Supplementary information

Deciphering the stigmatic transcriptional landscape of compatible and self-incompatible pollinations in *Brassica napus* reveals a rapid stigma senescence response following compatible pollination

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Material and Methods

Plant Material, RNA isolation, cDNA and Affymetrix Gene Expression Microarrays

Brassica napus canola cultivars spp., compatible Westar and self-incompatible W1, were germinated on soil and maintained at standard growth conditions (22°C, 16 h/8 h day/night cycle). Two biological replicates of un-pollinated, self-pollinated and compatible-pollinated stigmas (200 for each treatment) were collected, frozen in liquid nitrogen and stored at -80°C till RNA isolation. For compatible and incompatible pollinations, self-incompatible S1 stigmas were hand-pollinated *in-planta* with either compatible Westar pollen or incompatible W1 pollen (self-pollen). Total RNA extracted using TRIzol was used to synthesize cDNA through Whole Transcriptome Amplification (Sigma-Aldrich WTA1-10Rxn). A total of 300 ng RNA was used for cDNA amplification using the WTA kit, which comprises of two steps. In the first step, the library was synthesized by mixing 300 ng of RNA with library synthesis and stabilization buffer in the presence of library synthesis enzyme. The whole reaction mix was then subjected to three step amplification in a thermal cycler using the following profile; 24°C for 15 min, 42°C for 2 h and 95°C for 5 min, respectively. The reaction was cooled down

immediately after amplification. In the second step, cDNA amplification was done using WTA amplification mix from the first step, WTA master mix, dNTPs and antibody inactivated hot-start Taq DNA Polymerase. Aminoallyl-dUTP was incorporated during the WTA amplification to enable dye labeling. This reaction was carried out in a thermal cycler using the following profile; 95°C for 3 min, 17 cycles (94°C for 20 seconds, 65°C for 5 min). PCR purification was done using Sigma Gene-Elute, PCR clean up kit (NA 1020) for removal of residual primers and nucleotides. The cDNA obtained from the WTA amplification was then labeled with Cy5 and Cy3 dyes and hybridized onto Agilent 4×44K *Brassica* Gene Expression Microarrays (G2519F). The microarray experiments were run in duplicates with two biological replicates and dye-swap. In each array, mRNA from unpollinated stigmas was used as the reference sample. The hybridization procedure was carried out according to the manufacturer's instructions (Agilent Technology, Santa Clara, CA). The microarrays were washed in 6X SSPE/0.005% sodium N-lauroylsarcosine at room temperature for 5 min followed by a second wash in pre-heated 42°C 0.6X SSPE for 2 min. The microarrays were scanned with a GenePix4200A scanner (Molecular Devices, Sunnyvale, CA). The raw microarray data was lowess normalized and the average log2 ratios with the corresponding t-test p values from the dye-swap experiments were obtained using the R Bioconductor Limma package. Heat map images of the microarray expression were constructed with Cluster 3.0 and Java Treeview 1.1.6r2. All the steps in the microarray analysis were performed as described in (Kwon et al., 2012).

Filtering of pollen genes from the differentially-expressed genes during self-incompatible and compatible pollination

The contribution of RNA from pollen in the total RNA is expected to be minimal due to the large difference in the proportion of RNA from stigma tissue compared to the RNA from pollen. Since all four microarray experiments compared relative gene expression ratios between treatment and unpollinated stigmas, only those genes exclusively present in the pollen would always show up as highly up-regulated since their experssion are absent in the unpollinated stigmas. To filter the pollen genes from the 621 differentials (Table S1), we used the public microarray database (http://bar.utoronto.ca/welcome.htm) to analyze the expression of these genes in mature pollen and stigma tissue. Those that were exclusively expressed in pollen and stigma were classified as either pollen alone or stigma alone genes. Those that displayed strong expression in both tissues were grouped as stigma and pollen genes. Any genes that displayed repression after polllination were considered to be contributed by the stigma. In addition to this, the exclusive pollen genes were further verified by comparing the list with the reported pollen transcriptome (Honys and Twell, 2003). Genes without any known expression in pollen were also grouped along with the stigma genes.

Real-time Quantitative RT-PCR (qPCR)

Total RNA from *Brassica napus* stigma samples (two biological replicates) were isolated using TRIzol reagent (Invitrogen, USA) and DNase-treated using RNase-free DNase I (Fermentas, USA). First-strand cDNA was synthesized from 5 µg total RNA using oligo $(dT)_{12\text{-}18}$ primer and SuperScript II Reverse Transcriptase (Invitrogen, USA) following manufacturer's instructions. The qPCR was performed using StepOnePlus Real-Time PCR System (Applied Biosystems, USA). Primer pairs are listed in supplemental S6.

Each PCR reaction contained 1× Fast SYBR Green Master Mix (Applied Biosystems, USA), 200 nM of each primer, and 0.5 µl cDNA in a final volume of 20 µl. PCR amplification was performed for 40 cycles at 95ºC, 3 s and 60ºC, 30 s with a preceding initial enzyme activation of 20 s at 95ºC. Relative expression levels were calculated by the Δ-ΔCt method, and all quantifications were normalized using *actin 7 (*At5g09810) mRNA as an internal control. For each target gene, the reactions were carried out in duplicate.

Propidium iodide staining

Pistils from pollinated and un-pollinated flowers were excised and incubated in a microfuge tube containing 500 µl of propidium iodide solution (20 µg /ml) for 5 min on a rotary shaker. The pistils were then briefly rinsed with water, placed on a microscope slide and mounted in 50% glycerol. The slides were visualized under a Leica DMR epifluorescence microscope.

Aniline blue assay

After pollination, *Brassica* pistils were fixed in 3:1 ethanol, glacial acetic acid for 30 min, followed by incubation in 1N NaOH at 60°C for 1 h. After 1 h, the pistils were washed with distilled water and stained with basic aniline blue (0.1 M K_3PO_4 0.1% aniline blue). The stained samples were mounted in 50% glycerol to visualize pollen germination and pollen tubes under a Leica DMR epifluorescence microscope.

Supplementary data

Table S1: List of all 621 genes represented in the clustergram (Fig. S3) and their log2 ratios in each microarray experiment

Table S2: List of all 621 differentials identified in this study, their grouping and log2 ratios in each microarray experiment

Table S3: List of all 287 genes represented in the clustergram (Fig. S4) and their log2 ratios in each microarray experiment

Table S4: List of all genes reported in the heat maps (Fig. 1B-E) and their log2 ratios in each microarray experiment

Table S5: List of all senescence-associated genes (Fig. 1F) and their $\log_2$ ratios in each microarray experiment

Table S6: Primers used for q-RT-PCR

Supplemental figure legends

Figure S1

Quantitative real time RT-PCR validation of the selected genes (right side) which showed differential regulation following pollination in the microarray (left side). Error bars are based on two replicates.

References

Kwon, E.J., Laderoute, A., Chatfield-Reed, K., Vachon, L., Karagiannis, J., and Chua, G. (2012) Deciphering the Transcriptional-Regulatory Network of Flocculation in Schizosaccharomyces pombe. PLoS Genetics **8,** e1003104.

Honys, D., and Twell, D. (2003). Comparative analysis of the Arabidopsis pollen transcriptome. Plant Physiology **132,** 640-652.

Fig. S1

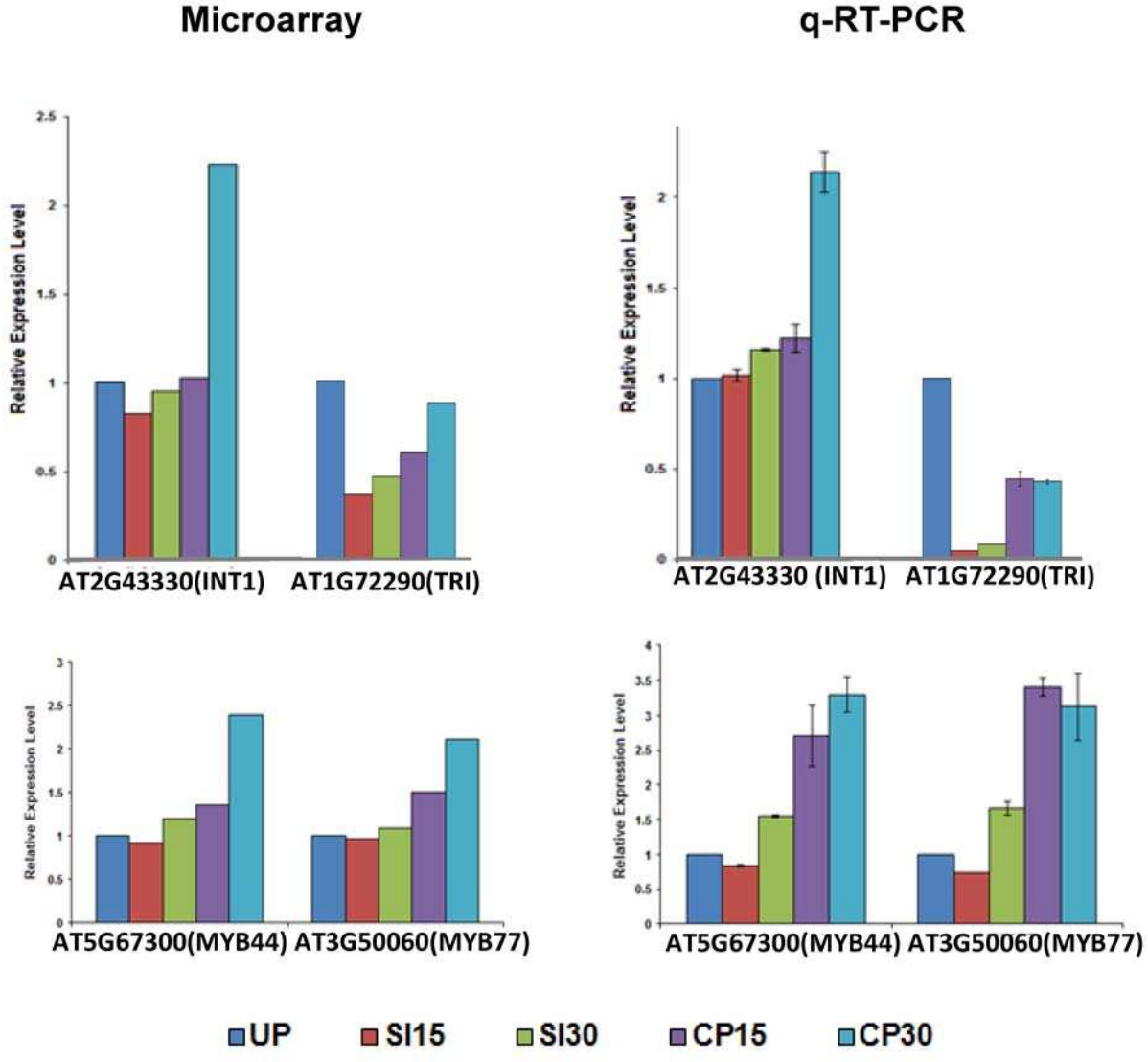
Microarray
q-RT-PCR
Relative Expression Level
AT2G43330(INT1)
AT1G72290(TRI)
AT2G43330 (INT1)
AT1G72290(TRI)
AT5G67300(MYB44)
AT3G50060(MYB77)
UP
SI15
SI30
CP15
CP30

Table S4: Log2 ratios of the genes induced or repressed under SI or compatible pollinations

Genes induced by SI pollination

ATG#	SI15/UP	SI30/UP	CP15/UP	CP30/UP	
AT3G43220	-0.2995	1.205	0.3493	0.658	Phosphoinositide phosphatase family protein
AT4G33990	-0.2758	1.009	0.2522	0.4532	pentatricopeptide repeat-containing protein
AT3G03740	-0.272	1.148	0.09567	0.4163	BTB/POZ and M4 domain-containing protein

Genes repressed by SI pollination

ATG#	SI15/UP	SI30/UP	CP15/UP	CP30/UP	
AT3G19970	-1.2	-0.03658	0.1456	0.1999	Arabidopsis thaliana uncharacterized protein
AT1G44446	-1.163	-0.0347	0.06041	0.1939	Arabidopsis thaliana chlorophyllide a oxygenase (CH1)
AT5G61820	-1.124	0.1453	0.1427	0.2667	Arabidopsis thaliana uncharacterized protein
AT5G25980	-1.065	0.08647	0.1641	0.3621	Arabidopsis thaliana myrosinase 2 (TGG2) (Thioglucoside glucohydrolase)
At1g27710	-1.011	-0.2326	0.02084	-0.0571	Arabidopsis thaliana Glycine-rich protein family
AT1G72290	-1.453	-1.111	-0.7508	-0.1885	Arabidopsis thaliana trypsin inhibitor (Kunitz) domain-containing protein
AT1G52030	-1.298	-0.9035	-0.7571	-0.2764	Arabidopsis thaliana myrosinase-binding protein 2 (MBP2)
AT5G48850	-1.221	-0.9344	-0.3019	-0.2348	Arabidopsis thaliana tetratricopeptide repeat domain-containing protein (ATSDI1)
AT3G31430	-1.182	-1.015	-0.3213	-0.2843	uncharacterized protein
AT3G08770	-1.109	-0.7224	-0.1196	-0.1063	non-specific lipid-transfer protein 6
AT5G26220	-1.217	-1.078	-0.06705	-0.2935	Arabidopsis thaliana ChaC-like family protein
AT5G24655	-1.135	-1.003	-0.1424	-0.1458	Arabidopsis thaliana response to low sulfur 4 (LSU4)

Genes induced by CP pollination

ATG#	SI15/UP	SI30/UP	CP15/UP	CP30/UP	
AT5G59310	-1.09	-0.401	-0.2029	1.505	Arabidopsis thaliana non-specific lipid-transfer protein 4 (LTP4)
AT3G56270	-0.7729	-0.2731	-0.4834	1.078	uncharacterized protein
AT5G65470	-0.6889	-0.1646	-0.405	1.083	Arabidopsis thaliana O-fucosyltransferase family protein
At5g13170	-1.014	0.1451	-0.5382	2.278	Arabidopsis thaliana senescence-associated protein 29 (SAG29)
AT4G20260	-0.7529	0.06603	-0.2936	1.165	Arabidopsis thaliana plasma-membrane associated cation-binding protein 1 (PCAP1)
AT1G07590	-0.6891	0.08994	-0.3638	1.126	Arabidopsis thaliana pentatricopeptide repeat-containing protein
AT2G19900	-0.997	0.06404	-0.8227	1.808	Arabidopsis thaliana malate dehydrogenase (oxaloacetate-decarboxylating)(NADP+) (NADP-ME1)
AT3G33187	-0.6017	0.008462	-0.6432	1.339	putative defensin-like protein 315
AT5G59320	-0.7194	-0.169	-0.04199	1.312	Arabidopsis thaliana non-specific lipid-transfer protein 3 (LTP3)
AT5G59330	-0.6532	-0.09421	0.1291	1.287	lipid binding protein
AT3G55840	-0.5933	-0.06801	0.2098	1.247	Arabidopsis thaliana Hs1pro-1 protein
AT1G21910	-0.3558	-0.1373	-0.1305	1.026	ethylene-responsive transcription factor ERF012
AT2G43330	-0.2814	-0.07704	0.0323	1.157	Arabidopsis thaliana putative inositol transporter 1 (INT1)
AT1G10550	-0.5615	-0.244	-0.854	1.039	Arabidopsis thaliana xyloglucan:xyloglucosyl transferase (XTH33)
AT2G18550	-0.5889	0.5401	-0.1199	1.285	Arabidopsis thaliana homeobox-leucine zipper protein ATHB-21 (HB21)
AT4G21680	-0.6647	0.3041	-0.1736	1.338	Arabidopsis thaliana nitrate transporter 1.8 (NRT1.8)
AT5G66400	-0.4697	0.3877	-0.324	1.365	Arabidopsis thaliana dehydrin Rab18 (RAB18)
AT1G70580	-0.4476	0.3093	-0.1855	1.344	alanine-2-oxoglutarate aminotransferase 2
AT2G38240	-0.2126	0.3383	0.1167	1.123	2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase-like protein
AT3G22600	-0.2785	0.2394	-0.00132	1.488	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
At1g23310	-0.1291	0.2592	0.06906	1.185	ALANINE-2-OXOGLUTARATE AMINOTRANSFERASE 1
AT5G45890	0.04559	0.2446	-0.1246	1.048	Arabidopsis thaliana senescence-associated protein 12 (SAG12)
AT4G10850	-0.3848	0.5554	0.4633	1.534	Arabidopsis thaliana nodulin MtN3-like protein
AT4G12520	-0.2306	0.2637	0.2406	1.024	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT4G08950	-0.23	0.3323	0.6633	1.764	Arabidopsis thaliana Phosphate-responsive 1 family protein (EXO)
AT5G67300	-0.1288	0.2654	0.4458	1.261	transcription factor MYB44
AT1G18360	-0.03078	0.3002	0.3652	1.17	Arabidopsis thaliana alpha/beta-hydrolase domain-containing protein
AT3G50060	-0.04495	0.1281	0.5846	1.08	myb domain protein 77; MYB77
AT3G41768	0.03968	-0.06776	0.1632	1.113	18SrRNA

Genes repressed by CP pollination

ATG#	SI15/UP	SI30/UP	CP15/UP	CP30/UP	
AT5G40780	-0.03137	-0.4815	-0.5932	-1.144	Arabidopsis thaliana Lysine histidine transporter 1 (LHT1)
At5g49360	-0.1768	-0.1106	-0.1139	-1.217	beta-xylosidase 1 (BXL1)
AT4G20320	-0.1244	-0.09659	-0.07546	-1.032	putative CTP synthase
AT1G55970	-0.02909	-0.1506	-0.1681	-1.99	ARABIDOPSIS THALIANA P300/CBP ACETYLTRANSFERASE-RELATED PROTEIN 2
AT5G17800	-0.07769	-0.09105	-0.09603	-2.681	Arabidopsis thaliana myb domain protein 56 (MYB56)
AT3G24850	-0.04895	0.03708	-0.0458	-1.725	uncharacterized protein
AT3G26125	-0.03574	-0.00745	-0.02233	-1.758	Arabidopsis thaliana cytochrome P450, family 86, subfamily C,polypeptide 2 (CYP86C2) mRNA, complete cds.
AT1G64810	0.00223	0.02092	-0.0816	-1.494	Arabidopsis thaliana APO protein 1 (APO1)
AT1G05830	-0.01675	0.1306	-0.05043	-2.118	Arabidopsis thaliana histone-lysine N-methyltransferase ATX2 (ATX2)
AT5G37380	0.1194	-0.01552	0.04273	-2.597	DNAJ heat shock N-terminal domain-containing protein
At1g27150	0.1225	0.03604	-0.05455	-1.149	Tetratricopeptide repeat (TPR)-like superfamily protein
AT1G07840	-0.06467	0.09118	0.1202	-1.182	Arabidopsis thaliana Sas10/Utp3/C1D family
At3g18780	0.02059	0.17	0.03941	-1.275	Arabidopsis thaliana actin 2 (ACT2) mRNA
AT3G15450	0.1832	-0.05058	-0.2978	-1.131	Arabidopsis thaliana aluminum induced protein with YGL and LRDR motif

Table S5: Senescence-associated genes differentially regulated by SI and CP

	SI15/UP	SI30/UP	CP15/UP	CP30/UP	
AT4G34410	0.472	0.1966	0.8415	1.194	Arabidopsis thaliana ethylene-responsive transcription factor ERF109
AT1G19210	0.6156	-0.09754	0.9082	1.025	Ethylene response factor ERF017
AT1G21910	-0.3558	-0.1373	-0.1305	1.026	ethylene-responsive transcription factor ERF012
AT2G46240	-0.01263	-0.9912	0.7494	1.238	Arabidopsis thaliana BCL-2-associated athanogene 6 (BAG6)
AT5G45890	0.04559	0.2446	-0.1246	1.048	Arabidopsis thaliana senescence-associated protein 12 (SAG12)
At5g13170	-1.014	0.1451	-0.5382	2.278	Arabidopsis thaliana senescence-associated protein 29 (SAG29)
AT5G59310	-1.09	-0.401	-0.2029	1.505	Arabidopsis thaliana non-specific lipid-transfer protein 4 (LTP4)
AT3G08770	-1.109	-0.7224	-0.1196	-0.1063	non-specific lipid-transfer protein 6
AT5G59320	-0.7194	-0.169	-0.04199	1.312	Arabidopsis thaliana non-specific lipid-transfer protein 3 (LTP3)
AT4G12520	-0.2306	0.2637	0.2406	1.024	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT3G22600	-0.2785	0.2394	-0.00132	1.488	bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin-like protein
AT5g59330	-0.6532	-0.09421	0.1291	1.287	lipid binding protein
AT2G43330	-0.2814	-0.07704	0.0323	1.157	Arabidopsis thaliana putative inositol transporter 1 (INT1)

Supplemental Table S6. The primer sequences of genes used in q-RT-PCR.

Gene name	AGI code	Probe ID	Forward primer (5′- 3′)	Reverse primer (5′- 3′)
Inositol transporter 1	AT2G43330	A_46_P054656	CCAGTTAACCGTTGCAGACA	TATGATGGAGGGCTCTACGG
MYB-related protein	AT5G67300	A_46_P309785	CACGATGTCGTTCAAGCAGT	TCACCTCCGCCTTAATCATC
R2R3-MYB transcription factor	AT3G50060	A_46_P360875	GAGGAGTTACATGGCGGAGA	TGCTCTTCAAATCCCCAAAC
Senescence-specific cysteine protease	AT5G45890	A_46_P163554	GGCGGTCTAATGGATACTGC	GATCTTGCAATTGGCGTCTT
Ethylene-responsive transcription factor ERF017	AT1G19210	A_46_P317025	CTTCGACGCTGCTCTCTTCT	CCGCCGAGTTTGAAGTAGAC
Wound-responsive AP2 like factor 1 (ERF109)	AT4G34410	A_46_P246649	TCATTAGGCTTTGCAGAGGAG	TGTCCTTTGCCCAATACAGTC
BCL-2-associated athanogene 6	AT2G46240	A_46_P059826	TTGAAGCGTTGGTTCTCCTC	ACACTGAAACAGCGAGCAAG
Senescence-associated protein 29 (SAG29)	AT5G13170	A_46_P188484	ACGGATCGCAGCTATGAAGT	CACGAGCCACGATCATTAGA
Trypsin Inhibitor (Kunitz) domain-containing protein	AT1G72290	A_46_P036011	AGAAGCACGAACGAAACAGC	TTCCGGTTAAGGTGGTCAAC