Molecular Plant
Letter to the Editor

CellPress
PARTNER JOURNAL

Flavonoids and ROS Play Opposing Roles in Mediating Pollination in Ornamental Kale (*Brassica oleracea* var. *acephala*)

Dear Editor,

In most flowering plants, the stigma of the flower represents the barrier that prevents any unrelated or incompatible (genetically similar) pollen from germination and fertilizing the ovule, leading to either defective or genetically unfavorable embryos. In Brassicaceae, self-incompatibility (SI) is a genetic mechanism in the stigmas that can discern compatible versus incompatible mate (pollen), in turn, rejecting self or incompatible pollen, while allowing non-self or compatible pollen to develop successfully to fertilize the ovules. Thus, stigmas have evolved complex mechanisms and specialized proteins to reject incompatible mates and to recognize and accept compatible pollen (Doucet et al., 2016). The fact that SI has been shown to exclusively operate in the stigmas temporally before and during anthesis (Kandasamy et al., 1993) indicates that multiple metabolic pathways essential for pollination exist in these stigmatic papillary cells. Despite identifying a few proteins from mature stigmas that are involved in mediating the SI response, a thorough analysis of the proteomic landscape during different stages of stigma development has been lacking. Identifying these proteins could lead to uncovering of the interactions between metabolic pathways and developmental programming that can orchestrate the process of pollination.

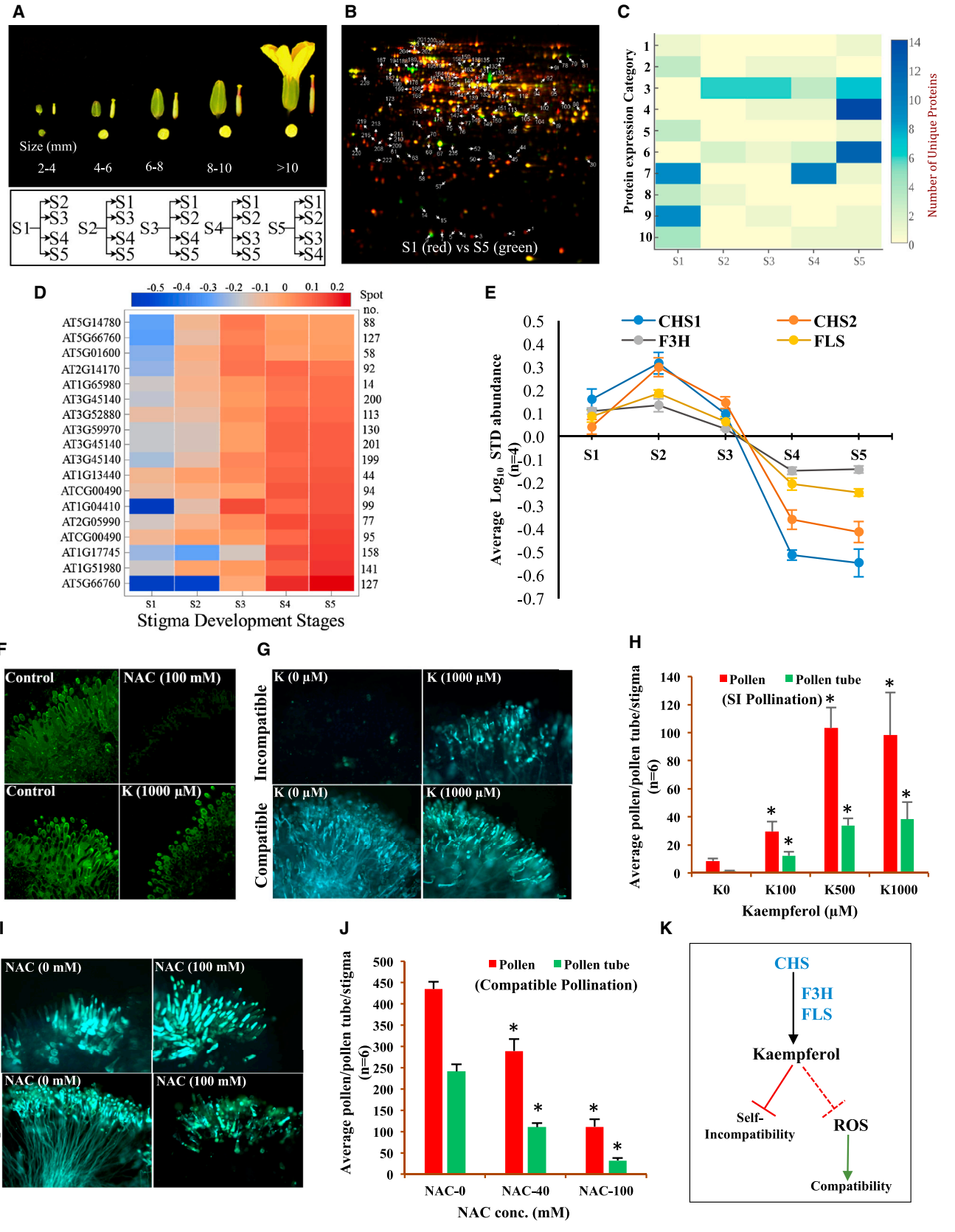
In order to address this, we adopted a robust proteomics approach using two-dimensional difference gel electrophoresis (2D-DIGE) (n = 4, pH 4–7) for separating proteins isolated from stigmas of self-incompatible ($S_{13\text{-}b}S_{13\text{-}b}$) ornamental kale (*Brassica oleracea* var. *acephala*) at five different developmental stages based on their size (S1–S5, 2 to >10 mm) (Figure 1A). To allow the normalization process, pair-wise comparisons were performed between the stages along with an internal control made up of an equal amount of proteins from all stages. Analysis of twenty 2D-DIGE gel images resolved a total of 4000 spots (Decyder analysis software); 240 protein spots showed at least a 1.5-fold change in their relative abundance ($p < 0.01$) (Figure 1B; Supplemental Figure 1; Supplemental Table 1). These 240 protein spots were further isolated and subjected to in-gel digestion, which led to the identification of 107 differentially expressed proteins (DEPs) after MALDI-TOF-TOF MS/MS and Mascot database analysis (MOWSE score >56) ($p < 0.01$) (Supplemental Tables 2 and 3).

To validate our approach, we first checked the changes of previously reported stigma factors involved in either SI signaling or stigma development in these 107 DEPs. Consistent with previous reports, the *S*-locus-specific glycoprotein (SLG), a previously characterized stigma-specific, developmentally regulated glycoprotein, was upregulated more than 40-fold in the S5 stage compared with S1 (Supplemental Table 3) (Stein et al., 1991; Doughty et al., 1998). Similarly, Glyoxalase I (GLO1), a known compatibility factor (Sankaranarayanan et al., 2015) in pollination, showed a gradual increase from S1 to S5 (Supplemental Table 3). An increase in the level of RGP1 (UDP-arabinopyranose mutase 1, AT3G02230) necessary for cell-wall additions and plant development (Rautengarten et al., 2011) was also observed (Supplemental Table 3). In order to further validate our approach, we generated antibodies against both GLO1 and RGP1 and subjected proteins from various stages to immunoblotting. The blots provided evidence for upregulation of these proteins during different stages of stigma development, further confirming our observations from the DIGE approach (Supplemental Figure 2).

A two-way functional clustering (five clusters) and categorization (10 groups) (Figure 1C) of these 107 DEPs revealed differential regulation of 32, 9, 10, 18, and 38 DEPs at S1, S2, S3, S4, and S5 stages of stigma development, respectively. The identified 32 DEPs in S1 are largely involved in signaling, transcription-related processes, protein synthesis, protein folding and turnover, cell structure, photosynthesis, and protein trafficking. Stage S2 clustered nine DEPs, of which the flavonoid biosynthesis-related proteins and MLP-like protein 328 (involved in vegetative growth and delayed flowering) and its homolog categorized under stress- and defense-related proteins, were upregulated. The S3 stigmas clustered ten DEPs that are involved in ROS metabolism, amino acid synthesis, and proteins involved in stress and defense. Stage S4 clustered 18 DEPs, which represented proteins related to signaling (Mitogen-activated protein kinase 4), protein folding and turnover, stress, and defense. S4 proteome also showed that an abundance of proteins related to carbohydrate and energy metabolism. However, the S5 stigmas represented the largest cluster of 38 DEPs showing upregulation of signaling (SLG), stress and defense-related proteins (GLO1, Probable monodehydroascorbate reductase, Formate dehydrogenase, Thiocyanate methyltransferase 1, Thiocyanate methyltransferase 2, Selenium-binding protein 2, MLP-like protein 423) and an increase in proteins involved in carbohydrate and energy metabolism.

These 107 DEPs were further matched with 79 unique homologs in *Brassica oleracea* and *Arabidopsis* by sequence BLASTing against the TAIR and NCBI(nr) database (Supplemental Tables 2 and 3). When these 79 genes were subjected to

Published by the Molecular Plant Shanghai Editorial Office in association with Cell Press, an imprint of Elsevier Inc., on behalf of CSPB and IPPE, SIBS, CAS.



(legend on next page)

gene ontology analysis using the Princeton GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder), an enrichment in the ROS metabolism genes was observed. We found a cluster of 17 proteins (Figure 1D) with oxidoreductase activity (21.5%, p = 0.00012) (Peroxiredoxin-2B, AT1G65980; Probable monodehydroascorbate reductase, AT3G52880; Formate dehydrogenase, AT5G14780), largely involved in ROS regulation. A heatmap generated for these proteins based on their relative abundance at different developmental stages (Figure 1D; Supplemental Table 4) showed a gradual upregulation of these proteins during stigma development. This increase in proteins involved in ROS metabolism suggested that ROS should accumulate in stigmas during maturity, and to balance this, a concomitant increase in ROS metabolic proteins is observed. To examine this possibility, we stained pistils at different developmental stages with the fluorescent ROS detecting agent, H_2DCF-DA, and recorded the fluorescence. As expected, a gradual increase in H_2DCF-DA fluorescence was observed from stage S1 to S5 stigmas, indicating the high-level ROS accumulation in mature stigmatic papillae close to flower opening (Supplemental Figure 3). This is consistent with previous reports on ROS accumulation in mature stigmas of angiosperms (Zafra et al., 2016). It is worth noting that this increase in ROS coincided with a dramatic reduction in proteins involved in flavonoid biosynthesis. Key enzymes (chalcone synthase, flavanone 3-hydroxylase, flavanol synthase) involved in the generation of flavonoids, such as kaempferol and quercetin, peaked at S2 and decreased significantly in the later stages (S3, S4, and S5) of development (Figure 1E; Supplemental Figure 4). This synchronized decrease in flavonoid enzymes, concomitant with an increase in ROS in the stigmas, suggests that this programming should be necessary for stigma functions such as stigmatic maturity, pollination, and fertilization events.

To investigate the cross-talk between flavonoids and ROS in pollen-stigma interaction, we systematically either supplemented or removed these metabolites and assessed the effects on SI and compatibility. For this, following treatments, self-incompatible ornamental kale ($S_{13\text{-}b}S_{13\text{-}b}$) flowers were either self-pollinated (SI response) or cross-pollinated (compatible response), and pollination was assessed. First, we tested if the flavonoid, kaempferol (KF), had an effect on ROS accumulation in S5 stigmas. When stigmas were pre-treated with KF and assessed for ROS accumulation, there was a reduction in ROS, but it did not completely abolish ROS accumulation unlike a potent antioxidant such as N-acetyl cysteine (NAC) (Figure 1F). We next examined if accumulation of KF in mature stigmas could alter pollen-pistil interactions. Treatment of S5 stigmas with KF did not alter the ability of the stigmas to accept compatible pollen, although there was a moderate reduction in pollen attachment (Figure 1G and Supplemental Figure 5). However, KF treatment led to the breakdown of SI response following self-pollination (Figure 1G). Compared with untreated stigmas, KF-treated self-incompatible stigmas were able to accept self-pollen and showed increased pollen attachment and pollen tube growth in a concentration-dependent manner (Figure 1G and 1H). This observation indicates that flavonoid accumulation in mature stigmas could compromise SI response and therefore lead to a reduction in the ability of plants to maintain genetic vigor. To determine if this observed phenomenon of reduced flavonoid enzymes at stigmatic maturity is specific to self-incompatible plants, we examined stigmas of a compatible ornamental kale line. The compatible kale in which SI response is broken down displayed a similar trend in accumulation of the flavonoid biosynthetic enzyme (CHS) during stigma development (Supplemental Figure 4), suggesting that this phenomenon likely pre-dates the breakdown of SI system in these lines.

To further investigate the role of ROS during pollen-pistil interactions, we treated S5 stigmas of self-incompatible ornamental kale ($S_{13\text{-}b}S_{13\text{-}b}$) with NAC concentrations that completely blocked ROS accumulation and assessed its ability to accept either self or cross-pollen. Lack of ROS in SI stigmas did not alter the self-rejection response, while it drastically reduced compatible pollen attachment and germination in a concentration-dependent manner (Figure 1I and 1J). Thus, ROS accumulation in stigmas is essential for compatible pollinations but has no influence on the SI reaction. Therefore, it seems likely that Brassicaceae species evolved to block accumulation of flavonoids in mature stigmas, since this could abrogate SI

Figure 1. Proteomic Landscape of the Self-Incompatible *Brassica oleracea* var. *acephala* Stigmas at Different Developmental Stages (S1–S5) Reveal an Interplay between Flavonoids and ROS during Pollination.

(A) The top panel shows different stages of the buds, pistil, and stigma based on their size. The lower panel illustrates the design of the 2D-DIGE experiment where proteins from different stages were compared (n = 4).

(B) A representative 2D-DIGE gel showing differential expression of proteins from stage 1 and stage 5 stigmas. The spots were resolved using 24 cm immobilized pH gradient (pH 4–7) strips followed by 12.5% SDS–PAGE. The DEPs identified in this study are shown in Supplemental Table 2.

(C) Heatmap showing a two-way functional categorization of 107 DEPs (10 groups; y axis) and clustering (five; x axis) of the DEPs based on the developmental stages of stigma. On the y axis: 1, signaling; 2, transcription related; 3, protein synthesis; 4, protein folding and turnover; 5, cell structure; 6, stress and defense; 7, carbohydrate and energy metabolism; 8, other metabolisms; 9, photosynthesis; 10, membrane and transport.

(D) Heatmap showing differential expression of proteins largely involved in ROS metabolism during stigma development. The proteins are represented by homologs from *Arabidopsis thaliana.* Respective spot numbers on the 2D-DIGE are also indicated on the right side.

(E) Chart showing downregulation of flavonoid-related proteins during stigma maturity. Values are expressed as $\log_{10}$ of the standard abundance (n = 4).

(F) Fluorescence images of S5 stigma treated with H_2DCF-DA before and after incubation with NAC (100 mM), top panel; and kaempferol (1000 μM), bottom panel.

(G and H) Aniline blue assay validating the critical role of kaempferol during SI responses. A significant increase in the mean number of pollen attachment and pollen tubes were observed **(H)** after kaempferol treatment compared with untreated self-incompatible stigma. Values are presented as ±SEM (*$p < 0.05$).

(I) Aniline blue assay showing the inhibitory effects of NAC on pollen attachment and pollen tube formation following compatible pollination.

(J) The data are presented as a graph. Values are presented as ±SEM (*$p < 0.05$).

(K) A simplified proposed model depicting the role of flavonoids and ROS in mediating SI and compatible signaling, respectively. Dashed line indicates weak inhibition.

response and lead to a reduction in genetic vigor. In addition, flavonoids can also function as antioxidants and reduce the ability of stigmas to accept compatible pollen.

Our observations allow us to conclude that during stigma developmental programming, proteins required for basic energy needs and development accumulate in the early stages of stigma, while at the later stages proteins required for defense and stress specifically accumulate. This coincides with flower opening when the stigmas have to be primed for pollination and at the same time defend the reproductive parts from biotic and abiotic insults. We further show that proteins required for flavonoid biosynthesis are turned off in mature stigmas, when the papillary cells begin to accumulate factors essential for SI response. We propose a model in which Brassicaceae stigmas have evolved this functionality of shutting down flavonoids at maturity to achieve two important and independent outcomes: (1) to prevent flavonoids from negatively regulating SI response, which is independent of the effect of flavonoids on ROS; (2) to mitigate the antioxidant properties of flavonoids that can inhibit ROS accumulation necessary for compatible pollination to occur (Figure 1K). Future research will aim to identify how flavonoids are able to break down SI response, and more importantly, to investigate the mechanisms behind how ROS accumulation functions as a prerequisite for successful pollination.

SUPPLEMENTAL INFORMATION

Supplemental Information is available at *Molecular Plant Online*.

FUNDING

This work was supported by Fundamental Research Funds for the Central Universities of China (no. DL13CA13) for X.L. and the Natural Sciences and Engineering Research Council of Canada for M.A.S. X.L. is supported by a fund from the China Scholarship Council.

AUTHOR CONTRIBUTIONS

X.Lan, J.Y., Y.L., and M.A.S. conceived and designed the experiments. X.Lan, J.Y., A.K., Y.N., and X.Li performed the experiments. X.Lan and M.A.S. analyzed the data. X.Lan, A.K., and M.A.S. wrote the manuscript.

ACKNOWLEDGMENTS

No conflict of interest declared.

Received: May 1, 2017
Revised: July 10, 2017
Accepted: August 7, 2017
Published: August 18, 2017

Xingguo Lan[1,2,3], Jia Yang[1,3], Kumar Abhinandan[2,3], Yuzhe Nie[1], Xiaoyu Li[1], Yuhua Li[1,] and Marcus A. Samuel[2,*]*

[1]College of Life Sciences, Northeast Forestry University, Harbin, Heilongjiang 150040, P R China
[2]Department of Biological Sciences, University of Calgary, Calgary, AB T2N 1N4, Canada
[3]These authors contributed equally to this article.
***Correspondence: Yuhua Li (lyhshen@126.com), Marcus A. Samuel (msamuel@ucalgary.ca)**
http://dx.doi.org/10.1016/j.molp.2017.08.002

REFERENCES

Doucet, J., Lee, H.K., and Goring, D.R. (2016). Pollen acceptance or rejection: a tale of two pathways. Trends Plant Sci. **21**:1058–1067.

Doughty, J., Dixon, S., Hiscock, S.J., Willis, A.C., Parkin, I.A., and Dickinson, H.G. (1998). PCP-A1, a defensin-like *Brassica* pollen coat protein that binds the *S* locus glycoprotein, is the product of gametophytic gene expression. Plant Cell **10**:1333–1347.

Kandasamy, M.K., Thorsness, M.K., Rundle, S.J., Goldberg, M.L., Nasrallah, J.B., and Nasrallah, M.E. (1993). Ablation of papillar cell function in *Brassica* flowers results in the loss of stigma receptivity to pollination. Plant Cell **5**:263–275.

Rautengarten, C., Ebert, B., Herter, T., Petzold, C.J., Ishii, T., Mukhopadhyay, A., Usadel, B., and Scheller, H.V. (2011). The interconversion of UDP-arabinopyranose and UDP-arabinofuranose is indispensable for plant development in *Arabidopsis*. Plant Cell **23**:1373–1390.

Sankaranarayanan, S., Jamshed, M., and Samuel, M.A. (2015). Degradation of glyoxalase I in *Brassica napus* stigma leads to self-incompatibility response. Nat. Plants **1**:15185.

Stein, J.C., Howlett, B., Boyes, D.C., Nasrallah, M.E., and Nasrallah, J.B. (1991). Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleracea*. Proc. Natl. Acad. Sci. USA **88**:8816–8820.

Zafra, A., Rejón, J.D., Hiscock, S.J., and Alché, J.D.D. (2016). Patterns of ROS accumulation in the stigmas of angiosperms and visions into their multi-functionality in plant reproduction. Front. Plant Sci. **7**:1112.