Euphytica (2008) 163:67–80
DOI 10.1007/s10681-007-9580-6

Identification of transcribed derived fragments involved in self-incompatibility in perennial ryegrass (*Lolium perenne* L.) using cDNA-AFLP

Inge Van Daele · Erik Van Bockstaele · Cindy Martens · Isabel Roldán-Ruiz

Received: 25 April 2007 / Accepted: 20 September 2007 / Published online: 10 October 2007

Abstract Self-incompatibility (SI) is a mechanism that prevents self-pollination and inbreeding in many flowering plant species. SI in *Lolium perenne* is controlled by two multi-allelic loci, S and Z. SI has important consequences for *L. perenne* breeding as it prevents the efficient production of inbred lines and hybrids. In this study, the cDNA-AFLP approach was used to identify key components of the SI response, S and Z, as well as genes involved in the signaling cascade triggered by a SI response in *L. perenne*. A total of 169 transcripts displaying an allele-specific expression profile were identified. Several of these transcripts displayed homology to genes involved in general cellular functions. In other cases, interesting homologies to proteins, such as kinases, known to be involved in SI in other plant families were found. A genome-wide expression analysis, on the other hand, allowed us to identify 515 transcript derived fragments as putatively related to SI in *L. perenne*. All the expression profiles were quantified using AFLP-QuantarPro™ and clustered using hierarchical and AQBC clustering methods. A subset of these genes was selected for sequencing and assigned into 10 functional categories. Homologies were found to proteins known to be involved in fertilization and SI processes in other plant families such as ubiquitin-related and calcium-related proteins.

Keywords Cluster · Functional category · Self-incompatibility · Transcript derived fragment (TDF)

I. Van Daele · E. Van Bockstaele · I. Roldán-Ruiz (✉)
Unit Plant, Applied Genetics and Breeding, Institute for Agricultural and Fisheries Research, Caritasstraat 21, 9090 Melle, Belgium
e-mail: isabel.roldan-ruiz@ilvo.vlaanderen.be

E. Van Bockstaele
Faculty of Bioscience Engineering, Department of Plant Production, Ghent University, Coupure Links 653, 9000 Ghent, Belgium

C. Martens
Faculty of Sciences, Department of Plant Systems Biology, Ghent University, Technologiepark 927, 9052 Zwijnaarde, Belgium

Introduction

Self-incompatibility (SI) is a genetically controlled mechanism that prevents self-pollination and inbreeding in many flowering plants, thus promoting genetic diversity. SI is based on the recognition between pollen and pistil. While the *S* locus encodes the determinants of specificity, additional factors are required for a complete SI response (McClure et al. 2000). Recently, exciting progress has been made in unraveling SI molecularly in the single-locus systems of Brassicaceae, Papaveraceae and Solanaceae members (de Graaf et al. 2006; Franklin-Tong and Franklin 2003; Hiscock and Mclnnis 2003; Hua and Kao 2006; McClure 2006; McClure and Franklin-Tong 2006; Sonneveld et al. 2005; Staiger and Franklin-Tong

2003; Thomas et al. 2006 and reviewed by Takayama and Isogai 2005).

In contrast, SI in the grass family is under the gametophytic control of the two multi-allelic loci, S and Z. Fertilization is prevented when the S and Z alleles present in the pollen are matched in the style (Lundqvist 1956). The SI reaction in grasses and the resulting inhibition of the pollen tube growth occurs almost immediately upon contact with the stigma (Shivanna et al. 1982). In addition to the S and Z components determining the pollen/stigma recognition, many other genetic factors are assumed to be involved in the signal cascade triggered by an incompatible reaction.

SI is widespread among outbreeding species of the Poaceae such as *Secale cereale* (rye), *Lolium* spp. (ryegrasses) and *Phalaris coerulescens*. The action of SI has important consequences for the design of breeding strategies as it prevents the efficient production of inbred lines and hybrids (Cornish et al. 1979; Fearon et al. 1983). Although SI can be overcome in grasses by high temperatures, resulting in pseudo-compatibility, this strategy is difficult to employ for large-scale seed production (Lundqvist 1975). Therefore, the manipulation of SI in the grasses requires a better knowledge of the genes that control the SI reaction. Molecular research in *P. coerulescens* resulted in the identification of a thioredoxin gene, which was initially thought to represent the S gene (Li et al. 1995). However, further experiments based on recombination found between the thioredoxin gene and S demonstrated that this thioredoxin gene was located 2 cM from S (Langridge et al. 1999). The chromosomal position of S and Z has been determined in rye (Lewis et al. 1980), *P. coerulescens* (Leach and Haymann 1987; Bian 2001) and ryegrass (Cornish et al. 1979; Thorogood 1991; Thorogood et al. 2002) using linkage analysis. The S locus is located on linkage group 1 (numbering of linkage groups follows the consensus map of the Triticeae) and the Z locus is located on linkage group 2 (Fuong et al. 1993). Recently, linkage analysis in rye has revealed one STS marker (TC116908) cosegregating with Z (Hackauf and Wehling 2005). Furthermore, the putative ubiquitin-specific protease gene, from which this marker was derived, was strongly expressed in rye pistils but not in leaves. Moreover, high resolution maps of the *P. coerulescens* S and Z regions have been generated using RFLP probes (Bian et al. 2004). Two markers (Xbm2 and Xbcd762) were found to fully cosegregate with S in a population with 844 individuals and one marker (Xbcd266) mapped 0.9 cM from Z in a population of 213 individuals (Bian et al. 2004). Whether the genes, from which these markers were derived, code the stylar or the pollen component of SI or whether they represent genetic factors involved in SI remains to be determined. Although this information opens new perspectives for the isolation of S and Z genes, additional efforts to identify candidate SI-related genes will contribute to a better understanding of SI in Poaceae and to the development of tools to manipulate SI.

The aim of the experiments described here was to identify genes involved in self-incompatibility in diploid *L. perenne*. First, a cDNA-AFLP experiment was designed to identify genes encoded at the S and Z loci. cDNA-AFLP analysis of (presumed) allelic variations in S and Z were exploited in an intent to identify the pistil components coded at these two loci. A mapping population characterized for S and Z genotypes was used for this purpose (Thorogood et al. 2002). A similar strategy was used by Kowyama et al. (2000) to identify S locus gene transcripts in mature stigmas of *Ipomoea trifida*, a species displaying a sporophytic SI response. In addition, a genome-wide transcript profiling of ryegrass pistils was carried out to identify genes putatively involved in the signaling cascade triggered by a SI reaction. For this purpose cDNA-AFLP profiles of non-pollinated, self-pollinated and cross-pollinated pistils were compared and differentially expressed tags were identified.

Material and methods

Plant materials

To identify genes coded by S and Z, we used the ILGI population (P150/112, Bert et al. 1999). The S and Z genotype of 139 offspring plants had previously been determined (Thorogood et al. 2002). As the population was derived from a cross between an anther culture-derived double haploid plant (female, $S_{11}Z_{11}$, according to the allele designation of Thorogood et al. 2002) and a fully-compatible heterozygous plant ($S_{23}Z_{23}$), only four distinct SI genotypes segregate in the offspring, namely, $S_{12}Z_{12}$, $S_{12}Z_{13}$, $S_{13}Z_{12}$ and $S_{13}Z_{13}$. Ten plants of each genotypic class were chosen for the experiments described here.

Table 1 Materials used for RNA extraction and cDNA-AFLP analysis: (a) Samples collected per SI genotypic class in the first experiment and (b) Samples collected per pair-cross (either LpM × LpN or LpT_6 × LpT_3) for the second experiment

(a)

Pistils	Genotypic class			
	$S_{12}Z_{12}$	$S_{12}Z_{13}$	$S_{13}Z_{12}$	$S_{13}Z_{13}$
Mature	X	X	X	X
Immature	X	X	X	X

(b)

Genotype	Pistils									Pollen	Leaf
	NP	SP (1 h)	SP (4 h)	SP (8 h)	SP (24 h)	CP (1 h)	CP (4 h)	CP (8 h)	CP (24 h)		
Female plant	X	X	X	X	X	X	X	X	X	X	X
Pollen donor										X	

$S_{12}Z_{12}$, $S_{12}Z_{13}$, $S_{13}Z_{12}$ and $S_{13}Z_{13}$: the four distinct SI genotypes segregating in the offspring. NP: non pollinated, SP1 h: self pollinated 1 h after pollination, SP4 h: self pollinated 4 h after pollination, SP24 h: self pollinated 24 h after pollination, CP1 h: cross pollinated 1 h after pollination, CP4 h: cross pollinated 4 h after pollination, CP8 h: cross pollinated 8 h after pollination and CP 24 h: cross pollinated 24 h after pollination...

For the second cDNA-AFLP experiment, the four *L. perenne* genotypes, LpM, LpN, LpT_3 and LpT_6 were used. LpM and LpN were two unrelated plants chosen at random from the ILVO breeding collection, while LpT_3 and LpT_6 were two full-sibs derived from a cross between two self-incompatible plants selected at random from the ILVO collection. To determine the compatibility relationships between LpM and LpN, on the one side and between LpT_3 and LpT_6 on the other side, controlled in vitro pollinations were carried out in a preliminary experiment using a slightly adapted version of the method described by Cornish (1979), in which pistils were transferred to agar in a Petri dish. To collect pollen, the inflorescences of the pollinators were excised and kept in water-filled bottles till flowering. The release of the pollen was stimulated by shaking the inflorescences over the receptive pistils, ensuring that only free-flowing, non-clumped, viable pollen was used. Reciprocal pollinations between LpM and LpN and between LpT_3 and LpT_6 resulted in fully compatible reactions. We concluded that these two pairs of genotypes were suitable for the isolation of pistils after compatible cross-pollination.

Sample collection

For the first experiment, we collected 100 non-pollinated pistils from 10 offspring plants of each genotypic class (10 pistils per plant). The flowers were first emasculated under a binocular to prevent unwanted self-pollination and enclosed in cellophane bags until sampling in order to prevent pollen deposition. As it was not known at which stage the mRNA of S and Z would be detectable in the pistils, we collected per genotypic class 100 immature and 100 mature pistils to construct separate bulks. Mature pistils were defined as fully developed receptive pistils, from which the stylodia come out of the spikelets. Immature pistils were not receptive for pollen and the stylodia were still in the spikelets. This resulted in 8 samples (2 × 4 bulks) to compare (Table 1). These materials were kept at −80°C until RNA extraction.

For the second experiment, non-pollinated, self-pollinated and cross-pollinated pistils were sampled (Table 1). For this purpose, controlled pollinations were carried out. LpM and LpT_6 were used as female parents and were therefore emasculated to avoid self-pollination. The emasculated inflorescences were enclosed in cellophane bags until the pistils were mature and receptive for pollination. The emasculated inflorescences were used to collect non-pollinated, self-pollinated (with pollen from the same genotype), or cross-pollinated (using LpN pollen for LpM and LpT_3 pollen for LpT_6) pistils. All pollinations were carried out on excised inflorescences, which were kept in water-filled bottles in the laboratory. To collect pollen, the inflorescences of the pollinator plants

were enclosed in cellophane bags. The release of the pollen was stimulated by shaking pollinator inflorescences above the receptive pistils.

Before sampling, the quality of the 'fertilization' was checked on 3 to 5 pistils. For this purpose, stylodia were separated from the ovaries with a scalpel on microscope slides. Stigmas were placed under a coverslip in a drop of decolourised aniline blue (0.2% water-soluble aniline blue in 2% K_3PO_4). Pollen tube growth was observed microscopically under UV light. If the expected results were observed (self-pollinations resulted in malformed pollen tubes and cross-pollinations resulted in fully grown pollen tubes), pistils were harvested at four different time-points after pollination. The first sample was taken within 1 h after pollination and the other samples were harvested 4, 8 and 24 h after pollination. Note that except for the non-pollinated case, each sample contained pistils and the pollen used for fertilization. Therefore, pollen (and leaf material) of the female plants and pollen of the pollinator plants were also sampled and included as negative controls in the experiment. This resulted in a total of 12 samples (Table 1). These materials were kept at −80°C until RNA extraction.

RNA extraction, cDNA synthesis and template preparation

Total RNA was extracted from 20 to 50 mg frozen plant material using the 'guanidinium thiocyanate method' described by Chomczynski and Sacchi (1987). Double stranded cDNA was synthesised from total RNA (between 25 µg and 50 µg) using a biotinylated oligo$(dT)_{25}$ primer according to standard protocols (Sambrook et al. 1989). The resulting double stranded cDNA was chloroform/isoamylalcohol extracted, ethanol precipitated and dissolved in a final volume of 20 µl water. The restriction enzymes *Bst*YI and *Mse*I (Invitrogen) were used as rare and frequent cutter, respectively, and the digestion was performed in two steps (Breyne and Zabeau 2001). After digestion with *Bst*YI, the restriction fragments carrying a biotinylated 3′ were collected using streptavidine magnetic beads. After digestion with the second enzyme, the restriction fragments, released from the beads, were collected and used as template in subsequent AFLP steps. Adaptors were ligated to the ends of the restricted fragments and 25 cycles of pre-amplification (30 s at 94°C, 1 min at 56°C and 1 min at 72°C) were performed on 5 µl of undiluted 'adaptor ligation mix', with primers without selective nucleotides and complementary to the anchors, following the standard AFLP procedure (Vos et al. 1995). The products of the preamplification were checked on 1.5% agarose gels (a smear between 100 and 1,000 bp indicated successful preamplification). The template was diluted 10-fold in TE (Tris-EthyleneDiamineTetraaceticAcid) buffer. The following adaptors and primers were used: *Bst*YI adaptor: 5′-CTCGT AGACTGCGTAGT-3′ and 5′-GATCACTACGCA GTCTAC-3′; *Bst*YI primer: 5′-GATCACTACGCA GTCTAC(N_{1-2})-3′; *Mse*I adaptor: 5′-GACGATGAG TCCTGAG-3′ and 5′-TACTCAGGACTCAT-3′; *Mse*I primer: 5′- ACGATGAGTCCTGAGTAA(N_{1-2})-3′, where N represents the selective nucleotides. For the PCR reactions, all 16 possible primers of two base extensions were combined, rendering a total of 16^2 (256) primer combinations. cDNA-AFLP primer combinations 1–105 were carried out on samples collected from the genotypes LpM and LpN (LpM as female plant and LpN as pollen donor). cDNA-AFLP primer combinations 106–256 were carried out on materials collected from LpT_6 and LpT_3 (LpT_6 as female plant and LpT_3 as pollen donor).

Radioactive cDNA-AFLP reactions

Radioactive labeling of the *Bst*YI primer was carried out as described by Vos et al. (1995). Thermocycling consisted of 35 cycles, including a 12 cycle touchdown (20 s 94°C, 30 s at 65°C, in which the annealing temperature was reduced from 65°C to 56°C in 0.7°C steps for 12 cycles, 55 s at 72°C) and then maintained at 56°C for 23 cycles and a final step for 15 min at 72°C. Samples were boiled after the addition of loading dye and 50% formamide and separated on 50 cm, 5% polyacrylamide gels. All gels were run at standard conditions (120 Watt and 48°C) with a 10-bp ladder (Sequamark, Genetic Research) as length marker. Gels were dried on Whatman 3 M paper using a slab gel dryer. Labeled DNA bands were visualized by autoradiography. Gels and films were positionally marked prior to development. Each two-nucleotide selective extension yielded between 50 and 70 labeled fragments per lane.

Quantitative measurements of the expression profiles and data analysis

The cDNA-AFLP profiles of the first experiment were scored visually and only qualitative differences (presence/absence) were considered. S and Z allele-specific transcripts were identified. For example, transcripts present in bulks $S_{12}Z_{12}$ and $S_{12}Z_{13,}$ but absent in other bulks, were selected as putatively associated to allele S_2; transcripts present in bulks $S_{12}Z_{13}$ and $S_{13}Z_{13,}$ but absent in other bulks were selected as putatively associated to allele Z_3. Alleles S_1 and Z_1 could not be identified in this experiment as they were present in all the samples compared.

For the second experiment, the gel images were analyzed quantitatively with AFLP-QuantarPro™ (Keygene, Wageningen, The Netherlands). All the differentially expressed cDNA-AFLP bands, displaying an interesting expression profile, were scored and individual band intensities were exported to Microsoft Excel spreadsheets. These raw data were used to estimate the quantitative expression of each transcript in each sample, after correction for lane to lane differences and standardization, as described by Breyne et al. (2003).

Cluster analysis of the expression data was carried out using two complementary approaches. First, the Quality-Based Clustering method (De Smet et al. 2002) was used (accessible for use at http://www.esat.kuleuven.ac.be/~thijs/Work/Clustering.html). This approach is similar to K-means clustering except that the number of clusters does not need to be defined in advance by the user and the expression profiles, which do not fit in any cluster at user-specified threshold values for specific 'quality' parameters, are not assigned to any cluster. As output, genes are grouped into different clusters and expression profiles are plotted. In this experiment the minimum number of tags in a cluster was set to 5 and the significance level (S) to 0.93 (De Smet et al. 2002). In addition, a hierarchical cluster analysis using average linkage (Saeed et al. 2003), as available at the TIGR MultiExperiment Viewer software v2.2 (freely available at http://www.tm4.org/mev.html), was performed. The statistical support of the tree nodes was calculated using the resampling strategies implemented in Support Trees (TIGR MultiExperiment Viewer software v2.2). For this new trees were generated by Jacknifing (resampling and leaving one gene out) the genes. The number of iterations was set at 1,000.

PCR-reamplification and sequencing of selected cDNA-AFLP bands

Bands corresponding to differentially expressed transcripts were excised from the gel and eluted in 200 µl of MilliQ water. About 5 µl of this elution was used for PCR amplification using the same conditions as for the preamplification, but with the primers used in the selective amplification step (see above). Direct sequencing reactions were performed in a total volume of 20 µl, with 50 ng of PCR product, 4 µl of Big Dyes v. 1.1 (Applied Biosystems), 3.2 pmol of the BSTY(+XX) primers and 2 µl 5× Sequenase buffer (Applied Biosystems). If direct sequencing was not successful, the reamplified fragments were cloned in the TOPO-vector with the TOPO TA cloning kit® from Invitrogen. For each cDNA-AFLP band at least 8 colonies were picked, indicated with a capital letter from A till H, and dissolved in MilliQ water. In a next step, direct colony PCR reactions were carried out and 5 of them were sequenced with an automatic sequencer (ABI Prism™ 377). Sequences were compared to the protein database using the BLASTx algorithm (Altschul et al. 1997). Significant candidate genes were selected not only based on the E-value as the cDNA-AFLP tags were very short, but also taking into account the percentages identity and similarity. A significant hit displays an E-value < E-05, or if an E-value > E-05, a percentage identity and similarity >60%. Sequence similarity is the extent to which two (nucleotide or amino acid) sequences are related and sequence identity is the extent to which two (nucleotide or amino acid) sequences are invariant (Altschul et al. 1997).

Results

cDNA-AFLP analysis and selection of TDFs for sequencing

First experiment: identification of allele-specific transcripts

To identify S and/or Z candidates in ryegrass cDNA-AFLP profiles of immature and mature pistils harvested from plants with known S and Z genotype were compared. As an example, part of a cDNA-AFLP gel showing the fingerprints of the 8 samples

analyzed with one primer combination is shown in Fig. 1. A total of 256 primer combinations were screened and cDNA bands ranging between 100 and 600 bp were considered. A total of 169 transcripts displayed one of the allele-specific expression profiles are shown in Table 2. As it was not known at which developmental stage the mRNA of S and Z are present in the pistil, we selected all the TDF (Transcript derived Fragments) displaying an allele-specific expression profile in the mature and/or immature pistil samples. For the definition of the categories one mismatch between mature and immature pistils was allowed, e.g. row 4 in Table 2. Twenty-two S_2 allele-specific TDFs, 53 S_3 allele-specific TDFs, 39 Z_2 allele- specific TDFs and 55 Z_3 allele-specific TDFs were identified (Table 2).

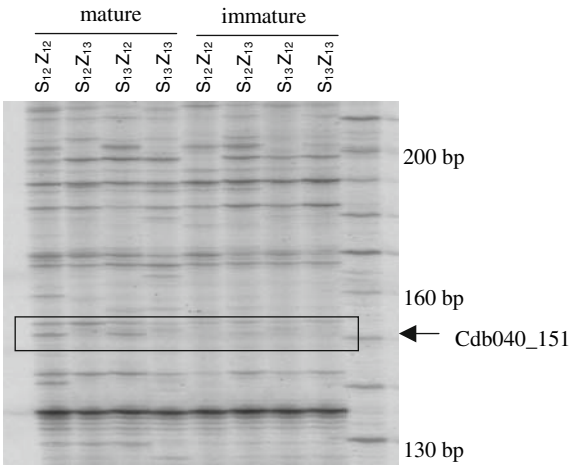

Fig. 1 Part of a cDNA-AFLP gel of the first experiment with primer combination BstY + GC/MseI + AT

Second experiment: genome-wide expression profiling

Self-pollinated (incompatible reaction) and cross-pollinated (compatible reaction) pistils were harvested at four time-points after pollination (1, 4, 8 and 24 h). Leaf and pollen samples were also taken as control as described in materials and methods. For each primer combination 50–70 bands, in the size range 60–600 bp, were visualized on gel. As a consequence, the 256 primer combinations allowed the screening of approximately 18,000 transcripts over the 12 different samples.

A total of 515 bands displaying differential expression were selected visually and scored with AFLP

Table 2 Overview of the different expression profiles identified in the cDNA-AFLP analysis of the first experiment

Mature pistils				Immature pistils				Number of TDFs	Allele
$S_{12}Z_{12}$	$S_{12}Z_{13}$	$S_{13}Z_{12}$	$S_{13}Z_{13}$	$S_{12}Z_{12}$	$S_{12}Z_{13}$	$S_{13}Z_{12}$	$S_{13}Z_{13}$		
+	+	−	−	−	−	−	−	11	S_2
+	+	−	−	+	−	−	−	5	S_2
−	−	−	−	+	+	−	−	4	S_2
+	+	−	−	+	+	−	−	2	S_2
−	−	+	+	−	−	−	+	3	S_3
−	−	+	+	−	−	−	−	35	S_3
−	−	+	+	−	−	+	+	8	S_3
−	−	−	−	−	−	+	+	7	S_3
+	−	+	−	−	−	−	−	23	Z_2
+	−	+	−	+	−	−	−	12	Z_2
+	−	+	−	+	−	+	−	4	Z_2
−	+	−	+	−	−	−	−	49	Z_3
−	+	−	+	−	−	−	+	4	Z_3
−	+	−	−	−	+	−	+	2	Z_3

Number: the number of selected fragments that displayed the corresponding expression profile. Allele: the S or Z allele, which corresponded to each expression profile. For the definition of the categories one mismatch between mature and immature pistils was allowed (e.g. a TDF present in sample $S_{12}Z_{12}$ of immature pistils but absent in sample $S_{12}Z_{13}$ of immature pistils was considered to represent allele S_2). Nomenclature of the samples as described in Table 1a

QuantarPro™. cDNA-AFLP bands amplified in leaf or in pollen control samples were not considered, as such bands probably represent genes not specifically involved in SI. Raw band intensities were first corrected for lane-to-lane differences. The corrected values were then standardized as described in Breyne et al. (2003). After normalization of the data, 36 of the 515 TDFs, which had been selected in the visual inspection, were excluded from the dataset as their coefficient of variation was smaller than 0.25 after correction and were considered to be constitutively expressed (Breyne et al. 2003).

The remaining TDFs were grouped according to their expression patterns using two alternative methods: the Adaptive Quality-Based Clustering (AQBC) method from De Smet et al. (2002) and the average linkage clustering method implemented in TIGR MultiExperiment Viewer (Saeed et al. 2003). Data on self-pollinated pistils 8 h after pollination were not included in the analyses as they contained too many missing data and distorted the calculations.

The AQBC analysis clustered 169 (35%) of the 479 TDFs into 11 clusters (Fig. 2). Cluster 1 (44 genes) and cluster 4 (36 genes) were the largest. All clusters, except numbers 9 and 10, showed clear differences between self-pollinated and cross-pollinated pistils. For example, cluster 1 comprises transcripts that are down-regulated during self-pollination (SI reaction) and display low but stable expression levels in the cross-pollinated pistils (compatible reaction). Clusters 2 and 3 contain transcripts that are down-regulated after self-pollination and almost absent in cross-pollinated pistils. As it is not known which kind of expression pattern is expected in genes activated or deactivated as consequence of a self-incompatible reaction, all clusters were considered to make a selection of genes to characterize further. The hierarchical average clustering grouped the 479 genes into approximately ten groups of co-expressed genes (data not shown). A high agreement was found between the two methods and most of the clusters identified by AQBC were also apparent in the average clustering tree. The between-group Jackknifing support values were, in general, very low. However, within group support values of up to 70% were found (data not shown).

We selected a subset of 168 TDFs for further characterization. These included representatives of the different AQBC clusters (31 TDFs) and TDFs that displayed similar expression profiles, but that had not been assigned to any cluster due to the stringent conditions used for classification (137 TDFs). This latter group of TDFs was selected using the results of hierarchical clustering.

Sequencing of cDNA-AFLP transcripts

The 169 TDFs of the first experiment and the 168 TDFs of the second experiment were excised from the gel (one band of a given sample per TDF), reamplified and sequenced.

First experiment: identification of allele-specific transcripts

For 55 out of 169 TDFs of the first experiment direct sequencing failed to get sequence information and therefore the TDFs had to be cloned. All the cloned TDFs corresponded to two to five different nucleotide sequences. A band in a gel can correspond to a single DNA fragment or a mixture of several fragments. The problem is that the excised band can be contaminated with unlabeled (invisible) DNA bands resulting in sequences of low quality. Therefore, a dataset of 261 sequences was compared to the sequences of the publicly available databases of the 'National Centre of Biotechnology Information (NCBI)'. For 48% (125/261) of the transcripts, no homology was found and for 18% (46/261) of the tags, homology was found to hypothetical proteins or to proteins for which no gene ontology information was available. For the other 34% of the sequences a significant hit against genes with known function was found. Several genes displayed homology to genes involved in general cellular functions, such as, genes involved in the basic metabolism of the cell, genes involved in the cell cycle and DNA replication and recombination processes, genes involved in transport, transcription, signaling and energy processes and genes involved in protein fate, synthesis and activity regulation processes. In other cases, interesting homologies to proteins known to be involved in SI were found. For example, TDF cdb39_250 displayed homology to ubiquitins, which are involved in protein degradation. Recent data demonstrate that the SI response in several gametophytic and sporophytic systems involves ubiquitin-related protein degradation. Furthermore, a STS derived from an ubiquitin-specific protease gene

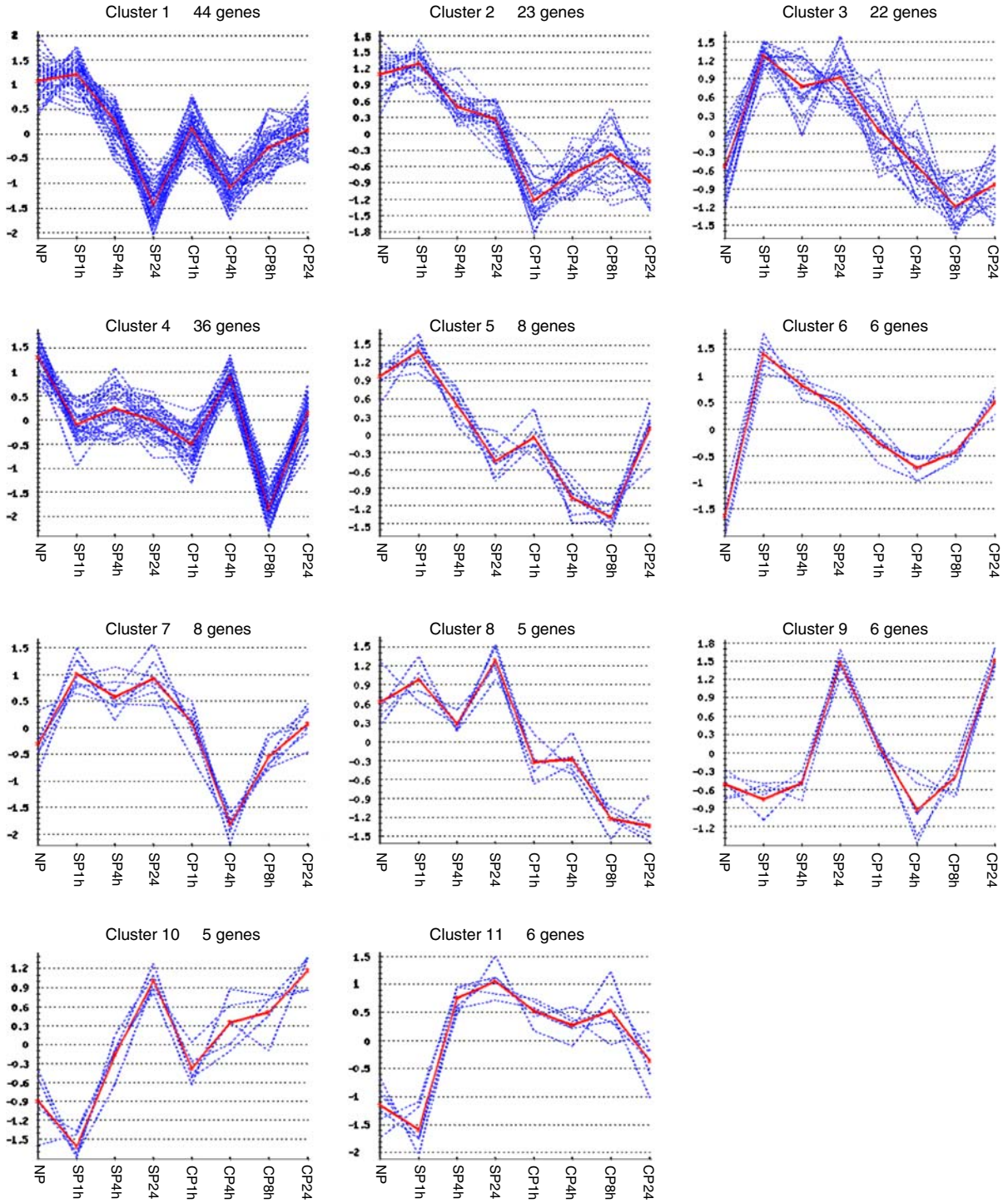

Fig. 2 Clusters identified using the adaptive quality-based clustering (AQBC) method of De Smet et al. (2002). Each graph summarizes the expression profiles of a set of genes which displayed similar expression patterns and which were assigned to the same cluster by AQBC. X-axis: samples, Y-axis: normalized expression values of the genes, which were classified by the clustering algorithm. NP: non pollinated, SP1h: self pollinated 1 h after pollination, SP4 h: self pollinated 4 h after pollination, SP24 h: self pollinated 24 h after pollination, CP1 h: cross pollinated 1 h after pollination, CP4 h: cross pollinated 4 h after pollination, CP8 h: cross pollinated 8 h after pollination and CP 24 h: cross pollinated 24 h after pollination

Table 3 Summary of the most relevant homologies of the putative S and Z allele-specific cDNA-AFLP fragments, identified in experiment 1

TDF	GenBank Accession	Putative allel	Most significant homology	Function	E-value	Identity (%)	Similarity (%)
cdb71_125	EX152686	Z_2	Putative CBL-interacting protein kinase 23, *Oryza sativa* (Q6ZLP5)	serine/threonine kinase activity	1E-08	92	100
cdb185_226A	EX152687	Z_2	Putative cyclin-dependent kinase B1-1, *Oryza sativa* (Q8L4P8)	serine/threonine kinase activity	1E-07	78	87
cdb183_344C	EX152688	Z_3	Putative receptor serine/threonine kinase PR5K, *Oryza sativa* (Q5ZC63)	serine/threonine kinase activity	1E-06	44	58
cdb136_175B	EX152689	S_2	Receptor protein kinase PERK1, *Brassica napus* (Q9ARH1)	serine/threonine kinase activity	2.1	62	70
cdb48_280	EX152690	S_3	Receptor protein kinase-like, *Oryza sativa* (Q5Z661)	serine/threonine kinase activity	3E-15	61	80
cdb98_509	EX152691	S_3	Serine/threonine protein kinase-like, *Oryza sativa* (Q69U57)	serine/threonine kinase activity	8E-22	69	84
cdb39_250	EX152692	S_3	Ubiquitin, *Triticum aestivum* (Q41570)	ubiquitinylation	7E-14	95	95

The selection was not only based on the E-value, but also taking into account the percentages identity and similarity

cosegregates with the Z locus in rye (Hackauf and Wehling 2005). A summary of the most relevant homologies, according to what is known about SI in other plant families, is given in Table 3. Several TDFs displayed homology to serine-threonine protein kinases.

Second experiment: genome-wide expression profiling

The 168 TDFs selected in the second experiment were cloned and sequenced. Of these, 57 corresponded to 2–4 different sequences, while for 111 TDFs, all clones rendered identical sequences. This indicates that the patterns of differential expression observed on cDNA-AFLP gels corresponded in most cases to single transcripts (66% or 111/168 TDFs). When different sequences were found for different clones of a given TDF, all were included in further analyses as it was not possible to determine which one (if any) represented the 'original' differentially expressed gene.

Finally, 259 sequences were compared with the protein database of NCBI. For 36% of the 259 sequences no significant hit against known proteins was found and 24% of the 259 tags remained unclassified as they showed homology to hypothetical proteins or to proteins for which no gene ontology information was available. The sequences that displayed homology to proteins with known function were classified into functional categories using the MIPS classification system. The annotated genes were assigned to 10 functional categories (Fig. 3). We checked whether transcripts clustering together according to their expression patterns were assigned to the same functional group, but no correlation between functional groups and clusters was found.

Discussion

The aim of the experiments described here was to identify key pistil components of the SI reaction. As the SI mechanisms are conserved among members of the same plant family (Nasrallah 2005), the results obtained for one given species of the Poaceae, such as *L. perenne*, would contribute to a better understanding of the SI response in other grass species.

Identification of allele-specific transcripts

The use of cDNA-AFLP (Bachem et el. 1996) in mature and immature pistils with known SI genotype to identify S and Z allele-specific gene transcripts relied on the assumption that allele-specific polymorphisms would be 'apparent' at the level of the cDNA sequence. Using this approach, we selected 169 putative candidates for S and/or Z. The number of differentially expressed genes identified using cDNA-AFLP depends strongly on the experimental set-up,

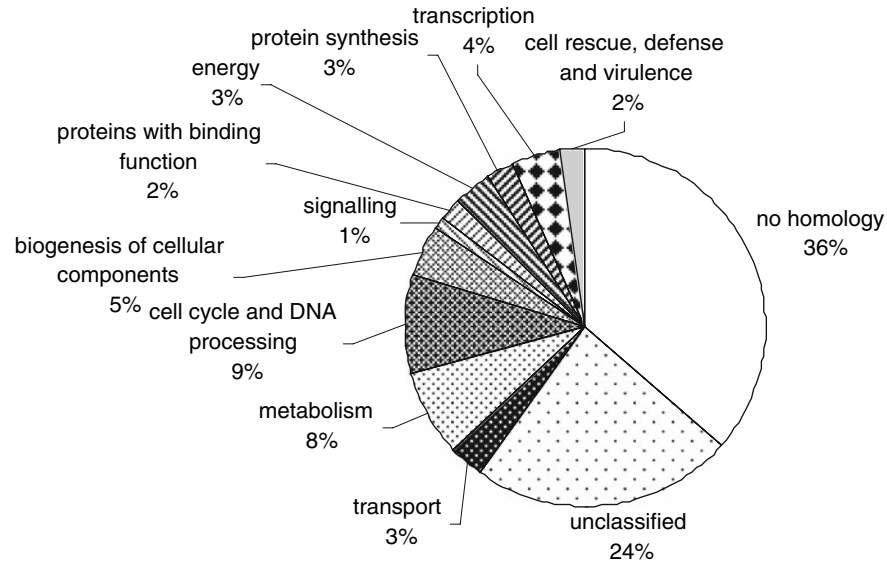

Fig. 3 Functional classification of the 256 TDFs selected in the second cDNA-AFLP experiment, according to the MIPS classification system

such as, the tissue type and trait analysed, the conditions used and the criteria for the selection of the candidates. However, our results are in line with data obtained in previous experiments. In an experiment similar to the one described here Kowyama et al. (2000) screened 64 cDNA-AFLP primer combinations and identified 11 S-allele specific fragments in *Ipomea trifida*. Durrant et al. (2000) identified 290 differentially expressed transcripts during Avr9- and Cf9-mediated defence response in tobacco cell lines. In tomato, Gabriëls et al. (2006) identified 442 transcript derived fragments putatively involved in the hypersensitive response. Bruggmann et al. (2005) identified 363 transcripts that were more expressed in powdery mildew-inoculated wheat leaves than in the control plants. Zheng et al. (2004), on the other hand, identified only 12 differentially expressed cDNA fragments putatively controlling rice blast resistance.

For 34% of the 261 DNA-sequences investigated a significant hit against genes with known function was found. Several TDFs displayed homology to genes involved in general cellular functions. Although nothing is known about the recognition components of the SI reaction in *L. perenne*, the TDFs displaying homology to receptor kinases may be interesting due to the specific features of the SI reaction in the Poaceae (Heslop-Harrison 1982). In the grass family, microscopic studies have shown that the inhibition of the pollen tube displays some similarities with the system active in *Brassica* (Heslop-Harrison 1982). It is well known that in members of the Brassicaceae the SI system is based on the action of a serine-threonine protein kinase coded at the S locus (the S locus Receptor Kinase, SRK) on the female side (Stein et al. 1991) and a cysteine-rich protein (SCR) on the male side (reviewed by Takayama and Isogai 2005). SRK is in fact a receptor with a serine/threonine kinase activity domain. Transgenic gain-of-function experiments in *Brassica rapa* have shown that a functional SRK alone determines S specificity in the stigma (Takasaki et al. 2000). Several of the TDFs identified in this study displayed homology to receptor kinases (Table 3) and showed allele-specific polymorphisms. The involvement of these genes in SI and their eventual role as the pistil SI component needs, however, to be verified yet. Linkage analysis can help to determine the map position of the selected genes as some of the genes involved in the signaling cascade resulting in a SI reaction will be located at either S or Z, but other genes may be dispersed all over the genome or clustered in certain genomic regions. In the Brassicaceae and Solanaceaece it is well known that S loci are located in chromosome positions where recombination is suppressed and that contain several genes interspersed with repetitive sequences and non-functional ORFs. These regions contain not only the genes coding the components determining SI-specificity, but also genes involved in SI and fertilization (Fukai et al. 2003 and Wang et al. 2004).

Genome-wide expression profiling

In a parallel experiment, we used cDNA-AFLP (Bachem et al. 1996) to analyze transcriptional changes in *L. perenne* pistils and to identify genes putatively involved in the signaling cascade triggered by a SI response, which finally results in the inhibition of the pollen tube growth. This genome-wide expression analysis allowed us to identify 479 TDFs displaying a differential expression pattern putatively related to SI in *L. perenne*. Although differentially expressed and constant bands can be discriminated by visual scoring, automated analysis with AFLP QuantarPro^{TM} proved more sensitive, allowing the generation of quantitative expression data, what made the technique very powerful (Breyne et al. 2003). These quantitative data were analyzed using clustering methods. A similar approach was applied for transcriptome analysis during cell division in plants (Breyne et al. 2001, 2002 and 2003), to get insights into the early response to ethylene (De Paepe et al. 2004) and lateral root initiation in *Arabidopsis* (Himanen et al. 2004). Quantitative analysis of the cDNA-AFLP expression profiles is a very good alternative to microarrays and reports demonstrate a good correlation between cDNA-AFLP and microarray results in *Saccharomyces cerevisiae* and *Arabidopsis* (Reijans et al. 2003 and De Paepe et al. 2004, respectively). The power of cDNA-AFLP lies in the fact that it can be used for 'gene discovery' in plant species for which no microarrays are available (Bachem et al. 1996).

The two analysis methods tested (AQBC and average linkage) grouped the TDFs similarly. The clusters probably correspond to groups of co-regulated genes as demonstrated in previous experiments (Branco-Price et al. 2005; Breyne et al. 2002; De Paepe et al. 2004; Himanen et al. 2004 and Vandenabeele et al. 2004). The main advantage of AQBC over clustering methods, such as K-means, is that there is no need for predefinition of the number of clusters and expression profiles that do not fit in any cluster, according to the predefined quality parameters, are rejected. It is a user-friendly and fast approach that renders clusters containing only tightly related expression profiles. In this experiment, AQBC only assigned 35% of the genes analyzed to clusters of co-expressed genes. Clusters 2 and 3 are probably the most relevant ones, as they contain transcripts with expression profiles fitting what is expected for genes involved in the SI-response. Both clusters contain transcripts down regulated in the self-pollinated pistils and absent in the cross-pollinated pistils. The main difference between the two clusters is the expression level in the non-pollinated pistils. Cluster 4 is probably less important with respect to SI as it contains transcripts that are constitutively expressed in self-pollinated pistils and displaying differential expression in the cross-pollinated pistils. These can be genes involved in (successful) pollination and fertilization. Clusters 7 and 8 are very similar and comprise genes with changing expression patterns, which are difficult to explain. Clusters 9 and 10 comprise genes displaying increasing expression levels in both self-pollinated and cross-pollinated pistils. These clusters probably contain the genes that are involved in more general processes, such as, degradation of the pistils at the end of the pollination.

The TDFs identified in this experiment were classified into 10 functional MIPS categories. The largest categories were Metabolism, Cell cycle and DNA processing and Biogenesis of cellular components. In many cases, homologies were found with components of general cellular functions. In other cases, interesting homologies to proteins known to be involved in fertilization and SI were found. These results were used to select a group of TDFs representing *L. perenne* genes that are putatively involved in SI and that should get priority in future research (Table 4). Interestingly, one TDF (Cdb11_255B) displayed homology to P-type ATPases, a superfamily of cation transport enzymes, including Ca^{2+}-transporting proteins. Other TDFs (Cdb15_200 and cdb22_230I) displayed homologies to actins. It has been demonstrated in several plant species that the actin skeleton and calcium gradients are very important factors for pollen tube growth (Franklin-Tong 1999). In *Papaver*, one of the first effects of a SI reaction is a fast and rapid rearrangement of the actin cytoskeleton of pollen tubes. The loss of the Ca^{2+} gradient, which characterizes the pollen tube tip, plays a role in the initial inhibition of pollen tube growth (Franklin-Tong et al. 1993 and 1995). Cdb16_320 represents a GTP-binding protein. It is known that Calcium, protein kinases and GTP-binding proteins are involved in signaling cascades important in different biological processes and probably also in SI (Clark et al. 2001).

Table 4 Overview of the genes identified using the cDNA-AFLP analysis in experiment 2 and which are putatively involved in SI

TDF	Genbank accession	AQBC cluster	Most significant homology	E-value	Identity (%)	Similarity (%)
cdb11_255B	EX152693	8	P-type ATPase (Fragment), *Hordeum vulgare* (Q94IN2)	5E-31	94	97
cdb15_200	EX152694	1	Actin 1, *Triticum monococcum* (AF326781)	4.8E-09	76	76
cdb16_320	EX152695	/	Putative GTP-binding protein, *Oryza sativa* (Q6K2P6)	6E-17	97	100
cdb22_230I	EX152696	1	Actin 2, *Oryza sativa* (P17298)	0.06	88	94
cdb43_200D	EX152697	8	Ubiquitin-like protein (At5g57860), *Arabidopsis thaliana* (Q8L8S0)	0.003	86	90
cdb117_300	EX152698	/	Allene oxide cyclase, *Oryza sativa* (Q8L6H4)	3E-21	97	97

Note that not all of the TDFs belong to a given AQBC cluster (second column). The selection was not only based on the E-value, but also taking into account the percentages identity and similarity

Interesting homologies were found to ubiquitin-related proteins (cdb43_200D). This TDF is of particular interest for further research as the SI response in several gametophytic and sporophytic systems involves ubiquitin-related proteins and presumably protein degradation (Franklin-Tong and Franklin 2003; Hackauf and Wehling 2005 and Sijacic et al. 2004). Although the specific molecular action of these proteins in SI is still unknown, evidence has accumulated recently that proteins necessary for pollen tube growth are degraded in incompatible pollen tubes (Thomas and Franklin-Tong 2004; Qiao et al. 2004 and Stone et al. 2003). In support of this theory an STS-derived fragment, which cosegregates with Z in rye, displayed homology to an ubiquitin-specific protease gene (Hackauf and Wehling 2005). Therefore, it is beyond any doubt that a better understanding of the involvement of these tags in the SI response should be the topic of further research.

Finally, TDF, cdb117_300 displayed homology to an allene oxide cyclase gene, which is important in jasmonic acid synthesis induced by mechanical wounding and as defense against pathogens (Creelman and Mullet 1995 and Creelman et al. 1992). Some of the processes activated in plants during fertilization and SI could be common to those triggered by pathogen attack. Although rather speculative at this stage, this hypothesis is very interesting if we take into account that the growth of the pollen tube through the transmitting tract of the style is a process with clear similarities to the growth of fungal pathogens in plants. In support of this hypothesis, SRC, the pollen determinant of SI specificity in crucifers (Kachroo et al. 2001), is similar in structure to defensins, the molecules of innate immunity that present the first line of defence to microbial challenge in plants and animals (Nasrallah 2005). Also 5% of the TDFs displayed homology to transcription factors, but till now nothing is known about which kind of transcription factors might be involved in SI.

In conclusion, our results demonstrate that cDNA-AFLP is a powerful technique to screen for genes controlling specific biological functions when no prior sequence information is available. Using cDNA-AFLP we were able to identify a set of genes displaying interesting expression profiles concerning SI. The involvement of these genes in SI is currently being checked.

Acknowledgments The authors thank Ariane Staelens, Carina Pardon, Lien De Smet and Cindy Merckaert for excellent technical assistance. We are grateful to Dr. Danny Thorogood, Institute of Grassland and Environmental Research (IGER), UK for providing plants from the ILGI population. This work was supported by the Flemish "Instituut voor de aanmoediging van Innovatie door Wetenschap en Technologie in Vlaanderen (IWT)".

References

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein search programs. Nucleic Acids Res 25(17):3389–3402

Bachem CWB, van der Hoeven RS, d Bruijn SM, Vreugdebhil D, Zabeau M, Visser RGF (1996). Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development. Plant J 9:745–753

Bert PF, Charmet G, Sourdille P, Hayward MD, Balfourier (1999) A high-density molecular map for ryegrass (*Lolium perenne*) using AFLPmarkers. Theor Appl Genet 99:445–452

Bian X-Y (2001) Towards cloning the self-incompatibility genes from *Phalaris coerulescens.* PhD Thesis, The University of Adelaide

Bian X-Y, Friedrich A, Bai J-R, Baumann U, Hayman DL, Barker SJ, Langridge P (2004) High-resolution mapping of the S and Z loci of *Phalaris coerulescens*. Genome 47:918–930

Branco-Price C, Kawaguchi R, Ferreira RB, Bailey-Serres J (2005) Genome-wide analysis of transcript abundance and translation in Arabidopsis seedlings subjected to oxygen deprivation. Ann Bot 96:647–660

Breyne P, Dreesen R, Cannoot B, Rombaut D, Vandepoele K, Rombauts S, Vanderhaeghen R, Inzé D, Zabeau M (2003) Quantitative cDNA-AFLP analysis for genome-wide expression studies. Mol Genet Genomics 269:173–179

Breyne P, Dreesen R, Vandepoele K, De Veylder L, Van Breusegem F, Callewaert L, Rombauts S, Raes B, Cannoot B, Engler G, Inzé D, Zabeau M (2002) Transcriptome analysis during cell division in plants. Proc Nat Acad Sci 12(99):14825–14830

Breyne P, Zabeau M (2001) Genome-wide expression analysis of plant cell cycle modulated genes. Curr Opin Plant Biol 4:136–142

Bruggmann R, Abderhalden O, Reymond P, Dudler R (2005) Plant Mol Biol 58(2):247–267

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Clark GB, Thompson G, Roux SJ (2001) Signal transduction mechanisms in plants: an overview. Plant Mol Biol 80(2):170–177

Cornish MA, Hayward MD, Lawrence MJ (1979) Self-incompatibility in ryegrass 1 Genetic control in diploid *Lolium perenne* L. Heredity 43:95–106

Creelman RA, Bell E, Mullet JE (1992) Involvement of a Lipoxygenase-Like Enzyme in Abscisic Acid Biosynthesis. Plant Physiol 99(3):1258–1260

Creelman RA, Mullet JE (1995) Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc Nat Acad Sci 92:4114–4119

De Graaf BH, Rudd JJ, Wheeler MJ, Perry RM, Bell EM, Osman K, Franklin FC, Franklin-Tong VE (2006) Self-incompatibility in Papaver targets soluble inorganic pyrophosphatase in pollen. Nature 444:490–493

De Paepe A, Vuylsteke M, Van Hummelen P, Zabeau M, Van Der Straeten D (2004) Transcriptional profiling by cDNA-AFLP and microarray analysis reveals novel insights into the early response to ethylene in *Arabidopsis*. Plant J 39:537–559

De Smet F, Mathys J, Marchal K, Thijs G, De Moor B, Moreau Y (2002) Adaptive quality-based clustering of gene expression profiles. Bioinformatics 18(5):735–746

Durrant WE, Rowland O, Piedras P, Hammond-Kosack KE, Jones JDG (2000) CDNA-AFLP reveals a striking overlap in Race-specific resistance and wound response gene expression profiles. Plant Cell 12:963–977

Fearon CH, Hayward MD, Lawrence MJ (1983) Self-incompatibility in ryegrass 5 Genetic control, linkage and seed set in diploid *Lolium multiflorum*. Heredity 50:35–45

Franklin-Tong VE (1999) Signaling and the modulation of pollen tube growth. Plant Cell 11:727–738

Franklin-Tong VE, Franklin FCH (2003) The different mechanisms of gametophytic self-incompatibility. Philos Trans Biol Sci 358:1025–1032

Franklin-Tong VE, Ride JP, Franklin FCH (1995) Recombinant stigmatic self-incompatibility (S-) protein elicits a Ca^{2+} transient in pollen of *Papaver rhoea.s* Plant J 8:299–307

Franklin-Tong VE, Ride JP, Read ND, Trewawas AJ, Franklin FCH (1993) The self-incompatibility response in *Papaver rhoeas* is mediated by cytosolic free calcium. Plant J 4:163–177

Fukai E, Fujimoto R, Nishio T (2003) Genomic organization of th S core region and the S flanking regions of a class-II S haplotype in *Brassica rapa*. Mol Genet Genomics 269:361–369

Fuong FT, Voylokov AV, Smirnov VG (1993) Genetic studies of self-fertility in rye (*Secale cereale* L) 2 The search for isozyme marker genes linked to self-incompatibility loci. Theor Appl Genet 83:619–623

Gabriels SH, Takken FL, Vossen JH, de Jong CF, Liu Q, Turk SC, Wachowski LK, Peters J, Witsenboer HM, de Wit PJ, Joosten MH (2006) CDNA-AFLP combined with functional analysis reveals novel genes involved in the hypersensitive response. Mol Plant Microbe Interact 19(6):567–576

Hackauf B, Wehling P (2005) Approaching the self-incompatibility locus Z in rye (*Secale cereale* L) via comparitive genetics. Theor Appl Genet 110(5):832–845

Heslop-Harrison Y (1982) Pollen stigma interaction and cross-incompatibility in the grasses. Science 161:230–237

Hiscock SJ, Mclnnis SM (2003) Pollen recognition and rejection during the sporophytic self-incompatibility response: *Brassica* and beyond. Trends Plant Sci 8(12):606–613

Himanen K, Vuylsteke M, Vanneste S, Vercrysse S, Boucheron E, Alard P, Chriqui D, Van Montagu M, Inzé D, Beeckman T (2004) Transcript profiling of early lateral root initiation. Proc Nat Acad Sci 101(14):5146–5151

Hua Z, Kao TH (2006) Identification and characterization of components of a putative Petunia S-locus F-box-containing E3 ligase complex involved in S-RNase-based self-incompatibility. Plant Cell 18(10):2531–2553

Kachroo A, Schopfer CR, Nasrallah ME, Nasrallah JB (2001) Allele-specific receptor-ligand interactions in *Brassica* self-incompatibility. Science 293:1824–1826

Kowyama Y, Tsuchiya T, Kakeda K (2000) Sporophytic self-incompatiblity in *Ipomoea trifida*, a close relative of Sweet Potato. Ann Bot 85(Suppl A):203–209

Langridge P, Baumann U, Juttner J (1999) Revisiting and revising the self-incompatibility genetics of *Phalaris coerulescens*. Plant Cell 11:1826

Leach CR, HAymann DL (1987) The incompatibility loci as indicators of conserved linkage groups in the *Poaceae*. Heredity 58:303–305

Lewis EJ, Humphreys MW, Caton MF (1980) Chromosomal location of two isozyme loci using primary trisomics. Theor Appl Genet 57:237–239

Li X, Niedl J, Hayman D, Langridge P (1995) Thioredoxin activity in the C terminus of *Phalaris* S-proteins. Plant Journal 8:133–138

Lundqvist A (1956) Self-incompatibility in rye I genetic control in the diploid. Hereditas 42:293–348

Lundqvist A (1975) Complex self-incompatibility systems in angiosperms. Proc R Soc Lond B Biol Sci 188(1092): 235–245

McClure BA (2006) New views of S-RNase-based self-incompatibility. Curr Opin Plant Biol 9(6):639–646

McCLure BA, Cruz-Garcia F, Beecher B, Sulaman W (2000) Factors affecting inter- and intra-specific pollen rejection in *Nicotiana*. Ann Bot 85(suppl A):113–123

McCLure BA, Franklin-Tong V (2006) Gametophytic self-incompatibility: understanding the cellular mechanisms involved in "self" pollen tube inhibition. Planta 224(2):233–245

Nasrallah JB (2005) Recognition and rejection of self in plant self-incompatibility: comparisons to animal histocompatibility. Trend Immunol 26(8):412–418

Qiao H, Wang H, Zhao L, Zhou J, Huang J, Zhang Y, Xue Y (2004) The F-box protein AhSLF-S_2 physically interact with S-RNases that may be inhibited by the Ubiquitin/26S proteosome pathway of protein degradation during compatible pollination in Antirrhium. Plant Cell 16:582–595

Reijans M, Lascaris R, Groeneger AO, Wittenberg A, Wesselink E, van Oeveren J, de Wit E, Boorsma A, Voetdijk B, van der Spek H, Grivell LA, Simons G (2003) Quantitative comparison of cDNA-AFLP, microarrays, and GeneChip expression data in *Saccharomyces cerevisiae*. Genomics 82:606–618

Sijacic P, Wang X, Skirpan AL, Wang Y, Dowd PE, McCubbin AG, Huang S, Kao T-H (2004) Identification of the pollen determinant of S-RNase-mediated self-incompatibility. Nature 429:302–305

Saeed AI, Sharov V, White J, Li J, Liang W, Bhagabati N, Braisted J, Klapa M, Currier T, Thiagarajan M, Sturn A, Snuffin M, Rezantsev A, Popov D, Ryltsov A, Kostukovich E, Borisovsky I, Liu Z, Vinsavich A, Trush V, Quackenbush J (2003) A free open-source system for microarray data management and analysis. Biotechniques 34(2):374–378 http://www.tigrorg/software/tm4/

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual (2nd edition). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Shivanna KR, Heslop-Harrison Y, Heslop-Harrison J (1982) The pollen-stigma interaction in the grasses 3 Features of the self-incompatibility response. Acta Botanica Neerlandica 31:307–319

Sonneveld T, Tobutt KR, Vaughan SP, Robbins TP (2005) Loss of pollen-S function in two self-compatible selections of *Prunus avium* is associated with deletion/mutation of an S haplotype-specific F-box gene. Plant Cell 17:37–51

Stein JC, Howlett B, Boyes DC, Nasrallah ME, Nasrallah JB (1991). Molecular cloning of a putative receptor protein kinase gene encoded at the self-incompatibility locus of *Brassica oleraceae*. Proc Nat Acad Sci (USA) 88:8816–8820

Staiger CJ, Franklin-Tong VE (2003) The actin cystoskeleton is a target of the self-incompatibility response in *Papaver rhoeas*. J Exp Bot 54(380):103–113

Stone SL, Anderson EM, Mullen RT, Goring DR (2003) ARC1 Is an E3 Ubiquitin Ligase and Promotes the Ubiquitination of Proteins during the Rejection of Self-Incompatible *Brassica* Pollen. Plant Cell 15:885–898

Takasaki T, Hatakeyama K, Suzuki G, Watanabe M, Isogai A, Hinata K (2000) The S receptor kinase determines self-incompatibility in *Brassica* stigmas. Nature 403:913–916

Takayama S, Isogai A (2005). Self-incompatibility in plants. Annu Rev Plant Biol 56:467–489

Thomas SG, Franklin-Tong VE (2004) Self-incompatibility triggers programmed cell death in *Papaver* pollen. Nature 429:305–309

Thomas SG, Huang S, Li S, Staiger CJ, Franklin-Tong VE (2006) Actin depolymerization is sufficient to induce programmed cell death in self-incompatible pollen. J Cell Biol 174(2):221–229

Thorogood D (1991) Self-fertility in *Lolium*. PhD Thesis, University of Wales

Thorogood D, Kaiser WJ, Jones JG, Armstead I (2002) Self-incomaptibility in ryegrass 12 Genotyping and mapping the S and Z loci of *Lolium perenne* L. Heredity 88:385–390

Vandenabeele S, Vanderauwera S, Vuylsteke M, Rombauts S, Langebartels C, Seidlitz HK, Zabeau M, Van Montagu M, Inzé D, Van Breusegem F (2004) Catalase deficiency drastically affects gene expression induced by high light in *Arabidopsis thaliana*. Plant J 39:45–58

Vos P, Hogers R, Bleeker M, Reijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, Zabeau M (1995) AFLP: a new technique for DNA fingerprinting. Nucleic Acids Res 23:4407–4414

Wang Y, Tsukamoto T, Yi K-W, Wang X, Huang S, McCubbin AG, Kao T-H (2004). Chromosome walking in the *Petunia inflata* self-incompatilbity (S-) locus and gene identification in an 881-kb contig containing S_2-RNase. Plant Mol Biol 54:727–742

Zheng X, Chen X, Zhang X, Lin Z, Shang J, Xu J, Zhai W, Zhu L (2004) Isolation and identification of a gene in response to rice blast disease in rice. Plant Mol Biol 54: 99–109