Arch Mol Biol Genet. 2022;1(2):34-42.

Archives of Molecular Biology and Genetics | Short Communication

Actors of ROS Homeostasis in Stigmatic Cells Essential for Plant Reproduction

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Received date: February 04, 2022, **Accepted date:** March 08, 2022

Citation: Kodera C, Fobis-Loisy I. Actors of ROS Homeostasis in Stigmatic Cells Essential for Plant Reproduction. Arch Mol Biol Genet. 2022;1(2):34-42.

Abstract

Reactive oxygen species (ROS) play important roles during development and responses to external stimuli. In Brassicaceae, the stigma epidermis accumulates a large amount of ROS. Moreover, regulating the stigmatic ROS status is crucial for Self-incompatibility (SI) mechanisms, to ensure self-pollen rejection while promoting compatible pollen. Here, scanning our transcriptomic data in light of recent advances in the Brassicaceae SI system, we identified Class III peroxidases that are highly expressed in mature stigma and might regulate stigma ROS homeostasis. We also found two Receptor Like Kinases from the CYSTEINE-RICH RECEPTOR-LIKE KINASES family (CRK31 and CRK41) strongly upregulated upon incompatible pollination. We proposed that these two CRKs might be part of the ROS-mediated SI response and serve to connect pollen recognition and ROS accumulation.

Keywords: Pollination, Self-incompatibility, ROS, Receptor like kinase, Brassicaceae

Abbreviations: PM: Plasma Membrane; SI: Self-incompatibility; ROS: Reactive Oxygen Species; H_2O_2: Hydrogen Peroxide; FER: FERONIA; RALF: RAPID ALKALINIZATION FACTOR; RBOH: RESPIRATORY BURST OXIDASE HOMOLOG; PCP: POLLEN COAT PROTEIN; FC: Fold Change; RLK: Receptor Like Kinase; nFPKM: Normalized Fragments Per Kilobase of exon per Million reads mapped; HPCA1: HYDROGEN-PEROXIDE-INDUCED Ca^{2+} INCREASES; CRK: CYSTEINE-RICH RECEPTOR-LIKE KINASES. SRK: *S*-LOCUS RECEPTOR KINASE; SCR: *S*-LOCUS CYSTEINE-RICH PROTEIN

Introduction

How sexual partners choose each other is greatly dependent on organisms, but this choice reveals particularly critical in angiosperms with predominantly sessile lifestyle and hermaphroditic flowers that greatly favors self-fertilization. Self/non-self-recognition mechanisms, known as SI systems, have evolved to prevent self-fertilization, hence promoting genetic variability [1]. In the Brassicaceae family, self/non-self-discrimination occurs when the male gametophyte (pollen grain), lands at the receptive surface of the female organ (stigma epidermis of the pistil). Compatible pollen (non-self-pollen) hydrate and germinate a pollen tube that penetrates the stigma epidermis and navigates within the pistil to transport the male gametes towards the ovules for fertilization. By contrast, self-pollen (also named incompatible pollen) is recognized through the interaction of the stigma plasma membrane (PM)-localized *S*-LOCUS RECEPTOR KINASE (SRK) and its cognate peptide ligand from the pollen surface, named the *S*-LOCUS CYSTEINE RICH PROTEIN (SCR) [2]. This recognition event triggers activation of a signaling cascade in stigmatic cells culminating in self-pollen inhibition. Indeed, accurate communication is required to precisely coordinate the stigmatic response, to promote compatible pollen whereas ensuring self-pollen rejection.

A significant advance in the understanding of the molecular dialogue established between pollen grains and the stigmatic cells comes from two recent exciting studies. Liu et al. [3] identified an autocrine signaling pathway that controls basal production of ROS in mature Arabidopsis stigma. Two Receptor Like Kinases (RLKs) (FERONIA, FER and ANJEA, ANJ) belonging

to the CrRLK1L (CATHARANTHUS ROSEUS RECEPTOR LIKE KINASE 1-LIKE PROTEINS) family, expressed in the stigma, sense the stigmatic peptide ligand RAPID ALKALINIZATION FACTOR 33 (RALF33). Ligand recognition in turn activates the plant NADPH oxidase, RESPIRATORY BURST OXIDASE HOMOLOG D (RBOHD), a PM-localized ROS-producing enzyme. The authors also demonstrated that peptide ligands from the POLLEN COAT PROTEIN B-CLASS (PCP-Bs), present at the surface of the pollen grain, compete with RALF33 for FER/ANJ receptor binding, thereby reducing RBOH-dependent ROS production to facilitate compatible pollen hydration. Zhang et al. [4] confirmed the existence of a FER/RBOH signaling module responsible for the basal ROS production in the stigma of the SI species *Brassica rapa*. In addition, the authors demonstrated that this module mediated a rapid ROS increase upon self-pollination resulting in the early inhibition of the self-pollen.

Recently, we developed an experimental procedure, coupled with a bioinformatic analysis, to comprehensively unravel the dynamic events that occur both in the stigma and pollen grain following compatible and incompatible pollinations [5]. Here, we reexamine these transcriptomic data in light of the recent advances in the field, mainly focusing on redox regulatory network and receptor-ligand complexes.

Materials and Methods

Transcriptomic design

Detailed procedure is described in the original article [5]. Briefly, using two transgenic *Arabidopsis* lines with restored SI response [6], we performed a time-course experiment of pollination and sequenced mRNAs extracted from stigmas harvested immediately (zero time point, C0), 10 or 60 minutes after pollen addition. We took advantage of the Single Nucleotide Polymorphism existing between two distinct *Arabidopsis thaliana* accessions, to differentiate pollen and stigma transcripts. Differential expression analysis of the whole transcriptome was performed using DESeq2. Then, we analyzed the relative abundance of each transcript using normalized Fragments Per Kilo base of exon per Million reads mapped (nFPKM) and retained genes expressed in mature stigma or pollen (at zero time point), those with a nFPKM >1 (Supplementary Table 1). We examined the transcriptomic changes in response to compatible (C10 and C60) and incompatible (I10 and I60) pollinations using the Fold Change (FC) calculation and retained genes exclusively upregulated (FC>2, padj < 0.1) in one pollination condition compared to the other (compatible *vs* incompatible and vice versa), in stigma and pollen (Supplementary Table 2).

Gene list construction

A list of 323 *Arabidopsis* Redox genes was retrieved from Oliveira et al. [7] (Supplementary Table 1).

A list comprising 472 genes encoding Arabidopsis RLKs was retrieved from Lee and Goring [8]. From our bibliographic analysis, we found 11 additional putative RLKs. We analyzed these 11 sequences with the Simple Modular Architecture Research Tool; two of them do not have obvious transmembrane domain and one encodes a truncated WAK-like kinase with a very short extracellular domain. These three sequences were not included in the RLK list and we ended up with a list containing 480 RLK genes (Supplementary Table 1).

Lease and Walker [9,10] described around 1000 putative peptides in Arabidopsis. In these studies, to allow some tolerance, the upper size limit was fixed at 250 amino acids based on the largest known signaling molecule, the tomato systemin precursor. Since 2010, a large number of studies has described the role of plant peptides in cell-to-cell communication. Thus, to update the Lease and Walker peptide database, we compiled published data together with information from The Arabidopsis Information Resource (TAIR) to generate a list of 958 signaling peptide/small protein genes based on the same criteria as those used by Lease and Walker (Supplementary Table 1).

Identification of Redox, RLK and peptide genes in our transcriptomic data

We screened our stigma and pollen transcriptomes with the Redox, RLK and Peptide lists using the Excel VLOOKUP function and made a Venn diagrams (https://bioinfogp.cnb.csic.es/tools/venny/) to represent the results.

Results

Stigmatic redox regulatory network

ROS, such as hydrogen peroxide (H_2O_2) and superoxide anion, are chemical compounds formed from oxygen. They are toxic byproducts of all aerobic organisms but at the same time they function as signaling molecules. Plants have a complex redox system, which comprises 323 antioxidant and ROS-generating enzymes to maintain redox homeostasis [7]. To better characterize the stigmatic redox network, we screened our transcriptomic data from the mature stigma (at zero time point) with the list of 323 plant redox genes. As the stigma is the epidermal tissue of the pistil organ, we decided to compare its redox network with that of another epidermis, obtained from laser microdissected leaves (RNAseq data retrieved from Berkowitz et al., [11]). Interestingly similar number of genes are expressed in both epidermal tissues, 14,220 genes in stigma (nFPKM>1) and 13,707 in leaf (Transcripts Per Million, TPM>=1) (Supplementary Table 1). We found 160 redox genes expressed in stigma and 156 in leaf (Figure 1, Supplementary Table 2). Although the number of redox genes expressed in both epidermis is equivalent, we found considerably more redox genes among the upmost expressed genes in stigma (four genes among the top12, Figure 2). Interestingly, three of

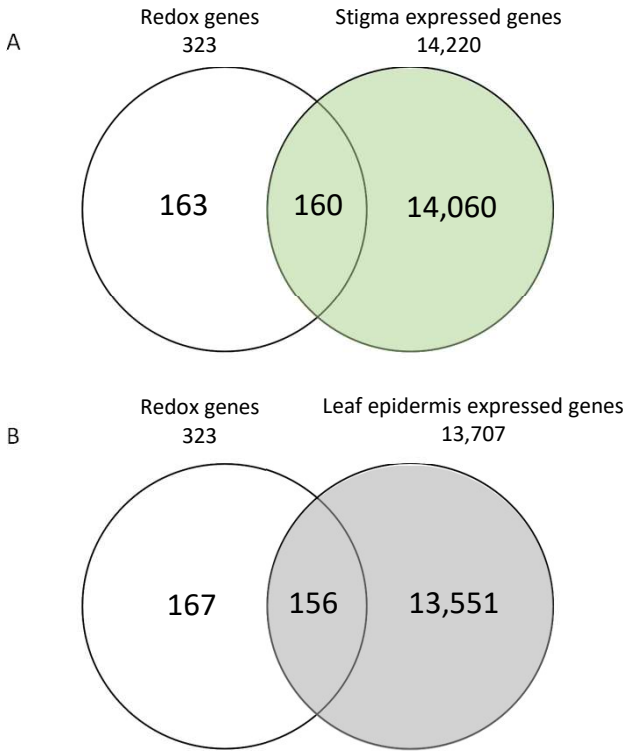


Figure 1: Redox genes expressed in mature stigma and leaf epidermis. (A, B) Venn diagrams showing the number of redox genes present in the lists of 14,220 genes expressed in mature stigma (at zero time point) **(A)** and 13,707 genes expressed in leaf epidermis (Berkowitz et al., 2021) **(B)**.

Top12 expressed genes in stigma			Top12 expressed genes in pollen			Top12 expressed genes in leaf epidermis		
gene ID	name	category	gene ID	name	category	gene ID	name	category
AT2G38540	LIPID TRANSFER PROTEIN 1	peptide	AT3G13400	SKU5 SIMILAR 13		AT1G67090	RIBULOSE BISPHOSPHATE CARBOXYLASE SMALL CHAIN 1A	
AT2G33850	E6-LIKE 1		AT1G02790	POLYGALACTURONASE 4		AT3G52700	hypothetical protein	
AT4G38770	PROLINE-RICH PROTEIN 4	peptide	AT2G47040	PECTIN METHYLESTERASE INHIBITOR		AT5G38410	RUBISCO SMALL SUBUNIT 3B	
AT5G02380	METALLOTHIONEIN 2B		AT3G28750	hypothetical protein		AT5G06043	hypothetical protein	
AT3G12000	S-LOCUS RELATED PROTEIN SLR1		AT1G61566	RAPID ALKALINIZATION FACTOR-LIKE 9	peptide	AT5G38420	RUBISCO SMALL SUBUNIT 2B	
AT4G11290	**CLASSIII-PEROXIDASE**	redox	AT1G61563	RAPID ALKALINIZATION FACTOR-LIKE 8	peptide	AT5G38430	RUBISCO SMALL SUBUNIT 1B	
AT3G26520	TONOPLAST INTRINSIC PROTEIN 2		AT3G01240	SPLICING REGULATORY GLUTAMINE		AT1G79040	PHOTOSYSTEM II SUBUNIT	
AT5G24780	VEGETATIVE STORAGE PROTEIN 1		AT3G07820	PECTIN LYASE-LIKE PROTEIN		AT2G45180	DISEASE RELATED NONSPECIFIC LIPID TRANSFER PROTEIN 1	peptide
AT5G20230	BLUE-COPPER-BINDING PROTEIN	redox	AT3G01270	PECTATE LYASE PROTEIN		AT2G07671	ATP SYNTHASE SUBUNIT C PROTEIN	
AT1G44970	**CLASSIII-PEROXIDASE**	redox	AT5G45880	POLLEN OLE E 1 ALLERGEN	peptide	AT2G38540	LIPID TRANSFER PROTEIN 1	peptide
AT2G34810	FAD-BINDING BERBERINE PROTEIN		AT3G57690	ARABINOGALACTAN PROTEIN 23		AT1G60950	FERREDOXIN 2	
AT5G19880	**CLASSIII-PEROXIDASE**	redox	AT5G19580	GLYOXAL OXIDASE-RELATED PROTEIN		AT2G05520	ARABIDOPSIS GLYCINE RICH PROTEIN 3	

Figure 2: **Top12 expressed genes in stigma, pollen and leaf epidermis**. Genes are classified from the largest to the smallest expression level and the top12 genes in each tissue are shown.

them (*PER39*, 2,528.79 nFPKM; *PER9*, 1,625.48 nFPKM; *PER58*, 1,569.97 nFPKM; in comparison the highest stigma-expressed gene has an abundance of 13,047.31 nFPKM, Supplementary Table 2) belong to the same gene family, the plant specific class III peroxidase family.

Plant PM-bound RBOHs are responsible for the major source of ROS production in the apoplast [12]. In Arabidopsis, there are 10 RBOH isoforms, referred to as RBOHA to RBOHJ [13]. We found *RBOHD* to be the highest expressed *RBOH* gene in mature stigma with a relative abundance of 340.48 (Supplementary Table 2). Two additional *RBOH* genes were expressed in mature stigma: *RBOHF* (16.70 nFPKM) and *RBOHC* (1.27 nFPKM). None of these three *RBOH* genes was expressed in mature pollen, whereas two other RBOH isoforms, *RBOHH* (45.99 nFPKM) and *RBOHJ* (10.56 nFPKM) were identified in our pollen transcriptome (Supplementary Table 2). These data are in accordance with previous studies where the *RBOHD* was identified as the most abundantly expressed *RBOH* gene in the stigma [3] and *RBOHH* and *RBOHJ* were found as expressed in mature tricellular pollen [14,15].

Identification of candidate RLKs for putative stigmatic functions

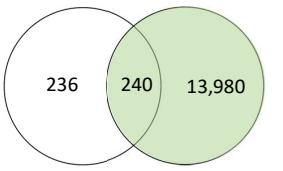
240 *RLK* genes were found within the 14,220 stigma-expressed genes and two *RLKs* linked to ROS sensing and signaling were among the highest *RLK* expressed genes (Supplementary Table 2, Figure 3): *FER* (180.02 nFPKM) and *HYDROGEN-PEROXIDE-INDUCED Ca^{2+} INCREASES* (*HPCA1*, 72.4 nFPKM).

We found 26 *RLK* genes specifically upregulated upon compatible pollination, whereas only seven were induced after the incompatible reaction (Supplementary Table 3). Interestingly, among the seven incompatible-induced *RLK* genes, two belong to the *CYSTEINE-RICH RECEPTOR-LIKE KINASES* family (*CRK31* and *CRK41*), whose several members are involved in the regulation of defense reactions and ROS signaling [16]. Both *CRK31 and CRK41* were strongly upregulated upon incompatible pollination with FCs around nine (Figure 3). This FC corresponds to a substantial induction as the highest FC for stigmatic genes is 12 and only two genes exhibited a greater FC upon pollination than those of the two *CRKs*. It is worth noting that neither *FER* nor *HPCA1* were upregulated upon pollination (Supplementary Table 3).

Signaling peptide and pollen-stigma interaction

We generated a list of 958 peptide/small protein genes (Supplementary Table 1). Their expression products are categorized into two classes: secreted and non-secreted peptides based on the presence of a N-terminal secretory signal sequence (Figure 4). Secreted peptides can be further divided into two major classes: CYSTEINE-RICH PEPTIDES that contain 4–16 cysteine residues necessary for the formation of intramolecular disulfide bonds, and post-translationally modified peptides that contain at least one modification such as tyrosine sulfation, proline hydroxylation, or hydroxyproline arabinosylation. Cell-to-cell signaling is mediated largely by secreted peptides, but there are also evidences that non-secreted peptides, released from damaged cells, or acting intracellularly, could support signaling functions. Each peptide

A



Top 12 RLK genes in mature stigma	
gene ID	name
AT2G41820	PXY/TDR-CORRELATED 3
AT3G51550	**FERONIA**
AT1G48480	RECEPTOR-LIKE KINASE 1
AT4G39400	BRASSINOSTEROID INSENSITIVE 1
AT1G66150	TRANSMEMBRANE KINASE 1
AT4G22730	LRR protein kinase family
AT5G49760	**HYDROGEN PEROXIDE SENSOR**
AT1G53440	LRR protein kinase family
AT1G73080	PEP1 RECEPTOR 1
AT4G00340	RECEPTOR-LIKE PROTEIN KINASE 4
AT5G56890	Protein kinase superfamily
AT2G02780	LRR protein kinase family

B

Top12 upregulated RLK genes after compatible pollination			7 upregulated RLK genes after incompatible pollination		
gene ID	name	FC C/C0	gene ID	name	FC I/C0
AT1G11050	Protein kinase superfamily	3,7	AT4G11470	**CYSTEINE-RICH RLK 31**	9
AT2G23770	LYSM RECEPTOR-LIKE KINASE	3,2	AT4G00970	**CYSTEINE-RICH RLK 41**	8,7
AT1G51820	STRESS INDUCED FACTOR 4	3,1	AT5G35370	S-locus lectin protein kinase	2,4
AT1G74360	NEMATODE-INDUCED LRR-RLK 1	3,1	AT1G18390	LEAF RUST 10 DISEASE-RESISTANCE LOCUS RECEPTOR- LIKE PROTEIN KINASE-LIKE 1.2	2,4
AT1G73080	PEP1 RECEPTOR 1	2,8	AT1G69990	BAK1-INTERACTING RECEPTOR-LIKE KINASE 4	2,2
AT1G69270	RECEPTOR-LIKE PROTEIN KINASE 1	2,8	AT5G20480	EF-TU RECEPTOR	2,2
AT1G14390	LRR protein kinase	2,8	AT3G59700	L-TYPE LECTIN RECEPTOR KINASE V.5,	2,1
AT1G68690	PROLINE-RICH EXTENSIN-LIKE RECEPTOR KINASE 9	2,7			
AT1G34420	LRR protein kinase	2,5			
AT2G48010	RECEPTOR-LIKE KINASE IN IN FLOWERS 3	2,5			
AT3G53810	L-TYPE LECTIN RECEPTOR KINASE IV.2	2,4			
AT1G21210	WALL ASSOCIATED KINASE 4	2,3			

Figure 3: RLK genes expressed in mature stigma and upregulated upon pollination. (A) Venn diagrams showing the number of RLK genes present in the lists of 14,220 genes expressed in mature stigma. RLK genes are classified from the largest to the smallest expression level and the top12 genes are shown. **(B)** Exclusively upregulated RLK genes in compatibility **(C)** are those with a FC>2 (related to the zero-time point, C0) in response to compatible pollination and a FC<2 upon incompatible pollination, and vise et versa for incompatibility (I). The 12 uppermost induced RLK genes in response to compatible response, sorted by FC from the largest to the smallest, are shown. Upon incompatible pollination, only seven RLK genes are exclusively upregulated.

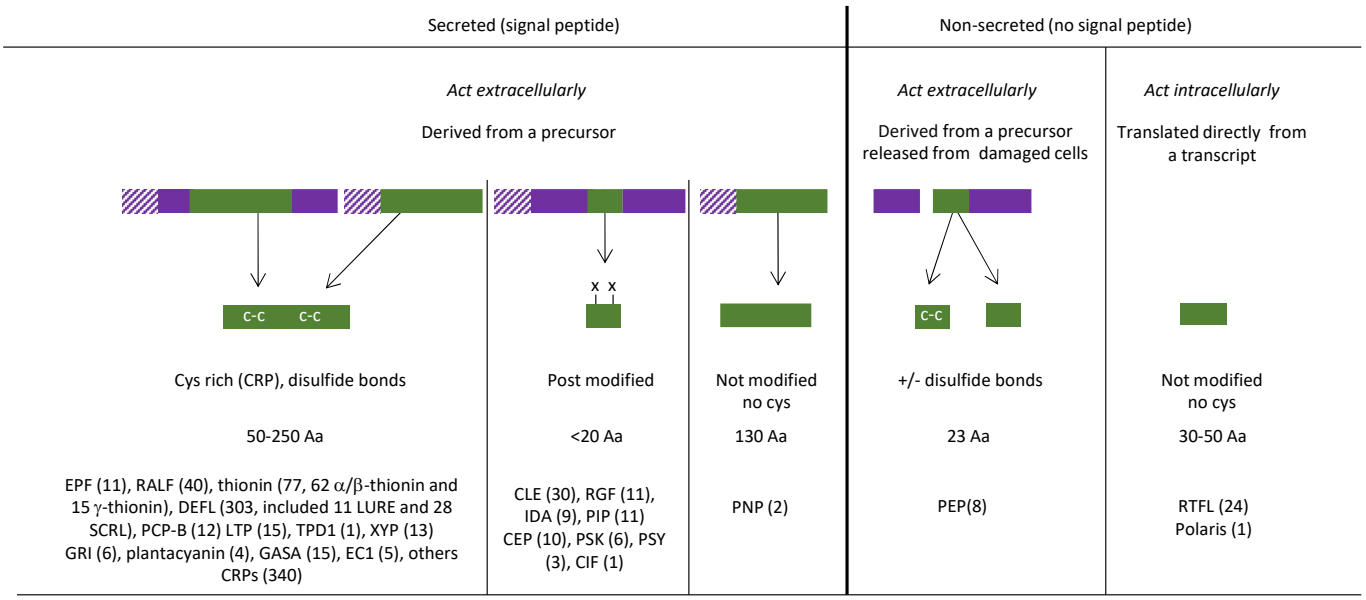


Figure 4: The diversity of plant signaling peptides/small proteins (adapted from Matsubayashi et al. [18]; Tavormina et al. [19], Gancheva et al., 2019). Plant peptides (green boxes) can be categorized into two major classes: secreted peptides and non-secreted peptides according to the presence or not of a N-terminal signal peptide (hatched purple box) respectively. They are synthesized from precursor proteins or directly translated from a transcript. Mature peptides can derive from (i) preprotein precursor upon removal of the signal peptide or (ii) proprotein precursor upon proteolytic processing (purple box). The proprotein can also harbor a signal peptide. Peptides can undergo intramolecular disulfide bonds (C-C) or posttranslational modifications (X). The typical size of mature peptides is indicated, Aa stands for amino acid, cys for cysteine residue. Representative peptides from each class are presented. Numbers in the brackets correspond to the number of genes in each family we found in our analysis. EPF (EPIDERMAL PATTERNING FACTOR, Takat et al., 2013), RALF (RAPID ALKALINIZATION FACTORS, Campbell and Turner, 2017), thionin are divided into the families of α/β-thionins (Almaghrabi et al., 2019) and γ-thionins also named plant DEFENSIN (DEF, Silverstein et al., 2005), DEFENSIN-LIKE (DEFL, Silverstein et al., 2005), SCRL (*S* LOCUS CYSTEINE-RICH-PROTEIN-LIKE, have homology to the male component of the self-incompatibility response in *Brassica*, Vanoosthuyse et al., 2001), LURE (Zhong et al., 2019), *PCP-B* (homologue of Brassica Pollen Coat Protein gene, Wang et al., 2016), LTP (LIPID TRANSFER PROTEIN also named STIGMA/STYLE CYSTEINE-RICH ADHESIN, SCA, Chae et al., 2010), ESF (EMBRYO SURROUNDING FACTOR, Costa et al., 2014; family members were retrieved from TAIR). TPD1 (TAPETUM DETERMINANT 1, Yang et al., 2003), XYP (XYLOGEN-TYPE, Kobayashi et al., 2011), GRI (GRI REAPER also known as STIG1-like Arabidopsis proteins, Wrzaczeka et al., 2009), plantacyanin (Dong et al., 2005, family members were retrieved from TAIR), GASA (plant-specific GIBBERELLIC ACID-STIMULATED ARABIDOPSIS also known as SNAKINS, Roxrud et al., 2007), EC1 (EGG CELL 1, Sprunck et al., 2012), others CYSTEINE RICH PEPTIDES (CRP, Silverstein et al., 2005), CLE (CLAVATA3/ESR, Strabala et al., 2006), RGF (ROOT GROWTH FACTOR also known as CLE-like, Meng et al., 2012), IDA (INFLORESCENCE DEFICIENT IN ABSCISSION, Vie et al., 2015), PIP (PAMP INDUCIBLE-SECRETED PEPTIDE, Vie et al., 2015), CEP (*C-TERMINALLY ENCODED PEPTIDE*, Delay et al., 2013), PSK (PHYTOSULFOKINE, Sauter, 2015), PSY (PEPTIDE CONTAINING SULFATED TYROSINE, Amano et al., 2007, family members were retrieved from TAIR), CIF (CAPARIAN STRIP INTERGRITY FACTOR 1, Nakayama et al., 2017), PNP (PLANT NATRIURETIC PEPTIDES, Turkey et al., 2014), PEP (ARABIDOPSIS THALIANA PEPTIDE, Bartel et al., 2013), RTFL (ROTUNDIFOLIA FOUR-LIKE, Guo et al., 2015), POLARIS (Chilley et al., 2006).

category has been well documented in several reviews [17-20].

We identified 124 and 54 peptide genes in mature stigma and pollen, respectively (Figure 5, Supplementary Table 2). Based on database screening and quantitative RT-PCR analysis, Liu et al. [3] determined that *RALF33*, a FER ligand implicated in ROS production, was expressed in stigma. Likewise, we found *RALF33* (96.91 nFPKM) to be the second highest expressed *RALF* gene in the stigma, *RALF8* (109.46 nFPKM) being the upmost expressed one. We identified a multitude of *RALF* genes expressed in pollen. *RALF9* (10,273.85 nFPKM) and *RALF8* (9,471.26 nFPKM) were the fifth and sixth upmost expressed pollen genes respectively (Supplementary Table 2). In addition to *RALF8* and *RALF9*, *RALF26* (2,049.09 nFPKM), *RALF4* (1,717.38 nFPKM) and *RALF19* (1,484.03 nFPKM) were among the top12 expressed peptide genes in pollen (Figure 5, Supplementary Table 2). *RALF13*, *RALF36*, *RALF6* and *RALF7* were upregulated upon compatible pollination (Supplementary Table 3). Although a function in reproduction has been described for two of them (*RALF4* and *RALF19*), the role of other pollen *RALFs* has not been reported yet.

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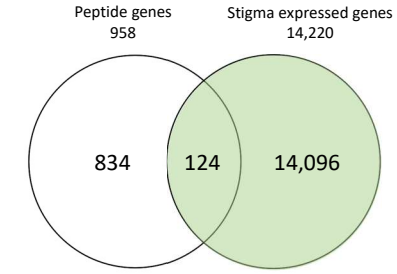

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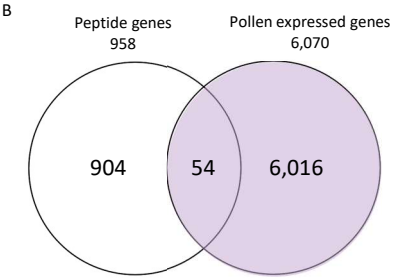

C

Top12 peptide genes in mature stigma		Top12 peptide genes in mature pollen	
gene ID	name	gene ID	name
AT2G38540	LIPID TRANSFER PROTEIN 1	AT1G61566	**RAPID ALKALINIZATION FACTOR-like 9**
AT4G38770	PROLINE-RICH PROTEIN 4	AT1G61563	**RAPID ALKALINIZATION FACTOR-like 8**
AT1G53130	STIGMA-SPECIFIC STIG1 PROTEIN	AT5G45880	POLLEN OLE E 1 ALLERGEN
AT4G21620	GLYCINE-RICH PROTEIN	AT4G18596	POLLEN OLE E 1 ALLERGEN
AT1G78040	POLLEN OLE E 1 ALLERGEN	AT5G26692	PLANT THIONIN FAMILY PROTEIN
AT1G55260	SEED STORAGE 2S ALBUMIN PROTEIN	AT3G25170	**RAPID ALKALINIZATION FACTOR-LIKE 26**
AT5G59310	LIPID TRANSFER PROTEIN 4	AT2G02140	LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 72
AT1G05850	CHITINASE-LIKE PROTEIN 1	AT1G28270	**RAPID ALKALINIZATION FACTOR-LIKE 4**
AT5G14920	A-STIMULATED IN ARABIDOPSIS 14	AT1G29140	POLLEN OLE E 1 ALLERGE
AT3G43720	SEED STORAGE 2S ALBUMIN PROTEIN	AT2G33775	**RAPID ALKALINIZATION FACTOR-like 19**
AT1G75750	GAST1 PROTEIN HOMOLOG 1	AT5G26717	PLANT THIONIN FAMILY PROTEIN
AT1G48750	2S ALBUMIN SUPERFAMILY PROTEIN	AT4G19038	LOW-MOLECULAR-WEIGHT CYSTEINE-RICH 15

Figure 5: **Peptide genes expressed in stigma and pollen.** (A, B) Venn diagrams showing the number of peptide genes present in the lists of 14,220 genes expressed in mature stigma (A) and 6,070 genes expressed in pollen (B). Peptide genes are classified from the largest to the smallest expression level and the top12 genes are shown.

Pollen peptides from the PCP B-class regulate the establishment of pollen-stigma compatibility through interaction with the FER receptor to reduce stigma ROS [3,21]. RNA-gel blot analysis and *in situ* hybridization showed that four *A. thaliana PCP-B* encoding genes are expressed in pollen at late stage of development. Surprisingly, we did not detect any expression of the *PCP B-class* genes (including the 12 *PCP-B-like* genes) in our pollen transcriptomes at zero time point or after pollination (Supplementary Tables 2 and 3).

Discussion

Maintenance of redox homeostasis in stigmatic cells

Class III peroxidases belong to a large multigenic family of 73 members in Arabidopsis [22], which all possess a signal peptide targeting the proteins into the secretory pathway [23]. Eight class III peroxidases are predicted to be PM-localized [23], among them we found the stigma-expressed gene *PER58*. Class III peroxidases, as other peroxidases, catalyze the oxidation of a variety of substrates by consuming hydrogen peroxide (H_2O_2), and indeed participate in the detoxification of ROS. It is now well established that stigma from angiosperms accumulate a large amount of ROS, believed to be associated with protection of the reproductive structures against pathogen [3,24,25]. Considering the activity of class III peroxidases in ROS detoxification, we may postulate that the three highly expressed isoforms, *PER9*, *PER39* and *PER58*, participate in protecting the stigmatic cells against oxidative damages caused by ROS excess. However, depending on the oxidized substrate, class III peroxidases can also provide ROS molecules from H_2O_2 and thus participate in the generation of ROS. Indeed, a ROS burst triggers upon pathogen attack is dependent on both the RBOHD enzyme and two *Arabidopsis* class III peroxidases, PER33 and PER34 [26]. Thereby, we may alternatively postulate that the three class III peroxidases highly expressed in the mature stigma, contribute to the basal level of stigmatic ROS in collaboration with the stigma-expressed *RBOHD*. Likewise, *PER9*, *PER39* and *PER58* might be part of the redox network responsive for the rapid and high induction of ROS levels to reject self-pollen. It is still not clear yet what are the determining factors that control peroxidases activity either as H_2O_2 scavengers or ROS producers. Understanding the role of class III peroxidases in stigma function and elucidating how their opposite activities are coordinated remains to be investigated.

ROS burst in SI response depends on RLK signaling

We found two *RLKs* (*HPCA1* and *FER*) linked to ROS sensing and signaling among the highest expressed *RLK* genes in stigma. HPCA1 belongs to the Leucine Rich Repeat subfamily and harbors a unique extracellular domain containing two cysteine pairs named the hydrogen peroxide domain. Activation of HPCA1 by H_2O_2 occurs via covalent modification of the cysteine residues, which leads to HPCA1 autophosphorylation and subsequently activation of Ca^{2+} channels in guard cells [27]. Interestingly, self-pollination in SI Brassicaceae plants induces an increase in cytoplasmic Ca^{2+} in papilla cells mediated by GLUTAMATE-GATED-Ca^{2+} channels [28]. FER has

emerged as a multifunctional regulator of diverse biological processes such as fertilization, cell growth, plant immunity, mechano-sensing and many of these processes require ROS production [29]. FER has two extracellular carbohydrate-binding (lectin) domains and, in addition to sense peptide ligands from the RALF and PCP-B families, FER binds to pectin wall components [30]. FER maintains a high ROS environment in stigmatic cells and stimulates ROS overproduction that allows rejection of self-pollen [3,4]. Similarly, FER is required for high ROS production in response to the bacterial flagellin. Flagellin induces association of its cognate RLK, FLAGELLIN SENSING 2 (FLS2), with the co-receptor BRASSINOSTEROID INSENSITIVE 1–ASSOCIATED RECEPTOR KINASE 1 (BAK1); formation of this immune complex is FER-dependent [31] and triggers activation of the RBOHD enzyme that enhances ROS production [32].

We identified two *RLK* genes (*CRK31* and *CRK41*) highly and specifically upregulated in the stigma after incompatible pollination. The extracellular domain of CRKs (44 family members, [33]) contains two cysteine-rich domains that correspond to DUF26 motifs (Cys-X_8-Cys-X_2-Cys). The molecular function of the CRK ectodomain remains unknown, it could either bind a ligand or be the target for redox regulation through cysteine modifications. Several studies highlighted the central role of CRKs in both biotic and abiotic stress responses. Recently, Kimura and collaborators [35] showed that CRK2 interacts and directly phosphorylates the plant RBOHD oxidase to generate a ROS burst essential to counteract pathogen infection. CRK36 also regulates ROS production interacting with and phosphorylating the RECEPTOR-LIKE CYTOPLASMIC KINASE *BOTRYTIS*-INDUCED KINASE (BIK1), a downstream effector of FLS2, that phosphorylates RBOHD [32,35,36]. In Addition, several CRKs associate with the FLS2 immune complex [37,38] suggesting that CRKs might act in concert with other RLKs. Beside their function in immune responses, CRKs have also been implicated in drought and salt tolerances [39,40].

In the Brassicaceae, self-pollen rejection clearly depends on the activation of a RBOH-dependent ROS production [4]. Moreover, the specificity of the SI response relies on the recognition of the incompatible pollen by the highly polymorphic stigmatic receptor SRK. It is tempting to speculate that cell surface ROS sensors we highlighted in this study, HPCA1 and/or CRK31/CRK41, might be part of the ROS-mediated SI response and serve to connect pollen recognition and ROS burst. In a first scenario, we propose that CRK31/CRK41 are phosphorylated by SRK following SCR perception. Activated CRKs then stimulate ROS production, by phosphorylating, directly or indirectly, RBOH enzymes. In a second scenario, SCR-SRK interaction triggers an initial ROS production in the apoplast. Apoplastic ROS are then sensed by HPCA1, CRK31 and/or CRK41 that in turn phosphorylate RBOHs, leading to a boost in ROS levels responsible for self-pollen inhibition.

Conclusion

Our work identified potential new molecular players to deeper understand the dialogue between the pollen grains and the stigmatic epidermis. Class III peroxidases might fine-tune ROS levels at the stigma surface and hence play an essential role in controlling ROS homeostasis in the mature stigma and/or mediating pollen discrimination during SI response. Self-pollen recognition relies on the specific interaction between the two highly polymorphic SRK and SCR proteins. A major question is how this initial step is connected to ROS production? We identified two RLKs (CRK31 and CRK41) highly induced upon incompatible pollination, known to activate RBOH enzymes and interact with other RLKs, as suitable candidates to link initial SRK activation and ROS burst during SI response. Whether SRK directly phosphorylates and activates CRK31/CRK41 and how FER fits in this signaling pathway remain elusive and required further investigations.

Acknowledgement

We thank Dr. Thierry Gaude for critical reading of the manuscript. This work was supported by the Centre National de la Recherche Scientifique and grant ANR-14-CE11-0021

Authors' Contributions

CK was responsible for all experiments and analysis performed in the original study. IFL designed the study, and performed additional analysis presented in this manuscript. CK and IFL wrote the manuscript and approved the final manuscript.

Availability of Data and Materials

All the generated data are included in this article

Competing Interests

The Authors declare that they have no competing interests.

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