Late-acting self-incompatibility (LSI) in Schima superba: pollen-ovary interactions and signaling to trigger PCD in incompatible pollen

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Article

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Abstract

Background

In angiosperm, Self-incompatibility (SI) is a common and widespread mechanism for plant preventing inbreeding risk and late-acting self-incompatibility (LSI) is even more ancestral conserved. In this work, we worked on *Schima superba*, one species in Theaceae, commercially important timer and fire-resistant tree species and revealed its LSI mechanism.

Results

The self-pollen tubes grew to the bottom of the stylus and enter ovary until 48h, but failed to penetrate the ovule. Meanwhile, the hormone and peroxidase levels were dramatically changed. Transcriptome and proteome analysis explored the molecular mechanisms of LSI and candidate genes related to LSI in *S.superba*. Overall 586.71 million reads were obtained and 79,642 (39.08%) unigenes were annotated. KEGG and GO showed that 4531 differentially expressed genes (DEGs) and 82 differentially expressed proteins (DEPs) were at 48h in self- (SP) vs outcross-pollination (OP). Among these, 160 DEGs and 33 DEPs involved in pollen-pistil interaction. The “pollen-pistil interaction”, “signal recognition” and “component of membrane” were downregulated in SP whereas the “cell wall and membrane biosynthetic process”, “oxidoreductase activity” were upregulated. The DEGs involved with S-RNases and SCF during SP suggested that the LSI was happened at 48h ovary, and the LSI in *S.superba* was under gametophyte control. The calcium ion increase-releasement and F-actin depolymerization initiated metacaspases-1 activity and lead to PCD progress. Also, mitochondrial function loss and ROS disruption further aggravated the PCD progress and cell death. Cell wall biosynthetic progress upregulated and cellulose synthase downregulated could lead to ovary xylification and withering.

Conclusions

The LSI of *S.superba* which happened at 48h after pollination was key time point. The incompatibility PT ceased growth in ovary because of the S-RNase recognition and PCD happened in this organ. This study highlight the LSI molecular mechanism in *S.superba* and also gave a reference to other species in Theaceae.

Background

In angiosperm, SI is a common and widespread mechanism to reduce inbreeding depression risk, and it usually under genetic control [–]. SI systems normally contained sporophytic SI (SSI) and gametophytic SI (GSI) [–]. The SSI is always happened in the stigma, and the pollen-specific S-locus cysteine-rich protein (SCR) and stigma-specific S-receptor Kinase (SRK) are both controlled the pollen tube (PT) growth, the novel species is in *Brassicaceae*[–]. As for the GSI systems, the S-RNase (the female determinant) and *S*-locus F-box protein (SLF/SFB, the male determinant) are interacted mediate the self-pollen tubes rejection response, and this pattern is most happened in the style in *Plantaginaceae*, *Solanaceae* and *Rosaceae*[–]. In *Papaveraceae*, another kinds of GSI is caused by the Ca^{2+} signaling cascade leading to apoptosis and ceased the pollen tube (PT) growth[]. Additionally, ovarian SI (OSI) or late-acting SI (LSI) will happen when the PT ceased growth in the ovary[]. LSI, an ancestral conserved mechanism of SI, exist widespread among *Narcissus papyraceus*, *Clivia gardenii* and *Aloe maculate*[–].

Since the abortion could occur before or after zygote formation plus much more complicated mechanism in postzygotic SI, the molecular mechanisms of LSI are poorly understood[14–]. The LSI could be controlled by sporophyte, gametophyte and the two joints together [3]. Studies showed that some growing molecular signals involved in PT attraction to the synergid cells and ovules would affect self-PT growth differentially in LSI species[–]. For example, SCR/s-locus protein 11 (SP11), which were synergid-derived cues (LUREs) belong to the super gene family as CRPs in *Brassica* pollen, comprise the male signaling ligand determinants[]. Whereas growth of self-pollination tube was halted in the style and SCF and *S-RNase* genes expressed in *Camellia sinensis* revealed the LSI was under gametophytic control [6]. Sage predicted that the LSI systems’s genetic basis is always gametophytic [5]. Diallel crosses with sibling progeny arrays suggested that multiple loci with multiple alleles controlled the LSI response[–].

Schima superba is a woody plant in tribes Schimeae in Theaceae, and the Theaceae is well-known by Camellia which in the tribes Theeae. These two tribes have phylogenetic similarity [,]. Nowadays, research on SI make a great progress and several metabolic pathways regulated by multiple genes in *Camellia sinensis*[,]. *S.superba* is widely distributed in southern China and its function mainly for biological fireproof and valuable timber [–]. Its florescence is from middle of May to the middle of July and the flower is belongs to small and middle type ($length_{corolla}$ =31.57mm). In our preliminary study, we detected that the PT of OP fast grew and the PT of SP slower grew from the first 2 h to12h. Then, they were all grew to the bottom of the styles and entered the ovary at 36h. The PT of OP penetrated the ovula at 48h, however, the PT of SP was ceased growth on the top of the ovary and did not penetrated into ovula. The fruit abortion rates were over 88% in SP treatment and seed yielded only 0.2%-0.4%. So we deduced the *S.superba* was an LSI species []. To make clear the LSI molecular mechanism of *S. superba*, we compared the hormone level between self- and outcross-pollination, and associating the transcriptome with proteome analysis to reveal the gene expression pattern. The results will gain better understanding on LSI mechanism in *S. superba* and refresh a knowledge in Theaceae.

Results

Hormone and Enzyme quantification in SP vs OP

A quantitative analysis of hormone levels in SP and OP were revealed by HPLC-MS/MS. The results indicated that IAA and ZT biosynthesis were decreased in SP from 24h to 120h (Fig. 1A, B), however, ABA were increased after 60h in SP (Fig. 1C). The changing of the hormone level showed that the 48h after pollination was a special time turning point, and at this time, the IAA, ZT, and ABA were downregulated by 52%, 36% and 38% respectively in SP than in OP ovaries.

The JA, MeJA and SA were important hormones in stress resistance and biotic stimulus. From 24h to 72h, we found that at 48h, the SA and JA/MeJA were sharply changed in quality. At this time point, the SA was 1.2-fold higher and the JA/MeJA were 5.4-fold lower in SP vs OP (Fig. 1D, E, F).

The activity of CAT, POD and SOD were all increased from 24h to 84h in both SP and OP (Fig. 2A, B). The activity of CAT and SOD were low; however, the activity of POD was high from 48h to 84h in SP vs OP, and the value was especially 1.3-fold at 48h in SP (Fig. 2C).

Degs In The Self- And Outcross- Ovaries

Illumina sequencing, de novo assembly and functional annotation

To identify genes changed during PT growth in the ovary at 48 h after OP and SP treatment in *S. superba*, six libraries were sequenced with Illumina HiSeq 2000 platform. 586.71 million bases of raw reads and 569 million bases of clean reads (Q30 > 92.86%) were generating. 85.36Gb of nucleotides, 203,767 unigenes, and 79,642 (39.08%) annotated unigenes were yielded (Table 1, Supplementary Table S1).

Table 1
Summary of functional annotation

	Number	Percentage (%)
Nr Annotated	61549	30.2
Nt Annotated	42395	20.8
Swiss-Prot Annotated	44414	21.79
KEGG Annotated	22088	10.83
GO Annotated	45663	22.4
Annotated in KOG/COG	21329	10.46
All annotated unigenes	79642	39.08
Total Unigenes	203767	100

A threshold of fold change ≥ 2.00 and p-value ≤ 0.05 were used to identify differentially expressed genes (DEGs). There were 4,531 DEGs were identified in the SP-vs-OP ovaries, in which 2,078 were upregulated and 2,453 were downregulated (Supplementary Table S2 and Supplementary Fig. S1). The gene ontology (GO) annotation identified these DEGs assigned with 2,453 GO terms and classified them into the cellular component (CC: 14%), molecular function (MF: 24%) and biological process (BP: 62%) (Supplementary Table S3). Furthermore, 62 putative transcription factors families were found in the DEGs which contained the AP2-EREBP were the most abundant (9) followed by bHLH (8), MYB (8) and ABI3VP1 (6) (Supplementary Fig. S2). 338 DEGs were categorized into 84 KEGG pathways and 11 of which were significantly enriched (corrected P value ≤ 0.05): phenylpropanoid biosynthesis (5.8%), plant-pathogen interaction (5.5%), Pentose and glucuronate interconversions (5.5%), plant hormone signal transduction (3.6%), Carotenoid biosynthesis (7.1%), Fatty acid elongation (7.0%), DNA replication (4.8%), Oxidative phosphorylation (2.9%), Limonene and pinene degradation (7.5%), ether lipid metabolism (4.9%), and monoterpenoid biosynthesis (9.1%) (Fig. 3). In addition, “ubiquitin mediated proteolysis”, “PCD” and “Calcium ion signaling” pathways related to SI were identified as up-regulated in SP.

Specifically, or preferentially expressed genes involves in SP and OP

To identify differentially expressed genes associated with LSI in *S. superba*, we compared the up and down DEGs in GO enrichment. Of the 4531 DEGs that were annotated into 2453 GO terms, the categories were upregulated related to “cell wall and membrane biosynthetic process” (13), “cuticle development” (1), “glycolipid metabolic process” (7) of BP; “host intracellular organelle and membrane” (10) of CC; “oxidoreductase activity” (16), “ribonuclease T2 activity” (3) and “binding” (21) of MF. However, “pollen-pistil interaction” (9), “recognition of pollen” (9), “response to stress” (8) and “glucose metabolic process” (7) of BP; “signal recognition” (2) and “component of membrane” (32) of CC; “transferase activity” (28), “cellulose synthase activity” (7) and “pheromone activity” (3) of MF showed downregulated in SP (S48 vs O48) (Supplementary Table S3 and Supplementary Fig. S3).

35 DEGs annotated in top 10 KEGG pathway and in top 15 GO enrichment analysis were related to ADP binding (5, K13459|K02133), cell wall (7, K13457|K01051), pollination (1, K15397), oxidoreductase activity (13, K09755|K00430|K09843|K00517|K02256), auxin (5, K14488), oligosaccharide (1, K01213) and phosphors signal (3, K14491|K14492|K14500) (Table 2). In addition, sesquiterpenoid and triterpenoid biosynthesis (oxidoreductase activity, c119780_g1), glycan biosynthesis (membrane lipid biosynthetic process, c89162_g1) and some immunity-related pathways were founded as up-regulated

KEGG pathways, and some amino acid metabolism (Cysteine, c113534_g5|c109638_g2|c123367_g5; Histidine, c109070_g5|c109070_g2; Tyrosine, c118577_g1), zeatin biosynthesis (c116756_g1) were down-regulated KEGG pathways related to LSI in *S. superba*.

Table 2
The DEGs were annotated in top 10 KEGG and top 15 GO analysis.

KEGG Term	P-value	UniGenes	KO	Ge
Plant-pathogen interaction	0.000	C120244_g1|c126261_g1|c77700_g2| c98405_g1|c111738_g3	K13457|K13459|K13459|K13459|K13459	RF
Phenylpropanoid biosynthesis	0.000	c113063_g3|c99127_g2|c109745_g1| c112534_g4|c87454_g1|c76665_g1| c127124_g5|c87454_g2| c49580_g1	K09755|K00430|K00430|K00430|K00430|K00430|K00430|K00430| K00430	CY E1
Pentose and glucuronate interconversions	0.015	c89170_g1|c125667_g1|c87333_g2| c63625_g1|c88144_g3|c88144_g2|c88144_g1	K01051|K01213|K01051|K01051|K01051|K01051|K01051|	E3
Plant hormone signal transduction	0.115	c119184_g1|c19937_g1|c113234_g5|c105124_g2| c98108_g1|c106334_g2|c104870_g1|c113246_g2	K14488|K14491|K14492|K14500|K14488|K14488|K14488|K14488	SA
Carotenoid biosynthesis	0.128	c98441_g1|c115612_g1|	K09843|K09843	E1
Fatty acid elongation	0.128	c84875_g1	K15397	KC
Oxidative phosphorylation	0.314	c26116_g1| c107429_g1	K02256|K02133	CC
Limonene and pinene degradation	0.332	c95477_g1	K00517	E1

Of 62 TFs expressed specifically in the self-ovaries of *S. superba*, one MYB (c123606_g4) were significantly upregulated, GRAS(c64954_g2), BBR/BPC (c102379_g1), HSF (c117518_g3), MBF1 (c110410_g3) were specifically upregulated, and FAR1(c107861_g4\|c55999_g2\|c112693_g3), C2H2 (c148204_g1\| c96967_g1), HB (c104947_g1), zf-HD (c95393_g1\| c87920_g1), G2-like (c29149_g1), PLATZ (c118044_g7), RWP-RK (c116010_g1), TCP (c104077_g2) and TRAF (c110218_g1) were specifically down regulated in SP. Among these, the FAR1, C2H2, GRAS, TCP were closely related to pollen development.

During the 48h in ovary's LSI, the growth-related hormones, such as auxin, cytokinin, ABA and JA/MeJA [] were significantly downregulated, and after this time, the ABA levels were increase and exceed the normal level (Fig. 1A, B and C). And at this time point, the plant hormone signal transduction genes and proteins had the corresponding changing, for example, the auxin-related DEGs (7), zeatin-related DEG (1), abscisic acid-related DEGs (4), JA biosynthetic enzymes-related DEGs (6) and cytokinin-related DEPs (2) were significantly down-regulated at 48h in SP (Supplementary Table S4).

RT-PCR validation

To test the transcriptome data's reliability, 23 DEGs, associated with "plant-pathogen interaction" (c126261_g1), "pyruvate metabolism" (c114981_g1) and "phenylpropanoid biosynthesis" (c72974_g2), etc., were selected (Supplementary Table S5). All these selected DEGs with highly repetitiveness could verify the accurate of the sequencing data (Supplementary Fig. S4).

Deps In The Self- And Outcross- Ovaries

DEPs were identified by fold change ≥ 2.0 and ≤ 0.5, and p-value ≤ 0.05 in S48 vs O48 (Fig. 4A, Supplementary Fig. S5 and Supplementary Table S6). In total, 82 DEPs were identified, and more down-regulated proteins (60) were observed in S48 (Fig. 4A). All these DEPs were assigned to molecular functions (193 GO terms), cellular components (142 GO terms) and biological processes (786 GO terms), and the significant terms were 47, 94 and 229, respectively (Fig. 4B, Supplementary Table S7). Most of these DEPs had ion binding, transferase, hydrolase, lyaseand oxidoreductase activity, and they were involved in development process, oxidation-reduction process, organic substance metabolic process, and cellular metabolic process.

The categories were significant up-expressed related to "oxidative phosphorylation" (COX6B-1|SS204; PDI|SS2022,SS7608; mitochondrial-like ATP synthase subunit d|SS2022, SS2015), and down-expressed related to "protein glutathionylation" (DHAR2|SS9106; GSTL2|SS2206), "glutamine biosynthesis" (GLNA|SS7412, SS8421), "aspartate family amino acid catabolic process" (MGL|SS8413; At5g55070|SS6511), "oxidation-reduction process" (PER12|SS2509, SS2613; At1g75280|SS8315; GLX1|SS3305, SS4306, SS4307; At1g60710|SS8318; SDH1-1|SS8808; PDH2|SS4407; At5g55070|SS6511; At1g53240|SS8319; At3g02090|SS7706; CTIMC|SS8214) (Supplementary Table S7).

82 DEPs were performed KEGG analysis and functional enrichment. In total, 30 pathways were enriched and 10 were significant enriched in metabolism and biosynthesis (Supplementary Table S7).

The Association Analysis Between Degs And Deps

The DEPs had a relatively lower correlation with DEGs in the 48h ovary of SP vs OP (y = 0.047x + 0.1854, R^2 = 0.0436, p = 0.2087, Fig. 5A). Only c113206_g1| SS8413 and c104041_g1| SS2022 were co-detected from the DEPs-DEGs association analysis (Fig. 5B). 41 proteins and genes annotated in 3 GO functional enrichment were aggregated into four clusters, and only cluster 3 was upregulated in cell (Supplementary Fig. S6A). 24 proteins and genes were categorized into 7 KEGG pathways were also aggregated into four clusters (Supplementary Fig. S6B). These pathways were mainly in Oxidation-reduction and basal metabolism.

Discussion

The SI is an important mechanism to protect flowering plants to overcome inbreeding depression and provides a high level of heterozygosity[]. When pollens grow from stigma to ovaries, penetrate the ovules and spread the sperms, form the zygote and success promote fruits and seeds, they undergo series of complex signaling control. Whereas the incompatible pollens could be killed in any stage during this journey. Based on our previous and the present study, the morphological, anatomical and gene expression forms of analysis have been comprehensively explored to unravel the complexity of LSI in *S.superba*. The 48h as implicated in this study was an important time point to explore the PTs elongation to ovary and penetrate ovule. The transcriptome and proteome data showed DEGs and DEPs were higher enrichment in “oxidation-reduction process”, “ribonuclease T2 activity”, “cell wall or membrane biosynthetic progress”, “transmembrane transport” in SP, while, “recognition of pollen”, “plant hormone signal transduction” and “Glycolysis” were downregulated (Supplementary Fig. S2 and Supplementary Table S8).

The Incompatible Pt (Ipt) Growth And Its Physiology Changes

Researchers had already revealed partly on the SI mechanism in some species in Theaceae [–]. Seth [] and Chen [] found the PTs of SP and OP of *Camellia sinensis* were all entered in the ovary at 48h, however, the IPT recognition system expressed in the style []. Zhang showed the PT growth in SP of *Camellia sinensis* was hindered at 24h in the style []. Whereas PT of SP and OP of *Camellia oleifera* were all enter ovary at 60h, but PT of SP failed penetrate the ovule. That’s means 24h in style’s SI or enter ovary’s LSI was the two features in Theaceae. In our previous research, we found the PT growth stopped and the IPT recognition system were happened in ovary at 48h after pollination []. We presumed the 48h in ovary’s LSI was the mechanism in *S.superba*. From the phylogenetic position of Theaceae and the fossil of *Schima*, it showed that the *Schima* possessed n = 18 symmetric karyotypes, 5 not 3 locules, no center axis, could confirm the Schimeae (≡ Gordonieae) belonged to an ancient taxon and occurs earlier than other genus in Theaceae[–]. So, *S. superba* should have preserved ancestral mechanism, like 48h in ovary’s LSI. However, this ancestral conserved mechanism was not effective, there was still 11.2% self-pollination fruit got []and *C. sinensis* only had 1.1% []. That maybe for the IPT abortion in ovary and this distance was much shorter than from the style to the ovule.

Reactive oxygen species (ROS) are the reactive products of oxygen that have potential to damage the living cell and they play a key role in diverse development stages, such as self-incompatibility during pollination to induce PCD in incompatible pollen []. In plant, enzymatic and non-enzymatic antioxidants (Proline, Carotenoids, Alpha-tocopherol, Glutathione, Ascorbic acid, Flavanoids and Carotenoids) act as ROS detoxicants. In our results, GSTs related proteins, such as the lambda GSTs (GSTL| SS2206) and dehydroascorbate reductases (DHARs| SS9106) which belong to the outlying minor GST classes, the lactoylglutathione lyase (GLX1|SS3305, SS4306, SS4307), in addition with glutathione (GSH| SS7412| SS8421| c112627_g1| c86003_g1| c90752_g1| c97760_g1), SOD, CAT were all down-regulated in 48h ovary of SP. These detoxification factors depression in SP would lead ROS accumulation and induced damage and lethal [–].

Pt Guidance And Ipt Recognition

The PT guidance involved in two progress: the pre-ovular and ovular guidance, and they were all happened in the ovary []. The researchers reported that the receptor like kinase (RLKs) have mediated signaling pathways in control of ovular guidance [–]. In our results, 16 G-type lectin S-receptor-like serine/threonine-protein kinase which included 5 RLK1 (c126066_g2| c100047_g1| c100047_g3| c103929_g1| c126606_g1) and one RKS1 (c106281_g1) were detected significantly down-regulated at 48h in SP ovary, and one PT reception gene rapid alkalinization factors (RALFs) c86988_g1 was also significantly down-regulated.

S-RNase, exclusively expressed in pistil, is associated with female determinants in GSI and implicated in genetically identical pollen and rejection of self-pollen and they would trigger mitochondrial collapse and IPT’s PCD []. As we showed in our results, three S-RNase genes, which contain catalytic domain of T2-type ribonuclease (PF00445), c110815_g1, c115031_g1(RNS1) and c94788_g1(RNS3) were significantly upregulated at 48h ovary of SP in our results. Compared these three genes with other S-RNase in Rosaceae, Plantaginaceae, Rutaceae, Solanaceae and Rubiaceae (Supplementary Fig. S7), we found the c110815_g1 had a close relationship with *Prunus* T2/S-type RNase. When comparing the deduced amino acid sequences of these three S-RNases with S-RNases reported for *Prunus* species, we were able to identify the Introns I, II and III, the five conserved regions and the hypervariable region located C2 and C3 (Supplementary Fig. S8). The three S-RNase possessed the HVa (RHV), HVb and RC4 regions, which mainly in Rosaceous S-RNase, are considered prime candidates for the *S*

specificity-determining region [].Interestingly, the c110815_g1 had three introns, the first one was next to the *Prunus*-specific intron and located in C1, the second one located the same position as in *Prunus* S-RNase in RHV, and the third one was in RC4. We presumed that this gene could have special function in the self- incompatibility.

The male determinant interact with S-RNase degradation is the S-locus region, which contained the novel F-box protein named SLF/SFBs []. The SLF/SFBs interacted with cognate S-RNase and prevented non-self S-RNase catalytic activity. When non-self S-RNase enters the PT, they will form a SCF complex and targets the S-RNase for ubiquitination and degradation, then the PT continues growth. As for self S-RNase, it binds to recognition domain not the active domain resulting dysfunction of SCF for polyubiquitination of self S-RNases. Then self S-RNases are released to trigger subsequent PT growth inhibition events []. In our results, 6 SFB putative genes were found, and they were F-box protein SKIP23-like (c88773_g1|c41648_g1), F-box LRR-repeat protein (c120445_g3| c103038_g1| c51202_g1| c110213_g1). The phylogenetic analyses of the *S. superba SFB* like genes together with Rosaceae, Solanaceae and Plantaginaceae *SFB* and *SKP* shown in Supplementary Fig. S9, revealed that the c51202_g1 gene clusters with *Prunus SFB* gene. Interestingly, c51202_g1 had 5 polymorphic SNP loci in F-box domain tightly linked to the RHV region (second intron) of c110815_g1 (Fig. 6). However, this gene is expressed in all tissues here analyzed (Supplementary Fig. S10), whereas the S-pollen gene(s) are mainly expressed in anthers/pollen only, and thus the c51202_g1 detected in *S. superba*, similar as *Prunus* species, may functioned as general inhibitor (GI) as in *Prunus*, and c41648_g1 and c110213_g1 may worked as SLF [,–]. QRT-PCR results showed that the c110815_g1 was mainly expressed in ovary and increased in SP ovary from 24h to 72h, and c51202_g1, c41648_g1 and c110213_g1 were significantly upregulated at 48h ovary in SP, accordingly (Fig. 7). We presumed that the c41648_g1 and c110213_g1 would interact with c51202_g1 and c110815_g1 to recognize the IPT and active the self S-RNase's cytotoxicity in 48h ovary, and the LSI in the *S.superba* could be gametophyte controlled (Fig. 8).

The Programmed Cell Death (Pcd) In Ipt

Apoptosis or PCD is a highly conservative mechanism removing unwanted cells in eukaryotes []. In 48h ovary's LSI in *S.superba*, the IPTs were ceased growth and happened to death. As mentioned above, the S-RNase would induce apoptosis or PCD by direct degradation of ribosome RNA or indirect phosphorylation of protein, destabilization of cytoskeletons and release of cytochrome *c* (cyt *c*) etc. (Fig. 8) []. The indirect progress related to Ca^{2+} signal which transduced by protein phosphorylation and this signal transduction mechanisms relates to mitogen activated protein kinase (MAPK) cascades. The Ca^{2+} concentration changing in cytosol could also triggered downstream sensors to form stimulus-specific information. In *S.superba*, one MAPK gene c120765_g5 was significantly upregulated and the "Ca^{2+} channel activity", "calcium ion transmembrane transporter activity" and "calcium ion transport" pathway related genes and proteins were also upregulated.

The important apoptosome was caspase, which was initiated by cyt *c* and apoptotic protease activating factor-1 (APAF-1) oligomerization. This complex engages caspase-9 activation, and then the caspases-3 and − 7 execute the cleavage of cellular proteins []. In plants the metacaspases with the same function as caspase-3 are involved in PCD []. In our results, there were 16 metacaspases and 11 of them had caspase domain (metacaspases-1) were downregulated in 48h ovary SP (Supplementary Table S9). One significantly downregulated gene c76797_g1, contained the LSD1 zinc finger domain and negatively regulates the PCD pathway. So we speculated the metacaspases-1 in *S.superba* had an important function to block IPT growth (Fig. 8). The changes in actin filament dynamics can stimulate activation of caspase-3-like enzyme and induce PCD in PT []. 7 out of 12 F-actin protein complexes were lower-expressed and c97073_g1 was significantly downregulated at 48h of SP. That means the F-actin protein was depolymerization and triggered the metacaspases-1 activity upstream of DNA fragmentation [].

The important prophase character of PCD is the ruin of mitochondrial outer membrane permeabilization (MOMP) and the mitochondria system loss it functions []. Then some soluble proteins, such as cyt *c*, diffused from the intermembrane space (IMS) to cytosol [,]. The Go analysis of DEGs showed that 30 GO class 14 DEGs related to mitochondrial, such as inner mitochondrial membrane protein complex, mitochondrial membrane organization, and mitochondrial proton-transporting ATP synthase complex, etc., were all down-regulated and none of such progress was up-regulated in 48h ovary SP (Supplementary Table S10). This proved that at 48h ovary SP, the membrane system of mitochondrial was dysfunction and PCD had already started (Fig. 8). In addition, during apoptosis, Bcl-2 family proteins regulated soluble proteins (e.g. cyt *c*) release from mitochondria [–]. In this result, the Bcl-2-associated athanogene like protein gene c122719_g2, which contained BAG domain, was 2.5-fold higher expressed in 48h ovary SP (Supplementary Table S9). Kang reported that the *AtBAG6*, which contained the BAG domain, overexpressed in Arabidopsis promoted mitochondrial fusion, lead to cyt *c* release into cytosol and induce PCD (Fig. 8) [,].

Cyt *c* leaking from the mitochondria into cytosol is an initial marker for PCD[,]. From our results, we found the concentration of cytochrome oxidase COX (SS204) and ATP synthase (SS2015) were 9.7- and 5.8-fold higher in 48h ovary SP, respectively. COX is the enzyme which catalyzed the oxidation of reduced cyt *c* by molecular oxygen. If the COX concentration exceeds the critical value, the activity is limited by either cyt *c* or ascorbate concentration[]. That means the COX's activity was inhibited by its higher concentration at 48h of SP and could lead to higher cyt *c* concentration at this time point. And this results also confirmed that the oxidation-reduction system had already disordered.

From DEGs and DEPs analysis, we found the cell wall biosynthetic process related genes and proteins were upregulated and cellulose synthase were downregulated at 48h in ovary SP. That means during the PCD in PT, the cell wall of the ovary cell were lignification and thickening. It's maybe the precursor of xylification and withering of ovary.

Conclusions

Analysis on PT growth characters, internal physiology changing, transcriptome and proteome of SP and OP ovary has revealed the LSI mechanism in *S.superba* and identified several candidate genes. The 48h after pollination in ovary's LSI in *S.superba* was different with 24h after pollination in style's SI in *C.sinensis*. The high levels of expression of 2 S-RNase genes and 3 SLF-related genes suggested that LSI in *S.superba* was under gametophyte control. The c51202_g1 may functioned as general inhibitor like *Prunus* SLFL/SFBL in recognized S-RNase in compatible PT. The self S-RNase induced PCD of IPT could by indirect transduction of Ca^{2+} signal, destabilization of F-actin and released of cyt *c* etc. Mitochondrial fusion and function loss caused the PCD, in addition, oxidation-reduction system disorder promoted cell death. This results revealed the LSI molecular mechanism in *S.superba* and gave the reference to other plants in the Theaceae family.

Methods

Plant materials and pollination treatment

This study was conducted by the Forestry Genetic and Breeding Lab at the Research Institute of Subtropical Forestry, CAF, State Forestry Administration, China (RISF-CAF). The State Forestry Administration is responsible for national parks and other protected areas. No specific permission was required for these locations/activities, as they were based on non-destructive collection of plant material. The species is not endangered or protected, and the locations were not privately owned or protected by law.

Two cultivars of 'JO59' and 'YX1', grown in Lanxi nursery, Zhejiang province, China, were used in this study. These two cultivars were identified, bred, and preserved by our institution. The scions were collected from selected trees of natural forest of *S. superba* from Jianou and Youxi in Fujian, then they were grafted on local stock and kept in Lanxi in 2013. The collection of the plant material was complied with institutional, national, and international guidelines. Field studies was conducted in accordance with local legislation.

In May 2019, at 9:00–11:00 am on sunny days, we performed pollination [self-pollination (SP) was 'JO59'×'JO59', outcross-pollination (OP) was 'JO59'×'YX1']. The styles and ovaries were collected at different intervals (2h, 4h, 8h, 12h, 16h, 24h, 36h, 48h, 60h, 72h, 84h, 96h, and 120h) after pollination.

Then the styles and ovaries of each sample with three replications were frozen in liquid nitrogen and stored at -80 ℃ for enzyme activity, hormone, Amino acid analysis, protein and RNA-seq analysis.

Enzyme Assays

Three defense-related enzymes, peroxidase (POD), catalase (CAT), and superoxide dismutase (SOD), were analyzed using SP and OP ovaries from 24 to 120 hours. The POD activity was determined using guaiacol-catalase activity method []. The SOD activity was measured by inhibition in photoreduction of nitro blue tetrazolium (NBT) []. The CAT activity was measured spectrophotometrically by monitoring the decrease in absorbance at 240 nm [].

Hormone Extraction And Determination

Using HPLC-MS/MS, the levels of auxin (IAA), abscisic acid (ABA), zeatin (ZT), jasmonic acid (JA), methyl jasmonate (MeJA) and salicylic acid (SA) were determined by Zoonbio Biotechnology Co., Ltd. (Nanjing, China)[]. The SP and OP ovaries, with three biological replicates from 24 to 120 hours, (0.1g fresh weight) were selected to extract the hormone, and then did the HPLC-MS/MS analysis (Agilent 1290). HPLC analysis was carried out using a poroshell 120 SB-C18 column (2.1 mm×150mm; 2.7μm). Parameters set as follows: spray voltage, + 4500 V; automizing temperature, 400 ℃; pressure of the air curtain, nebulizer, and aux gas were 15, 65, and 70 psi, respectively. SAS v8.0 (SAS Institute, Cary, NC, USA) was used to analysis the data.

Transcriptome Analysis

The total RNA from S48 and O48 ovaries were extracted using RNAprep pure Plant Kit (Dingguo, Beijing, China). The RNA quality and quantity were verified using 1% agarose gels and Agilent 2100 (Agilent Technologies, CA, USA). The mRNA was enriched and purified by magnetic beads with oligo T (dT), cleaved and synthesized first-strand cDNA using random hexamers. Then the double-strand cDNA libraries were purified by AMPure XP system (Beckman Coulter, Beverly, USA). The transcriptome sequencing was performed by Illumina HiSeq™ 2000 sequencing platform in Novogene (Tianjin, China). The adaptor and low-quality sequences (Q < 20 or less than 35 bp) were removed in raw reads. Then the clean reads were assembled into Trinity assembler[]. The functions of the unigenes were annotated using Blastx searches with 1e-5 against protein databases. Blast2GO software[] was using for gene Ontology (GO) term analysis. Plant Transcription Factor Database (PlnTFDB) (version 3.0) was for determined for the transcription factors (TFs)[]. The FPKM was using for estimating each gene expression[], and DESeq R package 1.10.1 was performed for estimating differential expression of the two treatments[]. The differentially expressed genes were determined with an adjusted P-value < 0.05 found by DESeq. The R-seq data were upload to the sequence read archive (accession no. SUB6596208).

Protein Extraction, Expression Analysis

With some modification, the protein extraction was performed as Isaacson's protocol []. The SP and OP ovaries at 48 hours were frozen and grinded to power and 1g sample power were used for protein extraction[]. Then 10 μg samples ran on 12% SDS-PAGE gel and visualized by CBB stain[]. The gel images with 300

dots per inch were scanned by the image scanner (GE healthcare, USA). Total 450 µl solution with 1500 µg protein sample using to finish the isoelectric focusing: 50uA per strip, rehydration at 50V for 8 hours, 100V for 1 hour, 200V for 1hour, 500V for 1 hour, 1000V for 1 hour, 1000–10000 V (gradient) for 1 hour, 10000V for 13 hours, 500V for 12 hours, temperature, 20°C. Using Ettan-DALT-Six system run for 45 min at 100V then at 300V for 6–8 hours. Using PDquest 8.0 software, all gel images were processed by three steps: spot detection, volumetric quantification, and matching. The differentially protein spots were selected using two thresholds ($p \leq 0.05$, fold change ≥ 2 or ≤ 0.5). The ABI 5800 MALDI-TOF/TOF Plus mass spectrometer (Applied Biosystems, Foster City, USA) was using for peptide MS and MS/MS detection and CalMix5 using to calibrate the instrument (ABI5800 Calibration Mixture). The GPS Explorer V3.6 software (Applied Biosystems, USA) with default parameters using for data integrated and the proteins were identified by the MASCOT V2.3 search engine (Matrix Science Ltd., London, U.K.).

Transcriptome And Proteome Association Analysis

The principle of association analysis: The corresponding transcript was found through the gene id of the proteome, obtained the corresponding relationship between the protein and the transcript, and then conduct a series of association analysis. Correlation analysis was conducted on the fold change (taken as log2) of genes (proteins) identified by transcriptome and proteome in the two omics. The drawing process was implemented by python. The difference multiples of corresponding transcriptome genes in the two omics. The difference multiples were taken as log2 and then plotted by the ComplexHeatmap of R package. The result of GO annotation of the transcriptome and proteome was used for GO function enrichment by the tool of wego (http://wego.genomics.org.cn/). The clustering heat map analysis of GO/KEGG function enrich was conducted on the fold change (taken as log2) of differentially expressed proteins identified by proteome and corresponding transcriptome genes. The drawing process was implemented by ComplexHeatmap.

RT-PCR

Using specific primers, which were designed by Beacon Designer 7.90 (Premier Biosoft International, Palo Alto, CA, Supplementary Table S5), the 22 expressed transcripts were validated, and the candidate genes expression level in the pollen, leaf, style and ovary at 0, 24, 48, 72 were also detected. 100 ng of total RNA in ovaries was used to synthesize cDNA (PrimeScript™ RT reagent Kit, Takara, Dalian, China). Then, RT-PCR was performed on the ABI Quantstudio 7 Flex Real-Time PCR System (Applied Biosystems) using a SYBR® Fast qPCR Mix (Takara, Dalian, China). The program was as follows: 95 ℃ for 30 s, 40 cycles at 95 ℃ for 15 s, 60 ℃ for 30 s. The reference gene set as *GADPH*. Three biological and technical replicates were performed and the gene expression relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method[].

Abbreviations

LSI
late-acting self-incompatibility
PCD
programmed cell death
HR
hypersensitive response
SI
self-incompatibility
SSI
Sporophytic SI
GSI
gametophytic SI
OSI
ovarian SI
SP
self-pollen
OP
outcross-pollen
DEGs
differentially expressed genes
DEPs
differentially expressed proteins
PT
pollen tube
IPT
incompatible PT
FAA
Formalin-Aceto-Alcohol
Lpt

PT length
Ls
total style length
KEGG
Kyoto Encyclopedia of Genes and Genomes
GO
gene ontology
TFs
transcription factors
POD
peroxidase
CAT
catalase
SOD
superoxide dismutase
NBT
nitro blue tetrazolium
IAA
indole-3-acetic acid
ABA
abscisic acid
ZT
zeatin
JA
jasmonic acid
MeJA
methyl jasmonate
SA
salicylic acid
HPLC
High-Performance Liquid Chromatography
CC
cellular component
BP
biological process
MF
molecular function
COX
cytochrome *c* oxidase
LRR-RLKs
leucine rich repeat receptor like kinase
RALFs
rapid alkalinization factors
SRK
S-locus receptor kinase
SCR
S-locus cysteine-rich protein
SCF
SKPl-Cullin1-Rbx1-F-box
S-RNase
S-locus-encoded ribonuclease
MAPK
mitogen activated protein kinase
cyt *c*
cytochrome *c*
IMS
intermembrane space
Bcl
B-cell lymphoma
DHAR

dehydroascorbate reductase
GSH
ascorbate-glutathione
GST
glutathione S-transferase
GSSG
oxidized glutathione
APAF
apoptotic protease activating factor.

Declarations

Ethics approval and consent to participate

All field studies were performed in accordance with the local legislation in China and complied with the convention on the trade in endangered species.

Consent for publication

Not applicable.

Data archiving statement

The RNA-seq and proteome datasets in this article are available in the supplementary files. All raw data were submitted to the NCBI Sequence Read Archive: BioProject ID PRJNA625576.

Competing interests

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Non-financial competing interests

The author(s) declare no non-financial competing interests.

Authors' Contributions

RZ conceived and designed the study. RZ and HY performed the experiments and wrote the paper. RZ, HY and ZZ reviewed and edited the manuscript. All authors read and approved the manuscript.

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Figures

Figure 1

Hormone content in SP and OP. (A) Zeatin, ZT. (B) Auxin, IAA. (C) Abscisic acid, ABA. (D) Salicylic acid, SA. (E) Jasmonic acid, JA. (F) Methyl jasmonate, MeJA.

Figure 2

The SOD(A), CAT (B) and POD (C) activities in SP and OP.

Figure 3

Statistic of pathway enrichment in KEGG of the DEG.

Figure 4

Volcano plot of DEPs (A) and GO terms classification (B). A, red plots means upregulated, green plots means downregulated.

Figure 5

Association analysis between DEGs and DEPs. A. Expression linear correlation between DEGs and DEPs. B. Venn diagram showing the overlaps of RNA information obtained by transcriptome and protein information identified by proteome.

Figure 6

LD plot among C41648_g1, C110815_g1, C51202_g1 and C110213_g1. Color for each box in LD plot represents LD relationship, showing increasing LD from white to red color.

Figure 7

qRT-PCR expression analysis of 3 SFB genes (A, B, and C) and 2 S-RNase (D and E) using GAPDH as internal control at time from 24h to 72h style and ovary in SP and OP. **, P-values (0.05).

Figure 8

Summarized illustration representing self-incompatibility and outcross-compatibility at 48h in ovary of *Schima superba*. The PT elongation within self and outcross-pollinated ovary (self PT, ceased-red, outcross PT fertilization-blue).

Supplementary Files

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- SupplementaryTableS1S10.xlsx
- TextTP13replacethesupplementaryfilename.pdf