interactions in outcrossing species? The evolution from outcrossing to selfing occurs under different selective mechanisms, such as reduced access to pollinators or population bottlenecks associated with colonization of new environments, and is associated with the loss of self-incompatibility in Brassicaceae species (reviewed in [92, 93]). The transition to selfing is also associated with a change in several floral traits termed the ‘selfing syndrome’ and includes changes in flower size and shape (small flowers) and reduced pollen numbers, as seen in comparisons between outcrossing *A. lyrata/A. halleri* and selfing *A. thaliana* [93–95]. Other changes associated with the ‘selfing syndrome’ include loss of pollinator attraction traits (reviewed in [93, 95]).

Specifically related to selfing, there are traits, in the addition to the loss of self-incompatibility, that can be modified to improve self-pollination in the transition to selfing. These include dichogamy (temporal differences between time of pollen release and stigma receptivity), herkogamy (height differences between to stigma and anther) and anther orientation (anther surface undergoing dehiscence is oriented away from stigma) [93, 95, 96]. *A. lyrata* and *A. halleri* are self-incompatible species, whereby they exhibit a tight control of outcrossing through the linked *S-locus protein 11/S cysteine-rich* and *S receptor kinase* polymorphic genes (reviewed in [16]). With both species carrying intact *BKN1* genes, it would be of interest to investigate a potential role in stigma-pollen interactions in the context of these different traits designed to avoid self-pollination. As well, some North American *A. lyrata*
populations around the Great Lakes region have also shifted towards self-compatibility, but show no significant changes towards the selfing syndrome [97–99]. These self-compatible A. lyrata may also be interesting to compare loss-of-function BKN1 mutations in the context of self-pollination. Finally, the recent discovery of a role for BKN2/SZE2 in plant immunity [91] opens another direction of inquiry. Related to this, dual roles have been uncovered for other signaling proteins in both plant reproduction and pathogen responses [5]. The relative ease with which CRISPR/Cas9 technology can be used to create loss-of-function mutants opens the door to asking these questions regarding BKN1 function in other A. thaliana ecotypes and Arabidopsis species in the future.

Conclusions
In this study, we have identified a novel family of Brassicaceae-specific pseudokinase genes, termed BRAS-SIKINs, and specifically focused on the function of the tandemly linked BKN1 and BKN2 genes, in the context of pollen-stigma interactions in A. thaliana Col-0. CRISPR deletion mutants were generated, and very mild hydration defects were observed for wild-type Col-0 pollen when placed on the bkn2 and bkn1/2 mutant stigmas. Polymorphisms leading to premature stop codons were uncovered for BKN1 in many A. thaliana ecotypes including Col-0 while absent in outcrossing Arabidopsis species. Thus, future studies should focus on examining BKN1 function in other A. thaliana ecotypes and Arabidopsis species.

Methods
Plant materials and growth conditions
Seeds for the A. thaliana bkn1–1 T-DNA insertion mutant (Col-0, SALKseq_039336), and the A. thaliana Hh-0 (CS76512), Västervik (CS78834), Dju-1 (CS78896) and Bela-1 (CS76696) ecotypes were obtained from Arabidopsis Biological Resource Center (ABRC). Seeds for the A. thaliana Col-0 ecotype and N. benthamiana were obtained from Dr. Nambara and Dr. Yoshioka, respectively (University of Toronto). A. thaliana seeds were sterilized and cold stratified for at least 2 days at 4 °C, then transferred to soil or plated on ½ Murashige and Skoog (MS) medium plates with 0.4% (w/v) phytoagar at pH 5.8 at 22 °C under 16 h light. After 7–10 days, seedlings were transferred to soil supplemented with 1 g/L 20–20–20 fertilizer and grown at 22 °C under 16 h light. For the A. thaliana bkn1–1 T-DNA insertion mutant (SALKseq_039336), homozygous mutants were confirmed by PCR, and the location of the T-DNA was verified by sequencing of PCR products. N. benthamiana seeds were cold stratified for several days and planted directly on soil, and grown at 22 °C under 16 h light conditions. Humidity was monitored and maintained at between 20 to 60% relative humidity in the growth chambers.

Plasmid construction and plant transformation
The 371 bp BKN1 predicted promoter consists of the untranslated region immediately following the BKN2 coding region to the BKN1 start codon. The BKN2 predicted promoter covers 465 bp upstream of the BKN2 start codon, including the 3′UTR for At5g11412. The BKN1 and BKN2 5′ predicted promoter regions were synthesized by GeneArt gene synthesis services (ThermoFisher Scientific). The promoters were cloned into the pORE-R2 vector upstream of the GUS coding region through Xho1 and NotI sites, [100], transformed into Arabidopsis thaliana Col-0 by floral dip [101]. T1 seeds were selected for kanamycin resistance on ½ MS medium plates containing 50 μg/ml kanamycin. Inflorescence or stage 12 flowers from several T1 plants were stained for GUS activity (see below).

For the CRISPR/Cas9 generated mutants, a two-sgRNA (single guide RNA) system was used to generate genomic deletions in the BKN1 and BKN2 genes [48]. The CRISPR sgRNA sequences targeting BKN1 or BKN2 were selected using the CHOPCHOP software to search for sequences adjacent to PAM sites and avoid potential off-targets in the A. thaliana genome [102]. PCR fragments containing the two sgRNAs (See Additional file 4: Table S3 for primer sequences), along with the promoter and terminator sequences were generated from the pCPC DT1T2 vector template using Phusion polymerase (ThermoFisher Scientific). The purified fragments containing the two sgRNAs were cloned into the final vector pBEE401E using a golden gate reaction with Bsal enzyme. This vector was modified to carry the Basta resistance marker (BlpR from pBUE411 [103]) rather than the original HygR marker in pHE401E [36, 48]. Constructs were transformed into Agrobacteria by electroporation, which were then used to transform A. thaliana Col-0 by floral dip [101]. T1 seeds were cold stratified and sown on soil as previously described [26]. Once seedlings had germinated, selection for Basta™ herbicide resistance was carried out, and resistant seedlings were transplanted, and PCR screened for the Basta™ selection marker and for genomic deletion. T1 plants were analyzed with primers pairs designed to amplify inside or outside of the deletion regions for BKN1/BKN2 to identify heterozygous mutants, and homozygous mutants carrying the respective gene deletions were identified in subsequent generations (T2–T5) for phenotyping. PCR products covering the deletions were sequenced to confirm the locations of each independent deletion mutation. For both BKNs, two constructs carrying different sgRNA target sites were
screened for deletions in the T1 (Additional file 4: Table S3). The BKN1 CR #1 and BKN1 CR #3 constructs produced the bkn1–2 and bkn1–3 mutants, respectively. The BKN2 CR #3 construct produced the bkn2–1 and bkn2–2 mutants. To generate double mutants, transgene free bkn1–3 mutants were transformed with the BKN2 CR #2 construct to produce three new bkn2 mutants, bkn2–3, bkn2–4 and bkn2–5, in the bkn1–3 background.

To clone cDNAs for A. thaliana BKN2 (Col-0, At5g11410), A. lyrata BKN1 (AL6G22040.t1) and A. lyrata BKN2 (AL6G22050.t1), RT-PCR was conducted on RNA extracted from top ½ pistil tissue. The A. thaliana Hh-0 BKN1 cDNA was cloned from stage 12 flower bud RNA. The BKN clones were introduced into the TOPO entry clone using the PCR8/GW TOPO cloning kit (ThermoFisher Scientific). To generate the Al-BKN2(G2A), AI-BKN2(C4A), and AI-BKN2(G2A, C4A) constructs, the myristoylation (G2) and palmitoylation (C4) sites at the N-terminus of A. lyrata BKN2 were disrupted by PCR with primers to replace the G2 and C4 sites (Additional file 4: Table S3). Gateway reactions were carried out using LR clonase II enzyme (ThermoFisher) into the destination vector pEARLEYGATE 101 containing a C-terminal YFP (Earley et al., 2006). Plasmids were then transformed into Agrobacterium taei, and visualized using a Leica TCS SP8 confocal microscope. Image processing was done using the Leica LAS lite software. Plasmolysis was achieved by treatment with 0.8 M mannitol as described by Lang et al. [106].

Confocal microscopy
At 24 to 48 h post-infiltration, leaf disks were cut from N. benthamiana and visualized using a Leica TCS SP8 confocal microscope. Image processing was done using the Leica LAS AF lite software. Plasmolysis was achieved by treatment with 0.8 M mannitol as described by Lang et al. [106].

Expression profiling, multiple sequence alignments and phylogenetic analyses
The BAR Expression Angler tool [39] (http://bar.utoronto.ca/) was used to search for stigma-enriched signaling proteins as previously described [36]. Briefly, the stigma-specific SLR1 gene as the bait (At3g12000, [40]) to search the AtGenExpress Plus-Extended Tissue Compendium dataset [39, 41]. Expression profiling of the BKN genes for Additional file 1: Figure S1 came from three additional transcriptome datasets: the TRAVA RNA-Seq dataset (http://travadb.org/ [44]), the stigmatic papillae RNA-Seq dataset [43] and the stigma microarray datasets [35]; and the data was displayed using the HeatMapper Plus tool [39].

For ecotype polymorphism searches of the 1135 genomes [50], two different databases were used to retrieve the BKN1 genomic sequences: 1001 Genomes (https://1001genomes.org/) and Salk Arabidopsis 1001 Genomes (http://signal.salk.edu/atg1001/index.php). Hh-0 was the first ecotype identified to carry the T128 and ΔA597 to encode a full-length BKN1 protein. The MEGA7 software [63] was used to produce multiple protein sequence alignments of the BKN1 genomic sequences retrieved from the 1135 ecotype genomes. The Col-0 and Hh-0 BKN1 cDNA sequences were included to remove introns and locate the position of the two SNPs (Additional file 5 and Additional file 6). The two SNP regions were copied from the alignment into an excel file for further analysis (see Additional file 3: Table S2). Genomic DNA samples were used to PCR amplify and sequence the BKN1 gene for the Västervik, Dju-1 and Bela-1 ecotypes (Additional file 1: Figure S5).

For the phylogenetic analysis of Brassicaceae BKNs (Fig. 6), amino acid sequences (Additional file 7) were obtained from TAIR (A. thaliana) [49]; Phytozone (A. lyrata, B. oleracea capitata, B. stricta, E. salsugineum - formerly T. halophila, C. rubella, C. grandiflora, C. papaya) [107]; NCBI (A. alpina, B. cretica, B. oleracea cv TO1000, T. hassleriana) [108]; EnsemblPlants (A. halleri, B. oleracea cv TO1000) [109]; BRAD (A. arabcum) [110]; or thellungiella.org (S. parvula - formerly T. parvula) servers using blastp or tblastn searches for genes similar to the BKNs or other RLCKs. For the phylogenetic analysis of Arabidopsis RLCK VII members (Additional file 1: Figure S8), the RLCK VII members defined by Lehti-Shiu and Shiu [51] were used, and amino acid sequences (Additional file 8) were retrieved from TAIR [49]. The MEGA7 software [63] was used to produce multiple protein sequence alignments using ClustalW [62]. The ClustalW alignments were trimmed at the N-and C-terminus and then used to generate a consensus tree by the Maximum Likelihood method [64] with 1000 bootstrap replicates [65] in MEGA7. Alignments in Additional file 1: Figure S3, S4, S5 and S6 were generated in MEGA7 and formatted with the Multiple Align Show tool (http://www.bioinformatics.org/sms111), using groupings of amino acids based on their side chains [112]. See additional files for all amino acid sequences and alignments.

RT-PCR and quantitative RT-PCR software
Anthers and pistil tissues (top-half: stigmas, bottom-half: ovaries) and were collected from stage 12 flower buds;
leaves and roots were collected from 2-week-old *A. thaliana* seedlings for RT-PCR and quantitative RT-PCR applications. RNA was extracted using a modified protocol of the SV total RNA extraction kit (Promega) which included vigorous grinding of plant tissue in liquid nitrogen. Next, cDNA synthesis was carried out using Superscript III reverse transcriptase (ThermoFisher) and oligo dT primers. The cDNA was then used in RT-PCR reactions with Taq polymerase, and quantitative RT-PCR reactions with PowerUp 2x SYBR Green master mix (ThermoFisher) (primers listed in Additional file 4: Table S3).

**Assays for pollen hydration, pollen adhesion and pollen tube growth, and seed set**

Stage 12 flower buds were emasculated and carefully wrapped with plastic wrap and allowed to mature overnight. For pollen hydration, the next day, pistils were mounted upright in ½ MS medium and hand-pollinated with a small amount of Col-0 pollen. Pictures were taken immediately at 0 min and again at 10 min post-pollination using a Nikon sMz800 microscope at 6x magnification with a 1.5x objective. Pollen grain diameter was measured laterally using the Nikon digital imaging software for 10 random pollen grains per pistil, 3 pistils per genotype. All pollinations were performed under an ambient humidity lower than 60% to avoid spontaneous water uptake from the surrounding environment.

For pollen adhesion and pollen tube growth, the next day, pistils were carefully unwrapped and lightly pollinated with Col-0 pollen, or transgenic pollen for reciprocal crosses. At 2 h post-pollination, pistils were collected, fixed and stained with aniline blue to stain the callose deposited by pollen tubes, as described by Safavian et al. (2015). Pollinated pistils were imaged using a Zeiss Axioskop2Plus microscope under bright-field to count the number of pollen grains adhered, and under UV fluorescence to assess pollen tube growth. Pollen adhesion was quantified for *n* = 10 pistils for each cross.

For seed set, late stage 12 buds were emasculated and hand-pollinated with Col-0 pollen for Col-0 pistils and for each transgenic line. Hand pollinations were marked with thread and siliques were allowed to mature fully over several days. Prior to senescence, green siliques were removed, and sliced longitudinally to count the number of developing seeds. 10 siliques were counted for each pollination.

In vitro BKN2 Palmitoylation assay

At-BKN2 and Ai-BKN2 gateway entry clones were recombined into the Gateway destination vector pYES-DEST52 (C-terminal V5 tagged, Invitrogen) to create yeast expression vectors pYES-AI-BKN2 and pYes-At-BKN2 respectively. Wild-type yeast BY4741 (MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) cells were transformed and grown at 25 °C in selective minimal media minus uracil to select transgenic yeast cells. To induce protein expression, the transformed yeast cells were grown in minimal liquid media containing 2% galactose. The palmitoylation assay was carried out by the Acyl-RAC method [82, 113]. Briefly, total proteins were lysed and recovered by acetone precipitation. Free –SH was blocked with 1% methyl methanethiosulfonate (MMTS), and samples were then treated with 1 M hydroxylamine, pH 7.5 (+ NH₂OH) to remove palmitoylate and to expose free thiols at the palmitoylation sites. In the negative control (−NH₂OH), 1 M Tris (pH 7.5) was added. Palmitoylated proteins were captured on thiopropyl sepharose beads (Sigma), and the presence of BKN2 proteins were detected by ECL western blotting with anti-V5 primary and HRP-conjugated secondary antibodies (CWBio, China).

**Supplementary information**

Supplementary information accompanies this paper at https://doi.org/10.1186/s12870-019-2160-9.

**Additional file 1:** Figure S1. Tissue expression profiles of the BKNs in different transcriptome datasets.

**Additional file 2:** Analysis of the bkn1–1 T-DNA mutant. Figure S2. Sequence Alignment of Arabidopsis BKN1 coding sequences.

**Additional file 3:** Arabidopsis genomic regions for the BKN genes and amino acid alignment for the BKN1 annotations.

**Additional file 4:** A. thaliana, A. lyrata and A. halleri amino acid alignments for BKN1, BKN2 and BKN3.

**Additional file 5:** Alignment of BKN1 coding sequences from four A. thaliana ecotypes.

**Additional file 6:** Pollen Hydration Assays in the Col-0 and Hh-0 ecotypes at 10 min post-pollination.

**Additional file 7:** Phylogenetic analysis of the Arabidopsis RLCK subfamily VII members.

**Additional file 8:** Pollen Hydration Assays in the Col-0 and Hh-0 ecotypes at 10 min post-pollination.

**Additional file 9:** Confocal microscopy imaging of *N. benthamiana* leaves infiltrated with C-terminal BKN:FYP fusion protein constructs and plasmolysed with 0.8 M mannitol.

**Additional file 10:** BAR Expression Angler using the stigma specific SUR1 gene as a bait.

**Additional file 11:** Polymorphism searches across the 1001 genomes for At-BKN1.

**Additional file 12:** RLCK Amino acid sequences for Fig. 6 phylogenetic tree.

**Additional file 13:** Primers Used.

**Additional file 14:** RLCK Amino acid sequences for Additional file 1: Fig. S7 phylogenetic tree.

**Additional file 15:** RLCK Amino acid sequences for Additional file 1: Fig. S7 phylogenetic tree.

**Additional file 16:** RLCK Amino acid sequences for Additional file 1: Fig. S7 phylogenetic tree.

**Abbreviations**

BKI: BOTRYTIS-INDUCED KINASE1; BKN: BRASSIKIN; CST: CASTAWAY; MMTS: Methyl methanethiosulfonate; MS: Murashige and Skoog; PBL: PBS1-Like; PCP-B: Pollen Coat Protein-B; RLCK: Receptor-Like Cytoplasmic Kinase; SPIK: SHAKER POLLEN INWARD K+ channel

**Acknowledgements**

We thank Rebecca Hytton and Betty Geng for technical assistance, and members of the Goring lab for critically reading this review. Thanks also to the Yoshiko lab for providing the CAM4:YPF construct. We thank the Arabidopsis Biological Resource Center (ABRC) for providing seeds for the
sequence-indexed Arabidopsis T-DNA insertion mutant and the Hh-0, Väster-vik, Dju-1 and Bela-1 ecotypes.

Authors’ contributions
JD and DRG design the research; JD, HKL, NU, JX, and BQ performed the research; JD, DRG, HKL and BQ conducted the data analysis; JD and DRG prepared the figures and wrote the manuscript; JD, HKL, DRG and BQ edited the manuscript. All authors read and approved the final manuscript.

Funding
J.D. and H.K.L. were supported by Ontario Graduate Scholarships (OGS). This research was supported by a grant from Natural Sciences and Engineering Research Council of Canada to D.R.G, and a grant from the Natural Science Foundation of China to B. Q. (grant number 31170233). The funders had no role in the design of the study and collection, analysis, and interpretation of data and in writing the manuscript.

Availability of data and materials
All data is included in this manuscript. Constructs and seeds are available upon request from the corresponding author.

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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Received: 4 June 2019 Accepted: 25 November 2019
Published online: 11 December 2019

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