Deciphering the *Theobroma cacao* self-incompatibility system: from genomics to diagnostic markers for self-compatibility

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Received 17 February 2017; Editorial decision 26 July 2017; Accepted 31 July 2017

Editor: Zoe Wilson, University of Nottingham

Abstract

Cocoa self-compatibility is an important yield factor and has been described as being controlled by a late gametosporophytic system expressed only at the level of the embryo sac. It results in gametic non-fusion and involves several loci. In this work, we identified two loci, located on chromosomes 1 and 4 (CH1 and CH4), involved in cocoa self-incompatibility by two different processes. Both loci are responsible for gametic selection, but only one (the CH4 locus) is involved in the main fruit drop. The CH1 locus acts prior to the gamete fusion step and independently of the CH4 locus. Using fine-mapping and genome-wide association studies, we focused analyses on restricted regions and identified candidate genes. Some of them showed a differential expression between incompatible and compatible reactions. Immunolocalization experiments provided evidence of CH1 candidate genes expressed in ovule and style tissues. Highly polymorphic simple sequence repeat (SSR) diagnostic markers were designed in the CH4 region that had been identified by fine-mapping. They are characterized by a strong linkage disequilibrium with incompatibility alleles, thus allowing the development of efficient diagnostic markers predicting self-compatibility and fruit setting according to the presence of specific alleles or genotypes. SSR alleles specific to self-compatible Amelonado and Criollo varieties were also identified, thus allowing screening for self-compatible plants in cocoa populations.

Key words: Candidate genes, diagnostic markers, fine mapping, gene expression, immunolocalization, self-incompatibility, *Theobroma cacao*. 

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Introduction

Theobroma cacao L. is a tropical tree originating from the humid forests of the northern part of South America, where large genetically diverse populations still exist in situ (Cheesman, 1944; Lanaud, 1987; Laurent et al., 1993, 1994; Figueira et al., 1994; Motamayor et al., 2002, 2008; Looi Solorzano et al., 2012). Theobroma cacao seeds are used to make chocolate, providing income for at least five million smallholders worldwide. Smallholder farmers typically cultivate between one and five hectares, achieving low yields. One important yield factor is the self-compatibility status of cocoa trees. Self-compatible (SC) young trees would produce an average of 66% more fruits than the self-incompatible (SI) trees (Cope, 1939a), and a higher proportion of self-compatible trees has been observed among higher-producing trees (Lachenaud et al., 2005). Various mechanisms of self-incompatibility (SI), preventing the self-fertilisation of plants and thus favouring plant heterozygosity, have been described in angiosperms (de Nettancourt, 1997; Takayama and Isogai, 2005; Rea and Nasrallah, 2008). These mechanisms act at the level of inhibition of pollen growth for most species, or at the ovary level, leading to a failure of embryo development. Several mechanisms of genetic control of SI have been described in plants, as follows.

The gametophytic S-RNase-based self-incompatibility system (GSI), which has been described in Solanaceae, Rosaceae, and Plantaginaceae, is controlled by a multi-allelic S-locus that includes at least two genes whose interaction will lead to pollen rejection. The female determinant codes for an extracellular ribonuclease (S-RNAse) and the male determinant codes for a protein containing the F-box motif SFB (S haplotype-specific F-box protein) (McClure, 2008; Sassa et al., 2010; McClure et al., 2011). The pollen is rejected when there is a match between the single S-haplotype in the haploid pollen and either of the two S-haplotypes in the diploid pistil.

The sporophytic self-incompatibility system (SSI), which has been well described in Brassicaceae, is controlled by one highly polymorphic S-locus that contains two tightly linked genes: the S-locus Cys-rich (SP11/SCR) gene, which is the male determinant, producing proteins that are secreted from the anther tapetum and localized at the surface of pollen grains (Schopfer et al., 1999; Suzuki et al., 1999), and the stigma S-locus receptor Ser/Thr kinase (SRK), which is the female determinant, localized in the stigma cells (Stein et al., 1991). During incompatible reactions, the self-incompatibility response is mediated by allele-specific interactions of the SP11/SCR and SRK genes. In the SSI system, the SI phenotype of the pollen is determined by its parental genotype where dominance relationships exist between alleles, and both possible pollen alleles express the same SI phenotype when interacting with the stigma cells.

A late-acting self-incompatibility system (LSI) has been also described in several species (for reviews see Seavey and Bawa, 1986; Gibbs, 2014). The SI mechanism in T. cacao has been described as a LSI system: in SC and SI reactions the pollen can reach the ovary with a similar rate of pollen-tube growth 4 h after pollination (Bouharmont, 1960), but the double-fertilization is completed after 24 h in SC reactions, whereas in SI reactions the male nuclei are released into the embryo sac, but fail to achieve gamete fusion in part of the ovules, resulting in floral abscission (Cheesman, 1927; Cope, 1939b, 1940, 1958, 1962; Posnette, 1940; Knight and Rogers, 1955; Bouharmont, 1960; Ford and Wilkinson, 2012). The genetic control of T. cacao SI has been studied by several authors (Cope, 1939b, 1940, 1958, 1962; Knight and Rogers, 1955; Glendinning, 1967), who hypothesized the existence of an S-locus and several alleles with dominance relationships between them, including an amorphous $S^f$ allele that is present in the SC variety Amelonado, and leading to self-compatibility when homozygous. Several authors have contributed to establishing the dominance relationships between the S alleles. Knight and Rogers (1955) first established the relationships between five S-alleles: $S_1>S_2=S_3>S_4>S_5$. Cope (1962) and Glendinning (1967) found additional alleles, and determined that $S_4$ and $S_5$ are recessive to the Amelonado self-compatible allele ($S^f$). The dominance relationships between all alleles were established as $S_0=S_1>S_2=S_3>S_4>S_5$.

In addition, two other loci involved in T. cacao self-incompatibility were also hypothesized (Cope, 1962), based on cytological studies showing 25%, 50%, or 100% of ovules without gamete fusion after selfing of SI cocoa trees. It was concluded that the genetic system of SI in cacao has both aspects of sporophytic and gametophytic control (Cope, 1958). Indeed, the success of gamete fusion depends to the interactions between specific male and female alleles, as in a gametophytic system, but it also depends on the interactions between alleles in the parental tissues, as in a sporophytic system. This leads to dominance relationships between the two alleles that either allow or prevent the activation of the incomaptibility reaction.

Two loci putatively involved in self-incompatibility as determined by the percentage of fruit setting have been localized by QTL analysis: one is localised at the top of chromosome 4 (Crouzillat et al., 1996; Royaert et al., 2010; Yamada et al., 2010), the other in chromosome 7 (Yamada et al., 2010). Failure of gamete fusion, observed in part of the ovules and associated with fruit abscission, was observed only at the embryo-sac level (Cope, 1939b; Bouharmont, 1960). However, fruits can reach maturity after self-pollination of a SI tree when compatible pollen is mixed with pollen from the SI trees (the ‘mentor pollen’ effect) (Opeke and Jacob, 1967; Bartley, 1969, 2005; Lanaud et al., 1987; Glendinning, 1960), allowing the development of fertilized ovules. With the help of molecular markers, the observation of skewed segregations potentially linked to SI is then possible among the progenies.

Here, we report the analysis of a large F$_2$ progeny produced from a SI tree using the ‘mentor pollen’ effect. A first molecular analysis, performed on a subsample of this F$_2$ progeny, revealed a skewed segregation at the level of CH1 and CH4 regions (Allegre et al., 2012). In this current work, we carried out fine mapping of these genome regions and identified candidate genes whose expression was then characterized during SC and SI reactions. In order to refine the existing predictive model for self-compatibility (Da Silva et al., 2016), we
searched for multi-allelic diagnostic simple sequence repeat (SSR) markers in the CH4 region identified by fine mapping.

**Materials and methods**

**Population used for fine mapping**

A large progeny was produced by CEPLAC, at Itabuna (Brazil), by selfing the clone TSH516, a hybrid of ICS 1 and Scavina 6 (SCA 6). ICS 1 is a SC Trinitario clone selected in Trinidad, a hybrid between Criollo and Amelonado (two SC varieties). SCA 6 is a SI Upper Amazon Forastero clone collected in Peru. In this study, a total of 877 individuals from this progeny were analysed.

TSH516 is a SI clone, but self-seeds were produced with mixed pollinations using pollen from *Herrania mariae* (closely related to the *Theobroma* genus) as mentor pollen, followed by pollination with TSH516 self-pollen, as described previously (Bartley, 1969, 2005; Opeke and Jacob, 1967). Only TSH516 self-fertilized seeds developed into mature seeds while embryos from the interspecific cross were aborted (flat seeds). The resulting progeny was considered as an F2 in this experiment, although TSH 516 parents are not pure lines.

**Analyses of potential skewed segregations in other progenies**

A progeny of 550 individuals was created at IRAD (Barombi-kang, Cameroon) from IMC 60, a SI clone originating from Peru. Pollinations were made using a mixture of pollens of IMC 60 and of Catongo (a SC Amelonado-type originating from Brazil) as mentor pollen. Self progenies were identified using molecular markers.

Another progeny of 96 plants, UF 676×ICS 95, planted in French Guiana, was analysed to observe the segregations in the CH1 locus: UF 676 (SI) and ICS 95 (SC) are two hybrid Trinitario plants, heterozygous for an Amelonado allele and a Criollo allele for the CH1 locus. This cross is compatible.

**Populations used for a genome-wide association study (GWAS) and to analyse the prediction efficiency of diagnostic markers**

A population of 710 individuals from different origins was used to assess the efficiency of prediction based on SSR genotypes and alleles. This population was made up as follows: 151 individuals belonging to several commercial varieties (full-sib progenies) selected within a cocoa farm in Cameroon; 125 individuals from a breeding population from IRAD; 270 individuals from a breeding population from CEPLAC; 97 individuals from a collection of Nacional-type accessions from INIAP, resulting from germplasm conservation or new breeding steps; nine other accessions from Upper or Lower Amazon regions from CIRAD; and 58 individuals from a germplasm collection of Trinitario- and hybrid-types from the CRC (Trinidad and Tobago), previously evaluated for SC (Bartley, 1970) and stored in the ICGD database (http://www.icgd.reading.ac.uk).

For determination of the SC status of individuals not previously evaluated, 30 to 60 floral buds were covered one day prior to hand-pollination, and for a 3-day period after pollination. Half of the flowers were pollinated using pollen from the same tree and the other half were pollinated using control SC pollen. The percentage of setting was observed after a 14-d period; trees showing less than 10% fruit set after self-pollination and more than 30% after cross-pollination were considered as SI.

A subset of 570 individuals from the populations was analysed by GWAS using single nucleotide polymorphism (SNP) markers.

**Samples for gene expression analyses**

Pollinations were carried out on the SCA 6 SI clone in the greenhouse of CIRAD/Montpellier using pollen from ICS 1 (SC) or from SCA 6 (SI), and ovaries with their pistil (termed simply ‘ovaries’ in the text) were collected at different times after pollination. RNA was extracted from unpollinated ovaries and from ovaries collected at 2, 5, 8, 12, 24, 48, and 72 h after SC and SI pollinations. RNA was pooled for samples taken at 2–8 h, 12–24 h, and 48–78 h after pollination. Quantitative PCR (qPCR) was conducted on three biological repetitions of each RNA extract.

**Genotyping**

**F2 progeny**

Our objective was to identify skewed regions with a total absence of recombinant plants, linked to the SI reaction, in the CH1 and CH4 regions of the F2 progeny. Several approaches were used to define new markers in these two regions. SSR markers were defined using the whole sequence of the Criollo genome V1 (http://cocoa-genome-hub.southgreen.fr/gbrowse) (Argout et al., 2011), and the tool ‘Search for SSR’ in the ESTTIK database (http://esttik.cirad.fr/cgi-bin/SSR_server.cgi) (Supplementary Table S1 at JXB online). PCR amplifications and genotyping of the different populations were performed according to Lloor Solorzano et al. (2012).

Two different strategies were used to identify deletions/insertions. First, deletions/insertions were identified in the CH4 region between the Criollo genome and the Amelonado genome (http://www.cacaogenomedb.org/) (Motamayor et al., 2013) after alignment of both sequence fragments using ClustalW2 implemented at the SimGene.com website (http://simgene.com/ClustalW2). Second, resequencing of the CH1 and CH4 regions was made by DNA capture in 24 accessions of different genetic origins. Capture oligos were designed by Roche NimbleGen using the targeted regions of the reference sequence of Criollo genome V1. The captures were carried out according to the protocol provided by NimbleGen (SeqCap EZ Library SR—User’s Guide version 4.0).

Primers amplifying fragments including the deletion were defined using the primer-BLAST online tool from NCBI (http://www.ncbi.nlm.nih.gov/tools/primer-blast) and checked for their polymorphism in the F2 progeny. Newly designed markers were named mSI-X. The mTcCIRx markers used in this study can be found in the TropGENE database (http://www.southgreen.fr/content/tropgene-database).

**Seedlings obtained from mixed pollinations performed on IMC 60**

We used four SSR markers to differentiate seedlings produced from self- or cross-fertilization within the IMC 60 progeny after mixed pollinations: mSI_303, mSI_458, mSI_460, and mTcCIR_7, discriminating IMC 60 from Catongo.

**GWAS population**

The GWAS population was genotyped by sequencing (GBS) using the DArTSeq technology after DNA restriction with *PstI* and *MseI* (Kilian et al., 2012).

**Cytological observation of the proportion of fertilized ovules**

Several SC trees from the F2 progeny, homozygous for the Amelonado allele for the CH4 locus and heterozygous for the CH1 locus, were self-pollinated or pollinated with a SC pollen (CCN 51) taken as a control. Young fruits were harvested 7 d after self-pollination and to determine the proportion of fertilized ovules, the fruits were boiled for 20 min in water and then dissected. Using a binocular microscope, the relatively large, white fertilized ovules could be distinguished from the poorly developed, yellow, aborted ovules.

**Genome-wide association studies**

GWAS was conducted using Tassel 5.2.31 software (Glazebolt et al., 2014) on 570 individuals assessed for SC/SI and coded as 0 (SI) and 1.
(SC). In addition, for a subsample of 388 individuals the percentage of fruit setting 14 d after self-pollination was determined as a quantitative variable to assess SC/SI status. The 570 individuals were genotyped by sequencing using 16480 SNPs stored in the TropGENE database (http://tropgened.bcid.fr/tropgene/) and with a minor allele frequency >0.05. The mixed linear model method was applied to identify marker/trait association using the population structure and kinship matrix as covariates (Bradbury et al., 2007). The structure of the population was determined with a subset of 150 SNPs distributed over all chromosomes, and using a Bayesian clustering method implemented in the STRUCTURE software (Pritchard et al., 2000), with a burning period of 100 000 iterations, 500 000 Markov Chain Monte Carlo repetitions, and 10 independent runs. The variances of Ln(PD) (Estimated Ln Prob of Data) were checked for each run, and runs for which the variance was not homogeneous with variances of the other runs with the same K value were eliminated.

A model with admixture and correlated allele frequencies was chosen, and with a K number of sub-populations between 5 and 12. The value of K was estimated as in Evanno et al. (2005). The contributions of the individuals to the K groups were then used as covariates in the analysis. The kinship matrix was constructed from the 16480 SNPs.

Significant associations between markers and SC/SI status were identified in two steps: first, the P values were computed, and second, to eliminate false-positive associations, the P-value threshold was determined using the QVALUE software to control the false discovery rate (FDR) (Storey, 2002). The corresponding P-value threshold was given by the QVALUE with a cut-off for rejection of the null hypothesis (q value) of 0.05 to ensure a 5% FDR.

Search for candidate genes

The Criollo cocoa genome sequence, version V1, available at the Cocoa Genome Hub (http://cocoa-genome-hub.southgreen.fr/gbrowse) (Argout et al., 2011), was used to identify candidate genes involved in the T. cacao SI system, on the basis of their homology with genes already known to be involved in SI systems in other species. Each ortholog to T. cacao genes annotated in the Criollo genome for the CH1 and CH4 genome regions without recombinant plants was checked for its potential role in self-incompatibility systems already described in the literature.

Gene expression analyses

The expression of candidate genes identified in the CH1 and CH4 regions was analysed in the SCA 6 accession during SC and SI reactions.

Ovaries were harvested at various time intervals after SC and SI pollinations (0 h; 2-5-8 h; 12-24 h, and 48-72 h) and conserved at −20 °C in RNA later solution (Sigma). Isolation of total RNA and cDNA synthesis were conducted according to Legavre et al. (2015).

Gene expression was assessed by qPCR using a Roche LightCycler 480 Real-time PCR System with a SYBR Green dye included in the supermix to detect dsDNA amplification products. qPCR was carried on 12 candidate genes together with two reference genes that were used for normalization, Tc04_g000050 (Isocitrate dehydrogenase) and Tc08_g003640 (Tubulin beta-6 chain), as previously described (Pinheiro et al., 2011). Primers were designed using the Primer3 program (http://bioinfo.ut.ee/primer3-0.4.0/) and qPCR was performed as described in Legavre et al. (2015) (see Supplementary Table S2). The absence of non-specific products was verified by a melting-curve analysis.

The amplification efficiency of each primer pair was determined using a standard procedure and integrated in the analysis. Differential expression of each candidate gene was calculated according to the 2−ΔΔCt method (Livak and Schmittgen, 2001). After normalization, the relative expression of candidate genes was analysed in relation to the unpolminated ovaries.

Immunolocalization of Tc01_g007270 and Tc01_g007290 proteins

Immunostaining was performed on 8-μm paraffin sections of unpolminated ovaries and SC-pollinated or SI-pollinated ovaries (8–24 h) after fixation in 3% paraformaldehyde/0.1% Triton X-100 in phosphate buffer and embedding in paraplast blocks (Brillouet et al., 2014).

Rabbit polyclonal antibodies anti-Tc01_g007270 and anti-Tc01_g007290 were produced by Eurogentec (Anti-peptide Speedy 28-Day, https://secure.eurogentec.com/speedy.html) using synthesized peptides as the antigen. The peptide sequence LGNDKTVRIWQTG Presents corresponds to residues 310–324 of the Tc01_g007270 protein and RSVDKSNDESEQVS corresponds to residues 478–492 of the Tc01_g007290 protein. Optimal antibody dilutions were determined by ELISA tests (final dilutions: 1:500 for anti-Tc01_g007270 and anti-Tc01_g007290, 1:200 for pre-immune serum). Controls were carried out with pre-immune serum. Immunolocalization was determined with an Alexa Fluor 488 dye conjugated goat anti-rabbit antibodies (Interchim, France, Montluçon).

Microscopic imaging was performed using the Montpellier RIO Imaging Platform (https://www.mri.cnrs.fr/fr/) with a confocal microscope (LSM510, Meta; Carl Zeiss Micro Imaging) or with a NIKON Eclipse Ni-E light microscope using the filters DAPI (340–380/400) and B2 A (450–490/505) for fluorescence.

Identification of diagnostic markers

To determine diagnostic markers, 11 SSR markers, identified in the CH1 and CH4 regions, were used to genotype the full population of 710 individuals, and to establish predictions for SC/SI according to the genotype or allele. Each marker was either considered alone or in combination with one or two other markers.

Allelic frequencies and identification of alleles specific to Amelonado and Criollo SC varieties

A set of 108 genotypes, capturing the diversity of the T. cacao genetic groups and planted in the international collection of the CRC, were genotyped with the 11 SSR markers. Allele frequencies were estimated for each genetic group, using the GENETIX software (http://www.genetix.univ-montp2.fr/genetix/constr.htm) (Belkhir et al., 2004), and a search was made for alleles specific to the SC varieties Amelonado and Criollo. Reference SSR profiles allowing cocoa breeders to characterize clones for potential SC/SI status were established for these SSRs (see Supplementary Table S3).

Prediction analyses were carried out according to genotype or allele presence, using SAS software modules (SAS Institute Inc., 2004):

Genotype analyses

All combinations were tested by Fisher’s Exact Test, using PROC FREQ. Probabilities associated to the tests were adjusted for multiple testing, using the QVALUE software (Storey, 2002). The probabilities of being SC given the individuals have (ProbSIs) or do not have (ProbSNo) the specified genotype were computed from a logistic regression computed through PROC LOGISTIC. Here, we tested the effects of all 36 939 combinations of genotypes (1 to 3 loci) in the self-incompatibility.
Results

Study of skewed segregations linked to the incompatibility system in the F2 progeny

Fine-mapping of restricted genome regions was conducted, including the skewed segregations in the F2 progeny. The alleles of TSH516 inherited from ICS 1 in both the CH1 and CH4 regions are ‘Amelonado alleles’. In these regions, three possible genotypes were expected: homozygous for the Amelonado allele (a), homozygous for the allele provided by SCA 6 (b), and heterozygous (h).

In the skewed CH1 region, 22 markers could be defined and were used to genotype the F2 progeny. Among the three possible types, the b and h genotypes were predominant in this region, with the ‘a’ Amelonado genotype totally absent in a small region of 70.3 kb delimited by the markers mSI_103 and mSI_375. A total of 761 individuals were analysed for this region, with the ‘a’ Amelonado genotype totally absent in the CH1 region with mSI_103, indicating the absence of gametic selection at the CH1 locus level.

In the skewed CH4 region, 26 markers segregating in the F2 progeny could be defined at the top of the chromosome (Table 1). The expected a/h/b genotype segregation for this F2 was 1/2/1, whereas only two genotypes, a and h, were observed at the top of CH4, within a region without recombinants delimited by the markers mSI_462 (1414 bp), and mSI_411 (258 684 bp) (Table 1), and with a segregation of 1/2. Part of this F2 population had been tested for self-incompatibility in a previous study (Yamada et al., 2010) and the QTL for self-compatibility, evaluated by the percentage of fruit set 14 d after self-pollination, was identified in the same region of CH4 as the skewed segregations, with the S/Amelonado allele providing self-compatibility when homozygous.

Interestingly, the origin of the absent genotype is not the same on the two loci located on CH1 (genotype homozygous for Amelonado allele) and CH4 (genotype homozygous for SCA 6 allele).

Skewed segregations observed in other progenies

Segregations were observed in several other progenies using three markers, mSI_103, mSI_107, and mSI_460, located in CH1 and CH4 regions, identified by fine mapping (Table 2).

Self-progeny from IMC 60

A total of 39 self-fertilized individuals were obtained from 550 seedlings produced after pollinations made on IMC 60, another SI clone, using a mixture of compatible and incompatible pollen. Skewed segregations, with a complete absence of one homozygous genotype, were only observed in the CH4 region with mSI_460, while the three possible genotypes were all observed in the CH1 region with mSI_103, indicating the absence of gametic selection at the CH1 locus level. Skewed segregations were observed in the chromosome 1 (CH1) and chromosome 4 (CH4) regions: 22 and 26 markers, respectively, defined in these regions, allowed the identification of restricted regions (in bold) with a complete absence of one of the three genotypes, expected from the F2. The number of each genotype is indicated at the level of each marker: (a) homozygous for the ICS1 allele (Amelonado type); (h) heterozygous; and (b) homozygous for the SCA 6 allele.

<table>
<thead>
<tr>
<th>Markers</th>
<th>Chromosome</th>
<th>Position (bp)</th>
<th>Number of individuals</th>
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<tr>
<td>mSI_26</td>
<td>CH1</td>
<td>3 377 732</td>
<td>5 228 118 351</td>
</tr>
<tr>
<td>mSI_88</td>
<td>CH1</td>
<td>3 499 444</td>
<td>5 224 111 340</td>
</tr>
<tr>
<td>mSI_89</td>
<td>CH1</td>
<td>3 525 756</td>
<td>5 233 114 352</td>
</tr>
<tr>
<td>mSI_32</td>
<td>CH1</td>
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<td>4 236 120 361</td>
</tr>
<tr>
<td>mTCgIR15</td>
<td>CH1</td>
<td>3 711 664</td>
<td>4 227 117 348</td>
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<td>4 291 138 429</td>
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<td>3 243 119 365</td>
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<td>10 127</td>
<td>198 380 0 578</td>
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<td>20 673</td>
<td>259 488 0 747</td>
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<td>183 348 0 531</td>
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<td>205 411 0 616</td>
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<td>193 383 0 576</td>
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<td>301 563 0 864</td>
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<td>259 506 0 765</td>
</tr>
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<td>CH4</td>
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<td>110 232 0 342</td>
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<td>277 421 1 699</td>
</tr>
<tr>
<td>mSI_42</td>
<td>CH4</td>
<td>343 424</td>
<td>109 233 2 345</td>
</tr>
<tr>
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<td>CH4</td>
<td>428 250</td>
<td>119 230 2 352</td>
</tr>
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<td>mSI_54</td>
<td>CH4</td>
<td>751 986</td>
<td>55 127 4 521</td>
</tr>
<tr>
<td>mSI_294</td>
<td>CH4</td>
<td>1 686 245</td>
<td>109 248 16 373</td>
</tr>
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</table>
Segregations observed in the SC F2 plants, homozygous for the CH4 loci (SI/SC) and heterozygous for the CH1 loci, after self-pollinations

Plants homozygous for the SI/SC CH4 locus are SC. In order to determine whether heterozygosity at the level of the CH1 locus could lead to skewed segregation even if associated with the SI/SC genotype at the CH4 locus, the F2 seedlings BR36 and BR59, homozygous SI/SC for the CH4 locus and heterozygous for the CH1 locus, were self-pollinated. For both the 109 seedlings from the selfing of BR59 and the 36 seedlings from BR36, and in common with the F2 progeny, there was a total absence of the ‘a’ genotype corresponding to plants homozygous for the Amelonado allele (mSI_103-223) for the CH1 locus. These results show clearly that even if the plants are SC due to the SI/SC CH4 genotype, the CH1 locus is still functional, and a genotypic selection could still be observed. The existence of genotypes homozygous for the CH1 locus (as for the Amelonado variety) shows that the CH1 allele is not a lethal one, and instead it is firmly involved in the SI system and leads to genotype selection even if the plant is SC.

Segregations observed in another progeny involving the Amelonado CH1 allele

We analysed the segregations in the progeny of UF 676×ICS 95. Each Trinitario parent of this progeny, a hybrid between Amelonado and Criollo genotypes, is heterozygous for the mSI_103 (223) Amelonado allele and the mSI_103 (228) allele that originates from Criollo. No skewed segregation was observed in this progeny (Table 2).

A lack of fertilisation between both gametes containing the Amelonado allele (mSI_103-223) was observed when the SCA 6 allele (mSI_103-225) was associated with the Amelonado allele in the parents of the progeny (for example BR59×BR59 and BR36×BR36). This was not the case when the Amelonado allele was associated with the Criollo allele (mSI_103-228) in the parents of the progeny (as in UF 676×ICS 95). These observations probably reflect a dominance effect of the SCA 6 allele towards the Amelonado allele that originates from Criollo. No skewed segregation could result from gamete non-fusion events, the CH1 region is expressed before the stage of gamete fusion, allowing the fertilization of all ovules in the ovary even in the case of the SI reaction. This is in contrast to the CH4 locus involved in fruit drop linked to gametic non-fusions in SI clones such as IMC 60 that has been observed by some authors.

Association studies for fruit setting linked to self-incompatibility

Using the Structure software, the number of sub-populations of the population of 570 individuals studied by GWAS was estimated as being K=9, and the contribution of individuals to the nine sub-groups was taken as a covariate in the GWAS analyses. Marker/incompatibility trait association studies using 16 480 SNPs were conducted on the 570 individuals coded with a 0/1 score for the SI/SC trait, and revealed positive associations for 11 SNP markers, all located at the top of CH4 from positions 189 447 bp to 1 328 172 bp (Table 3, Fig. 1). Only one marker, 3673000|F|0–37:G>A located at position 189 447 bp, was within the restricted region identified by fine mapping, which contained 36 other SNP markers subjected to GWAS. This marker is located inside the Tc04_ g000230 gene, an ortholog of the GEX1 gene of Arabidopsis thaliana, and it had one of the two lowest values for P and q resulting from the statistical analysis, indicating that the candidate gene could be strongly linked to this marker. No association was detected in CH1. Similar results were obtained with the 388 individuals evaluated for SC/SI by the percentage of fruit setting, with eight positive associations located from positions 189 447 bp to 558 838 bp, and the same positive marker 3673000|F|0–37:G>A located in the region of fine mapping. These observations revealed that only the CH4 locus is associated with fruit drop, not the CH1 locus.

Search for candidate genes potentially involved in the self-incompatibility system in the CH1 and CH4 genome regions and analysis of their expression

Searches for candidate genes potentially involved in cocoa self-incompatibility were first made in the CH1 and CH4 regions identified by fine mapping (Fig. 2), and the expression of 12 candidate genes was analysed during SC and SI reactions (see Supplementary Table S4).

CH1 region

No recombinant plants were identified within a region of 70.3 kb delimited by the markers mSI_103 and mSI_375, respectively located at the positions 4 021 267 bp and 4 091 577 bp of chromosome 1 (with a total absence of the ‘a’ genotype). In this region, only nine genes have been annotated in the whole-genome sequence V1 of Criollo (Argout et al., 2011), from Tc01_g007220 to Tc01_g007300. Among them three genes are considered as possible candidate genes, as follows.
Deciphering the Theobroma cacao self-incompatibility system

**Table 2. Segregations observed at the level of CH1 and CH4 loci for other progenies**

<table>
<thead>
<tr>
<th>Progeny</th>
<th>Marker</th>
<th>Chromosome</th>
<th>Parental genotypes</th>
<th>Number of seeds analysed</th>
<th>Expected genotypes</th>
<th>Number of seeds having the genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>IMC60×IMC60</td>
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<td>133–145 × 133–145</td>
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<td>19</td>
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<td></td>
<td>mSI_107</td>
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<td>233–245 × 233–245</td>
<td>38</td>
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<td></td>
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<td></td>
<td>233/245</td>
<td>19</td>
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<tr>
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<td>CH4</td>
<td>161–161 × 161–161</td>
<td>34</td>
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<td>109</td>
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<td></td>
<td>mSI_107</td>
<td>CH1</td>
<td>239–245 × 239–245</td>
<td>87</td>
<td>239–239</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>245–245</td>
<td>26</td>
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</table>

Segregations were observed for the loci in CH1 and CH4 for several progenies. The self-progeny obtained from the self-incompatible clone IMC60, using a pollen mentor, showed a total absence of one of the three expected genotypes, but only at the level of the CH4 locus. Two self-progenies were produced from BR36 and BR59, which are two self-compatible clones from the F2 progeny only heterozygous for the CH1 locus, with an Amelonado allele associated with a Scavina 6 allele: a total absence of the genotype homozygous for the Amelonado allele was observed for the locus of CH1. The last progeny (UF676×ICS95) was produced from a compatible cross between two Trinitario genotypes, both heterozygous for the CH1 locus, and associating an Amelonado allele with a Criollo allele: no skewed segregation was observed in this progeny, and all three expected genotypes were present. The complete absence of expected genotypes is highlighted in bold.

**Tc01_g007220** is an ortholog of *A. thaliana* BAM1. BAM1 (At5g65700) and BAM2 (At3g49670) encode CLAVATA1-related leucine-rich repeat receptor-like kinases, which have an important role in early anther development (Hord et al., 2006). DeYoung et al. (2006) have shown that BAM1 and BAM2 are involved in the development of both male and female gametophyte development. **Tc01_g007220** is differentially expressed between SC and SI reactions at later stages, with a higher expression during SC reactions (Fig. 3A).

**Tc01_g007270** is homologous to the COMPASS-like H3K4 histone methylase component WDR5a from *A. thaliana* (At3g49660). It is a transducin WD-40 repeat-containing protein acting as a site of protein–protein interactions and plays central roles in biological processes (Stirnimann et al., 2010). It has also been reported that WD40 could be involved in the last step of the ubiquination cascade that leads to protein degradation, an important step in the self-incompatible reactions (Wang et al., 2004).
**Te01_g007290** encodes a putative transmembrane transporter, part of the major facilitator superfamily (MFS-1), and is homologous to the *A. thaliana* At5g65687 gene, a probable sphingolipid transport spinnor homolog 1. According to the STRING network (http://string-db.org/), this gene is also predicted to interact with several genes involved in ubiquination. *Malus domestica* is characterized by a S-RNase-based GSI (Cheng et al., 2006), and when S_Rnase is unable to enter in the pollen tube, due to the silencing of the transporter MdABC (localized in the pollen tube membrane), this leads to a SC reaction.

**Te01_g007270** and **Te01_g007290** are differentially expressed between SC/SC reactions at early stages (2 to 8 h), with a much higher expression observed for **Te01_g007290** during SI reactions (Fig. 3B–C). Observations at later stages (48–72 h) showed that **Te01_g007290** expression levels decreased and became similar to those observed in unpollinated ovaries (control). In contrast **Te01_g007290** expression remained at a high level and there was differential expression between the SI and SC reactions, with increased expression for SI reactions. **Te01_g007290** could be involved in transport of molecules during the SI mechanism.

Immunolocalization experiments conducted with **Te01_g007270** and **Te01_g007290** showed that these genes are expressed in the cell layers of the style where pollen tubes are growing, and in cell layers surrounding the embryo sac. **Te01_g007270** was mostly expressed in the inner-integument cells and also in the nucellus during SC and SI reactions. Its expression can be also detected, but to a lower extent, in the same cell layers of unpollinated ovaules. Its expression was not observed in pollen tubes at 8 or 24 h after SC pollination of SCA 6, but it could be observed in the style cells where pollen tubes were growing (Fig. 4).

**Te01_g007290** was expressed in the nucelus and in the inner-integument cells, with a stronger expression observed in the cell layer separating the two. No difference was visible between SC and SI reactions. Its presence in pollen tubes was not observed at 8 or 24 h after SC pollination, or at 24 h after SI pollination, but it was observed in the style cells where pollen tubes were growing (Fig. 5).

**CH4 region**
The region of 257.270 kb identified by fine mapping includes 30 genes annotated in the Criollo genome V1. Among them, nine genes were identified as candidates potentially involved in the SI reactions, as follows.

**Te04_g000160** is homologous to the voltage-dependant L-type calcium channel subunit alpha-1F gene from *Gossypium arboreum*. This gene could play a role in Ca2+ influx pathways, as has been shown in humans (Kotturi et al., 2003) and in plants. It was demonstrated that Ca2+ influx into stigma papilla cells mediates SI signalling in the Brassicaceae, and it could be a common event in other types of SI systems. This gene is up-regulated during incompatible reactions (Iwano et al., 2015). **Te04_g000160** is differentially expressed between SC and SI reactions at early stages (2–8 h). Its higher expression during SI reactions is in agreement with the Ca2+ pathway described in the Brassicaceae during SI reactions (Fig. 3D).

### Table 3. Significant marker/self-incompatibility trait associations identified by GWAS

<table>
<thead>
<tr>
<th>Marker</th>
<th>Chromosome</th>
<th>Position</th>
<th>F</th>
<th>P-value</th>
<th>q-value</th>
<th>Marker effect</th>
</tr>
</thead>
<tbody>
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<td>SNP-SI/SC (0/1)</td>
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<td></td>
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<td>3673000</td>
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<td>CH4</td>
<td>432 186</td>
<td>24.16</td>
<td>9.17 e–11</td>
<td>7.56 e–7</td>
</tr>
<tr>
<td>3228506</td>
<td>F0–14:T–C</td>
<td>CH4</td>
<td>322 464</td>
<td>12.55</td>
<td>4.69 e–6</td>
<td>1.19 e–2</td>
</tr>
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<td>F0–38:G–T</td>
<td>CH4</td>
<td>325 666</td>
<td>12.47</td>
<td>1.23 e–3</td>
<td>7.69 e–1</td>
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<td>CH4</td>
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<td>4.30 e–7</td>
</tr>
<tr>
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<td>2.65 e–5</td>
</tr>
<tr>
<td>3253808</td>
<td>F0–32:G–C</td>
<td>CH4</td>
<td>581 127</td>
<td>11.36</td>
<td>1.46 e–5</td>
<td>2.67 e–2</td>
</tr>
<tr>
<td>3233009</td>
<td>F0–66:T–A</td>
<td>CH4</td>
<td>1 528 172</td>
<td>11.16</td>
<td>1.73 e–5</td>
<td>2.85 e–2</td>
</tr>
</tbody>
</table>

**A GWAS was carried out with the mixed linear model procedure implemented in Tassel 5.2.31 software for 570 individuals evaluated for self-incompatibility, with the trait coded as (0) for self-incompatible, SI, or by (1) for self-compatible, SC. Among them, 389 were also evaluated by the percentage of fruit setting after self-pollination. The population genotyping was carried out with 16 480 SNPs obtained by GBS. Significant associations were identified for a False Discovery Rate cut-off <0.05 after computing q-values from the P-values.**

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Deciphering the *Theobroma cacao* self-incompatibility system

Fig. 1. Genome-wide association study (GWAS) of self-incompatibility traits. GWAS-derived Manhattan plots showing significant $P$-values associated with self-incompatibility traits. Left: a qualitative variable was used with a score of 0 for self-incompatible trees (less than 10\% of fruit setting after self-pollination) and 1 for self-compatible trees (more than 30\% of fruit setting after self-pollination). Right: a quantitative variable: the percentage of fruit setting, assessed 14 d after self-pollination. A total of 16 480 genome-wide SNP markers identified by GBS were used for this analysis to generate the data, which was analysed using the MLM procedure of the Tassel software. Significant associations for CH1 to CH10, and for unmapped SNP (11) are shown by dots for a threshold of $P$-values $<3\text{e}^{-5}$ [or $-\log_{10}(P$-value$)=5.3$], corresponding to a FDR cut-off $q$-value $<0.05$.

$Tc04\_g000170$ is an ortholog to a KANADI transcription factor, potentially interacting with an auxin factor response 3. It is not differentially expressed between SC/SI reactions. $Tc04\_g000190$, $Tc04\_g000230$, $Tc04\_g000240$ and $Tc04\_g000260$ are homologous to *A. thaliana* GEX1 (GAMETE EXPRESSED PROTEIN, At5g55490) genes potentially involved in early embryo development. They have a dual function during male and female gametophyte development and early embryogenesis, and are required for correct pollen maturation. In *A. thaliana*, GEX1 is expressed in several tissues and several steps of embryogenesis: in the embryo sac before cellularization, the egg cell after cellularization, the zygote/embryo immediately after fertilization, and the male gametophyte, in both the vegetative cell and in the sperm cells (Alandete-Saez et al., 2011). Using the STRING network, it was shown that in *A. thaliana*, GEX1 interacts with the HAPLESS2 gene involved in male fertility, and is essential for pollen tube guidance, successful gamete attachment, and fertilization.

Among $Tc04\_g000190$, $Tc04\_g000230$, $Tc04\_g000240$ and $Tc04\_g000260$ had a level of expression similar to those of unpollinated ovules, while the two others displayed a differential expression between SC/SI reactions compared to unpollinated ovules (Fig. 3G–J). $Tc04\_g000240$ showed a higher expression level, but only at early stages of the SI reaction. $Tc04\_g000190$ was significantly more expressed during the SC reaction during the period 12–24 h.

$Tc04\_g000300$ is an ortholog to a ferredoxin thioredoxin reductase, and was not differentially expressed between SC/SI reactions.

$Tc04\_g000320$ is an ortholog of a zinc finger AN1 domain-containing stress-associated protein I2 (PMZ) gene from *A. thaliana*. According to the STRING network, PMZ interacts with several genes associated with protein degradation through the ubiquination complex. PMZ also interacts with *AtCDC48B*, a gene involved in cell division and growth processes that interacts with SNAREs, transmembrane proteins that are mainly responsible for membrane fusion and exchanges in the cell (Martens and McMahon, 2008). $Tc04\_g000320$ was significantly more expressed at the stage 12–24 h during the SC reaction, and also constitutes a good candidate gene potentially involved in variations in self-incompatibility (Fig. 3E).

$Tc04\_g000330$ is an ortholog of the ARM repeat-containing protein ARC1 gene from *Brassica napa* (AGP76183.1). The ARC1 protein is involved in SI signalling in *Brassica* and targets proteins for degradation during the SI response, promoting the ubiquitination and proteasomal degradation compatibility factors in the pistil (Stone et al., 2003). In our experiments, $Tc04\_g000330$ was differentially expressed at the stage 12–24 h, where a higher expression was observed for the SC reaction compared to unpollinated ovaries or the SI reaction, which is not in agreement with what is expected during the SI reaction (Fig. 3F).

Self-compatibility/incompatibility predictions

Using SSR markers located in the CH1 and CH4 regions, we genotyped a population of 710 individuals assessed for self-incompatibility in order to test for genotypes or genotype combinations that are able to predict SC or SI plants.

Genetic diversity and allele specificity

Two old *T. cacao* varieties, Amelonado and Criollo, are the ancestors and the main sources of self-compatibility alleles in the species. Both are completely homozygous, and are the origin of most of varieties under cultivation worldwide. Specific Amelonado and Criollo alleles were identified among the CH4 markers, predicting SC trees homozygous for these alleles.

The 161-bp allele of mSI_460 is specific to Amelonado. The presence of this allele with a low frequency in the Nanay and Nacional groups probably reflects introgression of Amelonado in some individuals of these two groups. Another allele of mSI_460, allele 149, is only shared by the SC Amelonado genotypes (mainly present in African Amelonado genotypes), and the Contamana SI genotypes (particularly SCA 6, a high-performance progenitor, widely used in breeding programs; see Supplementary Table S5). The combined use of markers mSI_303 and mSI_460...
enables discrimination between the two haplotype origins: the mSI_460-149 allele is shared by the Amelonado and Contamana genotypes, whereas the mSI_303-227 allele is present in Amelonado genotypes but absent in the Contamana group, and the mSI_303-229 allele can be present among Amelonado genotypes but is always absent in Contamana group.

The mSI_460-159 allele is shared by some Amelonado and Purus genotypes; however, the mSI_303-229 allele may allow discrimination between Amelonado and Purus genotypes in some cases.

The allele 217 of mSI_303 is specific to Criollo and could be used to screen for SC plants.

**Genotype analysis**

Allele combinations other than those identified in Amelonado and Criollo could result in SC plants. Dominance relationships exist between S-alleles and the multi-allelic status of...
SSR markers allows a better discrimination of S-haplotype interactions.

When only one genotype was considered, 24 genotypes from seven markers were significantly associated with cocoa self-incompatibility by Fisher's Exact test ($q$-value $< 0.05$). Among those genotypes, when present, 16 resulted in a higher frequency of SC plants and 10 resulted in a higher frequency of SI plants (Supplementary Table S6). Among those associated with SI, when present in a given individual, the genotypes 231/231 (locus mSI_303), 194/197 (mSI_7), and 155/161 (mSI_460), resulted in a chance of at least 95.6% of being SI (Supplementary Table S6). Trees with the six other genotypes resulted in a chance of at least 74.8% of being SI. Among the 16 genotypes associated with a higher number of SC plants, the genotypes 161/161 (mSI_460), 225/227 (mSI_303), 292/318 (mSI_458), and 132/149 (mSI_460) presented at least a 91.7% chance of being SC, with a very low chance (<8.3%) of being SI. The allele 161 of mSI_460 is specific to the SC Amelonado variety, which is homozygous for this allele, and 100% of individuals homozygous for this allele are SC. When two genotypes were considered, 178 combinations of genotypes were significantly associated with SC/SI status in cacao, with 134 combinations resulting in more SC clones and 44 in more SI clones. The addition of one more marker in some cases improved the discrimination of SC/SI individuals. For some specific marker combinations, the SI/SC ratio of trees was lower than 1/56; the discrimination of SI was also improved with some other genotype combinations (Supplementary Table S6). Predictions of SC/SI status obtained from a combination of three genotypes were not significantly more efficient than predictions based on one or two genotypes.

The use of any of the 26 genotypes listed in Supplementary Table S6 will result in an increased frequency of SC plants in the population. However, the loci mSI_460 and mSI_303 with 22 and 19 alleles, respectively, appear as the best predictors of SC/SI status. Combining both markers could greatly increase the efficiency of early selection of SC or SI genotypes.

**Allele effect**

Among the 128 alleles tested, 43 were significantly associated with SC/SI status by the Fisher's Exact test (Supplementary Table S7). It can be seen that the probability of a plant being...
SC is rarely higher than 50%. In contrast, the presence of one or two copies of several alleles seems to confer SI to cocoa plants. This is the case for the allele 225 of mSI_103, the presence of which in the F2 progeny and in BR36 and BR59, when associated with mSI_103-223, prevents the formation of a homozygous plant for the other allele (mSI_103-223). In the population studied for predictions, the probability of an individual having two copies of this allele being SI was 90%. Fifteen alleles from different markers give plants a probability of being SI that is greater than 80%. The allele effects could reflect the relationships of dominance that exist between incompatibility alleles: the presence of particular SSR alleles.

Fig. 4. Immunolocalization of the Tc01_g007270 protein. (A–F) Confocal microscope images of sections of ovules, ovary, and style of the Scavina 6 accession either self-pollinated (self-incompatible, SI) or pollinated by a compatible pollen (SC) and probed with a Tc01_g0007270 antibody (labelled green). (A) Controlled hybridization labelled with pre-immuned rabbit serum for an ovule subjected to SC pollination. (B–F) Sections of ovules and ovary probed by a Tc01_g0007270 antibody: (B) unpollminated, (C) SC at 8 h, (D) SI at 8 h, (E) SC at 24 h, and (F) SI at 24 h. The Tc01_g0007270 protein appeared mostly present in the inner integument cells for the SC and SI reactions. (G–L) Images (Nikon microscope) of pollen tube germination at 8 h (J–L) and 24 h (G–I, same style as shown in image E) probed with Tc01_g0007270 antibody and stained with anilin blue. (I, K) Visualization of pollen tube germination (light blue), (J, L) Tc01_g0007270 protein immunolocalization, (G, J) merged images. The Tc01_g0007270 protein did not seem to be present in the pollen tubes themselves, as observed at 8 or 24 h after pollination, but seemed to be localized in the style tissues surrounding them. Abbreviations: es, embryo sac; s, synergid cell; sg, starch grains surrounding the polar nucleus; n, nucellus; ci, outer integuments; ii, inner integuments; m, micropyle; ce, chalazal end; pt, pollen tube.
such as mSI_7/197 or mSI_460/155, are probably linked to some dominant incompatibility alleles, giving the plants a low probability (0.07) of being SC.

**Discussion**

*Theobroma cacao* has a complex incompatibility system under the control of several genes. It has already been described as a late incompatibility system having both gametophytic and sporophytic aspects and dominance relationships between alleles (Cope, 1958, 1962; Glendinning, 1960). Our results confirmed its gametophytic and sporophytic features and led to the identification of two independent loci involved in the SI system through two different mechanisms. Indeed, the CH1 locus, identified for the first time in this study, does not function as a LSI system expressed at the stage of gametic non-fusions, nor as classical GSI or SSI systems. The CH1 locus has traits of both gametophytic and sporophytic systems: it depends upon the nature of the alleles present, as for a GSI (for example, when selfing BR36 or BR59, only the homozygous genotype mSI_103-223 was eliminated, in contrast to the homozygous genotype mSI_103-225 which could be observed in the progenies), but it depends also upon specific interactions between them, as in a SSI (for example, the mSI_103-223 allele generates a SI reaction only when associated with the mSI_103-225 allele and not when associated with itself, as in the SC Amelonado variety, or when associated with the mSI_103-228 allele, as in ICS95).

The proteins of candidate genes of the CH1 region (*Tc01_g0007270* and *Tc01_g0007290*) did not seem to be present in the pollen tubes observed at 8 or 24 h after pollination, but were apparent in the style cells where there were growing pollen tubes. The expression of these genes in the female tissues could result from interactions between male and female tissues that depend upon the specific allele combination and parental allele interactions, and result in the prevention of
fertilization between some gametes. This could explain the CH1 locus segregation observed in BR36 and BR59 after self-pollination, with 2/3 heterozygous plants and 1/3 homozygous plants. Moreover, in these cases all ovules were fertilized which reflects a gametic selection that happened prior to the gamete fusion step. This selection could involve a lack of penetration or migration of the sperm nucleus in the embryo sac as was already observed in SCA 24 (closely related to SCA 6) (Ford and Wilkinson, 2012).

A region of 70.3 kb without recombinants plants, identified by fine mapping, contained only nine genes. Three of them were differentially expressed: Tc01_g007220 is ortholog to a gene involved in the development of the gametophyte, and Tc01_g007270 and Tc01_g007290 are orthologous to genes known to be involved in GSI in other species and involved in protein-protein interactions and protein degradation, respectively, and also in transport of S-RNase in the pollen tube. Through qPCR analysis, it was determined that Tc01_g007270 and Tc01_g007290 are up-regulated in the earlier stages of SI reactions (2 to 8 h) compared to SC reactions, which is in accordance with their potential interactive role with putative gametophytic S-locus genes not present in this locus. In *Solomonum chacoense*, a single dominant gene (Sli) with sporophytic action that inhibits gametophytic S gene expression in the pollen has been identified (Hosaka and Hanneman, 1998). The candidate genes identified for the CH1 locus could have a similar role as modifier genes, in interaction with gametophytic genes located in other genome regions.

The CH4 locus is involved in the SI reaction through a different mechanism, strongly associated with fruit drop. All positive associations identified by the GWAS were determined in the CH4 region where a gametic selection (probably linked to the observations on gamete non-fusions made by several authors: Cheesman 1927; Cope 1939b, 1940, 1958, 1962; Posnette, 1940; Knight and Rogers, 1955; Bouharmont, 1960; Ford and Wilkinson, 2012) was also observed in the F2 progeny under study, as in the self-progression of IMC 60, another SI clone. In these two progenies, only the use of mentor pollen, which prevented the fruit from dropping, had allowed the seed development in ovules where gametic fusions were effective. A region of 257.270 kb, without recombination and containing 30 genes was also identified in CH4. A GWAS conducted on a large population of 570 individuals identified 11 SNP markers significantly associated with fruit setting in the top of CH4. However, only one SNP marker (3673000|F|0–37:G>A), significantly associated with fruit setting in the CH4 region identified by fine mapping and thus probably close to the gene controlling the CH4 locus. Among the other three closely linked TcGEX1 orthologous genes (Tc04_g000190, Tc04_g000240, and Tc04_g000260), Tc04_g000190 was the most expressed in SC reactions in comparison with unpollinated ovules, at the stage 12–24 h. The GEX1 genes, involved in male and female gametophyte development and in early embryo development in *A. thaliana*, could be involved in the late incompatibility reaction of *T. cacao* acting at the ovule level and controlled by the CH4 locus. Recently, it has been observed that the non-fusion of gametes, as observed after pollination of *T. cacao* using incompatible pollen, could result from two different and independent mechanisms: an incomplete migration of the sperm nucleus, which fails to reach the female nuclei, or a successful sperm nucleus migration and reaching of the female nuclei, but followed by a non-fusion of gametes (Ford and Wilkinson, 2012). The role of *GEX1* genes, interacting with the *HAPLESS2* gene that is known to be essential for pollen tube guidance, successful gamete attachment and fertilization (von Besser et al., 2006; Mori et al., 2006), could be determinant in this late manifestation of SI in *T. cacao*. Until now, few results have been reported on molecular mechanisms potentially involved in LSI (Zhou and Zheng, 2015) and the *GEX1* genes constitute good candidates for its control. However, Tc04_g000160, Tc04_g000320, and Tc04_g000330, orthologs to genes involved in SI signalling in *Brassica* and protein degradation during the SI response, cannot be ruled out and could also participate in the pathway leading to self-incompatibility.

The practical output from this study is the identification of markers that could help breeders to select for SC plants in cocoa populations at an early stage in the breeding process. Indeed, the identification of highly polymorphic SSR markers in the CH4 region identified by fine mapping gives the potential to have a strong linkage disequilibrium between SSR alleles and incompatibility alleles, leading to a good ability of SSR to predict SI or SC genotypes. Specific Amelonado and Criollo alleles, identified in this study, will be particularly efficient in predicting SC varieties.

*T. cacao* has developed an efficient system to maintain genetic variability, based on two different and independent mechanisms of gamete selection. However, beyond the progress already achieved in deciphering the *T. cacao* SI system, made possible by advances in the fields of genomic technology and whole-genome sequencing, many questions still remain about its mechanism, which involves several loci for which no clear orthology with classical GSI or SSI S-locus receptors was found. Further investigations will be needed to study their potential interactions with other loci of the whole genome and to establish the links between incompatibility alleles and structural variations of candidate genes. This increased knowledge will help to achieve better identification of S-alleles and consequently will allow for better control of selection for the self-compatibility trait in *T. cacao*.

**Supplementary data**

Supplementary data are available at *JXB* online.

- Table S1. Primer pairs defined in CH1 and CH4 regions.
- Table S2. Primer pairs defined in candidate genes differentially expressed.
- Table S3. Reference SSR profiles for a collection of genetic resources.
Table S4. Relative expression of candidate genes.
Table S5. Allelic frequencies of SSR markers located in CH1 and CH4 regions in the *T. cacao* genetic groups and populations studied.
Table S6. Significant predictions of self-compatibility/incompatibility based on genotype presence.
Table S7. Significant predictions of self-compatibility/incompatibility based on allele presence.

Acknowledgements

This project was supported by Agropolis Fondation under the reference ID 1403 46 through the ‘Investissements d’avenir’ programme (Labex Agro: ANR-10-LABX 001 I). We also thank Mondelēz International for their financial contribution to this project. We thank Marc Lartaud and Geneviève Conéjéro for their helpful advice regarding immunolocalization experiments and imaging. We thank Nea Ngwa Akongwi, for his help in managing field pollinations in Cameroun and Semi Melliti, and Gerald Oliver for the management of cocoa plants in the greenhouse in Montpellier.

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