Comparative Proteomic Analysis of the Resistant Response in *Brassica rapa* Root Culture to the Clubroot Disease Agent *Plasmodiophora brassicae*

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Summary

Comparative proteome analysis of the resistance responses in cultured roots of *Brassica rapa* (turnip) on the treatment with resting spore of *Plasmodiophora brassicae*, which is the causal agent of clubroot disease, was performed by 2-D PAGE. Using cultured roots from a susceptible and a resistant cultivar, 251 protein spots were clearly observed in 2D-PAGE and protein profiles were compared between resistant and susceptible roots as well as spore-treated and non-treated roots. In comparison between resistant and susceptible non-treated roots, 11 and 8 spots were significantly higher in intensity in resistant and susceptible roots, respectively. The treatment with resting spores of *P. brassicae* affected protein profiles of 2D-PAGE in a different manner between susceptible and resistant roots. In spore-treated susceptible roots, concentration of 6 protein spots increased but 7 were reduced compared to those in untreated control. These proteins are possibly involved in infectious events necessary for root hair infection or for defense responses in non-pathogen-specific stresses. On the other hand, in the spore treated resistant roots, 10 and 13 spots were up- and down-regulated, respectively. The protein profile of the resistant roots treated with the spore was clearly different from that of the susceptible ones, suggested that the fluctuated proteins of resistant roots should include the one(s) specific to resistant responses.

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Key words : Brassica rapa, clubroot resistance, *Plasmodiophora brassicae*, root culture, 2-D PAGE

Plasmodiophora brassicae is an obligatory soil-borne pathogen causing clubroot disease in economically important crops of the Brassicaceae. Roots of infected plants exhibit abnormal division and expansion of cells due to pathogen proliferation, leading to the formation of galls. Severe disease causes a delay of crop growth followed by a decay of the infected tissue. To control the disease, CR (clubroot-resistant) cultivars have been bred by conventional breeding, and recently several CR loci have been identified from Brassica plants (Suwabe *et al*., 2003; Hirai *et al*., 2004; Piao *et al*., 2004). However, little is known about molecular events in the incompatible interaction.

Plants have resistance system against microbial pathogens. In general, the system is triggered by recognition of a pathogen-derived elicitor, followed by induction of signal transduction pathways, e.g., Ca^{2+} uptake, kinase cascade, oxidative burst, etc. These early responses further induce hypersensitive response (HR). In previous studies, we found several cultivar-specific resistant responses against *P. brassicae* treatment, e.g., elevation of phenylalanine ammonia lyase activity, cell death and alkalization of medium for turnip callus and adventitious roots (Takahashi *et al*., 2001, 2002, 2006). However, the mechanism to confer the resistance has not clarified at all. Here we report our preliminary results of proteome analysis to reveal molecular events in resistance response against *P. brassicae* using cultured adventitious root.

MATERIALS AND METHODS

Plant materials and *P. brassicae*

Seeds of turnip (*Brassica rapa* L.) cultivars Natsumaki 13-gou kokabu (clubroot susceptible) and CR Takamaru (clubroot resistant) were purchased from The Musashino Seed Co., Ltd. (Tokyo, Japan). Resting spores of *Plasmodiophora brassicae* were kindly given by Dr. Akira Kiso (The Musashino Seed Co., Ltd.) and determined as race IV as described previously (Takahashi *et al*., 2001).

Root culture

Roots were excised from aseptically-grown young seedlings of the turnip and cultured in liquid MS medium supplemented with 25 μM 4-(3-indolyl)butyric acid, 29 nM gibberellin A_3 and 3% sucrose as described previously (Takahashi *et al*., 2006).

Resting spore preparation

Resting spore of *P. brassicae* was aseptically prepared from infected calli. The susceptible turnip was cultivated on soil and infected with *P. brassicae* as described elsewhere (Ishikawa *et al*., 2007). Infected roots were harvested on 35-40 days after inoculation, sterilized with 70% ethanol for 10 min and 10% sodium hypochlorite for 20 min. The damaged surface of the tissue was removed and the inner part was excised into 3-5 mm cube. The pieces were put onto 0.8% (w/v) agar plates of MS medium supplemented with 27 μM 1-naphthaleneacetic acid, 44 nM 6-benzylaminopurine and 3% (w/v) sucrose and cultured described previously (Takahashi *et al*., 2001). Induced calli were grown for 2 months. Well

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grown calli were homogenized with a sterile mortar and pestle, and the homogenate was gently layered on sterile 40% (w/w) sucrose. After sedimentation at 4 ℃ for 3 days, the intermediate layer containing spores was collected and centrifuged at 1,000 × g for 20 min at 4 ℃ . The precipitated spores were resuspended in sterile distilled water and centrifuged again. The wash was repeated four times and finally the spore was resuspended in small amount of sterile distilled water. Spore concentration was determined using a hemocytometer and adjusted to 10^7 mL^{-1}. The spore suspension was stored at 4 ℃ under the dark for up to 1 week.

Spore treatment

Three-week-old cultured root was transferred to a fresh medium and pre-cultured under the same condition as shown above. After 3 days, the culture was inoculated with *P. brassicae* spores at the final concentration of 10^4 mL^{-1} and the culture was lasted further 4 days under the same condition. The treated roots were harvested at designated days after spore treatment and stored at -80 ℃ .

Protein sample preparation for 2-D PAGE

One hundred mg root was homogenized in 1 mL of ice-cold 50 mM glycylglycine extraction buffer containing 1 mM EDTA, 0.5 M mannitol, 1 mM phenylmethylsulfonyl fluoride with a mortar and a pestle. The homogenate was passed through 8-layers of cheesecloth and centrifuged at 10,000 × g for 30 min at 4 ℃ . The supernatant was centrifuged again at 100,000 × g for 30 min at 4 ℃ , and the resulted supernatant was added with a 1/10 volume of 100% (w/v) trichloroacetic acid. After 20 min-placing on ice, the mixture was centrifuged at 18,000 × g for 10 min at 4 ℃ . The precipitate was rinsed with ethanol three times and dried under air at room temperature. The dried pellet was stored at -80 ℃ until use as the protein sample for 2-D PAGE analysis.

2-D PAGE

First dimensional IEF was performed using an IPGphor instrument (Amersham Biosciences Co., Piscataway, NJ). Two hundred μL of lysis buffer (8 M urea, 2% (w/v) CHAPS, 5% (v/v) 2-mercaptoethanol, 1.25% (v/v) ampholine pH 3.5-10) was added to the protein pellet prepared as above and allowed to stand for 1 h at room temperature. The suspension was well resuspended and allowed to stand for 30min at room temperature. The suspension was added with 5 μL of 5% (w/v) Orange G and centrifuged at 18,000 × g for 5 min at 4 ℃. The supernatant was applied onto an Immobiline Drystrip pH 3.5-10 (Amersham Biosciences Co.). After 10 h rehydration, IEF was carried out using a voltage gradient condition as follows: 0-500 V, 1 h; 500-1,000 V, 1 h; 1,000-8,000 V, 1 h; 8,000 V, 7 h. The strip was then equilibrated twice with 5 mL of 60 mM Tris containing 2.5% (w/v) SDS, 5% (v/v) 2-mercaptoethanol, 10% (v/v) glycerol and 0.1% (w/v) Orange G for 10 min each at room temperature with gentle stirring.

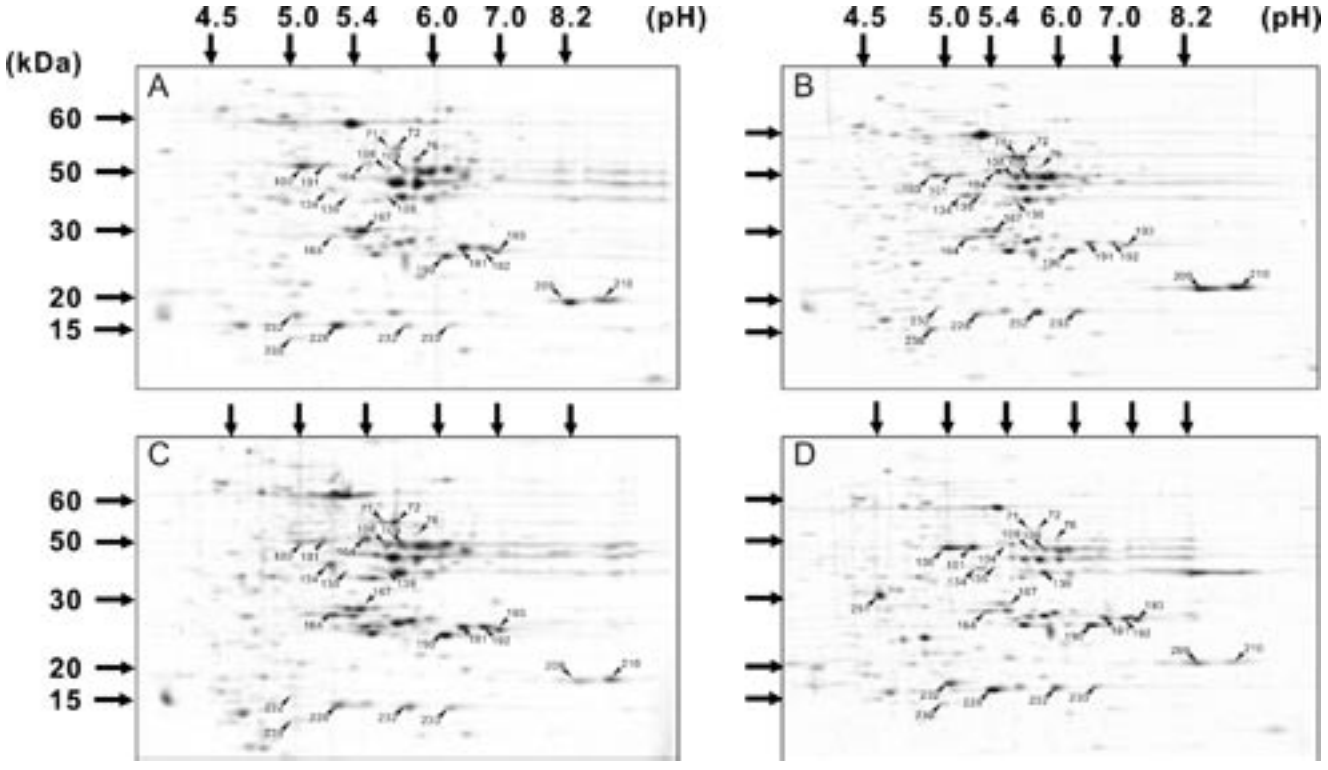

Fig. 1. 2-D PAGE analysis. A: Susceptible root non-treated. B: Resistant, non-treated. C: Susceptible, spore-treated. D: Resistant, spore-treated. Protein spots showing different concentration among samples are indicated with arrow heads having numbers corresponding to the Spot Nos. listed in **Table 1.**

Table 1. Protein profile showing quantitative differences in expression ratio among the variously conditioned roots

Spot No.	MW($\times 10^3$)	pI	[a]Differences (> 2-fold)		
			RNT / SNT	ST / SNT	RT/ RNT
71	43.1	5.62	+		-
72	43.0	5.69	+	+	-
76	41.2	5.81	-	-	
100	39.3	4.90	-	-	+
101	39.7	5.13			+
104	40.4	5.74	+	+	-
108	38.9	5.64	+		-
109	38.8	5.69	+		-
123	37.2	8.22			+
124	37.2	8.49			+
134	37.3	5.22		+	-
135	37.7	5.31	+		-
139	35.8	5.63		+	+
164	27.8	5.28	+	+	
167	28.5	5.66	-	-	-
190	25.9	6.09		+	
191	27.0	6.45	-		+
192	27.0	6.81	-		+
193	26.5	7.08	-		+
209	19.5	8.42	+	-	-
210	19.5	8.50	+	-	-
227	18.1	5.08	-	-	+
228	17.2	5.28	-	-	+
232	17.5	5.66	+		-
233	17.5	6.21			-
239	16.8	4.91	+		-
251	32.0	4.38			+ +

[a]Differences are expressed as follows; +, ratio > 2; -, ratio < 0.5; ++, newly detected.
Cultured roots were variously treated as followings: non spore-treated resistant and susceptible (RNT/SNT), spore-treated and non-treated susceptible (ST/SNT), and spore-treated and non-treated resistant (RT/RNT). RNT, resistant root non-treated; RT, resistant root spore-treated; SNT, susceptible root non-treated; ST, susceptible root spore-treated.
Molecular weight (MW), pI value, and spot quantification and comparison were performed using a PDQuest software.

Electrophoresis at second dimension was performed as described previously (Laemmli *et al.,* 1970) using a 16% (w/v) polyacrylamide gel. Proteins on the gel were CBB-stained and analyzed using the PDQuest software (Bio-Rad Laboratories, Inc., Hercules, CA).

RESULTS AND DISCUSSION

2-D PAGE analysis

To reveal molecular events that occur in the incompatible interaction between a CR cultivar of turnip and *P. brassicae*, we tried comparative proteome analysis using cultured roots from highly resistant cultivar CR Takamaru and a highly susceptible cultivar Natsumaki 13-gou kokabu.

Fig. 1 shows the results of 2-D PAGE analyses. Two hundreds fifty one spots were obtained from the four sample roots, non-treated susceptible (**Fig. 1A**), spore-treated susceptible (**Fig. 1B**), non-treated resistant (**Fig. 1C**) and spore-treated resistant roots (**Fig. 1D**). Twenty seven spots out of above protein spots, showed more than 2-fold differences in concentration in comparisons of susceptible to that of resistant roots or spore treated to that of non-treated roots. The 27 spots were indicated with the numbers in **Fig. 1** and listed with estimated molecular weights and pI values in **Table 1**.

Comparative study of protein profiles in 2-D PAGE

Comparison of profile of the proteins obtained from normally grown of cultured roots from the resistant to that from the susceptible cultivar

In comparison of protein profiles in 2-D PAGE of resistant roots to susceptible one in non-infected samples, 11 spots (Nos. 71, 72, 104, 108, 109, 135, 164, 209, 210, 232, 239) were abundant in the resistant roots and 8 spots (Nos. 76, 100, 167, 191, 192, 193, 227, 228) were contrarily in higher concentration in the susceptible ones. We had observed the constitutive differences in levels of PAL activity (Takahashi *et al*., 2001) and endogenous contents of lignin and salicylic acid (Takahashi *et al*., unpublished data) in both the calli and the cultured roots originated from two cultivars. Thus, the proteins rich in those non treated roots as above mentioned may be involved in the secondary metabolism.

Comparison of profile of the proteins obtained from spore-treated susceptible cultured roots to that from non treated one

In non-treated susceptible roots, 6 spots (Nos. 72, 104, 134, 139, 164, 190) were up-regulated but 7 spots (Nos. 76, 100, 167, 209, 210, 227, 228) were down-regulated in the treated roots.

Comparison of profile of the proteins obtained from spore-treated resistant cultured roots to that from non treated one

In non-treated resistant roots, 10 spots (Nos. 100, 101, 123, 124, 139, 191, 192, 193, 227, 228) were up-regulated but 13 spots (Nos. 71, 72, 104, 108, 109, 134, 135, 167, 209, 210, 232, 233, 239) were down-regulated in the treated roots. Interestingly, one spots, No. 251, was detected with intensive signal in the treated resistant roots but even faint signal was never detected in non-treated resistant roots and in neither non-treated nor treated susceptible roots.

CONCLUSION

We achieved 2-D PAGE to detect differences in protein profiles between resistant and susceptible cultured roots in the early responses against the given *P. brassicae* resting spores. These proteins, especially in up-regulated ones in resistant roots, must include specific agents involved in the resistance to clubroot infection. In addition, fluctuated proteins in susceptible roots may include the crucial agents for the completion of infection of the pathogen. Further analyses on these proteins will contribute to understanding the molecular mechanism of clubroot resistance.

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Brassica rapa 培養根の根こぶ病病原菌 *Plasmodiophora brassicae* に対する抵抗反応のプロテオーム解析

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要　約

根こぶ病菌感染初期反応を解明するため、根こぶ病菌感受性と抵抗性の２系統のカブから誘導した培養根に根こぶ病菌 *Plasmodiophora brassicae* の胞子を処理し、タンパク質の消長を2-D PAGE で検出した。無処理区及び胞子処理区の感受性、抵抗性両系統由来培養根からは、251スポットのタンパク質が検出された。無処理区で比較すると、251のうち11スポットは抵抗性系統で強発現し、８スポットは感受性系統で強発現していた。感受性培養根では胞子処理で６スポットの発現量が増大し、逆に７スポットでは低下した。これらのタンパク質はおそらく、根毛での感染に必要な反応あるいは病原菌非特異的な防御反応に関与していると思われる。一方胞子処理区の抵抗性培養根では、10スポットの発現が増大し13スポットで減少した。これら抵抗性培養根における胞子処理に対するタンパク質の消長は感受性のそれとは明らかに異なっており、この中に抵抗性反応特異的なタンパク質があると考えられた。

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キーワード：*Brassica rapa*、*Plasmodiophora brassicae*、二次元電気泳動、根こぶ病抵抗性、培養根

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