Plant Biotechnology 30, 83–87 (2013)
DOI: 10.5511/plantbiotechnology.12.1129a

Note

Proteome analysis of infection-specific proteins from Japanese birch (*Betula platyphylla* var. *japonica*) plantlet No.8 infected with *Inonotus obliquus* strain IO-U1

Yuya Takashima[1], Futoshi Ishiguri[2], Kazuya Iizuka[2], Nobuo Yoshizawa[2], Shinso Yokota[2,*]

[1] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, Fuchu, Tokyo 183-8509, Japan; [2] Faculty of Agriculture, Utsunomiya University, Utsunomiya, Tochigi 321-8505, Japan
* E-mail: yokotas@cc.utsunomiya-u.ac.jp Tel: +81-28-649-5539 Fax: +81-28-649-5545

Received October 13, 2012; accepted November 29, 2012 (Edited by K. Kato)

Abstract This study aimed to identify the proteins produced specifically in *Betula platyphylla* var. *japonica* plantlet No. 8 by infection with *Inonotus obliquus* strain IO-U1. Sterile Japanese birch plantlets were infected with the fungus, and the protein samples obtained at 2 days post-infection were subjected to two-dimensional electrophoresis to detect infection-specific proteins. The specific proteins were analyzed using MALDI-TOF-MS and identified by peptide mass fingerprinting with MASCOT software. Among the 735 protein spots detected in the infected plantlets, 169 spots were recognized as infection-specific proteins. Of these spots, 91 were analyzed using MALDI-TOF-MS, with two heat shock proteins (Hsp70 and Hsp60) being identified as infection-specific proteins. Hsp70 and Hsp60 may cooperate to refold proteins that were denatured by the infection of *I. obliquus* strain IO-U1 in the birch plantlets. It is suggested that these proteins are expressed in *B. platyphylla* plantlet No. 8 by infectious stress with *I. obliquus* strain IO-U1.

Key words: *Betula platyphylla* var. *japonica*, *Inonotus obliquus*, host-pathogen interaction, heat shock protein.

Betula platyphylla var. *japonica* belongs to Betulaceae and is distributed throughout the subalpine zone in Honsyu and Hokkaido, Japan. *Inonotus obliquus* is a white rot fungus classified into Hymenochaetaceae of Basidiomycotina. The fungus causes stem heart rot of birch and produces a black solid sclerotium called a sterile conk or canker-like body. Formation of the sclerotium requires several years (Hirt 1949; True et al. 1955; Zabel 1976). Recently, Cha et al. (2011) reported that basidiocarps of *I. obliquus* were observed at the margin of a sterile conk on the stem of a living paper birch tree (*Betula papyrifera*) in North America. Although some studies have been performed with regard to wood decay in *Betula* species infected with *I. obliquus* (Blanchette 1982; Terho 2009; Terho et al. 2007; Yamaguchi et al. 2001), reports are limited on the interactions between birch trees and *I. obliquus* at the initial stage of fungal infection. We previously investigated the anatomical and histochemical characteristics of living *B. platyphylla* var. *japonica* (Tohoku) plantlets infected with the *I. obliquus* strain IO-U1 for clarifying the host-pathogen interactions (Rahman et al. 2008). Our research clarified that phenolics were first deposited at the cut margin tissues and subsequently in the vessels after 4 h of infection; thereafter, their deposition extended to the other xylem elements, cortex, and pith. In addition, a necrophylactic periderm was formed at 30 days post-infection. Based on these results, we considered that phenolic deposition and necrophylactic periderm formation occur as infection-induced responses in Tohoku birch plantlets infected with *I. obliquus* IO-U1.

Proteome analysis is considered to be a powerful method to resolve vital phenomena in organisms (Chevalier 2010). Proteome analysis has been applied to clarify host-pathogen interactions in phytopathology studies (Amey et al. 2008; Casado-Vela et al. 2006; Chivasa et al. 2006; Mahmood et al. 2006; Marsh et al. 2010). In a susceptible grapevine (*Vitis vinifera* 'Cabernet Sauvignon') infected with powdery mildew (PM) (*Erysiphe necator*), a comparative analysis of the differentially expressed proteins was conducted, with 63 proteins being significantly altered in abundance at 24, 36, 48, and 72 h post-infection. These PM-responsive proteins were classified functionally into groups involved in photosynthesis, metabolism, disease/defense, protein destination, and protein synthesis, suggesting that PM-susceptible Cabernet Sauvignon is able to initiate a basal

This article can be found at http://www.jspcmb.jp/
Published online March 19, 2013

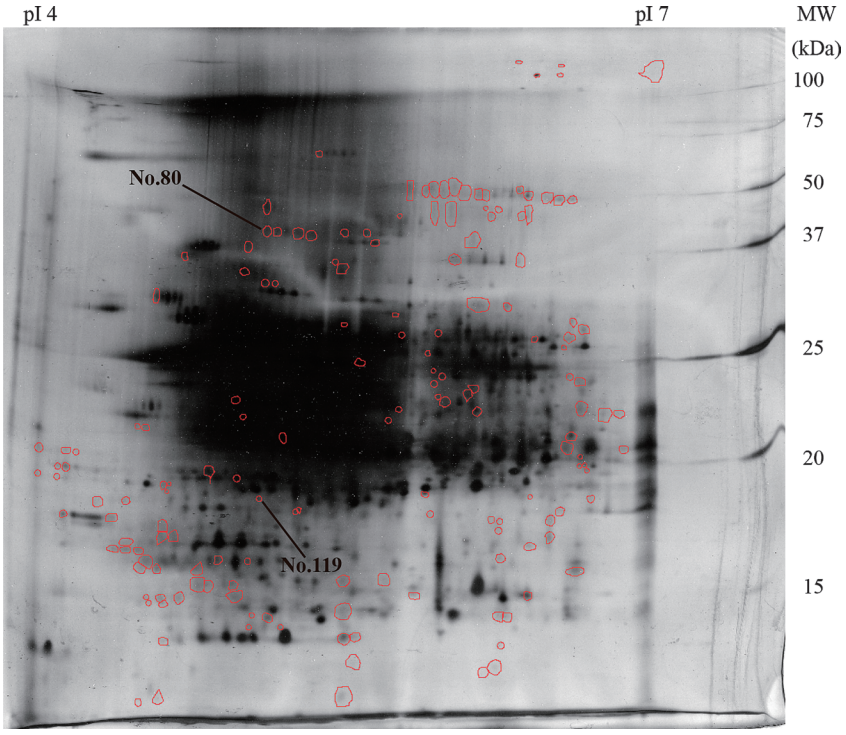

Figure 1. Silver-stained 2-DE gel of the proteins from *B. platyphylla* var. *japonica* plantlet No. 8 infected with *I. obliquus* strain IO-U1. The infection-specific proteins are shown by circles. A total of 169 protein spots were detected as infection-specific proteins. Of 169 the protein spots, 91 proteins were excised from the gel and used as the samples for the mass spectrometric analysis. Proteins of spot ID Nos. 80 and 119 were identified as heat shock 70 kDa protein (Hsp70) and heat shock 60 kDa protein (Hsp60), respectively.

defense but unable to restrict fungal growth or slow disease progression (Marsh et al. 2010). Thus, proteomics has been evolving as a useful tool to understand plant-microbe interactions at the molecular level.

Because the proteomic analysis of woody plants has rarely been performed, the present study attempted to understand woody plant immunity, even though the complete genome sequence of *B. platyphylla* has not yet been elucidated. The purpose of this study is to identify the proteins produced specifically in *B. platyphylla* var. *japonica* plantlet No.8 infected with *I. obliquus* strain IO-U1 and to clarify the responses of infected Japanese birch plantlets.

Aseptic Japanese birch (*Betula platyphylla* var. *japonica*) plantlet No. 8, which was provided by Forestry and Forest Products Research Institute, Tsukuba, Japan, was used in this study. The plantlets were grown on Murashige and Skoog medium (Murashige and Skoog 1962) containing 2.5 μM indole-3-butyric acid and 0.1 μM 1-naphthaleneacetic acid at 25°C with a 16 h photoperiod. For the propagation of the plantlets, axillary buds were subcultured in same medium every three months. The fungus *Inonotus obliquus* strain IO-U1 used in this study was provided by Forest Resource Biology, Forest Resource Science, Division of Environmental Resources, Graduate School of Agriculture, Hokkaido University. The fungus was cultured on potato-dextrose-agar slant medium at 24°C in the dark. After the surface of the slant medium was covered with the mycelium, the fungus was kept at 4°C in the dark. The third internode from the apex of a three-month-old plantlet was cut at a depth of approximately 1 mm using a surgical knife, and a small plug of mycelium was placed on the wound. Intact and wounded sterile plantlets were also prepared as controls.

Two days after the treatments, each plantlet was deep-frozen in liquid nitrogen, and the material was powdered using a mortar and pestle. The proteins were extracted from the powder with 6–10 ml buffer containing 1.5% (w/v) Trizma Pre-Set Crystals (pH 7.5) (Sigma-Aldrich Co., USA), 3.01 mM Na_2EDTA (Dojin Laboratories, Japan), 20% (v/v) glycerol, 1% (v/v) Tween 80, and 10 mM DTT and mixed with 100 mg Polyclar (Wako, Japan) and a small amount of quartz sand. The crude homogenates were centrifuged at 10,000×*g* for 1 h at 4°C. The supernatants were precipitated with 10% (w/v; final concentration) trichloroacetic acid aqueous solution for 1 h at −30°C, followed by centrifugation at 10,000×*g* for 30 min. The pellets obtained were washed three times with cold acetone, and the acetone was evaporated at room temperature. The dried samples were resuspended in a solution containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS (GE Healthcare, England), 20 mM DTT, and 2% (v/v) IPG buffer (pH 4–7; GE Healthcare). The resuspended samples were stored at −30°C. The protein concentration of the samples was determined using

the Bradford method (Bladford 1976) with ovalbumin (Sigma-Aldrich Co.) as a standard.

Isoelectric focusing (IEF) was performed using IPG strips (Immobiline DryStrip pH 4–7, 13 cm; GE Healthcare) with an Ettan IPGphor unit (GE Healthcare). A 300 μg sample of protein was focused at 75 kVh with four step voltages from 500 V to 8,000 V. The second-dimension electrophoresis was performed on a 12.5% polyacrylamide gel using a Hoefer SE600 Ruby apparatus (GE Healthcare). After the two-dimensional electrophoresis (2-DE), the gels were stained with a Silver Staining Kit (GE Healthcare), and the gel images were acquired using a scanner (GT-9700; EPSON, Japan). The spot detection, spot edition, and gel-to-gel matching were performed using ImageMaster 2D Platinum ver. 5.0 software (GE Healthcare). Two-DE was carried out three times for intact, wounded, and infected plantlets, respectively. In addition, the infection-specific protein spots from *B. platyphylla* var. *japonica* were confirmed not to be overlapped on those from *I. obliquus* by comparing their 2-DE gels.

The spots specifically expressed in the samples from the infected plantlets were selected and excised from the silver-stained gel. The gel pieces were destained with a mixture of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate (Gharahdaghi et al. 1999). The in-gel digestion of the proteins by trypsin (Promega, USA) was performed according to the method of Shevchenko et al. (1996).

Prior to the mass spectrometric analysis, all of the peptide samples were desalted with ZipTip μ-C18 tips (Millipore Co., USA). For the mass spectrometric analysis, the peptide samples were mixed with α-cyano-4-hydroxycinnamic acid (CHCA) on an AnchorChip (Bruker Daltonics Inc., USA) according to the method of Gobom et al. (2001). The mass spectra of the peptides were recorded using MALDI-TOF-MS (Autoflex; Bruker Daltonics Inc.). All of the analyses were performed using a reflector positive mode, and the mass range was set from 600 to 3,000 *m*/*z*. The protein identification was performed by peptide mass fingerprinting (PMF) using MASCOT software (www.matrixscience.com). The used databases were NCBInr and SwissProt. MASCOT search was performed using the following parameters: taxonomy, all entries; enzyme, trypsin; number of missed cleavage sites, up to 2 missed; fixed modification, carbamidomethylation of cysteine; variable modifications, oxidation of methionine; peptide tolerance, $\pm$50 ppm; peptide mass value, MH^+ and monoisotopic mass.

The number of protein spots detected from the 2-DE gels of the intact plantlets, wounded plantlets, and infected plantlets were 641, 526, and 735, respectively. Some protein spots were condensed at the ends in the pI 4 and pI 7 ranges. A comparison of the gels between the intact and wounded plantlets showed that a total of 119 protein spots were expressed as injury-specific proteins. In contrast, a comparison of the 2-DE gels between the infected and wounded plantlets revealed that a total of 169 protein spots were infection specific (Figure 1).

Among the 169 infection-specific protein spots, 78 protein spots were too vaguely stained to excise from the gel. Hence, the remaining 91 protein spots were excised from the gel and used as samples for the mass spectrometric analysis. Among the 91 protein spots, only two protein spots were identified by PMF using MASCOT search. Although mass spectra were obtained from the remaining 88 protein spots, these 88 proteins

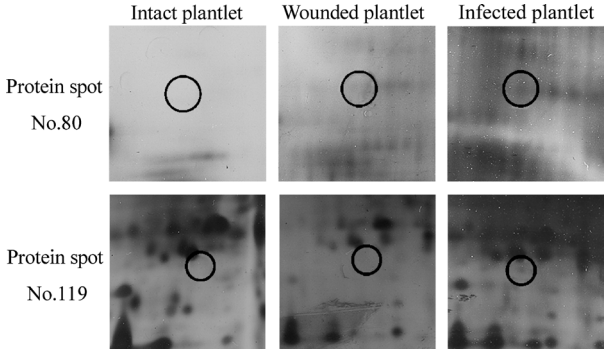

Figure 2. Expressive comparison of three protein spots between intact, wounded, and infected plantlets of *B. platyphylla* var. *japonica* No. 8. Proteins of spot ID Nos. 80 and 119 are shown by circles, respectively.

Table 1. List of identified proteins differentially expressed in the *B. platyphylla* var. *japonica* plantlets No. 8 infected with *I. obliquus* strain IO-U1.

Spot ID	Annotation	Source	pI value		Matched peptide mass (*m*/*z*)		Matched peptide no.	Sequence coverage (%)
			Observed	Theoretical	Observed	Theoretical		
80	Heat shock 70 kDa protein (Hsp70)	*Malus domestica*	5.22	5.17	1197.6510	1196.6877	6	13
					1228.6320	1227.6207		
					1487.7010	1486.6940		
					1675.7320	1674.7234		
					1680.8220	1679.8267		
					2658.1800	2657.2609		
119	Heat shock 60 kDa protein (Hsp60)	*Mycobacterium shimoidei*	5.18	4.72	1230.6800	1229.7231	2	19
					1284.7600	1283.7812		

could not be identified. Protein spot Nos. 80 and 119 (Figure 2) were identified as partial peptides of heat shock 70 kDa protein (Hsp70) and heat shock 60 kDa protein (Hsp60), respectively, by the MALDI-TOF-MS analysis and subsequent database search with MASCOT software (Figures 1 and 2, and Table 1). However, adequate mass spectra could not be obtained from the other protein spots.

In this study, the quantitative analysis of the protein expression in three treated plantlets could not be performed because the silver staining intensity varied due to the differences in protein structure. However, a total of 169 protein spots were detected as infection-specific proteins. Thus, infection with *I. obliquus* caused physiological changes in the *B. platyphylla* plantlets.

Protein spots Nos. 80 and 119 were identified as Hsp70 and Hsp60, respectively, by the MALDI-TOF-MS analysis using the MASCOT PMF search. Hsp70 and Hsp60, known as molecular chaperones, assist in the folding of newly synthesized proteins. Under normal conditions, Hsp70 plays a role in assisting the folding of some newly translated proteins, guiding translocating proteins across organellar membranes, disassembling oligomeric protein structure, facilitating the proteolytic degradation of unstable proteins, and controlling the biological activity of folded regulatory proteins, including transcription factors (Bukau and Horwich 1998). In addition, Hsp70 most likely contributes to the stable refolding of proteins that are denatured by heat shock and other various stresses (Sung et al. 2001). For example, several members of the Hsp70 family in spinach are regulated by a light/dark signal that is circadian rhythm independent (Li and Guy 2001). In addition, it was found that pea seed-borne virus induces the expression of PsHsp71.2 (Aranda et al. 1996). Regarding Hsp60, Langer et al. (1992) reported that Hsp70 and Hsp60 cooperated functionally to regulate the maintenance of proteins in cells. Based on the results of our study, therefore, it is suggested that Hsp70 and Hsp60 cooperate to refold the proteins in birch plantlets that are denatured by the infection of *I. obliquus* strain IO-U1.

In the present study, the proteins which could be identified by MALDI/TOF/MS with PMF were only two proteins, Hsp70 and Hsp60, although 169 spots were recognized as infection-specific proteins on the 2-DE gel. These results indicate that the protein identification using MALDI/TOF/MS with PMF requires certain amounts of peptide samples, and that this method has limitation to identify the proteins derived from woody plants. Thus, to identify the proteins related to the interactions between *B. platyphylla* var. *japonica* and *I. obliquus*, further study is needed using LC/MS/MS with peptide sequence tags.

Acknowledgements

The authors acknowledge Professor Hamako Sasamoto, Faculty of Environment and Information Sciences, Yokohama National University, for providing *Betula platyphylla* var. *japonica* plantlet No. 8. The authors are grateful to Professor Minoru Terasawa and Associate Professor Yutaka Tamai, Graduate School of Agriculture, Hokkaido University, for providing the fungus, *Inonotus obliquus*. The authors would like to thank Associate Professor Kenichi Nihei, Faculty of Agriculture, Utsunomiya University, for his kind guidance with regard to MALDI-TOF-MS. This study was supported by JSPS KAKENHI Grant Numbers 15580119 and 19580162.

References

Amey RC, Schleicher T, Slinn J, Lewis M, Macdonald H, Neill SJ, Spencer-Phillips PTN (2008) Proteomic analysis of a compatible interaction between *Pisum sativum* (pea) and the downy mildew pathogen *Peronospora viciae*. *Eur J Plant Pathol* 122: 41–55

Aranda MA, Escaler M, Wang D, Maule AJ (1996) Induction of HSP70 and polyubiquitin expression associated with plant virus replication. *Proc Natl Acad Sci USA* 93: 15289–15293

Blanchette RA (1982) Progressive stages of discoloration and decay associated with the canker-rot fungus, *Inonotus obliquus*, in birch. *Phytopathology* 72: 1272–1277

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254

Bukau B, Horwich AL (1998) The Hsp70 and Hsp60 chaperone machines. *Cell* 92: 351–366

Casado-Vela J, Sellés S, Martínez RB (2006) Proteomic analysis of tobacco mosaic virus-infected tomato (*Lycopersicon esculentum* M.) fruits and detection of viral coat protein. *Proteomics* 6 (Suppl 1): S196–S206

Cha JY, Lee SY, Lee SY, Chun KW (2011) Basidiocarp formation by *Inonotus obliquus* on a living paper birch tree. *For Pathol* 41: 163–164

Chevalier F (2010) Highlights on the capacities of "Gel-based" proteomics. *Proteome Sci* 8: 23

Chivasa S, Hamilton JM, Pringle RS, Ndimba BK, Simon WJ, Lindsey K, Slabas AR (2006) Proteomic analysis of differentially expressed proteins in fungal elicitor-treated *Arabidopsis* cell cultures. *J Exp Bot* 57: 1553–1562

Gharahdaghi F, Weinberg CR, Meagher DA, Imai BS, Mische SM (1999) Mass spectrometric identification of proteins from silver-stained polyacrylamide gel: A method for the removal of silver ions to enhance sensitivity. *Electrophoresis* 20: 601–605

Gobom J, Schuerenberg M, Mueller M, Theiss D, Lehrach H, Nordhoff E (2001) α-cyano-4-hydroxycinnamic acid affinity sample preparation. A protocol for MALDI-MS peptide analysis in proteomics. *Anal Chem* 73: 434–438

Hirt RR (1949) Decay of certain northern hardwoods by *Fomes igniarius*, *Poria obliqua*, and *Polyporus glomeratus*. *Phytopathology* 39: 475–480

Langer T, Lu C, Echols H, Flanagan J, Hayer MK, Hartl FU (1992) Successive action of DnaK, DnaJ and GroEL along the pathway of chaperone-mediated protein folding. *Nature* 356: 683–689

Li QB, Guy CL (2001) Evidence for non-circadian light/dark-regulated expression of Hsp70s in spinach leaves. *Plant Physiol* 125: 1633–1642

Mahmood T, Jan A, Kakishima M, Komatsu S (2006) Proteomic

analysis of bacterial-blight defense-responsive proteins in rice leaf blades. *Proteomics* 6: 6053–6065

Marsh E, Alvarez S, Hicks LM, Barbazuk WB, Qiu W, Kovacs L, Schachtman D (2010) Changes in protein abundance during powdery mildew infection of leaf tissues of Cabernet Sauvignon grapevine (*Vitis vinifera* L.). *Proteomics* 10: 2057–2064

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiol Plant* 15: 473–497

Rahman MM, Ishiguri F, Takashima Y, Azad MAK, Iizuka K, Yoshizawa N, Yokota S (2008) Anatomical and histochemical characteristics of Japanese birch (Tohoku) plantlets infected with the *Inonotus obliquus* IO-U1 strain. *Plant Biotechnol* 25: 183–189

Shevchenko A, Wilm M, Vorm O, Mann M (1996) Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal Chem* 68: 850–858

Sung DY, Kaplan F, Guy CL (2001) Plant Hsp70 molecular chaperones: Protein structure, gene family, expression and function. *Physiol Plant* 113: 443–451

Terho M (2009) An assessment of decay among urban *Tilia*, *Betula*, and *Acer* trees felled as hazardous. *Urban For Urban Green* 8: 77–85

Terho M, Hantula J, Hallaksela AM (2007) Occurrence and decay patterns of common wood-decay fungi in hazardous trees felled in the Helsinki city. *For Pathol* 37: 420–432

True RP, Tryon EH, King JF (1955) Cankers and decays of birch associated with two *Poria* species. *J For* 53: 412–415

Yamaguchi T, Sasaki K, Sakamoto Y (2001) Evaluation of a stress-wave timer for the minimally destructive detection of decay in living trees in Northern-Japanese forests. *J For Res* 6: 117–120

Zabel RA (1976) Basidiocarp development in *Inonotus obliquus* and its inhibition by stem treatments. *For Sci* 22: 431–437