Plant Biotechnology 30, 199–205 (2013)
DOI: 10.5511/plantbiotechnology.13.0312b

Note

Cationic peroxidase related to basal resistance of *Betula platyphylla* var. *japonica* plantlet No. 8 against canker-rot fungus *Inonotus obliquus* strain IO-U1

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Received November 15, 2012; accepted March 12, 2013 (Edited by Y. Watanabe)

Abstract This study investigated the time-course changes of the *in situ* peroxidase (POD) distribution and expression of POD isozymes in *Betula platyphylla* var. *japonica* plantlet No. 8 infected with a canker-rot fungus, *Inonotus obliquus* strain IO-U1. Intact (C1), wounded (C2), and infected (T) plantlets were collected at 2 h up to 30 d. *In situ* POD activity was detected in the C2 and T plantlets, and the POD activity in the T plantlets was more widely distributed compared to the C2 plantlets. In addition, the area of POD activity localization was almost the same as that of phenolic compounds, although a time lag was found between the appearance of POD activity and phenolic compounds. POD isozymes were clearly detected within the basic range (pI>8.5) in isoelectric focusing electropherograms. The activity of cationic POD isozymes in the C2 and T plantlets was induced strongly compared to the C1 plantlets. In addition, the pattern of time-course changes in the activities of *in situ* POD and POD isozymes was different between the C2 and T plantlets, suggesting that the responsive mechanisms against fungal infection are different from the responses to wounding. The obtained results suggest that cationic POD isozymes are related to the basal resistance in *B. platyphylla* var. *japonica* plantlet No.8 against infection with *I. obliquus* strain IO-U1.

Key words: *Inonotus obliquus*, *Betula platyphylla* var. *japonica*, cationic peroxidase, tree pathology.

Plant peroxidases (PODs) are involved in the lignification and suberization of the cell wall and the cross-linking of cell wall proteins to limit pathogen invasion through cell wall reconstitution. Lignin and suberin are polymerized from phenolics via POD-mediated oxidative reactions in the presence of H_2O_2. Additionally, the generation of H_2O_2 is also catalyzed by PODs through the oxidation of NADH. POD-mediated phenolic oxidation also synthesizes anti-pathogenic phenolics, such as phytoalexins, in addition to polymerizing phenolic monomers into cell wall components (Almagro et al. 2009; Kawano 2003; Marjamaa et al. 2009; Passardi et al. 2004). Therefore, PODs have very important roles in plant defense mechanisms.

Inonotus obliquus is a white rot fungus classified into Hymenochaetaceae of Basidiomycotina and causes stem heart rot of *Betula* species, producing a black solid scerotium called as sterile conk or canker-like body (Cha et al. 2011; Shigo 1969; Zabel 1976). In our previous study, host-pathogen interactions were investigated using the *Betula platyphylla* var. *japonica* plantlet Tohoku infected with a canker-rot fungus, *I. obliquus* strain IO-U1 (Rahman et al. 2008). We found that phenolic deposition and necrophylactic periderm formation occur as infection-induced responses in *B. platyphylla* var. *japonica* plantlet Tohoku as a result of infection with strain IO-U1.

The purpose of this study is to observe the time-course changes of the *in situ* POD distribution and POD isozyme expression in *B. platyphylla* var. *japonica* No. 8 plantlets infected with canker-rot fungus *I. obliquus* strain IO-U1. In addition, phenolic compound accumulation was also evaluated.

Three-month-old *B. platyphylla* var. *japonica* No. 8 plantlets and *I. obliquus* strain IO-U1 were used for the experiments. The preparation of the plantlets and fungus and the treatments of intact (C1), wounded (C2), and infected (T) plantlets were performed according to the methods of our previous report (Rahman et al. 2008). After the treatments, the plantlets were grown for 2, 4, 6, and 12 h and 2, 10, and 30 d. Stem samples (1 cm in length) were collected from the third internode or treated position in the C1, C2, and T plantlets to observe the *in situ* POD activity and phenolic compound accumulation.

This article can be found at http://www.jspcmb.jp/
Published online June 11, 2013

In addition, the same samples at 2, 10, and 30 d were used for analyzing the POD isozymes.

The fixation of the samples, staining for peroxidase activity, and section preparation were performed according to the methods of De Vecchi and Matta (1988). Longitudinal radial sections (20 μm in thickness) were obtained using a simple hand microtome (Nippon Optical Works Co., Japan). These sections were mounted with 75% glycerin and observed using optical light microscopy (BX 51, Olympus, Japan). The evaluation of phenolic compound accumulation by observation using fluorescence microscopy and optical light microscopy was performed by the methods described in our previous paper (Rahman et al. 2008).

For analyzing the POD isozymes, samples (1 cm in length) were cut from the stems of C1, C2, and T plantlets and then immediately deep-frozen in liquid N_2. The frozen samples were homogenized in an extraction buffer (EXT) composed of EXT-1, EXT-2, and EXT-3 in a volume ratio of 3 : 2 : 1. The components were as follows: EXT-1–3.0% Trizma Pre-Set Crystal (Sigma-Aldrich, USA) (w/v) (pH 7.5), 0.22% EDTA-2Na (w/v), and 40% glycerol (v/v) in distilled water; EXT-2–3% Tween 80 (v/v) in distilled water; and EXT-3–0.926% dithiothreitol (w/v) in distilled water (Shiraishi 1987). The homogenates were centrifuged at 10,000×*g* for 30 min at 4°C, and the supernatants were deionized using MicroSpin column G-25 (GE Healthcare, England) and centrifugation at 735×*g* for 2 min at 4°C. The obtained samples were used for the POD isozyme analysis.

The protein concentration in each sample was determined according to the method of Bradford (Bradford 1976). Isoelectric focusing (IEF) of the protein preparations was conducted using a Multiphor II Electrophoresis System (GE Healthcare) and PowerPac HV (Bio-Rad, USA) with native PAGE [T=5% and C=3%, containing 2.2% Pharmalyte (pH range 3.5–9.5, GE healthcare)] (Westermeier 1997). The isoelectric points were estimated using protein standards (IEF Standards, pI 4.45–9.60, Bio-Rad). Aspartic acid (0.04 M) was used as the anolyte, and 1 M NaOH was used as the catholyte. The samples were focused at 1,500 V, 50 mA, 25 W, and 3,000 Vh. After IEF, the gel was stained with staining solution to detect the POD isozymes. The staining solution was composed of B-POD, POD-1, and POD-2 in the volume ratio of 80 : 20 : 1. The components were as follows: B-POD–0.151% 2-amino-3-hydroxymethyl-1,3-propanediol (w/v) and 0.162% (v/v) acetone in distilled water; POD-1–0.21% 3-amino-9-ethylcarbazole (w/v) and 0.145% 2-naphthol (w/v) in ethanol; and POD-2–3% H_2O_2 (v/v) in distilled water (Shiraishi 1987). After drying, photographs of the stained gels were taken using a digital camera.

Figures 1 to 3 show the time-course changes of the *in situ* POD activity, specific autofluorescence of phenolic compounds, and phenolic compound accumulation. In the T plantlets, specific POD activity was first detected in the cortical layer, cambium zone, lumen of vessels, and pith area at 2 h post-infection (hpi). Thereafter, the localization area of specific POD activity continuously expanded up to 30 d post-infection (dpi), and activity was also detected in the wound-induced callus at 10 and 30 dpi in the T plantlets (Figure 1). Specific autofluorescence of phenolic compounds was detected in the periderm at 2 hpi and then in the cortex and cambium at 1 dpi, in the pith at 10 dpi, and in the outer layer of the wound-induced callus at 30 dpi in the T plantlets (Figure 2). The presence of phenolic compounds was confirmed in the cortical layer and lumen of vessels at 2 hpi; thereafter, phenolic compounds were found in the cambial zone at 12 hpi and in the pith area at 2 dpi in the T plantlets. The deposition of phenolic compounds was also observed in the wound-induced callus at 30 dpi in the T plantlets (Figure 3). The tissues that accumulated phenolic compounds at 2 dpi continued to exhibit these compounds up to 30 dpi. In addition, phenolic compounds were observed only at 2 hpi in the T plantlets (Figure 3), whereas they were observed at 4 h after wounding in the C2 plantlets (data not shown).

POD localization and the accumulation of phenolic compounds were more rapidly and extensively observed in the T plantlets compared to the C2 plantlets (Figures 1 to 3). Although a time lag was found for POD localization and accumulation of phenolic compounds, the POD localization area was almost the same as that of phenolic compound accumulation. Moreover, POD was activated more rapidly than phenolic compound accumulation in all the treated plantlets (Figures 1 to 3). These results were in agreement with the previous reports that PODs are involved in polymerization of phenolics, and synthesis of phenolic compounds in relation to plant defense responses against infection and wounding (Bruce and West 1989; Deborah et al. 2001; Egea et al. 2001; Gayoso et al. 2010; Lagrimini 1991; Morkunas and Gmerek 2007). However, *B. platyphylla* var. *japonica* plantlet could not prevent itself from progress of mycelial growth of *I. obliquus* strain IO-U1. In our previous report, the fungal localization was observed in *B. platyphylla* var. *japonica* plantlet Tohoku infected with *I. obliquus* strain IO-U1. In that study, the fungal hyphae were detected at 10 dpi in cortex, xylem ray, and pith area, although the hyphae did not detected at 2 dpi (Rahman et al. 2008). Therefore, *B. platyphylla* var. *japonica* plantlets No. 8 and Tohoku are considered to be compatible to *I. obliquus* strain IO-U1. In addition, POD activation and phenolic compound accumulation are considered to be related to basal defense in *B. platyphylla* var. *japonica* plantlet No. 8 as responses to the infection with *I. obliquus* strain IO-U1.

The isoelectric focusing electropherograms of POD

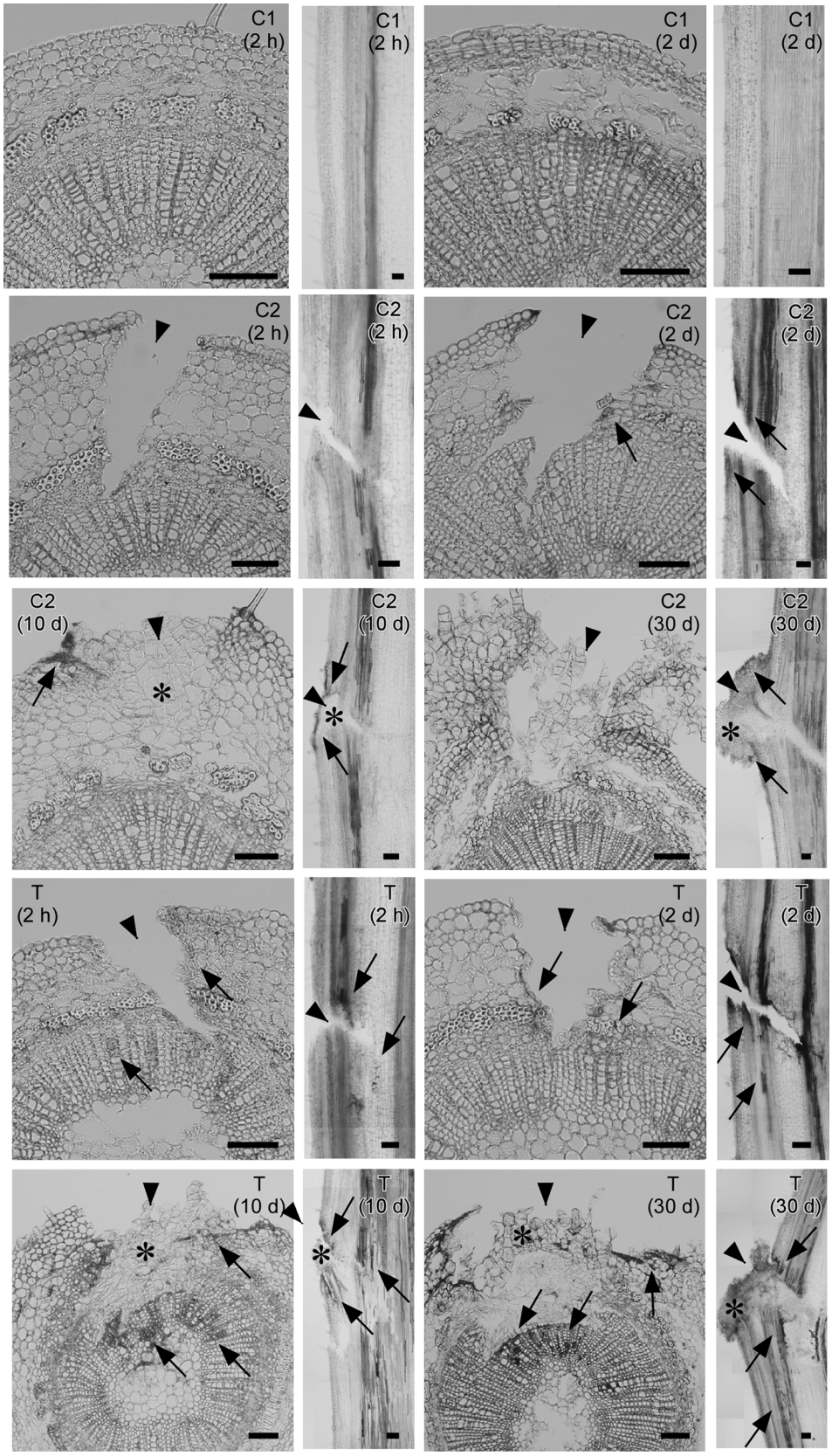

Figure 1. *In situ* peroxidase distribution in transverse and longitudinal sections of C1-, C2-, and T-treated *B. platyphylla* var. *japonica* No. 8 plantlets after 2 h to 30 d. The arrows indicate the localization of peroxidase; the arrow-heads indicate the wound and infection sites; the asterisks indicate the callus; Bar = 100 μm.

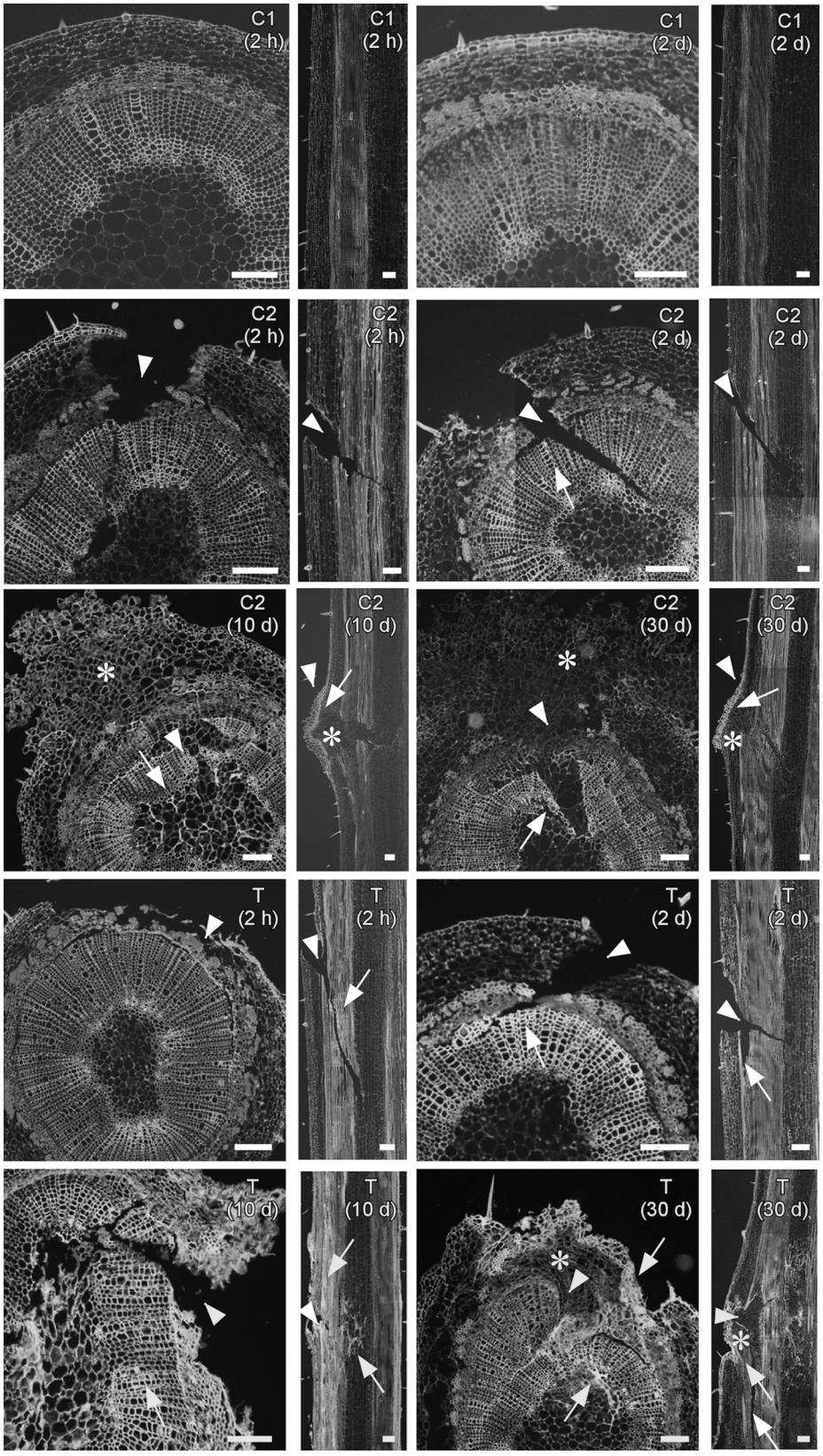

Figure 2. Fluorescence micrographs of phenolic compounds in transverse and longitudinal sections of C1-, C2-, and T-treated *B. platyphylla* var. *japonica* No. 8 plantlets after 2 h to 30 d. The arrows indicate the specific autofluorescence of phenolic compounds; the arrow-heads indicate the wound and infection sites; the asterisks indicate the callus; Bar = 100 μm.

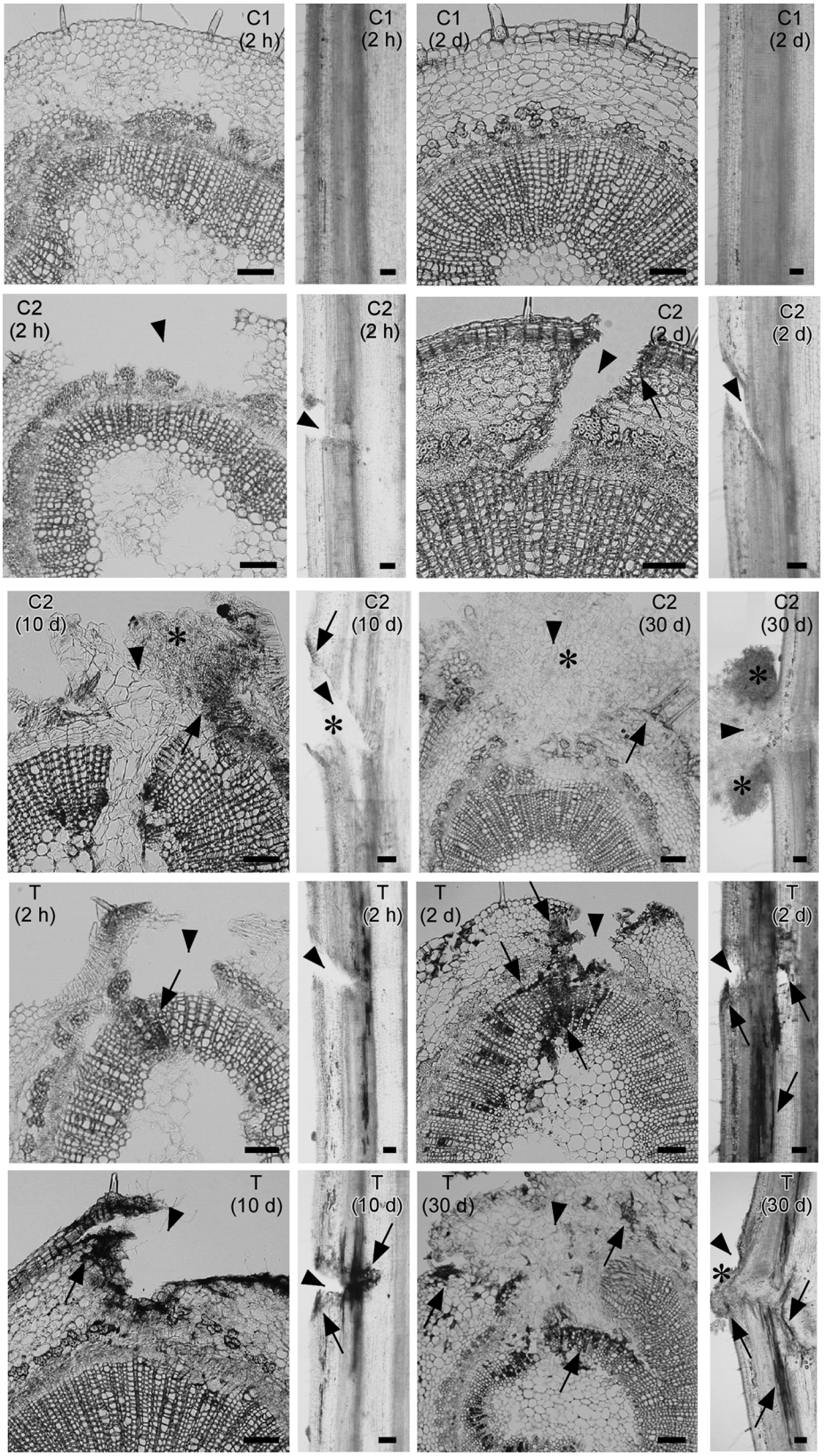

Figure 3. Phenolic compound accumulation in transverse and longitudinal sections of C1-, C2-, and T-treated *B. platyphylla* var. *japonica* No. 8 plantlets after 2 h to 30 d. The arrows indicate the accumulation of phenolic compounds; the arrow-heads indicate the wound and infection sites; the asterisks indicate the callus; Bar=100 μm.

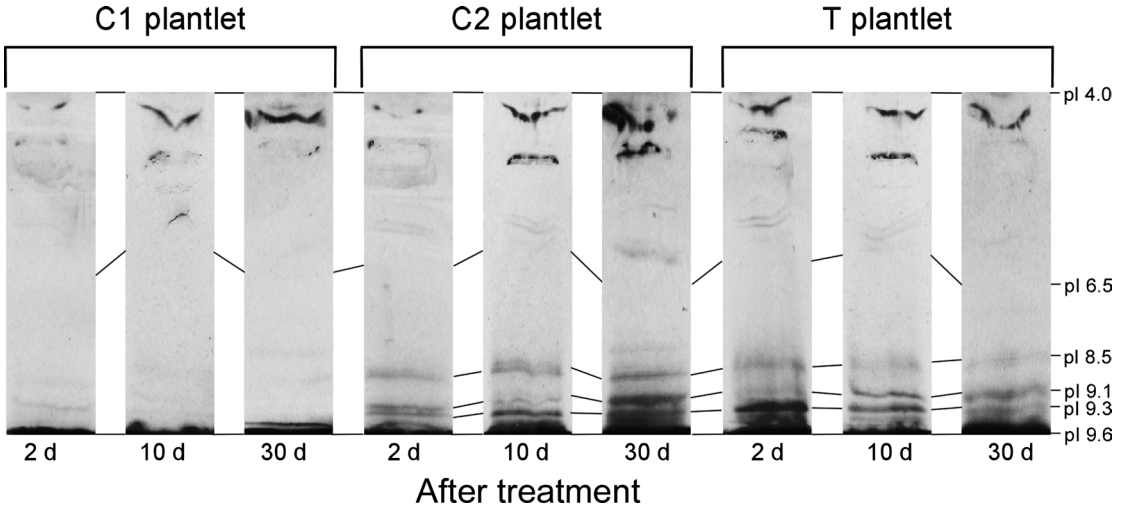

Figure 4. Isoelectric focusing electropherograms of peroxidase isozymes expressed in C1-, C2-, and T-treated *B. platyphylla* var. *japonica* No. 8 plantlets at 2, 10, and 30 d.

isozymes in the C1, C2, and T plantlets at 2, 10, and 30 d after treatment are shown in Figure 4. Clear isozyme bands were observed in the basic region (pI>8.5), and three POD isozymes (pI 8.5, pI 9.1, and pI 9.3) were induced by wounding and fungal infection. The absence of cationic POD isozymes in the C1 plantlet reflects the observation of the *in situ* POD activity. In the C2 plantlets, the activity of PODs with pI 9.1 and pI 9.3 increased with time after wounding, and the activity of POD with pI 9.1 also increased at 10 to 30 dpi in the T plantlets. However, the activity of POD with pI 9.3 was strongly induced within 2 dpi and then decreased gradually up to 30 dpi in the T plantlets.

It has been reported that anionic POD is involved in responses to wounding (Bernards et al. 1999; Espelie et al. 1986), elicitor treatments (Egea et al. 2001; Fernandes et al. 2006; Kukavica et al. 2012), and pathogen attack (Lagrimini and Rothstein 1987; Ye et al. 1990). However, some studies have revealed that cationic POD is also related to resistance responses to abiotic and biotic stress (Quiroga et al. 2000; Ros Barceló et al. 1996; Wally and Punja 2010). Wally and Punja (2010) examined the mechanisms of disease resistance in a transgenic carrot (*Daucus carota* L.) line (P23) that constitutively over-expresses rice cationic peroxidase *OsPrx114*. When the carrot suspension-cultures were treated with cell wall fragments of the fungal pathogen *Sclerotinia sclerotiorum* as an elicitor, the transcript levels of pathogenesis-related (PR) genes were dramatically increased in line P23 compared to the controls. Simultaneously, H_2O_2 accumulation was reduced in line P23 despite the observation of the typical medium alkalization associated with oxidative burst responses. According to these results, particular cationic PODs may contribute to the enhancement of disease resistance through increased PR transcript accumulation, rapid removal of H_2O_2 during the oxidative burst response, and enhanced lignin formation. On the other hand, it has been also reported that some PODs contribute to basal resistance in plant (Johrde and Schweizer 2008). In barley (*Hordeum vulgare*) infected with the powdery-mildew fungus *Blumerina graminis* f. sp. *hordei* (*Bgh*), a new POD mRNA, *HvPrx40*, was specifically expressed in *Bgh*-attacked epidermis. The results of transient overexpression and transient-induced gene silencing of *HvPrx40* showed that HvPrx40 is indeed a factor of basal resistance in barley (Johrde and Schweizer 2008).

In the present study, cationic POD isozymes (pI 8.5, pI 9.1, and pI 9.3) were activated by wounding and fungal infection in *B. platyphylla* var. *japonica* plantlet No. 8. Therefore, these cationic PODs are considered to be involved in the responses to wounding and fungal infection especially as basal resistance in T plantlets, even though the changes in the POD isozyme activity did not exactly correspond to the changes in the *in situ* POD activity.

In addition, the POD with pI 9.3, which was rapidly induced by fungal infection, might be correlated with the basal resistance in the No. 8 plantlet. Furthermore, as shown in Figure 1, the patterns of the time-course changes in the *in situ* POD activity observed using a histochemical method were different between the C2 and T plantlets, suggesting that the responsive mechanisms against fungal infection are different from the responses to wounding. Based on the results obtained, it is considered that cationic POD isozymes are involved in the basal resistance of *B. platyphylla* var. *japonica* plantlet No. 8 against infection with *I. obliquus* strain IO-U1.

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 Emeritus Professor Minoru Terasawa and Associate Professor Yutaka Tamai, Graduate School of Agriculture, Hokkaido University for providing the *Inonotus obliquus* strain IO-U1. This study was supported by JSPS KAKENHI Grant Number 19580162.

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