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Transcriptional Characteristics and Differences in Arabidopsis Stigmatic Papilla Cells Pre- and Post-Pollination

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Regular Paper

Pollination is an important early step in sexual plant reproduction. In *Arabidopsis thaliana*, sequential pollination events, from pollen adhesion onto the stigma surface to pollen tube germination and elongation, occur on the stigmatic papilla cells. Following successful completion of these events, the pollen tube penetrates the stigma and finally fertilizes a female gametophyte. The pollination events are thought to be initiated and regulated by interactions between papilla cells and pollen. Here, we report the characterization of gene expression profiles of unpollinated (UP), compatible pollinated (CP) and incompatible pollinated (IP) papilla cells in *A. thaliana*. Based on cell type-specific transcriptome analysis from a combination of laser microdissection and RNA sequencing, 15,475, 17,360 and 16,918 genes were identified as expressed in UP, CP and IP papilla cells, respectively, and, of these, 14,392 genes were present in all three data sets. Differentially expressed gene (DEG) analyses identified 147 and 71 genes up-regulated in CP and IP papilla cells, respectively, and 115 and 46 genes down-regulated. Gene Ontology and metabolic pathway analyses revealed that papilla cells play an active role as the female reproductive component in pollination, particularly in information exchange, signal transduction, internal physiological changes and external morphological modification. This study provides fundamental information on the molecular mechanisms involved in pollination in papilla cells, furthering our understanding of the reproductive role of papilla cells.

Keywords: *Arabidopsis thaliana* • Laser microdissection • Papilla cell • Pollination • RNA sequencing • Transcriptome.

Abbreviations: ARC1, arm repeat containing 1; CP, compatible pollinated; DEG, differentially expressed gene; GO, Gene Ontology; IP, incompatible pollinated; KEGG, Kyoto Encyclopaedia of Genes and Genomes; KO, KEGG Orthology; LM, laser microdissection; RIN, RNA integrity number; RNA-seq, RNA sequencing; RPKM, reads per kilobase of exon per million mapped reads; SI, self-incompatibility; SRK, *S*-locus receptor kinase; UP, unpollinated.

Introduction

In angiosperms, sexual reproduction comprises a complex series of events, regulated by multiple processes, which must be completed effectively for successful seed production. When mature pollen grains land on a stigma, an adhesive interaction between pollen and stigma is initiated, and strong adhesion is established by formation of a structure called the foot (Gaude and Dumas 1984, Dickinson 1995). After this, pollen is recognized, and accepted or rejected by pollen recognition systems, such as self-incompatibility (SI) and cross-compatibility. Accepted pollen rapidly starts hydration by receiving water and resources for germination and pollen tube elongation from the pistil. The pollen tube precisely penetrates, elongates and arrives at the vicinity of the ovule by growth through the stigma, style and ovarian transmitting tract. The tube tip next invades synergid cells by following the pollen tube guidance signal (Cheung et al. 1995, Franklin-Tong 1999, Lord 2000, Shimizu and Okada 2000, Takeuchi and Higashiyama 2011). Finally, two sperm cells are released from the pollen tube tip, which fertilize an egg cell and central cell, respectively, a process termed double fertilization (Dickinson and Elleman 1994, Stephenson et al. 1997).

Pollination is an important initial step for successful sexual reproduction in plants. Brassicaceae species have a dry type of stigma, and pollination events occur on the stigmatic surface, i.e. on the papilla cell (Heslop-Harrison and Shivanna 1977). The papilla cell is a large single cell with two layers of cell wall, a cuticle and a pectocellulosic layer (Elleman et al. 1988, Elleman et al. 1992). Pollen recognition, pollen hydration and pollen tube guidance to the stigma are thought to be regulated by interactions between papilla cells and pollen. Pollen recognition in many Brassicaceae species involves a sporophytic SI system. SI is a sophisticated system for pollen selectivity to prevent self-fertilization and is genetically controlled by a single, highly polymorphic *S* locus (Bateman 1995). *S*-locus receptor kinase (SRK) and *S*-locus protein 11 (SP11)/*S*-locus cysteine-rich protein (SCR) have been identified as the female and male

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S-determinant factors, respectively (Stein et al. 1991, Schopfer et al. 1999, Takasaki et al. 2000, Takayama et al. 2000). In addition, *M*-locus receptor kinase (MLPK) and arm repeat containing 1 (ARC1) have been identified as downstream signaling factors that interact with SRK (Gu et al. 1998, Stone et al. 1999, Stone et al. 2003, Murase et al. 2004, Kakita et al. 2007a, Kakita et al. 2007b). Exo70A1, a subunit of the exocyst complex, is a target of ARC1 and has been identified as a mediator of water transport to pollen from the papilla via vesicle trafficking (Samuel et al. 2009). Prevention of pollen hydration and inhibition of pollen tube penetration have been observed to occur in the *Brassica* SI response (Zuberi and Dickinson 1985, Dickinson, 1995). Recently, it has been reported that pollen hydration is regulated by a balanced combination of multiple components of hydration, dehydration and rehydration, which was identified by time-series observations of pollen behavior on pollination (Hiroi et al. 2013). Thus, pollen hydration is controlled by a complex water transport system in the papilla cells, and vacuolar dynamics in the papilla have been predicted to be involved in water transport for pollen hydration (Iwano et al. 2007). After pollen hydration, the germinating pollen tube precisely penetrates the papilla cell, an event thought to be regulated by its own cell wall-degrading enzymes, triggering tube elongation between cell walls of the papilla cell. A plantacyanin, belongoing to a subfamily of blue copper proteins, has been identified as a regulator of in vitro pollen tube orientation in lily (Kim et al. 2003), and pollen tube guidance at papilla cells is disrupted by overexpression of plantacyanin in *Arabidopsis thaliana* (Dong et al. 2005).

Although many of the events in pollination and the relevant expressed genes have been identified (reviewed in Suwabe et al. 2010, Watanabe et al. 2012), it is still not possible to describe fully the overall molecular systems involved in pollination, and the specific molecular components are also largely unclear. To improve this situation, transcriptome analysis is proposed to be an effective approach for establishing a knowledge base to understand molecular mechanisms and factors affected in pollination. Recently, we established a cell type-specific transcriptome system in Brassicaceae papilla cells by a combination of laser microdissection (LM) and RNA sequencing (RNA-seq) (Osaka et al. 2013). This method is widely applicable to various conditions and tissues, including steps in pollination, and thus functional analysis at the transcriptional level in papilla cells has the potential to provide a basis for understanding molecular systems during pollination.

A. thaliana is a self-compatible, predominantly selfing species, belonging to the Brassicaceae. From a comparative genomic analysis with the outcrossing species *A. lyrata* and *A. halleri*, close relatives of *A. thaliana*, the genetic basis of loss of SI has been revealed (Kusaba et al. 2001, Shimizu et al. 2008). By ecological and molecular analyses, pseudogenization of the male *S*-determinant gene *SCR* has been proven to be the first mutation conferring self-compatibility of *A. thaliana*, and it is nearly fixed in geographically wide European accessions (Tsuchimatsu et al. 2010). Indeed, an experimental reversal of SI is successful by restoration of *SCR*, on particular *A. thaliana* accessions. By taking advantage of this, in this study we report the characterization of gene expression profiles in unpollinated (UP), compatible pollinated (CP) and incompatible pollinated (IP) papilla cells in *A. thaliana* by LM–RNA-seq analysis. We used these papilla cell-specific gene expression profiles, acquired pre- and post-pollination, to investigate the molecular systems and factors involved in pollination. By bioinformatics analysis, the biological characteristics and differences in papilla cells pre- and post-pollination, and between compatible and incompatible pollination, were also examined.

Results and Discussion

RNA sequencing analysis

To establish the data sets of genes expressed in papilla cells pre- and post-pollination, total RNA was extracted from 68–97 sections of UP, CP and IP papilla cells. The quality of these samples for RNA-seq was confirmed to be acceptable by RNA integrity number (RIN), a quality value for total RNA on a scale from 1 to 10; the values were 7.6, 6.1 and 6.2 for UP, CP and IP papilla cells, respectively (**Supplementary Fig. S1**). In plants, RNA with a quality value of more than RIN = 6 is acceptable for gene expression analysis (Takahashi et al. 2010, Osaka et al. 2013). After linear amplification of mRNAs obtained from extracted total RNA, fragment libraries were sequenced on an Ion Proton platform. As a result of RNA sequencing, 74,572,833, 77,704,982 and 72,870,933 raw reads were obtained from UP, CP and IP papilla cells, respectively, and 90.15, 92.12 and 86.97% of these were mapped to the Arabidopsis reference genome by aligning with raw reads to *A. thaliana* genome sequences (TAIR10) (**Table 1**). Expected genes were calculated from mapped reads, and when there were ≧10 reads for at least one transcript isoform they were defined as expressed genes. As a result, 15,955, 18,057 and 17,533 genes were found to be expressed in UP, CP and IP papilla cells, respectively. Expressed genes were further classified into protein-coding genes, pseudogenes, transposable element genes and non-coding RNAs, and we regarded both protein-coding genes and pseudogenes as genes expressed in papilla cells, because whether a particular gene is functional or not can depend on the ecotype or line, e.g. *SRK* acts as a pseudogene in Col-0 but as a functional gene in Old-1 (Shimizu et al. 2008, Tsuchimatsu et al. 2010). Consequently, 15,475, 17,360 and 16,918 genes were identified as papilla cell-expressed genes in

Table 1 Results of RNA sequencing in unpollinated, compatible pollinated and incompatible pollinated papilla cells

Type of papilla cell	Total reads	Mapped reads to the genome	Genes
Unpollinated	74,572,833	67,223,751 90.15%[a]	15,955 47.48%[b]
Compatible pollinated	77,704,982	71,581,421 92.12%[a]	18,057 53.74%[b]
Incompatible pollinated	72,870,933	63,376,799 86.97%[a]	17,533 52.18%[b]

[a] Percentage of mapped reads to the genome per total reads
[b] Percentage of genes per total genes in *A. thaliana* (33,602 genes)

Table 2 Categorization of papilla cell expressed genes in unpollinated, compatible pollinated and incompatible pollinated papilla cells

Type of papilla cell	Protein-coding genes	Pseudogenes	Transposable element genes	Non-coding RNAs
Unpollinated	15,356	119	287	193
Compatible pollinated	17,209	151	468	229
Incompatible pollinated	16,782	136	413	202

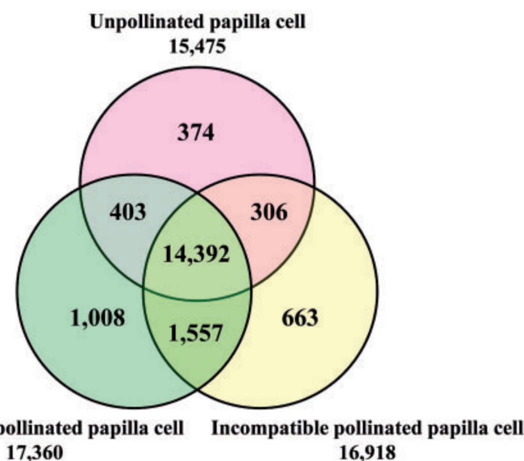

Fig. 1 Comparison of papilla-expressed genes pre- and post-pollination. The Venn diagram shows the number of genes in UP, CP and IP papilla cells.

UP, CP and IP papilla cells, respectively (**Table 2**). These data are consistent with our previous estimate of approximately 17,000 genes expressed in papilla cells of Brassicaceae (Osaka et al. 2013).

Among the expressed genes in UP, CP and IP papilla cells, 14,392 genes were common to all data sets, corresponding to 93% of the expressed genes in UP, 82.9% in CP and 85.1% in IP papilla cells, and 374, 1,008 and 663 genes were specific to UP, CP and IP papilla cells, respectively (**Fig. 1**). This result indicates that the majority of expressed genes commonly function in pre-, post-, compatible and incompatible pollination. Because a simple comparison of expressed gene data sets does not take into account the levels of gene expression, we consider that this does not provide sufficient information for a complete investigation of functional changes in gene expression pre- and post-pollination or for compatible and incompatible pollination. We therefore next examined differentially expressed gene (DEG) analysis in more detail.

Identification of DEGs and Gene Ontology analysis

Functional changes in papilla cells for pollen recognition, acceptance or rejection of pollen grains, and pollen tube guidance are controlled by complex cellular systems that are regulated by diverse molecular players, most of which are still unidentified. Therefore, to investigate the molecular basis of these functional changes during pollination, DEG analysis was conducted for CP and IP compared with the UP papilla cell-expressed gene data sets (**Fig. 2**; **Supplementary Table S1**). First, some DEGs, such as *AtS1* (Dwyer et al. 1994), peroxidase superfamily protein (AT3G03670) (Tung et al. 2005), *ACA13* (Iwano et al. 2014) and *AtPUB2* (Zhang et al. 2014), have previously been predicted to be expressed in Arabidopsis stigmas, demonstrating the validity and reliability of the DEGs. Secondly, 278 up-regulated and 122 down-regulated genes were commonly detected for pre-, post-, compatible and incompatible pollination, and a total of 400 genes, which correspond to 2.3% of the expressed genes in CP and 2.4% in IP papilla cells, showed a difference in expression during pollination (Fisher's exact test, P-value ≤ 0.05, fold change ≥ 3). Thirdly, 147 and 71 genes were up-regulated in the CP and IP data sets compared with UP, corresponding to 0.85% of the expressed genes in CP and 0.42% in IP. Fourthly, 115 and 46 genes were down-regulated in CP and IP data sets compared with UP, corresponding to 0.66% of the expressed genes in CP and 0.27% in IP. These results indicate that there was little difference in the set of genes expressed pre- and post-pollination and therefore suggest that the functional changes of papilla cells upon pollination depend on either a small number of genes or alteration of gene expression.

To investigate molecular and biological functions of DEGs, DEGs were functionally classified by Gene Ontology (GO) analysis. The 10 most highly represented GO terms in each category of cellular components, molecular functions and biological processes are shown in **Fig. 3**. In the cellular components category, GO terms associated with the nucleus, plasma membrane and extracellular region were predominant in all DEGs (**Fig. 3A**). In the nucleus category, transcription factor genes were also present: this was expected as genes for the MYB family, typical transcription factors, are required in *A. thaliana* for pollen tube and synergid cell differentiation for successful fertilization (Kasahara et al. 2005, Liang et al. 2013, Leydon et al. 2013). In the plasma membrane category, the GO term 'protein kinases', which includes proteins such as protein kinase superfamily protein and calcium-dependent protein kinase, were mostly represented as up-regulated genes. In the category of extracellular region, the GO term 'small peptide', identifying such proteins as rapid alkalinization factor and secretory small cysteine-rich protein (CRP), functioning in signal transduction, were predominant. In plant reproductive process, signal recognition and transduction through interaction between CRP and protein kinase, also known as receptor-like kinase, are critical in many steps, such as pollen recognition, pollen tube guidance and fertilization. The finding from the GO analysis that molecular factors categorized as associated with the plasma membrane and extracellular region are predominant in papilla cells indicates that information exchange with pollen and the external environment actively functions in papilla cells, and regulation of expression of these genes is one of the keys to successful reproduction, i.e. pollen–pistil interaction, and maintenance and protection from external attacks.

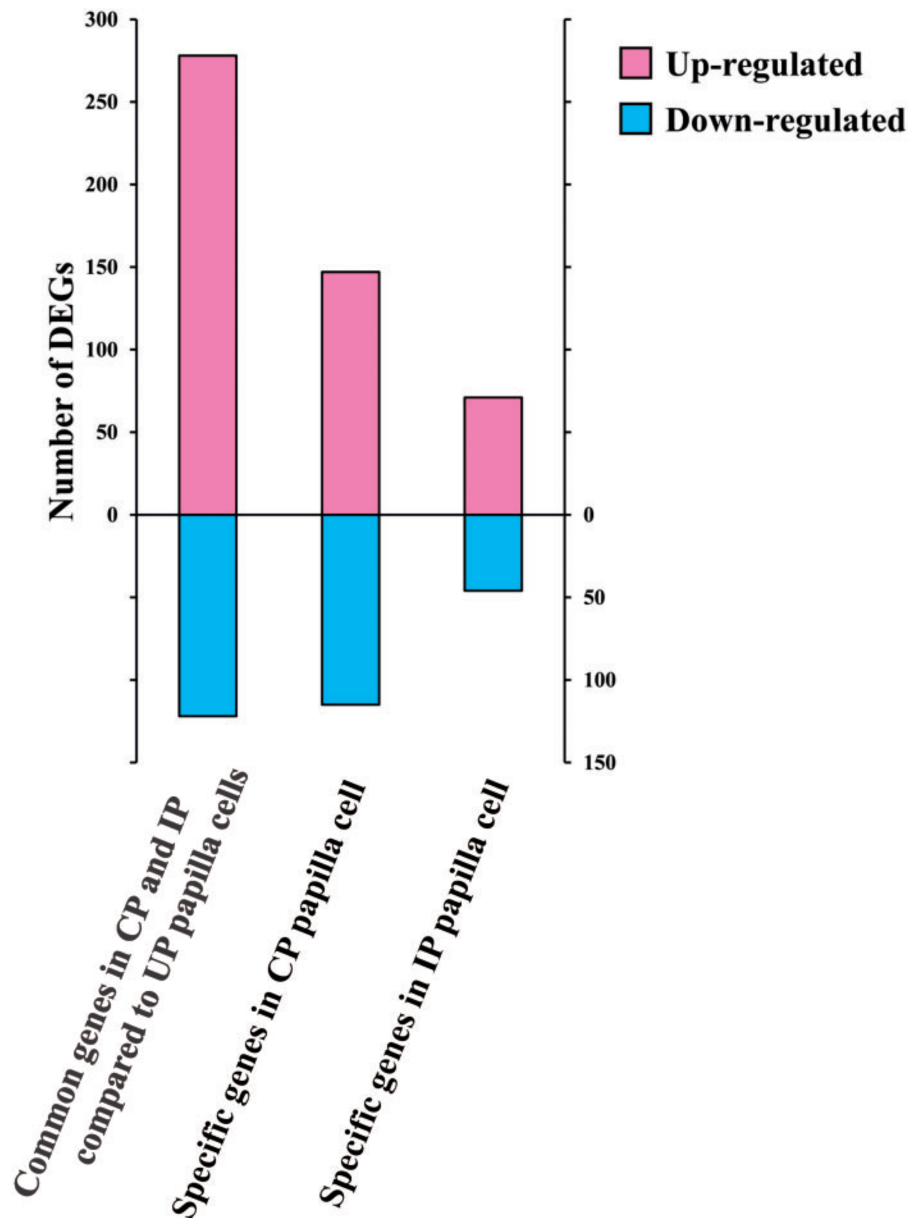

Fig. 2 Genes differentially expressed pre- and post-pollination. Genes were classified according to comparisons between CP and IP papilla cells. The numbers of up-regulated (pink) and down-regulated (blue) genes are shown in the bar chart.

The predominant GO terms in molecular functions were 'zinc ion binding' and 'calcium ion binding' (**Fig. 3B**). In 'zinc ion binding', some genes were specifically up-regulated in CP and others were down-regulated in both CP and IP papilla cells. Zinc ions are essential for stabilization of E3 ubiquitin ligase, in which the RING (really interesting new gene) finger domain is essential for ubiquitination of the target protein for degradation by the proteasome (Smalle and Vierstra 2004). Active protein degradation by the proteasome is known to play a key role in the *Brassica* SI system, i.e. ARC1, an E3 ubiquitin ligase, has been identified as interacting with the SRK domain in *B. napus* (Gu et al. 1998, Stone et al. 1999, Stone et al. 2003). Exo70A1, a member of the exocyst subunit family, is a target of ARC1 and mediates Golgi-derived vesicles to target membranes to transport water to pollen grains (Samuel et al. 2009). In compatible pollination, ARC1 is not active and Exo70A1 can act in the supply of water to pollen from the papilla cell by exocytosis. In contrast, in incompatible pollination, ARC1 activated by SRK degrades Exo70A1 by the proteasome system and the supply of water is aborted, leading to pollen rejection. Thus, the water transport system from papilla cell to pollen is regulated through down-regulation of ARC1 and up-regulation of Exo70A1 in the pre-pollination state and also in the compatible pollination state and, once incompatible pollination is initiated, ARC1 is activated to inhibit the supply of water from papilla cell to pollen through ubiquitination and degradation of Exo70A1.

In the term 'calcium ion binding', expression of genes coding for calcium-binding EF-hand family proteins and calcium-dependent protein kinase was up-regulated in all

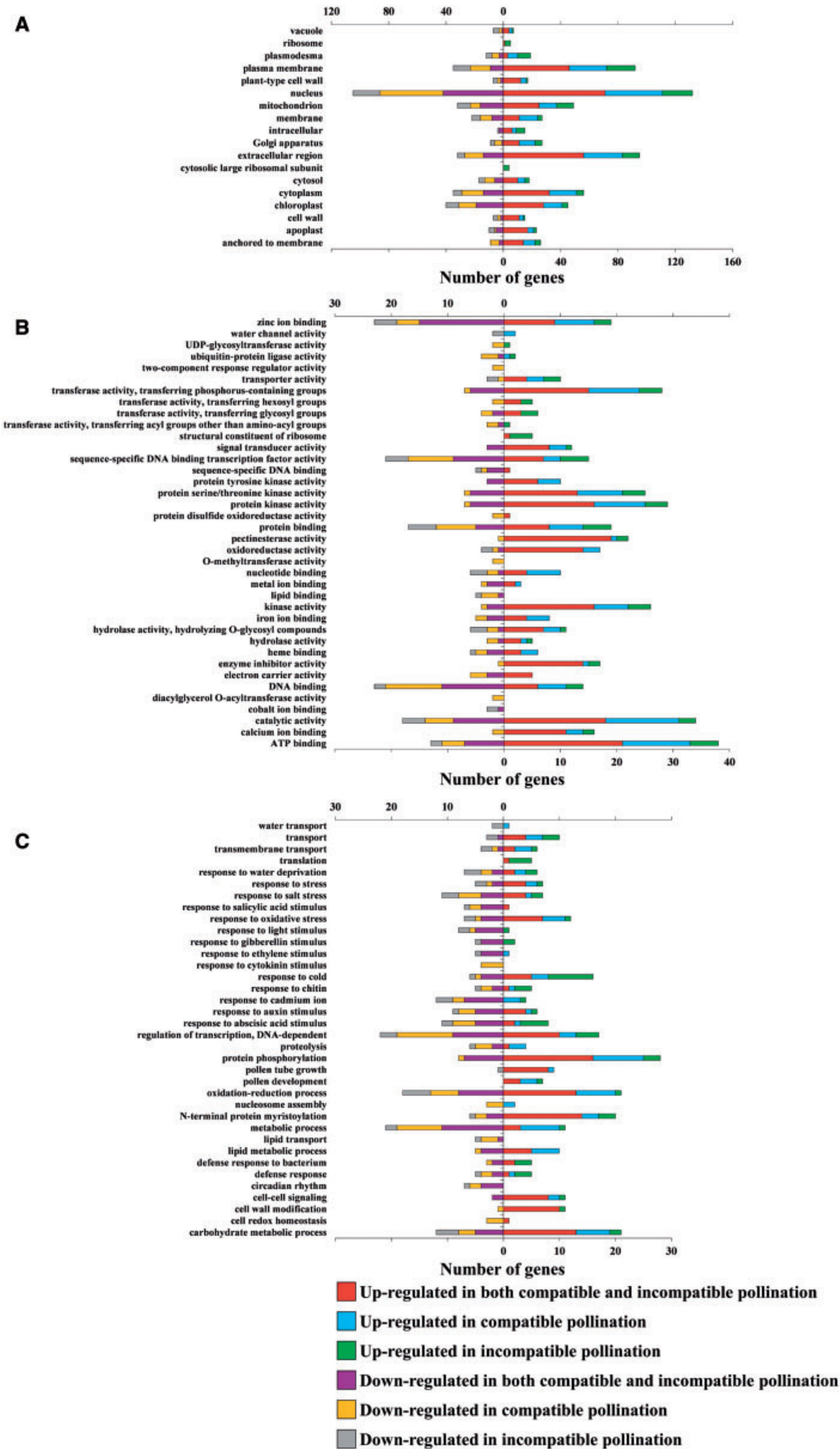

Fig. 3 Functional classification of DEGs pre- and post-pollination. Six sets of DEGs in compatible, incompatible and both pollinations were classified into three Gene Ontology (GO) categories: (A) cellular components, (B) molecular functions and (C) biological processes. Right and left bars indicate the number of up-regulated and down-regulated genes, respectively. The proportion of DEGs that were up- or down-regulated in the different pollinations is shown by six colors: red, up-regulated in both compatible and incompatible pollination; blue, up-regulated in compatible pollination; green, up-regulated in incompatible pollination; purple, down-regulated in both compatible and incompatible pollination; yellow, down-regulated in compatible pollination; and gray, down-regulated in incompatible pollination.

DEGs (Fig. 3B). Products of these up-regulated genes would act in Ca^{2+} signaling in papilla cells. Together with the fact that the concentration of cytoplasmic Ca^{2+} increases at the pollen tube germination site and tip region after compatible pollination (Iwano et al. 2004), it is clear that cytoplasmic Ca^{2+} is one of the key factors for various cellular processes in both male and female reproductive functions.

In addition to the above observations, GO terms involved in 'transferase activity', 'catalytic activity', 'metabolic process' and 'oxidation–reduction process' were highly represented in all DEGs, in both molecular functions and biological processes (Fig. 3B, C). This result indicates that various metabolic and biosynthesis pathways are actively involved in both compatible and incompatible pollination. Therefore, we conducted pathway analysis of DEGs to investigate their detailed function in pollination at the level of the metabolic pathway.

Pathway analysis of DEGs

To characterize metabolic pathways active in papilla cells during pollination, DEGs were mapped to metabolic pathways using the KEGG mapper tool (Kyoto Encyclopaedia of Genes and Genomes: KEGG). Unmapped DEGs were annotated with KEGG Orthology (KO) numbers by BlastKOALA and attempts were made to map these to metabolic pathways. Among these, 97 DEGs were mapped to various metabolic pathways, and these were categorized into seven metabolic pathways (Fig. 4; Supplementary Table S2). Genes belonging to carbohydrate metabolism were present in up-regulated genes in CP and IP papilla cells, and these were, in particular, in the starch and sucrose metabolism pathway and the pentose and glucuronate interconversion pathway (Fig. 5). β-Glucosidase (EC 3.2.1.21) was represented in up- and down-regulated genes in CP papilla cells, and invertase (EC 3.2.1.26) was represented in up-regulated genes in both compatible and incompatible pollination. These enzymes are involved in α-/β-D-glucose biosynthesis (Fotopoulos 2005, Ketudat Cairns and Esen 2010). Genes related to trehalose biosynthesis from UDP-glucose were down-regulated in both compatible and incompatible pollination. In the galactose metabolism pathway, the gene coding for UDP-D-glucose/UDP-D-galactose 4-epimerase (EC 5.1.3.2) was up-regulated in CP and down-regulated in IP papilla cells. The gene coding for β-galactosidase 2 (EC 3.2.1.23), a catalytic enzyme for hydrolysis of lactose into D-galactose and α-D-glucose (Gantulga et al. 2009), was down-regulated in IP papilla cells. These results indicate that sugar metabolism has an active role in both CP and IP papilla cells, and glucose and UDP-glucose seem to be accumulated in papilla cells after pollination in a compatible pollination. Glucose-specific monosaccharide transporters AtSTP9 and AtSTP11 have been found to function in pollen tubes in *A. thaliana* (Schneidereit et al. 2003, Schneidereit et al. 2005) and, in our data, some genes related to sugar transport were up-regulated in CP papilla cells and others were down-regulated in IP papilla cells. UDP-glucose is a source of callose, an essential material of the pollen tube wall and the callose plug in pollen tubes (Schlüpmann et al. 1994, Nishikawa et al. 2005, Park et al. 2010). Growing pollen tubes require an optimal supply of nutrients from surrounding female tissues for their activity, because pollen is basically a sink for products of photosynthesis and cannot independently produce carbohydrates as an energy source. Thus, co-ordination of sugar production and metabolism in papilla cells and export to pollen by transporters is critical for pollen activity in the reproduction process. In addition, components of the sugar metabolic pathways and transporters that were up-regulated in CP papilla cells were, in contrast, down-regulated in IP papilla cells. Thus, pollen acceptance or rejection on papilla cells may be regulated by alteration of the energy source available for pollen tube growth via sugar metabolism and transport systems.

Another sugar metabolic pathway identified from the analysis of DEGs was that associated with pectin and cellulose metabolism (Fig. 5). Genes related to pectin and cellulose degradation pathways were up-regulated in CP papilla cells, and pectin esterase (EC 3.1.1.11), synthesizing pectate by hydrolysis of pectin, was up-regulated in IP and down-regulated in CP papilla cells. The major part of the plant cell wall is composed of proteins and polysaccharides such as pectin, cellulose and hemicellulose (Cosgrove 2005, Lehner et al. 2010). In Brassicaceae, after pollen recognition, pollen hydration and pollen germination, the pollen tube penetrates the outer layer of the cell wall of the papilla cell and grows in the apoplastic space down to the ovary to deliver sperm cells for fertilization. In this process, the cell wall of the papilla is softened and expanded prior to pollen tube penetration, and pectinase and expansin, functioning in cell wall modification in the papilla cell, should contribute to loosening of the papilla cell wall. Penetration of pollen tubes into the papilla cell is considered to be controlled by cell wall-modifying enzymes such as pectinase and polygalacturonase, which are derived from pollen (Dearnaley and Daggard 2001). In contrast, in *B. napus*, it has been reported that serine esterases, identified in pollen tube and stigma, are required for pollen tube penetration into the papilla cell (Hiscock et al. 2002). In tobacco, a pistil-specific β-expansin, PPAL, is secreted in the stigmatic exudate and acts as a cell wall-loosening factor (Pezzotti et al. 2002). Therefore, from our results and previous reports, loosening of the papilla cell wall for pollen tube penetration is probably controlled by cell wall-modifying enzymes derived from the papilla cell itself, in addition to enzymes derived from pollen. Activation of cell wall remodeling of the papilla cell appears to be one of the differences between CP and IP papilla cells, i.e. wall degradation occurred in CP papilla cells for acceptance of the pollen tube, and wall composition occurred in IP papilla cells for rejection of pollen. In connection with this idea, genes related to secondary metabolites of phenylpropanoid biosynthesis were also represented in up- and down-regulated genes in CP papilla cells (Fig. 6). Genes coding for caffeoyl coenzyme A *O*-methyltransferase 1 (CCOAOMT1; EC 2.1.1.104) and *O*-methyltransferase 1 (OMT1; EC 2.1.1.68), involved in lignin biosynthesis, were down-regulated in CP papilla cells. Various genes in the peroxidase superfamily (EC 1.11.1.7) were found to be up- and down-regulated in CP papilla cells; their gene products also mediate lignin biosynthesis, which metabolizes lignin from monolignol. Deposition of lignin, a phenolic macromolecular compound, increases the strength of plant tissue, and thus these results

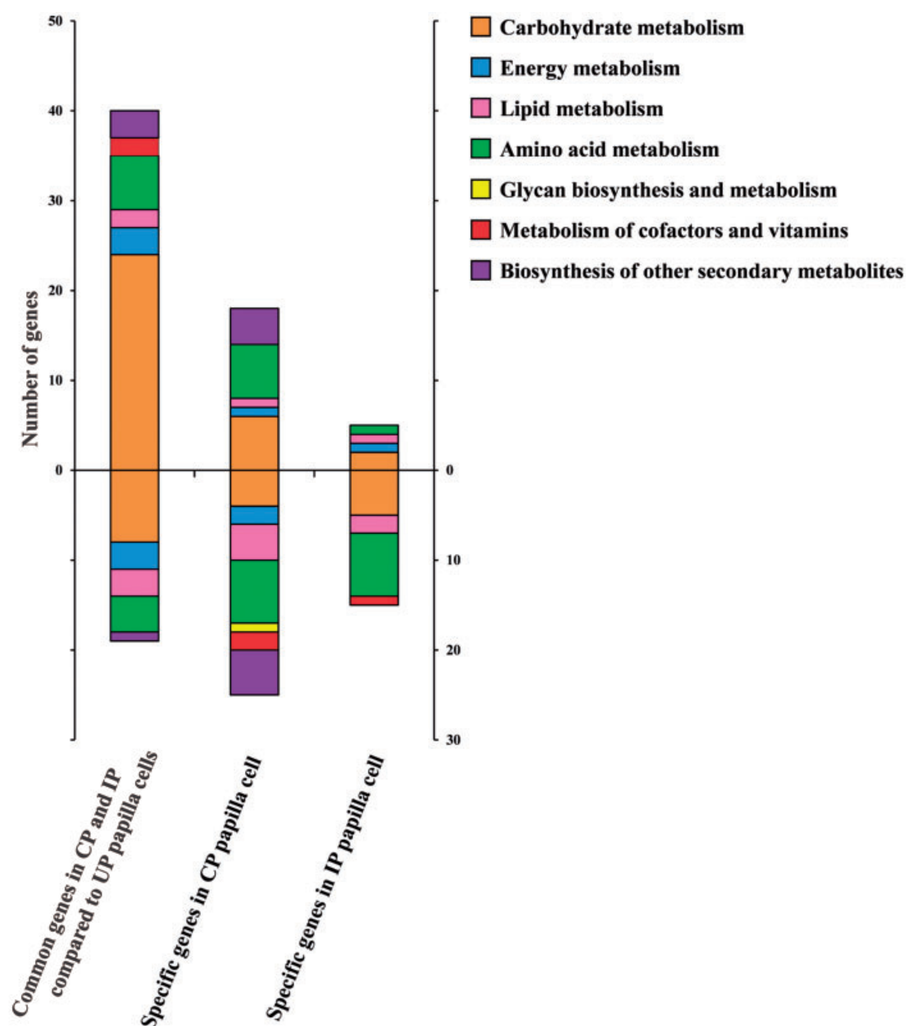

Fig. 4 Classification of metabolic pathways in DEGs pre- and post-pollination. Metabolic pathways were classified into seven categories: orange, carbohydrate metabolism; blue, energy metabolism; pink, lipid metabolism; green, amino acid metabolism; yellow, glycan biosynthesis and metabolism; red, metabolism of cofactors and vitamins; and purple, biosynthesis of other secondary metabolites. Upper and lower bars indicate the number of up-regulated and down-regulated genes, respectively.

suggest that balanced cell wall remodeling of papilla cells, via pectin and cellulose metabolism and lignin biosynthesis, is an important element in successful pollination, especially in relation to pollen tube reception and rejection.

Conclusion

In this study, we characterized transcriptional differences in papilla cells pre- and post-pollination, and between compatible and incompatible pollination. The molecular and biological functions of papilla cells during pollination were also examined via DEG analysis and metabolic process analysis. This has demonstrated the characteristic features of the papilla cell as a female reproductive component in pollination, which includes a variety of cellular systems: information exchange with pollen and the external environment; signal transduction through ligand–receptor interaction; internal physiological changes for supply of water and nutrition to the pollen; and external morphological modifications to accept or reject pollen grains. These are regulated by diverse molecular factors and sophisticated, well-orchestrated systems as an early step for successful reproduction. Expression of this wide variety of genes is essential to ensure the correct cellular, physiological, developmental and reproductive processes in papilla cells, and, from our transcriptome data, they appear to be regulated by regulation at the gene expression level, rather than by regulation of transcription.

Materials and Methods

Plant materials

A. thaliana ecotype Oldenburg (Old-1) and transgenic SI Old-1 (Matsuda et al. unpublished results) were grown in a growth chamber with an 8 h light/16 h dark cycle at 22°C. Old-1 is self-compatible, categorized as haplotype A-t4 (Shimizu et al. 2008), but still retains the female SI function, although the male SI gene *SCR* is disrupted (Tsuchimatsu et al. 2010). Thus, pistils of wild-type Old-1 can be used to analyze both compatible and incompatible

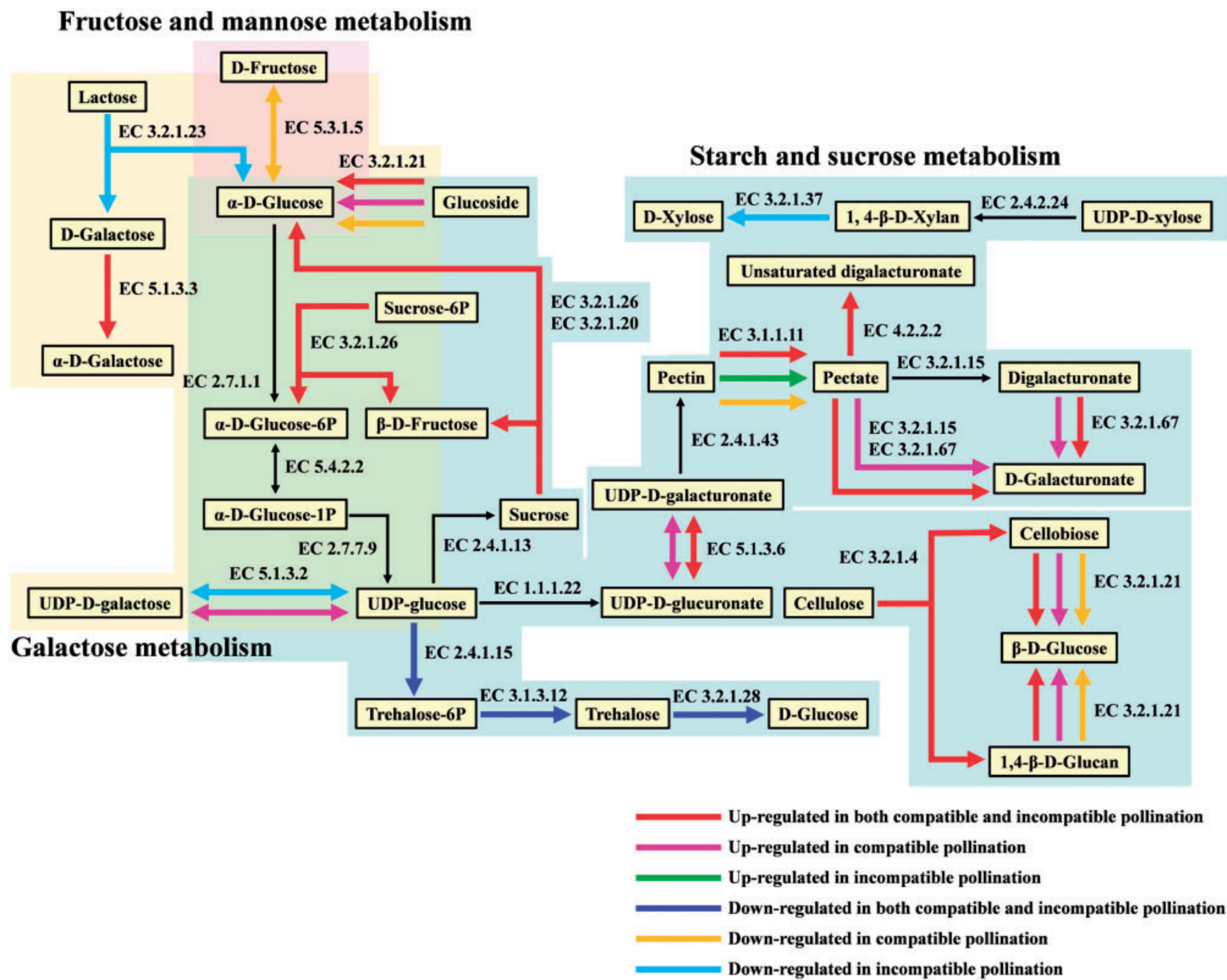

Fig. 5 Changes in sugar metabolic pathways pre- and post-pollination. Pathway maps of starch and sucrose metabolism, galactose metabolism, and fructose and mannose metabolism were partially extracted from the KEGG pathways and merged. Black arrows indicate pathways not mapped from DEGs. Pathways mapped from DEGs that were up- or down-regulated in the different pollinations are shown by six colored arrows: red, up-regulated in both compatible and incompatible pollination; purple, up-regulated in compatible pollination; green, up-regulated in incompatible pollination; blue, down-regulated in both compatible and incompatible pollination; yellow, down-regulated in compatible pollination; and light blue, down-regulated in incompatible pollination.

pollination reactions. For hand pollination, flower buds of wild-type Old-1 were emasculated at developmental stage 12 (Smyth et al. 1990) and incubated overnight at 22°C on 1% agar medium. Emasculated wild-type pistils at developmental stage 14 were pollinated with pollen either of wild-type Old-1 (compatible pollen donor) or transgenic SI Old-1 (incompatible pollen donor). At 1 h after pollination, pollinated pistils were collected and immediately fixed in an ethanol/acetate 3 : 1 (v/v) solution on ice. Unpollinated pistils at developmental stage 14 were collected and were placed immediately in this fixative on ice.

Preparation of paraffin-embedded sections

Tissue fixation, paraffin embedding and sectioning of paraffin-embedded samples were conducted according to methods of Osaka et al. (2013). Briefly, fixed samples of UP, CP and IP pistils were serially dehydrated in 70, 80, 90 and 100% ethanol using a LabPulse H2850 microwave processor (Energy Beam Sciences). For paraffin embedding, each fixative was replaced by 50% paraffin/50% 2-propanol by microwaving, and samples were then embedded in Paraplast X-TRA paraffin wax (Fisher Scientific). The embedded tissue specimens were cooled to room temperature, and the paraffin blocks were stored at 4°C. The paraffin-embedded tissues were cut into 6 µm thick sections using an RV240 microtome (YamatoKoki) within 1 d of paraffin embedding. Serial paraffin sections were mounted on PEN membrane frame slides (Life Technologies), with RNasecure Reagent (Ambion) diluted approximately 25-fold using nuclease-free water (Invitrogen, Life Technologies), and spread onto a heating plate at 57°C for 30 s to 1 min. One PEN membrane frame slide was used per two paraffin-embedded pistils. After removing RNasecure Reagent from PEN membrane frame slides using RNase-free paper, paraffin sections were dried and placed at 4°C for at least 1 h.

Isolation of papilla cells, extraction of total RNA and sample preparation for RNA sequencing

For total RNA extraction, papilla cells were collected from paraffin sections of UP, CP and IP pistils using an Arcturus XT Laser Capture Microdissection System (Applied Biosystems, Life Technologies). Extraction and quality checking of total RNA and linear amplification of mRNAs were conducted according to Osaka et al. (2013). Total RNA was extracted from 4–6 paraffin-embedded pistils for each sample and their quantity and quality assessed using an Agilent 2100 Bioanalyzer and RNA 6000 Pico kit (Agilent Technologies), with the Eukaryote Total RNA Pico parameter. Total RNA samples with a quality

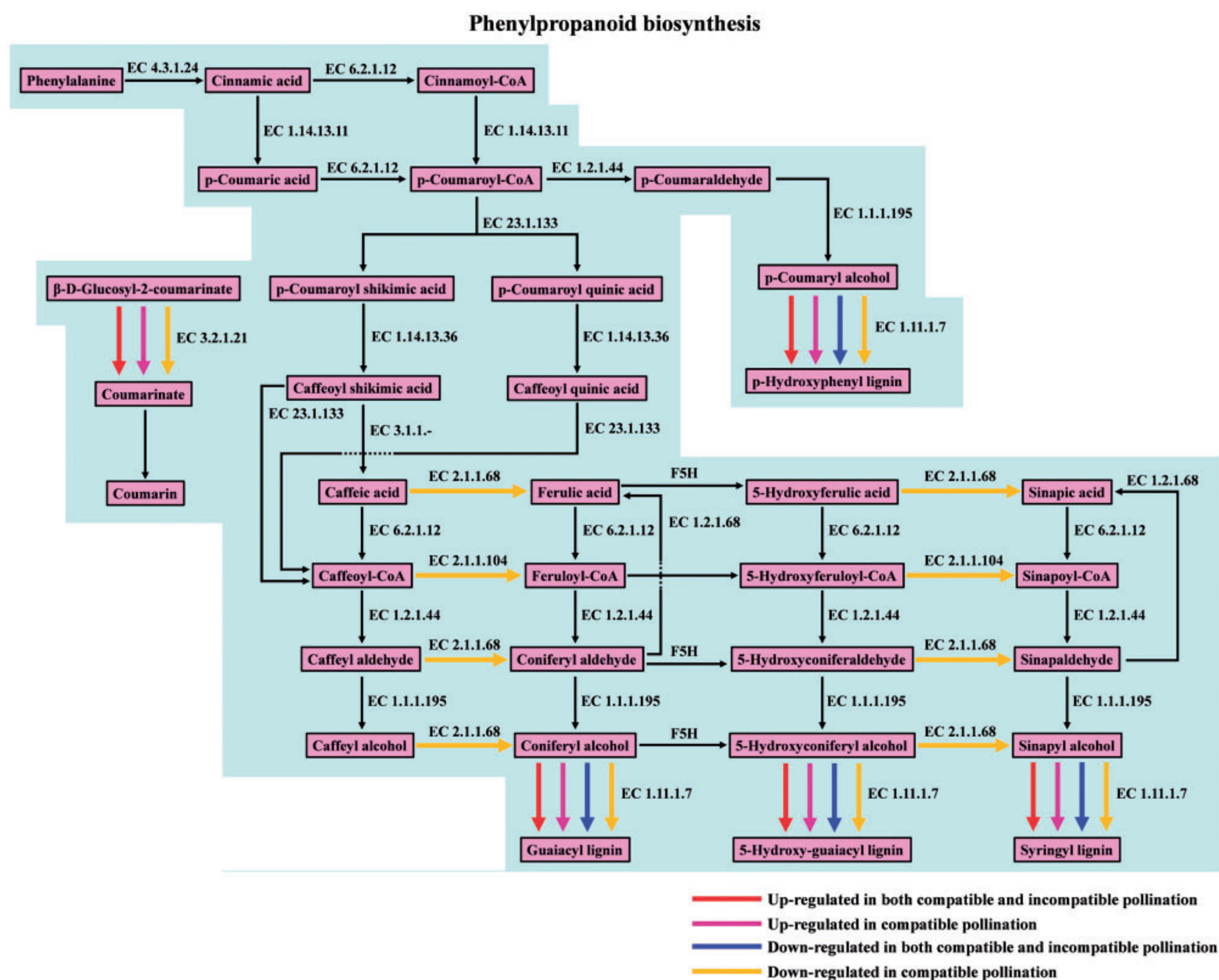

Fig. 6 Changes in the phenylpropanoid biosynthesis pathway pre- and post-pollination. Pathway maps were partially extracted from the KEGG pathways. Black arrows indicate pathways not mapped from DEGs. Pathways mapped from DEGs that were up- or down-regulated in the different pollinations are shown by four colored arrows: red, up-regulated in both compatible and incompatible pollination; purple, up-regulated in compatible pollination; blue, down-regulated in both compatible and incompatible pollination; and yellow, down-regulated in compatible pollination.

value greater than RIN = 6 were used for linear amplification by a RiboAmp HS PLUS RNA amplification kit (Life Technologies).

RNA sequencing

After amplification, preparation of a fragment library for Ion Torrent sequencing was carried out following the manufacturer's instructions (Life Technologies) using the Ion Plus Fragment Library Kit for AB Library Builder System and AB Library Builder System (Life Technologies). A cDNA fragment library was prepared by the Ion OneTouch 2 System using the Ion PGM Template OT2 200 Kit (Life Technologies). Prepared libraries were sequenced by the Ion Proton platform using Ion PI Sequencing 200 Kit v3, with one Ion Proton I Chip per sample. Sequence reads were aligned to *A. thaliana* genome sequences (TAIR10) by Ion Torrent Suite Software 4.2.1 (Life Technologies) with the RNA-seq parameter.

Sequence data processing and DEG analysis

After aligning sequences, the resulting BAM files were sorted by SAMtools 0.1.19 (Li et al. 2009). Sorted BAM files were imported into Partek Genomics Suite version 6.6 and the reads were mapped to genomic regions using an *A. thaliana* annotation file (TAIR10), a GFF3 format file downloaded from Ensembl Plants (http://plants.ensembl.org). Read counts per gene locus were calculated from reads mapped to the genome, and their expression values were normalized by reads per kilobase of exon per million mapped reads (RPKM). To identify DEGs pre- and post-pollination, statistical analysis was carried out using Fisher's exact test by R 3.01 software (Ihaka and Gentleman 1996). We defined DEGs as genes with an RPKM value showing a ≧3-fold change in expression at a *P*-value $\leq$ 0.05. Functional categorization of DEGs by GO was performed using GO annotations from the TAIR website (http://www.arabidopsis.org). In the annotation of GO terms for DEGs, GO terms were confirmed and categorized by GO evidence codes, and GO terms flagged as Inferred from the Reviewed Computational Analysis and the Non-traceable Author Statement were removed. Metabolic pathway analysis of DEGs was carried out based on the KEGG database (http://www.genome.jp/kegg/), and DEGs were mapped onto metabolic pathways using the KEGG Mapper tool. Unmapped DEGs were annotated with KO numbers by BlastKOALA and an attempt was made to map to metabolic pathways based on KO number.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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