In Vitro Self-incompatible-like Response Applied for Protein Identification and Gene Expression Analysis in Citrus Cultivars, Banpeiyu and Hyuganatsu

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ABSTRACT. Self-incompatibility (SI) is an important mechanism in higher plants that promotes outcrossing and prevents self-fertilization. ‘Banpeiyu’ (Citrus maxima) and ‘Hyuganatsu’ (Citrus tamurana), two of the Citrus cultivars distributed in Kyusyu, Japan, show gametophytic SI. In this study, we used the Citrus mature pollen culture system and stylar crude protein extracts to simulate compatible (C) and SI responses in ‘Banpeiyu’ pollen tubes. We analyzed the protein changes in pollen tubes with the C- and SI-like treatments by nano-liquid chromatography–mass spectrometry (nano-LC-MS); 14 and 27 proteins were identified in C- and SI-like treatments, respectively. We picked up some candidate genes that were particularly prevalent in SI-like treatment and analyzed their expression level changes during C- and SI-like treatments in ‘Banpeiyu’ and ‘Hyuganatsu’ pollen tubes. The expression levels of copper/zinc superoxide dismutase (Cu/Zn SOD), manganese SOD (Mn SOD), catalase (CAT), and cysteine protease (CYP) increased after SI-like treatment. We used a fluorescent probe to visualize reactive oxygen species (ROS) level changes in ‘Banpeiyu’ and ‘Hyuganatsu’ pollen tubes after C- and SI-like treatments and found that 2-hour SI-like treatment induced ROS levels to increase in the pollen tubes of both cultivars. These results suggest that an ROS increase could be one of the key phenomena in the SI response of Citrus and that gene expression changes were responses to ROS generation.

Self-incompatibility in angiosperms is known as a mechanism for preventing self-fertilization and promoting outcross pollination by arresting pollen tube growth. One of the SI systems, RNase-mediated gametophytic SI (GSI), is based on the recognition and degradation of self-pollination by interaction between S-locus-encoded proteins from pistil and pollen (Foote et al., 1994; Hiratsuka et al., 2012; Lai et al., 2002; Lee et al., 1994; Murfett et al., 1994; Wheeler et al., 2009).

In recent years, several studies have investigated the cytosal alterations in pollen tubes exposed to SI response. Reactive oxygen species, a potent signaling molecule, was easily affected by SI response in pollen tubes of Papaver rhoeas (Wilkins et al., 2011) and Pyrus pyrifolia (Wang et al., 2010). The increase of ROS was thought to be a stress response, and a following influx of Ca2+ was detected (Rentel and Knight, 2004). Peroxynitrite, a powerful oxidizing and nitrating agent, was generated in the pollination of Olea europaea, increasing the protein nitration in SI pollen tubes (Serrano et al., 2011). It has been proved that the toxicity of excessive ROS induced by SI response triggers a cascade reaction and finally results in programmed cell death (PCD) in incompatible pollen tubes. PCD is a crucial process to selectively eliminate unneeded or damaged cells for development and tissue homeostasis (Fuchs and Steller, 2011). In apoptosis, one form of PCD, relocation of cytochrome c is considered the beginning of the PCD process in mammals; cytochrome c functions as an activator of procaspase in the apoptosome (Gray, 2004). Although caspase-3 is only present in animal PCD, the caspase-3-like/DEVDase displays DEVD (Asp–Glu–Val–Asp) specificity in the SI response of P. rhoeas pollen tubes (Thomas and Franklin-Tong, 2004). Mitochondria collapse, degradation of DNA, ROS burst, and cytoskeleton depolymerization have been observed as SI responses in pollen tubes, and these phenomena are common characteristics of PCD (Geitmann et al., 2000; Roldán et al., 2012; Wang et al., 2009a; Wilkins et al., 2011).

The Citrus is one of the typical GSI plants, and S-RNase homologues have been cloned from mandarin cultivars, which
kept the same conserved regions with S-RNase as Solanaceae (Miao et al., 2011). However, it has not been confirmed whether these homologues have functions for GSI. Proteomics and genomics analyses have been widely applied to identify the SI mechanism of Citrus. Distefano et al. (2009) has compared the transcript profiles of style and stigma with/without self-pollination in the Clementine mandarin (Citrus clementina). Although 96 unigenes were identified between the SI ‘Comune’ and identified the proteins between the C and SI treatments. SI-related proteins in the pollen tubes, we obtained the protein in Kyusyu, Japan, and produce few seeds with self-pollination pollen tubes. These are economically important fruit cultivars relate to as well as purified S-RNase protein in vitro (Meng et al., 2014). It has also been demonstrated that stylar crude protein extracts induced SI response in apple pollen tubes, as well as purified S-RNase protein in vitro (Meng et al., 2014).

In this study, we used strong GSI Citrus cultivars Banpeiyu pummelo and Hyuganatsu, a chance seeding cultivar that may relate to Citrus yuzu, for investigation of the SI response in the pollen tubes. These are economically important fruit cultivars in Kyusyu, Japan, and produce few seeds with self-pollination (Wakana et al., 2004; Yamamoto et al., 2006). To detect the SI-related proteins in the pollen tubes, we obtained the protein expression profiles in ‘Banpeiyu’ pollen tubes by nano-LC-MS, and identified the proteins between the C and SI treatments. Transcriptional changes were also analyzed in the genes predicted to participate in the SI responses.

Materials and Methods

PLANT MATERIALS. One day before anthesis, buds of ‘Banpeiyu’ (collected in Spring 2013 and 2014), ‘Hyuganatsu’ (collected in Spring 2014), and ‘Hassaku’ (Citrus hassaku) (collected in Spring 2013) were collected from mature trees growing in the experimental field of the University of Miyazaki, Miyazaki, Japan. Anthers of ‘Banpeiyu’ and ‘Hyuganatsu’ were separated with tweezers and dried with silica gel overnight in an incubator at 25 °C until anther dehiscence. Mature pollen grains were stored at −40 °C until use. The styles were separated from fresh pistils and prepared for protein extraction as soon as possible.

STYLAR CRUDE PROTEIN EXTRACT PREPARATION. The styles from ‘Banpeiyu’, ‘Hassaku’, and ‘Hyuganatsu’ were prepared for the isolation of crude protein extracts using extraction buffer as described by Li et al. (2015). All extraction operations were done on ice, and isolated protein solution was divided into 100-µL portions and stored at −80 °C until use. The concentration of crude protein extracts was determined using Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA).

POLLEN CULTURE AND NANO-LC-MS ANALYSIS BY C- AND SI-LIKE TREATMENTS. Pollen grains of ‘Banpeiyu’ were cultured in the Citrus mature pollen culture system as described by Uchida et al. (2012a), for 4-h initial cultivation in the dark at 25 °C. Stylar crude protein extracts, derived from the styles of ‘Hassaku’ or ‘Banpeiyu’ were added into the medium to reach 50 µg·mL⁻¹ final concentration, as C- or SI-like treatment, respectively. Protein extraction buffer was added into the culture medium as a control. After 2-h treatments, 100 pollen tubes with normal development were picked up by glass capillary, according to the method described in the work of Hirano and Hoshino (2010), and transferred into new clean tubes. All pollen tubes were resuspended in 75 µL of the extraction buffer containing 10-mM dithiothreitol (DTT), 50-mM pH 8.0 Tris base, 10-mM ethylenediaminetetraacetic acid, 0.5% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, and 0.5-mM phenylmethanesulfonyl fluoride (Sigma, St. Louis, MO). The samples mixed with micro glass beads (425 to 600 µm in diameter) were treated by vortex for 30 s, and then kept in an ice bath for 30 s; this process was repeated five times. The crude protein solution was then purified with a 2-D Clean-Up Kit (GE Healthcare Life Science, Little Chalfont, UK). The purified protein samples were dissolved in 50 µL of 10-mM DTT and 25-mM NH₄HCO₃. Then, the samples were treated by reductive alkylation using a solution containing 55-mM iodoacetamide (Sigma) and 25-mM NH₄HCO₃ for 30 min at room temperature in the dark. Finally, the samples were treated by adding 50 µL trypsin [10 ng·mL⁻¹ (Sigma)] for 12 h at 37 °C, and diluted with formic acid to a final concentration of 0.1%.

The protein samples were analyzed using nano-LC-MS (LTQ Orbitrap; Thermo Fisher Scientific, Waltham, MA), and spectra were processed and exported by extract_msn.exe program (BioWorks 3.2 software; Thermo Fisher Scientific). Extracted spectra were internally calibrated using trypsin autoproteolysis products. Protein identification was accomplished by comparing the mass list with the Citrus database in NCBI using MASCOT software (version 2.2; Matrix Science, Boston, MA). The proteins detected in the control were excluded in both C- and SI-like treatments.

Expression analysis of candidate genes related to SI-LIKE RESPONSE BY SQRT-PCR AND qRT-PCR. ‘Banpeiyu’ and ‘Hyuganatsu’ pollen grains were resuspended in the Citrus mature pollen culture system at a density of 10⁶ grains/mL for 4-h initial cultivation at 25 °C in the dark. Pollen tubes of ‘Banpeiyu’ were exposed to ‘Banpeiyu’ or ‘Hyuganatsu’ stylar crude protein extracts as SI- or C-like treatment, respectively; vice versa, ‘Hyuganatsu’ pollen tubes were exposed to ‘Banpeiyu’ or ‘Hyuganatsu’ stylar crude protein extracts as C- or SI-like treatment, respectively.

The pollen tubes of ‘Banpeiyu’ and ‘Hyuganatsu’ were collected after 0, 1, 2, and 4 h of C- and SI-like treatments. Total RNA was extracted by RNeasy Plant Mini Kit (Qiagen, Venlo, The Netherlands), and cDNA were synthesized from 1 µg of total RNA using the Superscript III kit (Life Technologies, Carlsbad, CA) with Oligo(dT)₃₀ primers. The primer sequences of putative SI-related genes in Citrus, including Cu/Zn SOD (AB981053), iron SOD [Fe SOD (AB981054), Mn SOD (AB981055), CAT (AB981056), CYP (AB981057), l-galactose-1-phosphate phosphatase [GPP (AB981061)], miraculin-like protein-1 [MLP-1 (AB981059)], and MLP-3 (AB981060) were obtained, and gene expressions after 4-h C- and SI-like treatments were analyzed by semiquantitative reverse transcription polymerase chain reaction (Sqrt-PCR). The primer sequences and SqRT-PCR procedures are listed in Table 1. Citrus constitutively expressed the actin gene of ‘Banpeiyu’ (accession no. GU911361) and ‘Hyuganatsu’ (accession no. XM_006432422) as an internal control. Real-time qRT-PCR was performed by a CFX manager real-time PCR detection system (Bio-Rad Laboratories) using the SYBR Fast qRT-PCR Kit (KapaBiosystems, Wilmington, MA) and corresponding primers (Table 2). The data were analyzed using CFX manager software (Bio-Rad Laboratories) using the 2⁻ΔΔCt method. The
experiments of pollen tube treatments and expression analysis were repeated three times for statistical analysis, individually (Tukey’s multiple range test).

ROS ANALYSIS IN POLLEN TUBES AFTER SI-LIKE TREATMENT. Pollen grains of ‘Banpeiyu’ and ‘Hyuganatsu’ were precultured for 4 h at 25 °C in the Citrus mature pollen culture system in the dark. CM-H2DCFDA (Life Technologies) was used for ROS visualization. The probe was diluted into anhydrous dimethyl sulfoxide to prepare 5-μM (CM-H2DCFDA) of working solution. Germinated pollen tubes were incubated with the probe in the dark for 30 min to load the probe into the pollen tubes. After labeling, the probe solution was discarded and pollen tubes were washed with culture medium three times. Then, pollen tubes were treated with C- and SI-like treatments for 2 h. Fluorescence of ROS in pollen tubes was investigated with a confocal laser microscope (LSM700; Carl Zeiss, Jena, Germany).

Results and Discussion

In this study, we applied this SI-like response system for molecular identification of SI-related proteins in Citrus pollen tubes. To detect the SI-related proteins, we isolated the proteins from the ‘Banpeiyu’ pollen tubes treated with C and SI stylar crude protein extracts and obtained the peptide spectral data by nano-LC-MS analysis for the pollen tube proteins. As a result of searching the spectral data against the Citrus database, different protein expression was observed in the C- and SI-like treatments in comparison with the control, and we successfully identified 14 putative proteins induced by the C-like treatment (Table 3) and 27 putative proteins induced by the SI-like treatment (Table 4). All these proteins were classified according to Gene ontology terms relating to biological processes and molecular functions. In the C-like treatment, the metabolic process (85.7%) was the most frequent category in the biological process, followed by the stress-response process, which accounted for the rest. On the other hand, in the category of biological process for the SI-like treatment, metabolic process accounted for 85.2%, followed by the C-like treatment, the metabolic process (85.7%) was the most frequent, accounting for 78.7%; hydrolases activity, another 7.15%. For the molecular function, catalytic activity was also the most frequent category in the biological process, followed by the catalytic activity, accounting for 7.1% (Fig. 1A). On the other hand, in the category of biological process for the SI-like treatment, metabolic process accounted for 85.2%, followed by the stress-response process, which accounted for 11.1%, and the glycolysis process, which accounted for the rest. For the molecular function category, catalytic activity was also the most frequent activity, accounting for 74.1%, followed by the stress-response process, which accounted for the rest. For the molecular function category, catalytic activity was also the most frequent activity, accounting for 74.1%, followed by the stress-response process, which accounted for 11.1%, and the glycolysis process, which accounted for the rest. For the molecular function category, catalytic activity was also the most frequent activity, accounting for 74.1%, followed by the stress-response process, which accounted for the rest.

Table 1. Primers and PCR procedures used for semiquantitative reverse transcription PCR (SqRT-PCR).

| Primer name | Primer sequences (5′-3′) | Procedure for SqRT-PCR |
|-------------|--------------------------|------------------------|
| Copper/zinc superoxide dismutase (Zn/Cu SOD) | For: AGGAGCCTCCTCTGGTCTCA Rev: CAGGAGGTAAAGTGTTGGGT | 94 °C, 5 min; 94 °C 30 s, 61 °C 30 s, 50 cycles; 72 °C 10 min |
| Iron SOD (Fe SOD) | For: GGAACGATCGATGGTGTTGGA Rev: AGTTCCACCAAGGCGTTTCC | 94 °C, 5 min; 94 °C 30 s, 60 °C 30 s, 50 cycles; 72 °C 10 min |
| Manganese SOD (Mn SOD) | For: CCGGCTCCTGTATGGCAAAA Rev: AAACCTCTACGTGGCTCC | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| Catalase (CAT) | For: GGGGCTCCTTGATGGCAAAA Rev: AAACCTCTACGTGGCTCC | 94 °C, 5 min; 94 °C 30 s, 58 °C 30 s, 50 cycles; 72 °C 10 min |
| Cysteine protease (CYP) | For: AGCTTCGACGACTCCAATCC Rev: GGAACCACAATAGCAGGGCA | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| L-galactose-1-phosphate phosphatase (GPP) | For: TCAAAGCAGATTTCACCCACG Rev: CTCGCGATGCAATTCCACAA | 94 °C, 5 min; 94 °C 30 s, 58 °C 30 s, 50 cycles; 72 °C 10 min |
| Miraculin-like protein-1 (MLP1) | For: GGAATAGCGGCGAGCACAAGG Rev: CACAACTCAGCAGTTGAGC | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| Miraculin-like protein-3 (MLP3) | For: GAGGCACAAGTGGAGATGTG Rev: AGCCTTCAGTCCAAAATGCCC | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |

Table 2. Primers and PCR procedures used for real-time quantitative PCR (qRT-PCR).

| Primer name | Primer sequences (5′-3′) | Procedure for qRT-PCR |
|-------------|--------------------------|------------------------|
| Copper/zinc superoxide dismutase (Zn/Cu SOD) | For: AGCAGTTGCAAGTCTTCCGTCG | 94 °C, 5 min; 94 °C 30 s, 61 °C 30 s, 50 cycles; 72 °C 10 min |
| Iron SOD (Fe SOD) | For: AGAAAGACTGTGGTGCGGAAAT Rev: CAGGGCCCAACCAAGAACAAAAA | 94 °C, 5 min; 94 °C 30 s, 60 °C 30 s, 50 cycles; 72 °C 10 min |
| Manganese SOD (Mn SOD) | For: CGATTACAGCAGCTTTGAGC Rev: GCACCCTCAGCCTACCTT | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| Catalase (CAT) | For: TTGTCGGTTTCTCTCCCTT | 94 °C, 5 min; 94 °C 30 s, 58 °C 30 s, 50 cycles; 72 °C 10 min |
| Cysteine protease (CYP) | For: TTGCTGATTGGAGCTGGGA Rev: GTACCCCAACAGCAAAGCAG | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| L-galactose-1-phosphate phosphatase (GPP) | For: TCAAAGCAGATTTCACCCACG Rev: CTCGCGATGCAATTCCACAA | 94 °C, 5 min; 94 °C 30 s, 58 °C 30 s, 50 cycles; 72 °C 10 min |
| Miraculin-like protein-1 (MLP1) | For: GGAATAGCGGCGAGCACAAGG Rev: CACAACTCAGCAGTTGAGC | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
| Miraculin-like protein-3 (MLP3) | For: GAGGCACAAGTGGAGATGTG Rev: AGCCTTCAGTCCAAAATGCCC | 94 °C, 5 min; 94 °C 30 s, 59 °C 30 s, 50 cycles; 72 °C 10 min |
Table 3. Result of protein identification in Citrus maxima ‘Banpeiyu’ pollen tubes exposed to compatible (C)-like treatment.

| Accession | Coverage | PSM* | Peptides | AA* | MW (kDa)* | calc. pl* | Score | Description |
|-----------|----------|------|----------|-----|-----------|-----------|-------|-------------|
| gi11596178 | 45.69 | 14 | 10 | 232 | 25.2 | 7.91 | 44.94 | Miraculin-like protein (Citrus ×paradisi) |
| gi289600010 | 13.26 | 7 | 5 | 445 | 47.8 | 5.78 | 13.92 | 2-phospho-D-glycerate hydrodase (Citrus trifoliata) |
| gi50199132 | 30.77 | 2 | 2 | 291 | 32.6 | 5.59 | 4.14 | Hypothetical protein (C. ×paradisi) |
| gi2213425 | 7.56 | 2 | 2 | 291 | 32.6 | 5.59 | 4.14 | Hypothetical protein (C. ×paradisi) |
| gi11596180 | 12.29 | 8 | 3 | 236 | 25.6 | 6.54 | 3.77 | Miraculin-like protein 2 (C. ×paradisi) |
| gi68138959 | 3.95 | 2 | 1 | 380 | 41.3 | 6.2 | 3.33 | Alcohol dehydrogenase (C. ×paradisi) |
| gi77417705 | 10.56 | 1 | 1 | 142 | 15.6 | 6.32 | 3.06 | Super oxide dismutase (Citrus trifoliata var. monstrosa) |
| gi16797799 | 10.47 | 4 | 4 | 277 | 30 | 6.68 | 2.52 | Chalcone synthase (C. jambhiri) |
| gi169160465 | 4.61 | 4 | 4 | 802 | 91 | 5.95 | 2.28 | Phospholipase D alpha (C. sinensis) |
| gi1170567 | 3.55 | 2 | 2 | 507 | 56.3 | 6.07 | 1.69 | RecName: Full = inositol-3-phosphate synthase, Short = MIP synthase; AltName: Full = myo-inositol 1-phosphate synthase, Short = IPS; Short = MI-1-P synthase |

Table 4. Result of protein identification in Citrus maxima ‘Banpeiyu’ pollen tubes exposed to self-incompatible (SI)-like treatment.

| Accession | Coverage | PSM* | Peptides | AA* | MW (kDa)* | calc. pl* | Score | Description |
|-----------|----------|------|----------|-----|-----------|-----------|-------|-------------|
| gi11596178 | 45.69 | 14 | 10 | 232 | 25.2 | 7.91 | 44.94 | Miraculin-like protein (MLP) (Citrus ×paradisi) |
| gi87299375 | 42.67 | 23 | 9 | 232 | 25.2 | 7.91 | 38.5 | MLP 1 (C. jambhiri) |
| gi289600010 | 20.9 | 10 | 7 | 445 | 47.8 | 5.78 | 24.63 | 2-phospho-D-glycerate hydrodase (Citrus trifoliata) |
| gi2213425 | 27.15 | 13 | 7 | 291 | 32.6 | 5.59 | 18.16 | Hypothetical protein (C. ×paradisi) |
| gi319739583 | 3.09 | 2 | 2 | 680 | 78.2 | 8.37 | 0 | RNA polymerase beta (C. sinensis) |
| gi254305423 | 16.67 | 6 | 4 | 277 | 30 | 6.68 | 2.52 | Chalcone synthase (C. trifoliata) |

SI response. Since the “stress-response process” in the biological process category and the “pathogenesis-related protein” in the molecular function category were only observed in the SI-like treatment (Fig. 1B), we focused on these proteins as candidates for SI-related proteins. We selected 8 genes, including Cu/Zn SOD, Fe SOD, Mn SOD, CAT, CYP, GPP, MLP-1, and MLP-3, as candidates and investigated their expressions in the pollen tubes of ‘Banpeiyu’ and ‘Hyuganatsu’ after 4-h exposure to C- and SI-like treatments by SqRT-PCR (Fig. 2). The genes, Cu/Zn SOD, Fe SOD,
Mn SOD, CAT, CYP, and GPP, were chosen for further investigation. We analyzed the expression levels in the pollen tubes during the C- or SI-like treatments by qRT-PCR. The expression profiles in ‘Banpeiyu’ (Fig. 3A) and ‘Hyuganatsu’ (Fig. 3B) showed similar trends, and the expression levels of Cu/Zn SOD, Mn SOD, CAT, and CYP after the SI-like treatment from 2 to 4 h were higher than those after the C-like treatment or control, except for Mn SOD and CYP in ‘Banpeiyu’ after 2-h SI-like treatment. In Mn SOD of ‘Banpeiyu’ and ‘Hyuganatsu’, the expression levels after 4-h C-like treatment were also higher than those after control treatment. The expression levels of Fe SOD in both ‘Banpeiyu’ and ‘Hyuganatsu’ pollen tubes showed no significant changes throughout 4-h C- and SI-like treatments. Although GPP expression level with 1-h SI-like treatment in ‘Hyuganatsu’ was higher than with C-like treatment and control, there was no significant difference from 2 to 4 h.

In the candidates, the gene expressions of CAT, Cu/Zn SOD, and Mn SOD were increased in the pollen tubes of ‘Banpeiyu’ and ‘Hyuganatsu’ by the SI-like treatment (Fig. 3). SODs (EC1.15.1.1) are a family of metalloenzymes that generally exist in vegetative and reproductive plant tissues as scavengers that protect cells against oxidative stress and maintain a balance between ROS generation and degradation (del Río et al., 2002; Wang et al., 2009b). CAT, as an antioxidant enzyme, can effectively convert H₂O₂ into H₂O and O₂ following the reaction of SODs. Therefore, it was predicted that ROS in the pollen tubes would correlate with the SI-like response. We investigated the ROS level changes after C- and SI-like treatments in ‘Banpeiyu’ and ‘Hyuganatsu’ pollen tubes. We detected the levels by ROS probe, CM-H₂DCFDA. Levels in the pollen tubes of ‘Banpeiyu’ and ‘Hyuganatsu’ were quite low in the control and C-like treatment (Fig. 4). On the other hand, the pollen tubes after SI-like treatment showed strong fluorescent signals in both ‘Banpeiyu’ and ‘Hyuganatsu’ (Fig. 4). These results suggest that ROS production is not induced by the addition of stylar crude proteins to the culture medium, but is caused by the reaction between specific proteins from the styles and some kind of factor in the pollen tubes.

ROS is an important signal molecule in plants and participates in cell signaling networks (Gadjev et al., 2008). In the pollen tubes of P. rhoesas, it has been reported that ROS increase is induced by the SI response following Ca²⁺ oscillations and that the depolymerization of actin in pollen tube cytoskeletons was also detected shortly thereafter (Wilkins et al., 2011). In ‘Banpeiyu’ and ‘Hyuganatsu’, ROS increase is also thought to be one of the key phenomena in the SI-like response, and the expression increases of CAT and SODs would be a feedback response to excessive ROS generation. In addition to the CAT and SODs, the expression level of CYP increases after SI-like treatment (Fig. 3). CYP is associated with the stress response (Bernoux et al., 2008; Chen et al., 2010), and high expression of CYP is considered an indicator of the early stages of PCD (Kuriyama and Fukuda, 2002). Since ROS has been proposed as a key inducer of PCD (De Pinto et al., 2012), it is possible that...
the ROS cascade reaction, which was induced by SI-like treatment in ‘Banpeiyu’ and ‘Hyuganatsu’ pollen tubes, is a trigger for the PCD process.

The \( S \) genotype of ‘Banpeiyu’ has been determined as \( S_1S_2 \) by number of pollination experiments, and the \( S \) genotype of ‘Hassaku’ has been estimated as \( S_4S_5 \) (Kim et al., 2011). Therefore, in this study, the candidates for SI-related proteins derived from the differences between SI- and C-like treatments with different \( S \) genotypes. The \( S \) genotype of ‘Hyuganatsu’ is \( S_1S_? \) and is known as

Fig. 3. Gene expression changes during compatible (C)-like and self-incompatible (SI)-like treatments. The pollen tubes of Citrus maxima ‘Banpeiyu’ (A) and Citrus tamurana ‘Hyuganatsu’ (B) were processed with C- or SI-like treatment for 0–4 h, and then the gene expression in the pollen tubes were analyzed by real-time quantitative PCR for Copper/zinc superoxide dismutase (Cu/Zn SOD), iron SOD (Fe SOD), manganese SOD (Mn SOD), catalase (CAT), cysteine protease (CYP), and L-galactose-1-phosphate phosphatase (GPP). Values are the mean of three biological replicates ± SD for each timing (\( n = 3 \)). Different letters represent significant differences at 5% level as determined by Tukey’s multiple range test (NS = nonsignificant difference).

Fig. 4. Effect of 2-h compatible (C)-like or self-incompatible (SI)-like treatments on reactive oxygen species level in Citrus maxima ‘Banpeiyu’ (A) and Citrus tamurana ‘Hyuganatsu’ (B) pollen tubes; Scale bar = 200 \( \mu \)m.
not including S₂ allele (Kim et al., 2011). In expression analysis, C-like setting of 'Banpeiyu' (S₁S₁) and 'Hyuganatsu' (S₃S₃) is interpreted as a semicompatible genotype. The gene expressions of SODs, CAT, and CYP showed significant differences between C- and SI-like treatments (Fig. 3), suggesting that the in vitro SI-like system would be applicable for S genotyping analysis in Citrus.

In conclusion, we successfully identified the proteins related to SI-like response by combining an in vitro SI-like response system and nano-LC-MS analysis, and revealed that ROS generation in the pollen tubes is an SI-like response. The gene expressions of CAT and SODs were increased in the pollen tubes by the SI-like treatment, also indicating that oxidative balance in Citrus pollen tubes is disrupted by the SI-like treatment. To clarify the SI mechanism in Citrus, further study of the SI response in pollen tubes is needed. In addition, the female and male determinants for SI need to be identified. The SI-like system has great potential for progressing our understanding of the SI response.

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