Deciphering the Stigmatic Transcriptional Landscape of Compatible and Self-Incompatible Pollinations in *Brassica napus* Reveals a Rapid Stigma Senescence Response Following Compatible Pollination

Dear Editor,

Self-incompatibility (SI) is a genetic mechanism through which flowering plants prevent self-pollination to ensure outcrossing and genetic diversity. In *Brassica* sp., this mechanism is controlled by the self-incompatibility (S) locus, in which, the stigmatic 'S-locus receptor kinase (SRK)' recognizes the 'S-locus cysteine rich protein (SCR)' from the self-pollen to elicit an active rejection response. This results in blocking of compatibility factors from being delivered to the site of pollen attachment leading to self-pollen rejection (Chapman and Goring, 2010). In contrast, following recognition of compatible signals from the cross-pollen or compatible pollen (CP), the stigma releases its resources such as water and nutrients to the dry pollen so that the pollen tube can germinate and penetrate the stigmatic cuticle leading to successful fertilization. Thus, an incompatible or self-pollen is fully capable of eliciting a compatible response, but is actively rejected before compatible responses can occur. Following landing of self-pollen or cross-pollen on stigmas of *Brassica napus* (canola), there is a latent period of 30 min when signals are exchanged between the highly lipophilic pollen coat proteins and the stigmatic components. CP tubes can be observed to emerge between 30 and 90 min after initiation of this interaction. Given that stigmas control the outcome of pollen acceptance or rejection, deciphering the transcriptional changes during this latent period would reveal genes involved in compatible and self-incompatible responses.

As expected, when self-incompatible W1 canola stigmas were stained with aniline blue to observe pollen tubes, SI-pollinated stigmas lacked any pollen attachment or pollen tubes at 30 min and 6 h after pollination (Figure 1A, right panel). The weakly attached pollen without any positive interactions is washed away during the staining process. Following compatible pollination, although pollen attachment and pollen tubes could be observed at 6 h, at 30 min after pollination, no pollen attachment could be observed (Figure 1A, right panel). This is due to lack of complete adhesion and pollen tube germination at 30 min after pollination. These observations suggest that analyzing the transcriptome changes 0–30 min following SI and compatible pollinations would likely reveal genes that are triggered by pollen landing on the stigma and could represent genes that are required for promoting SI and compatible responses, respectively. To identify the genes that are differentially regulated by SI and compatible pollinations (Figure 1A, left panel), RNA extracted from self-incompatible W1 stigmas, pollinated with self-pollen or cross-pollen for either 15 or 30 min, were compared against RNA from unpollinated (UP) stigmas through transcriptome profiling, using the Agilent 4×44K *Brassica* Gene Expression Microarrays (G2519F). Following normalization, filtering based on *P*-values (<0.001) and then by two-fold up-regulation in at least one of the microarray experiments, we identified 621 genes that were differentially regulated. Clustering of these genes (Supplemental Table 1) clearly indicated strong up-regulation of multiple genes across all four treatments. This suggested to us that these were likely expressed pollen genes when SI and compatible pollinated stigmas were compared with UP stigmas that lacked any pollen. Utilizing the high sequence similarity between *Arabidopsis* and *Brassica*, we identified the orthologous *Arabidopsis* genes for the 621 canola genes to facilitate further bioinformatic analyses. Following filtering of the pollen genes from the differentially expressed genes in the microarray experiments (see Supplementary Data), the 621 genes were subdivided into stigma genes (287), pollen genes (181), and stigma–pollen genes (153) (Supplemental Table 2). Since the focus of this study was to identify stigmatic genes specifically regulated by SI and CP, we focused our attention on the 287 stigmatic genes. Clustering the stigmatic genes (Supplemental Table 3) revealed a clear pattern of changes between SI and compatible pollination.

Heat maps were generated for the subset of genes that were specifically induced or repressed in SI and compatible pollination (Figure 1B–1E and Supplemental Table 4).

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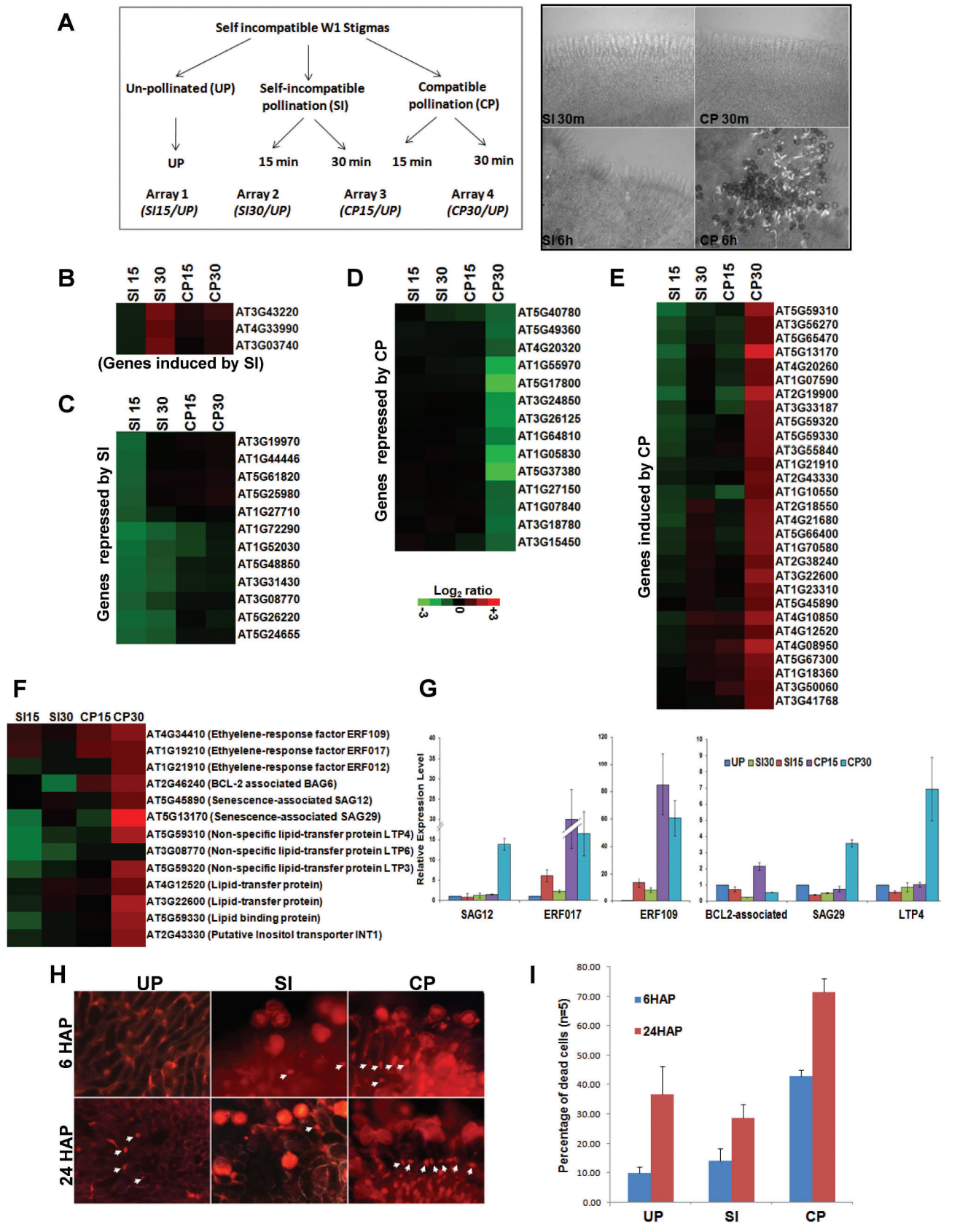

Figure 1. Transcriptome Comparison of *Brassica napus* Stigmas Following Compatible and Self-Incompatible Pollinations.

There were only three genes that displayed specific up-regulation following SI pollination (Figure 1B). These included AT3G43220/SAC3, a phosphoinositide phosphatase; AT4G33990, a pentatricopeptide repeat-containing protein; and AT3G03740, a BTB/POZ domain-containing protein that serves as a protein adaptor for cullin-based E3 ligase-mediated proteasomal degradation in plants. The major pathway through which SI functions is through ARC1 (U-box E3 ligase)-mediated proteasomal degradation of compatibility factors leading to pollen rejection (Samuel et al., 2008). Rapid induction of AT3G03740 would suggest that, once SI is activated through ARC1, the AT3G03740 could function to enhance the proteasomal degradation pathway to synergistically influence the SI response. The lack of up-regulation of many SI-specific genes following SI pollination is quite consistent with the proteomics study that reported only down-regulation of multiple proteins following SI without any differential up-regulation (Samuel et al., 2011). This observation further strengthens the hypothesis that most of the SI-specific events are posttranslational events so that the rejection response occurs at a faster pace than the compatible response. Out of the 12 genes that were specifically repressed following SI pollination (Figure 1C), AT1G72290, a Kunitz domain-containing protease (trypsin) inhibitor, was particularly interesting, since it showed rapid and consistent down-regulation at 15 and 30 min following SI pollination. Protease inhibitors are small proteins that interact with proteases and maintain the fully active proteases in a partially active or inactive state that can be activated by release of these inhibitors (Habib and Fazili, 2007). Following SI, a sudden burst of proteasomal activity and ubiquitination is known to occur (Stone et al., 2003). The Kunitz trypsin inhibitor could potentially maintain the proteasomal proteases at a low active state prior to pollination and, following self-pollen attachment, this inhibition could be relieved to potentiate a rapid induction of proteasomal activity.

Following compatible pollination, 14 genes were specifically down-regulated (Figure 1D). These included transcription factors (TF) AT3G24850, a DUF domain TF and MYB56 (AT5G17800), cell wall modifying enzyme beta-xylosidase (AT5G49360 (BXL1)) and AT1G05830 (ATX2), a histone–lysine N-methyltransferase that was down-regulated more than four-fold at 30 min following compatible pollination. Reduction of ATX2 could release the transcriptional inhibition imposed by the tightly wound chromatin resulting in a sudden burst of transcriptional activity following compatible pollination.

A number of genes (29) including MYB44 (AT5G67300), MYB77 (AT3G50060), and citric acid cycle-associated genes were strongly up-regulated following compatible pollination (Figure 1E and Supplemental Figure 1), indicating a system preparing itself for pollen tube penetration. When these 29 genes were subjected to gene ontology analysis using the Princeton GO Term Finder (http://go.princeton.edu/cgi-bin/GOTermFinder), these genes were enriched in genes for lipid transport (17.2%, *p*-value = 0.0002). The representation of genes involved in lipid-transfer and lipid binding (AT5G59310, AT5G59320, AT5G59330, and AT3G22600) is quite consistent with previous observations that have reported a role for long-chain lipids in pollen hydration. The identities of the genes involved in this process of adding lipids to the cuticular surface of the papillary cells, to form a hydraulic conduit for transport of water to the pollen, have remained unknown (Dickinson, 1995; Samuel et al., 2008). The identification of multiple genes involved in phosphoinositol metabolism or transport sheds light on a possible early role for phosphatidylinositol phosphates (PtInsPS) signaling, which have been previously implicated to have a stigmatic role in supporting pollen hydration (Chapman and Goring, 2011). The inositol transporter, INT1 (AT2G43330) (Supplemental Figure 1), and the plasma membrane-localized PcaP1 (AT4G20260), proposed to be involved in release of PtInsPS (Kato et al., 2010), were up-regulated by compatible pollination. Interestingly, following SI pollination, induction of PtIns-phosphatase (AT3G43220) was observed, suggesting that SI could likely negate the generation of the PtInsPS (IP3) required to induce calcium influx essential for compatible pollinations (Iwano et al., 2004).

Several senescence-associated genes were up-regulated following compatible pollination (Figure 1F and Supplemental Table 5). Senescence-associated SAG12 and SAG29, lipid-transfer proteins (LTP3 and LTP4), ethylene-responsive TF (ERF012, ERF017, and ERF109), BCL-2 athanogene 6 (BAG6), and inositol transporter 1 (INT1) have been shown to be induced under

(A) Flow chart showing the pollination treatments used for this study and the four different microarray experiments performed (left panel). Pollen attachment on stigmas after 30 min and 6 h of compatible and self-incompatible pollination (right panel).
(B) Heat map showing the genes up-regulated following incompatible pollination.
(C) Heat map showing genes down-regulated following incompatible pollination.
(D) Heat map showing genes down-regulated following compatible pollination.
(E) Heat map showing genes up-regulated following compatible pollination.
(F) Heat map showing the list of senescence-related genes up-regulated following compatible pollination.
(G) Validation of the CP-induced senescence genes through q-RT–PCR. Transcript levels are reported relative to the abundance in un-pollinated stigmas. Error bars are based on two biological replicates.
(H) Comparison of the extent of papillary cell death in un-pollinated, self-incompatible, and compatible stigmas after 6 and 24 h of pollination as visualized by propidium iodide staining (white arrows indicate nuclear staining).
(I) Bar graph comparing the percentage of papillary cell death in un-pollinated, self-incompatible, and compatible stigmas after 6 and 24 h of pollination ($n = 5$).

senescence responses (Gepstein et al., 2003). These genes were either down-regulated (SAG29, LTP3, 4, and 6) or had no noticeable differential expression after self-incompatible pollination, as evidenced by their expression in the transcriptome analysis and validation through qRT–PCR (Figure 1F and 1G). To examine whether compatible pollination induced a rapid senescence process in the papillary cells, we stained SI and compatible pollinated stigmas at various times after pollination with propidium iodide, followed by fluorescence microscopy. The observations indicated that compatible pollination induced a rapid senescence process with >40% papillary cells showing cell death at 6 h and >70% at 24 h, while UP and SI-pollinated stigmas displayed <40% and <30% dead cells, respectively, at 24 h (Figure 1H and 1I). Lack of cell death following SI pollination indicates that the stigma, despite being pollinated, prevents the senescence programming from occurring to stay receptive for the right mate.

Thus, our study has revealed that, within 30 min after landing on the stigmatic surface, self-pollen and cross-pollen trigger a unique transcriptional program that would culminate in either rejection or acceptance of the pollen, respectively. Identification of these genes unveils a number of previously unknown mechanisms through which pollination can be controlled by the stigmas.

SUPPLEMENTARY DATA

Supplementary Data are available at *Molecular Plant Online*.

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