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Identification of self-incompatibility related proteins in the pistil of Japanese pear [*Pyrus pyrifolia* (Burm.f.)] by proteome analysis

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Abstract

The differences between stylar protein of the Japanese pear (*Pyrus pyrifolia* (Burm.f.)) cultivars 'Kosui' (S^4S^5) and 'Kikusui' (S^2S^4) were compared by two-dimensional difference gel electrophoresis (2-D DIGE), and were labelled and visualized with different fluorescent dyes (IC3-OSu, IC5-OSu) on a single 2-D gel. The individual different expressed proteins spots were subjected to identification. The proteins were analyzed by matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry (MALDI-TOF/MS) to identify proteins related to gametophytic self-incompatibility (GSI). S^4-RNase and thaumatin-like protein 1 were successfully detected as expected in the pistils of 'Kosui' and 'Kikusui'. S^5-RNase was also detected in the pistils of 'Kosui'. However, we could not detect S^2-RNase in 'Kikusui' in this study, possibly because the level of expression of S^2-RNase might be minuscule, or the estimated isoelectric point (pI) of S^2-RNase (pI:9.26) was more basic than S^4-RNase (pI: 9.17) and S^5-RNase (pI: 9.01). These results indicate that proteomic studies are effective tools for detection of the expected proteins and might be helpful for finding the unknown key proteins related to the mechanism of self-incompatibility (SI) in many other SI plants.

Keywords: protein analysis; Two-dimensional difference gel electrophoresis; MALDI-TOF/MS; gametophytic self-incompatibility; Rosaceae; 2D-DIGE; Peptide Mass Fingerprint; fluorescent dye.
Abbreviations: BP- band pass; BPB- bromophenol blue; CHAPS-3-[(3-Cholamidopropyl) dimethylammonio]propanesulfonic acid; DIGE-difference gel electrophoresis; DTT-dithiothreitol; GSI-gametophytic self-incompatible; IEF-isoelectric focusing; MALDI-TOF/MS- matrix-assisted laser desorption/ionization-time-of-flight mass spectrometry; MS-mass spectrometry; MS/MS-tandem mass spectrometry; pI-isoelectric point; PMF-peptide mass fingerprint; SAGE-serial analysis of gene expression; SDS-sodium dodecyl sulfate; SFB-S-haplotype-specific F-box gene; SI- self-incompatibility; 2-D- two- dimensional.

Introduction

SI is a mechanism which prevents self-fertilization and promotes out-crossing (De Nettancourt, 1977). Among the species showing SI, those belonging to Solanaceae, Rosaceae and Plantaginaceae exhibit GSI, which is controlled by glycoprotein with RNase (S-RNase) activity expressed in the pistil. S-RNase is encoded by the *S*-locus gene (McClure *et al.* 1989; Sassa et al., 1996), and another *S*-locus gene named *SFB* (*S*-haplotype-specific F-box gene) in the Rosaceae (Ushijima et al., 2003) determines pollen-specific protein, F-box protein. *SFB* is tightly linked to the S-RNase gene (Yamane et al., 2003 a). Until now, SI in Rosaceae has mainly been studied in Japanese pear (*Pyrus pyrifolia* (Burm.f.)) and almond (*Prunus dulcis* (Mill.) D.A.). Many molecular biological techniques such as isoelectric focusing (IEF) (Yamashita et al., 1987; Hiratsuka et al. 1986, 1995), two-dimensional (2-D) polyacrylamide gel electrophoresis (Tao et al., 1997, 1999), staining for RNase activity (Sassa et al., 1992; Boskovic et al., 1999), immunoassay (Sassa et al., 1993, 1998; Ushijima et al., 2001), PCR (Guerra et al., 2009; Rahemi et al., 2010) and RT-PCR (Yamane et al., 2003a, b; Ma and Oliveira, 2000; Okada et al., 2008; Sanzol, 2009) have been used for the investigation of SI in Rosaceae. However, studies of the translational level of the S-RNase gene in the Rosaceae are scarce. Recently, mass spectrometry (MS)-based proteomics have been popular due to the availability of gene and genome sequence databases and technical development in many categories (Takasaki et al., 2008; Amato et al., 2010; Gammulla et al., 2011), such as the methods of protein ionization and mass analysis (Aebersold and Mann, 2003). Proteins can be identified directly in sodium dodecyl sulfate (SDS) gels by excising the stained band and performing in-gel digestion, typically with trypsin (Shevchenko et al., 1996), followed by analysis with MALDI-TOF/MS or electrospray ionization-tandem mass spectrometry.

Peptide identification is mainly performed by entering the mass list into the peptide mass fingerprint (PMF) database and tandem mass spectrometry (MS/MS) ions search database. In this study, we compared the protein differences between the Japanese pear cultivars 'Kosui' and 'Kikusui' by 2-D-DIGE and MALDI-TOF/MS to identify proteins related to SI. Additionally, we discuss the efficacy of the proteome techniques with regard to the results.

Materials and Methods

Plant materials

Two varieties of Japanese pear with known S-genotypes, 'Kosui' (S^4S^5) and 'Kikusui' (S^2S^4), were used. The styles were dissected from flower buds at the 'balloon' stage of development and then rapidly frozen in liquid N_2 and stored at -80°C until use.

Protein extraction and labeling

The proteins were extracted from the pistils as described by Damerval et al. (1986) with slight modifications. Precipitated proteins were resuspended in an extraction buffer containing 7.0 M urea, 2.0 M thio-urea, 2.0% 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) and 2.0% n-dodecyl-β-D-maltoside (Valcu and Schlink, 2006). The concentration of total protein was determined using Bio-Rad Protein Assay Dye Reagent Concentrate (Bio-Rad Laboratories, Hercules, CA, USA) according to the procedure described. The extracted proteins were labeled using IC3-OSu ('Kosui') and IC5-OSu ('Kikusui') (Dojindo Laboratories, Kumamoto, Japan), respectively. In brief, 50 μg of protein was incubated with 1.0 μl 400 pM of either IC3-OSu or IC5-OSu for 1 h at room temperature in the dark, and then freshly dissolved in
dimethyl sulfoxide. The reaction was stopped by the addition of 2.0 μl 10 mM lysine that was treated for 15 min at room temperature in the dark. Equal volumes of each of the labeled samples were mixed, which were then cleaned up to remove contaminant (e.g. salt, surfactant, phenol and lipid). Trichloroacetic acid (TCA) was added to the samples so that final concentration of TCA was 10%, and incubated 1 h at room temperature. Sodium deoxycholate was added up to a final concentration of 0.02%, incubated for 15 min at room temperature and centrifuged. One M ammonium bicarbonate was added to the obtained pellets, which were then vortexed. After adjusting the samples to the neutral pH, 1 ml pre-cooled acetone was added and incubated for 2 h at -30°C. For IEF, the cleaned up samples were centrifuged and then added with 100 μl of a lysate buffer containing 7.0 M urea, 2.0 M thio-urea, 2.0% CHAPS, 2.0% *n*-dodecyl-β-D-maltoside, 0.45 M DL-dithiothreitol (DTT) and 1.0% IEF buffer BIOLITE 3/10 (Bio-Rad).

2-D DIGE and image analysis

Fifty micrograms of rehydrated proteins were loaded onto a 7 cm nonlinear IPG Ready Strip pH 3–10 NL (Bio-Rad). The strip was rehydrated in the above-mentioned lysate buffer containing 0.1% bromophenol blue (BPB) at room temperature. The first dimension IEF was performed automatically at 250 V for 30 min, 4,000 V for 1 h, and 10,000 Vh at 4,000V, at 20°C on a Protean IEF CELL system (Bio-Rad). Next, the focused strips were treated in equilibration buffer (7.0 M urea, 2.0 M thio-urea, 2.0% CHAPS, 2.0% *n*-dodecyl-β-D-maltoside) containing 2% (w/v) DTT and 2.5% iodoacetamide. The second dimension separation was performed on 10% sodium dodecyl sulfate (SDS) polyacrylamide gel. The gels were run at a constant current of 25 mA/gel for 70 min until the BPB dye front had run off the bottom of the gel. Labeled proteins were visualized using a FLA3000 imager (Fuji Film, Tokyo, Japan). The IC3-OSu images were scanned using a 532 nm laser and a 580 nm band pass (BP) 30 emission filter, whereas the IC5-OSu images were scanned using a 633 nm laser and a 670 nm BP30 emission filter. All gels were scanned at 100 mm resolution. The scanned images were then analyzed using a PD Quest (Bio-Rad). The labeled proteins were visualized as different images, i.e. IC3-OSu image (red), IC5-OSu image (green) and overlaying IC3-OSu and IC5-OSu images (yellow). Proteins were post-stained with 0.1% coomassie brilliant blue-R250 and the spots of interest were excised manually and subjected to mass spectrophotometric protein identification.

Protein identification

Protein spots which varied in expression in the area of estimated appearance of S-RNase from the database (MW:25kDa, pI:9) were identified. They were excised from the gel, and then in-gel digestion with trypsin was performed using a modified method of Shevchenko et al. (1996). After trypsin digestion, the peptides were spotted onto a MALDI target plate (AnchorChip™ Target Plates with Transponder Technology, Bruker Daltonik GmbH, Bremen, Germany) using 1.0 μl of a 3% solution of matrix saturated in acetone with recrystallized α-cyano-4-hydroxycinnamic acid and analyzed by a MALDI-TOF mass spectrometer (AutoFLEX III TOF/TOF, BRUKER DALTONIK GmbH). These mass spectrometers were operated in a positive reflector mode, and the spectra were internally calibrated using trypsin autoproteolysis products. Protein identification was finally performed by entering the mass list into a MASCOT PMF database (Mascot search, http://www.matrixscience.com).

Results and Discussion

Typical 2-D DIGE gel images of pistils from 'Kosui' and 'Kikusui' are shown in Fig. 1. Protein spots which varied in expression in the area of the estimated appearance of S-RNase from the database were analyzed, and three proteins related to SI were identified (Fig.2 and Table 1). S^4-RNase and thaumatin-like protein 1 were detected in the pistils of both 'Kosui' and 'Kikusui'. S^5-RNase was also detected in the pistils of 'Kosui'. S-RNase is essential for SI. In the pistils, S-RNase inhibits protein synthesis of internal incompatible pollen tubes by degradation of rRNA stored within pollen grains (McClure et al., 1990), and finally arrest pollen tube growth (Matton et al., 1994). In addition, recent study assumes that SFB protein ubiquitinates non-self S-RNases to lead to degradation and specifically interacts with self S-RNase to escape ubiquitination, leading to arrest of the growth of self-pollen tubes (Ushijima et al., 2003). Thaumatin-like protein 1 is an acidic glycosylated protein which has signal peptides for secretion to extracellular spaces. The members of thaumatin proteins have a cognitive function in signal molecules (Grenier et al., 1999). It is suggested that thaumatin-like protein 1 plays a role in the recognition of signals from pollen (Grenier et al., 1999; Sassa and Hirano, 1998). In the present study, S^2-RNase, which should be expressed in 'Kikusui,' could not be detected. It was reported that the amounts of S-RNase were different among each *S*-haplotype (S^3>S^1>S^5>S^4>S^6>S^2>S^7), and that the amounts of S-RNase produced das from the same *S*-haplotype

Table 1. Identified stylar proteins in Japanese pear cultivar, 'Kosui' and 'Kikusui'.

Cultivar name	Spot No.	Accession No. Swiss-Prot	Protein name	MW [z] (Da)	pI [y]	Score of Mascot Search	Peak No.	Identified peptides
' Kosui ' (S^4S^5)	A	RNS5_PYRPY	S^5-RNase (*Pyrus pyrifolia*)	26,059	9.01	114	A1	R.ALLDIENAIR.N
							A2	K.LLEPQLAIIWPNVFDR.T
							A3	K.HGTCGYPTIDNENHYFETVIK.M
							A4	K.LFTVHGLWPSSMAGPDPSNCPIR.N
							A5	K.SGEHFIDCPHPFEPISPHYCPTNNIK.Y
							A6	K.SGEHFIDCPHPFEPISPHYCPTNNIK.Y
	B	RNS4_PYRPY	S^4-RNase (*P. pyrifolia*)	25,858	9.17	141	B1	R.YFCPANVK.Y
							B2	R.SLVDIENAIR.S
							B3	K.FINCPHGPPK.G
							B4	K.LFTVHGLWPSNR.N
							B5	R.TTTELVEVTLCSNR.D
' Kikusui ' (S^2S^4)	C	TLP1_PYRPY	Thaumatin-like protein1 (*P. pyrifolia*)	25,308	5.07	76	C1	K.NQCPQAYSYAYDDK.S
							C2	K.SSTFTCFGGPNYEITFCP.-
							C3	K.SACLALNQPQYCCTGAYGTPDTCPPTDFSK.V
	D	RNS4_PYRPY	S^4-RNase (*P. pyrifolia*)	25,858	9.17	132	D1	R.YFCPANVK.Y
							D2	R.SLVDIENAIR.S
							D3	R.SDHVGFWER.E
							D4	K.FINCPHGPPK.G
							D5	K.LFTVHGLWPSNR.N
							D6	R.TTTELVEVTLCSNR.D

[z]:MW was cited from a MASCOT PMF database (Mascot search, http://www.matrixscience.com). [y]:pI was cited from a UniProt datebase(http://www.uniprot.org/).

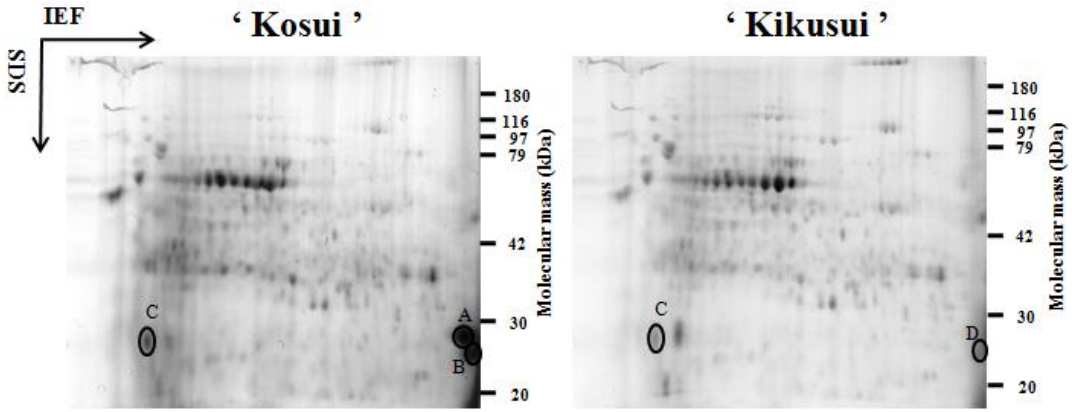

Fig 1. 2D-DIGE gel images of protein extracted from the the pistils in Japanese pear cultivar, 'Kosui' and 'Kikusui'. The extracted proteins were labeled using IC3-OSu ('Kosui') and IC5-OSu ('Kikusui'), respectively. Circled protein spots were detected in the area of estimated appearance of S-RNase from the datebase (MW:25kDa, pI:9) were identified.

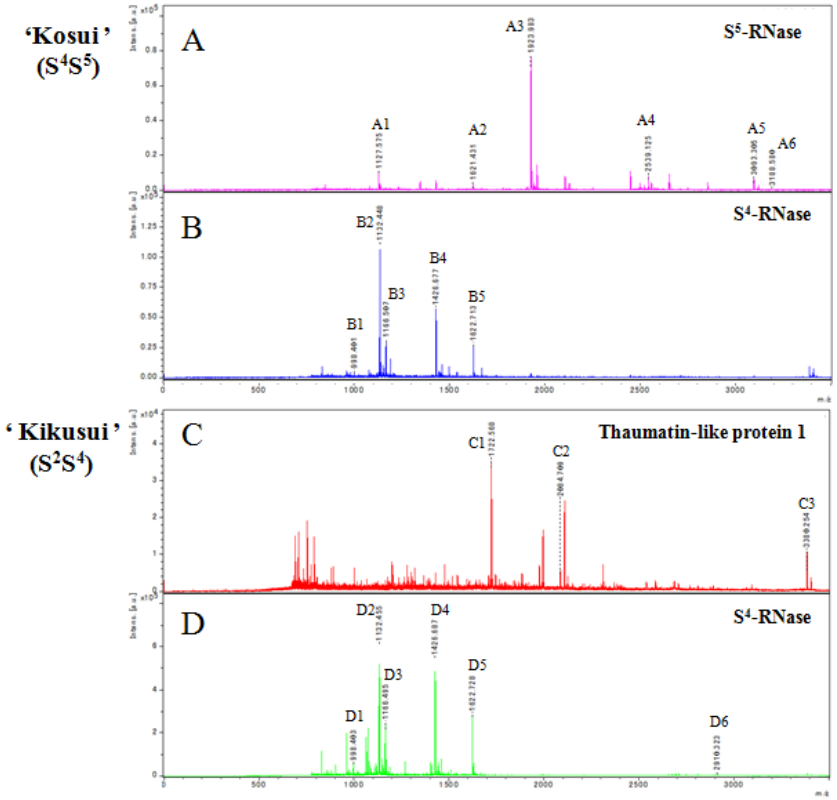

Fig 2. The MALDI-TOF/MS spectrum of tryptic digest of the spots from CBB-stained 2D-gel of style protein in Japanese pear cultivar, 'Kosui' and 'Kikusui'. After trypsin digestion, the peptides were analyzed by a MALDI-TOF mass spectrometer (AutoFLEX III TOF/TOF, BRUKER DALTONIK GmbH). These mass spectrometers were operated in a positive reflector mode, and the spectra were internally calibrated using trypsin autoproteolysis products. Protein identification was finally performed entering the mass list into a MASCOT Peptide Mass Fingerprint (PMF) database (Mascot search, http://www.matrixscience.com).

differed among cultivars (Zhang and Hiratsuka, 1999). Moreover, the concentration of S^2-RNase was about half that of S^4-RNase in 'Kikusui' (Zhang and Hiratsuka, 2000). Because the expression level of S^2-RNase might be minuscule, we could not detect it in 'Kikusui' in the present study. Furthermore, in this study, the proteins were loaded onto a gel strip with a range of pH from 3 to 10 at IEF, and spots of S-RNases (pI S^4: 9.17, S^5: 9.01) were thus detected at the end of the SDS polyacrylamide gel at the second dimension separation. The estimated pI of S^2-RNase was more basic than S^4-RNase and S^5-RNase (pI:9.26), which might be a possible reason that the S^2-RNase spot was not separated. In a future study, we will use two-dimensional electrophoresis to investigate the amount of protein and pH range of the gel in IEF. Gene expression and function can be studied through several techniques such as cDNA microarrays and serial analysis of gene expression (SAGE). However, the success of these approaches depends on the progress of genomic research and transcriptome techniques such as cDNA microarrays are still restricted to model plants. Moreover, even generally performed transcription analysis in non-model plants requires some information about the orthologous genes. However, a proteomic study can analyze translation products directly, so that it is possible to perform transcription analysis for non-model plants, which hold poor genomic information. Proteome techniques have become a powerful tool for researching various mechanisms such as pigment biosynthetic processes (Muccilli et al., 2009), pathogen interactions (Garavaglia et al., 2010), high chilling tolerance (Zhang et al., 2010), induction of drought resistance (Macarisin et al., 2009), and regulation of fruit senescence (Qin et al., 2009) in many important crops. Moreover, Feng et al. (2009) compared the protein profiles of self-compatible and self-incompatible cultivars of apricot (*P. armeniaca* L.) using LC-ESI-MS/MS technique, and identified S-RNase only in SI pistils. Further proteomic studies might be helpful for confirming the unknown key proteins related to unclear mechanisms in many SI plants. In conclusion, we identified proteins related to SI, such as S^4-RNase, S^5-RNase and thaumatin-like protein 1 in the SI cultivars of Japanese pear, 'Kosui' and 'Kikusui', by proteome analysis. However, key enzymes of SI remain unclear in other plant species with SI. Therefore, it may be possible to use proteome analysis to elucidate unclear mechanisms of SI system in other SI plants.

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