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Purification and characterization of three neutral extracellular isoperoxidases from rye leaves

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Abstract

Rye (*Secale cereale* L.) seedlings; contain two major flavone glucuronides, luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide (L3GlcUA) (**1**) and luteolin 7-*O*-diglucuronide (L2GlcUA) (**2**) in abundance in the apoplast of primary leaves; express a large number of peroxidase isoenzymes; and release H_2O_2 into the apoplast during primary leaf development. We purified and characterized three neutral extracellular peroxidase isoenzymes (rPOXs N1, N2, and N3) that can oxidize L2GlcUA as a natural substrate. The isoelectric points and molecular weights of rPOXs N1, N2, and N3 were 6.1, 7.2, and 6.3, and 42, 37, and 51 kDa, respectively. The optimum pH of the rPOXs N1, N2, and N3 were 5.5, 5.5, and 8.5, respectively, and their optimum temperatures ranged from 45 to 50 °C for all isoenzymes. rPOXs N1, N2, and N3 recognized flavonoids with 3′, 4′-OH groups as potential substrates, but not flavonoids with a glycosylated 4′-OH group or those without a 3′-OH group. The activities on phenol-type substrates were high in the order of guaiacol > catechol > *o*-cresol for all isoenzymes. rPOXs N1, N2, and N3 exhibited broad reactivity with endogenous hydrogen donors including luteolin glucuronides derived from the apoplast of rye primary leaves.

Keywords: Rye (*Secale cereale* L.); Poaceae; Enzyme purification; Peroxidase; Flavonoid; Luteolin glucuronide

1. Introduction

Plant peroxidase (EC 1.11.1.7) is a heme-containing enzyme that catalyzes oxidation of hydrogen donors in the presence of H_2O_2. Higher plants usually contain a large number of peroxidase isoenzymes classified as acidic, neutral, or basic based on their isoelectric points. Plant peroxidases can oxidize various phenolics, lignin precursors, and various secondary metabolites as a hydrogen donor, and have been known to be multi-functional, participating in a broad range of physiological processes such as auxin metabolism, lignin and suberin formation, cross-linking of cell wall components, and defense against pathogens or cell elongation (reviewed in Passardi et al., 2005; Kawano, 2003).

Flavonoids are major secondary metabolites in plants. They consist of a C6–C3–C6 basic skeleton, and vary in type and content according to the plant species, tissue, and growth stage. To date, more than 6000 flavonoids have been described, and the number is increasing (Harborne and Williams, 2000). Recent reports have shown some of their important roles in plants, for example, in regulation of polar auxin transport (Peer et al., 2004), providing pigmentation for flowers, fruits, and seeds (Koes et al., 2005), protecting against UV light (Li et al., 1993), defending against pathogenic microorganisms (Dixon and Paiva, 1995), in plant fertility and germination of pollen (Guyon et al., 2000), as signal molecules in plant–microbe interactions (Novak et al., 2002), and in defending against herbivorous insects (Simmonds, 2003). Understanding the correlation between peroxidases and flavonoid metabolism will therefore give further insight into the defense mechanisms and development regulation of plants.

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Rye (*Secale cereale* L.) seedlings show tissue-specific flavonoid accumulation in the shoot epidermis and mesophyll, with a close correlation between flavonoid metabolism and leaf development (Strack et al., 1982; Schulz and Weissenböck, 1988b). The rye mesophyll contains two major flavone glucuronides, luteolin 7-*O*-diglucuronyl-4′-*O*-glucuronide (L3GlcUA) (**1**) and luteolin 7-*O*-diglucuronide (L2GlcUA) (**2**) (Fig. 2a). It has been shown that L3GlcUA (**1**) synthesized intracellularly is transported into the apoplast and converted to L2GlcUA (**2**) with a specific β-glucuronidase (Schulz and Weissenböck, 1987; Schulz and Weissenböck, 1988a; Anhalt and Weissenböck, 1992). In this study, we purified and characterized three neutral extracellular peroxidase isoenzymes that can oxidize L2GlcUA (**2**) as a natural substrate from the apoplast of rye primary leaves.

2. Results and discussion

2.1. Peroxidase isoenzyme accumulation and H_2O_2 release in the apoplast of rye primary leaves

Whole extracellular peroxidase isoenzymes present in rye primary leaves were detected. Extracellular soluble fractions derived from 3-9-day-old rye seedlings were loaded onto isoelectric focusing-PAGE (IEF-PAGE), and peroxidase isoenzymes were actively stained using 3,3′-diaminobenzidine (DAB) as a non-specific substrate (Fig. 2a). The peroxidase isoenzymes increased in number and activity during primary leaf development, and their isoelectric points were diverse, ranging from over 9.6 to 4.45, with the neutral isoenzymes (p*I* values from 8 to 5) exhibiting higher activities.

We also detected H_2O_2 release into the apoplast with 2′,7′-dichlorofluorescin (DCFH), which is oxidized by H_2O_2 and converted into the fluorescent substance 2′,7′-dichlorofluorescein (DCF) (Rodriguez et al., 2002). Rye seedlings aged 3, 5, 7, and 9 days were embedded in a medium containing DCFH with fluorescence detected using UV irradiation (Fig. 1b). Strong DCF fluorescence was detected around the seeds and roots of the seedlings, with a slight fluorescence at the tip of the coleoptiles. The spontaneous generation of extracellular H_2O_2 is necessary for plant defense and growth. Release of H_2O_2 into the apoplast by growing plant tissues has been reported in the embryonic axes of soybean (Puntarulo et al., 1988), as well as in germinating radish seeds and roots (Schopfer et al., 2001). Rodriguez et al. (2002) also reported the generation of H_2O_2 by maize seedlings in the expanding region of leaves. The expansion zone of rye exists inside the coleoptile, and the fluorescence detected is thought to have derived from H_2O_2 leaking from there. These observations indicate that rye primary leaves express a large number of peroxidase isoenzymes and vigorously release H_2O_2, which is necessary for oxidation of substrates by the peroxidase, into the apoplast during primary leaf development.

2.2. Changes in luteolin glucuronide content in the apoplast of rye primary leaves

The changes in luteolin glucuronide content in the apoplast of rye primary leaves were also examined (Fig. 2). L3GlcUA (**1**) content reached a maximum on day 4 after seeding (21.8 nmol/g leaf), and gradually decreased with leaf development. On the other hand, the L2GlcUA (**2**) content resulting from hydrolysis of L3GlcUA (**1**) with a specific β-glucuronidase (Schulz and Weissenböck, 1987)

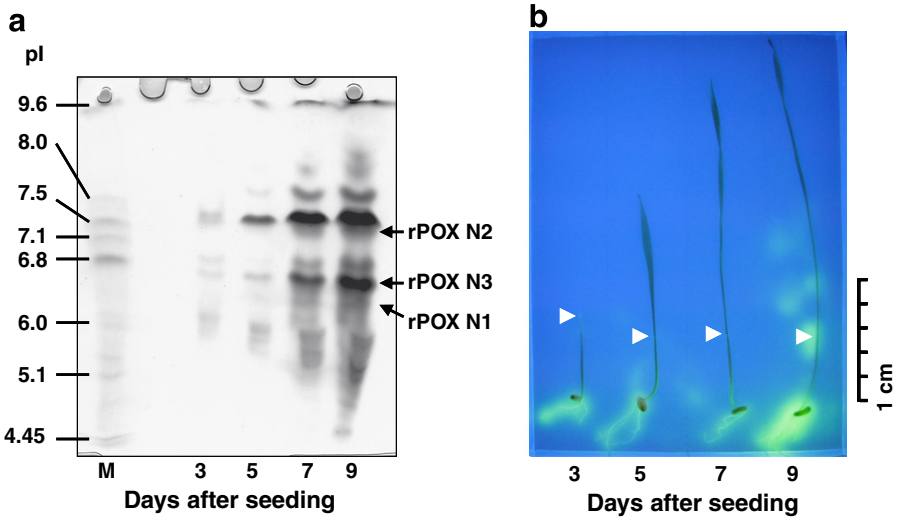

Fig. 1. Peroxidase isoenzyme accumulation and H_2O_2 release in the apoplast of rye primary leaves. (a) Peroxidase isoenzyme accumulation during primary leaf development. Apoplastic solution derived from 3 to 9-day-old rye primary leaves was loaded onto IEF-PAGE with 800 ng protein/lane. Peroxidase isoenzymes were then visualized with DAB and H_2O_2. M: IEF standard marker. (b) Detection of H_2O_2 release from rye seedlings. The yellow–green fluorescence indicates the conversion of DCFH into DCF by extracellular H_2O_2 released from the rye seedlings. The *white arrows* indicate the tip of the coleoptile.

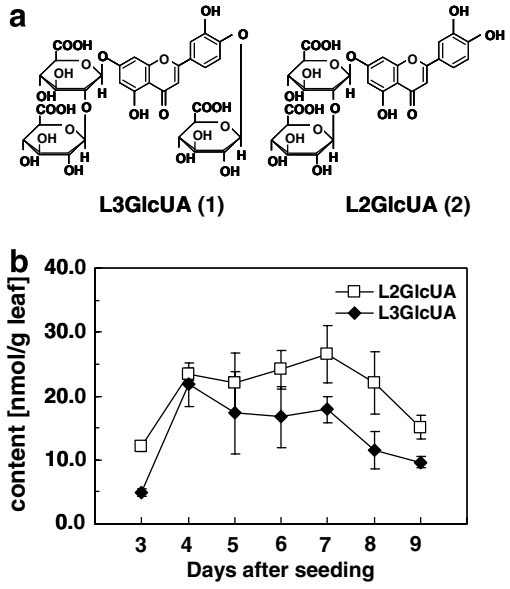

Fig. 2. Changes in luteolin glucuronide **1** and **2** content in the apoplast of rye primary leaves. (a) Structures of luteolin glucuronides **1** and **2**. (b) Changes in L2GlcUA (**2**) and L3GlcUA (**1**) content in the apoplastic space of rye primary leaves. Values represent the means ± SD ($n = 3$).

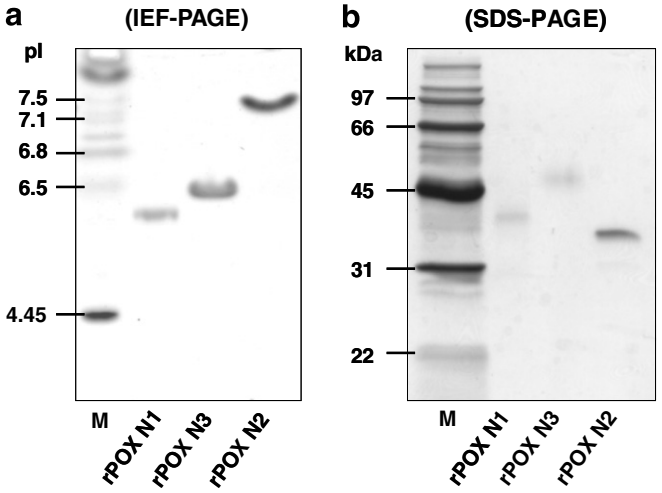

Fig. 3. IEF-PAGE and SDS–PAGE of purified rye peroxidase isoenzymes. (a) IEF-PAGE. M, IEF standard marker. (b) SDS–PAGE. M, Molecular-weight standard marker.

gradually increased until reaching a maximum on day 7 (26.6 nmol/g leaf) and then decreased. These results suggest that luteolin glucuronides colocalize with peroxidase isoenzymes and H_2O_2 during primary leaf development.

2.3. Separation and purification of rye peroxidase isoenzymes

Extracellular peroxidase isoenzymes were separated and purified using L2GlcUA (**2**) as a hydrogen donor for the enzyme assay. Seven active peaks were separated from the extracellular soluble fraction using anion exchange, chromatofocusing, and gel filtration columns, respectively. Furthermore, three isoenzymes of seven peroxidases were purified (with the separation and purification protocol described in the Section 3). The isoelectric points of the purified isoenzymes were 6.1, 7.2, and 6.3 (for rPOXs (rye peroxidase neutral isoenzymes) N1, N2, and N3 in Fig. 3a, respectively), and these bands corresponded to those of the major isoenzyme activities in Fig. 1a. The total and specific activities of rPOXs N1, N2, and N3 were 552 pkat and 100 pkat/μg protein, 553 pkat and 78.6 pkat/μg protein, and 2814 pkat and 733 pkat/μg protein, respectively (Table 1). While the specific activity of rPOX N3 towards L2GlcUA (**2**) is about 5-fold higher than the extracellular soluble fraction, rPOX N1 and N2 exhibit lower activity, suggesting that rPOXs N1 and N2 do not have a particular affinity for L2GlcUA (**2**). The molecular weights of rPOXs N1, N2, and N3 were 42, 37, and 51 kDa, respectively, on SDS–PAGE (Fig. 3b). In general, the molecular weight of plant peroxidases is diverse, approximately ranging from 33 to 55 kDa (Kvaratskhelia et al., 1997; Morimoto et al., 1998; de Marco et al., 1999; Deepa and Arumughan, 2002).

2.4. Optimum pH and temperature of rPOXs N1, N2, and N3

The enzymatic properties of the purified rye peroxidase isoenzymes were also examined. The pH optima of rPOXs N1, N2, and N3 were 5.5, 5.5, and 8.5, respectively (Fig. 4), whereas the temperature optima ranged from 45 to 50 °C for all isoenzymes. The optimum pH of other plant peroxidases tends to range from 4.5 to 6.5 (Kvaratskhelia et al., 1997; Morimoto et al., 1998; Christensen et al., 1998; de Marco et al., 1999; Deepa and Arumughan, 2002). Generally, plant peroxidases localize in apoplasts or vacuoles, where the pH is weakly acidic. The pH optima of rPOXs N1 and N2 were therefore thought to be suitable for the environment in which they are localized; however, it is a rare finding that the optimum pH of rPOX N3 is alkaline.

2.5. Activities of rPOXs N1, N2, and N3 on different substrates

The enzyme activities of rPOXs N1, N2, and N3 on several hydrogen donors were also examined (Fig. 5). When enzyme activity was measured using five flavonoids as hydrogen donors, all isoenzymes recognized L2GlcUA (**2**), luteolin (**3**), and quercetin (**4**), which have 3′,4′-OH groups (Fig. 5a). In contrast, L3GlcUA (**1**) and apigenin (**5**), which have a 4′-OH group glycosylated with glucuronic acid and no 3′-OH group, were not recognized as hydrogen donors. These results suggest that the ability of flavonoids to act as hydrogen donors for peroxidase depends entirely on their B-ring structure, and moreover, that the conversion of L3GlcUA (**1**) into L2GlcUA (**2**) by β-glucuronidase is necessary for the provision of the peroxidase substrate. Bors et al. (1990) suggested three structural features that are important determinants of the radical-scavenging potential of flavonoids: (a) the *o*-dihy-

Table 1
Purification summary of rye peroxidase isoenzymes

Fraction	Total protein [μg]	Total activity [pkat]	Specific activity [pkat/μg protein]
Extracellular soluble fraction	5493	776484	141
$(NH_4)_2SO_4$ precipitation (0–80%)	2093	362020	173
HiTrap Q-bound proteins (peak 3)	49	6691	137
HiTrap Q-non-bound proteins	1241	337752	272
Mono P-bound proteins (peak 4)	60	3882	65.2
Mono P-non-bound proteins	155	91547	591
Superdex 200 rPOX N1	6	552	100
Superdex 200 rPOX N2	7	553	78.6
Superdex 200 rPOX N3 (peak 6)	4	2814	733

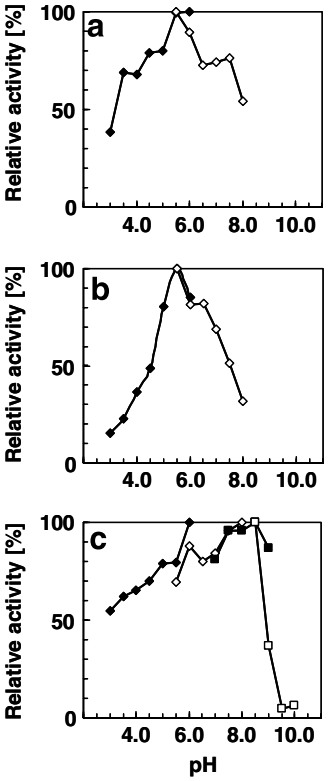

Fig. 4. Effects of pH on rPOXs N1, N2 and N3 activities. Enzyme activities were measured with buffers of various pHs. (♦); citrate buffer (pH 3.0–6.0); (◇) Tris–maleate buffer (pH 5.5–8.0); (■) Tris–HCl buffer (pH 7.0–9.0); (□) glycine–NaOH buffer (pH 8.5–10.0). (a) rPOX N1. (b) rPOX N2. (c) rPOX N3.

droxy (catechol (**7**)) structure in the B ring; (b) the 2, 3-double bond in conjunction with 4-oxo function; and (c) the presence of 3- and 5–OH groups. Similarly, the present results indicate that the hydrogen-donating activity of flavonoids to peroxidases is dependent on these structures.

We also examined three structurally different phenol-type substrates (Fig. 5b). The specific activities of these substrates were high in the order of guaiacol (**8**) > catechol (**7**) > *o*-cresol (**6**) for all isoenzymes. Guaiacol (**8**) (*o*-methoxyphenol) is often used as a substrate in peroxidase enzyme assays, and because the same substructure is also found in one of the monolignols (Blee et al., 2003), polyphenols with such an *o*-methoxyphenol structure are thought to be a suitable substrate for peroxidases. rPOX N3 exhibited markedly higher activities against these substrates, while in contrast, rPOXs N1 and N2 showed lower activities against phenol-type substrates, even though the concentrations of these substrates were 5-fold higher than those of flavonoids. These results suggest that rPOX N3 has a broader substrate specificity than the other isoenzymes and could also be involved in lignification. In addition, Fig. 5 also shows that the respective specific activities of rPOXs N1, N2, and N3 against all the tested substrates always followed the same proportions, rPOX N2 being the least active and rPOX N3 the most.

2.6. Detection of endogenous hydrogen donors in the apoplast of rye primary leaves

rPOXs N1, N2, and N3 showed relatively lower activity against L2GlcUA (**2**) compared with several other substrates (Fig. 5). To ascertain whether L2GlcUA (**2**) is a major hydrogen donor for rPOXs N1, N2, and N3 *in vivo*, we attempted to detect the endogenous hydrogen donor in rye apoplastic solution using reversed-phase HPLC (Fig. 6). When authentic samples of luteolin (**3**) and its glucuronides was applied to HPLC, the retention times of L3GlcUA (**1**), L2GlcUA (**2**), and luteolin (**3**) were 11.2, 12.7, and 18.2 min, respectively (Fig. 6a). Deproteinized rye apoplastic solution was then analyzed under the same HPLC conditions (Fig. 6b). Major peaks of the components in the apoplastic solution were detected at a retention time of 10–16 min, and MS analysis confirmed that the peaks at retention times of 11.1 and 12.6 min corresponded to L3GlcUA (**1**) and L2GlcUA (**2**), respectively. However, no peak of luteolin (**3**) was detected. rPOXs N1, N2, and N3 displayed 6-, 4-, and 4-fold higher specific activity against luteolin than L2GlcUA (**2**), respectively (Fig. 5a). Yamasaki et al. (1997) reported that the aglycones of flavonoids, quercetin and kaempferol, showed higher hydrogen-donating activity to peroxidase than its glycoside. However, they also reported that it is unlikely that aglycones act as substrates for soluble peroxidase *in vivo* because they are localized in the nonaqueous phase. It can therefore be presumed that luteolin does also not act as a substrate

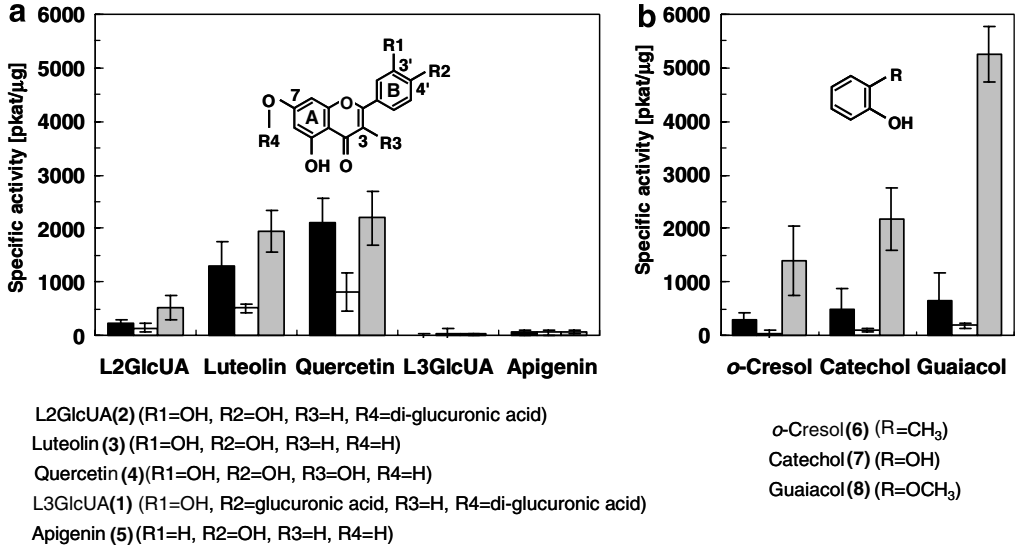

Fig. 5. Activities of rPOXs N1, N2, and N3 with several hydrogen donors. (a) Specific activities of rPOXs N1, N2, and N3 with flavonoid-type hydrogen donors. The donor concentration was 50 μM in all cases. (b) Specific activities of rPOXs N1, N2, and N3 with phenol-type hydrogen donors. The donor concentration was 250 μM in all cases. *Black columns*, rPOX N1; *White columns*, rPOX N2; and *Gray columns*, rPOX N3. Values represent the means ± SD ($n = 3$).

for peroxidase isoenzymes *in vivo*. When H_2O_2 and rPOX N3 were added to the apoplastic solution, the major peaks at a retention time of 10–16 min, which included L2GlcUA (**2**) and L3GlcUA (**1**), were greatly reduced (Fig. 6c). Moreover, small peaks that seemed to correspond to the reaction products also appeared (e.g., at retention times of 9.52, 16.05, 17.21, 18.13, and 18.59 min). The same results were shown in a reaction using rPOXs N1 and N2 (data not shown). These results suggest that L2GlcUA (**2**) is almost certainly one of the endogenous hydrogen donors for rPOXs N1, N2, and N3. L3GlcUA (**1**) was not recognized as a hydrogen donor in Fig. 5a, but was reduced by the reaction in Fig. 6c; however, the reason for this is not clear.

The existence of numerous endogenous hydrogen donors for rPOXs N1, N2, and N3 besides luteolin glucuronides was also suggested. Rye primary leaves contain various phenolic secondary metabolites in their apoplast, all of which are potential peroxidase substrates. For example, it is well known that hydroxamic acids such as 2,4-dihydroxy-1, 4-benzoxazin-3-one (DIBOA) and its precursor are also abundant in rye leaves (Rice et al., 2005; Sue et al., 2000). DIBOA elicits a wide variety of biological activities including antifungal and mutagenic (Hashimoto and Shudo, 1996; Rice et al., 2005), and is probably oxidized by peroxidases.

2.7. Conclusions

Rye primary leaves accumulate luteolin glucuronides during primary leaf development. In this study, three neutral extracellular peroxidase isoenzymes that can oxidize L2GlcUA (**2**) in the apoplast of rye primary leaves were purified and characterized. In recent years, several studies have shown that various flavonoids can negatively regulate polar auxin transport *in vivo* (Brown et al., 2001; Buer and Muday, 2004; Murphy et al., 2000; Peer et al., 2004). Thus, the potential physiological significance of L2GlcUA (**2**) oxidation by peroxidases is thought to be regulation of cellular auxin levels by reduction of the flavonoid concentration. To examine this, study of the specific inhibition of luteolin-metabolizing enzymes, including rPOXs N1, N2, and N3, using the RNA interference method and analysis of reaction products of L2GlcUA oxidized by rPOXs N1, N2, and N3 *in vivo* are necessary.

3. Experimental

3.1. Plant material

Rye (*S. cereale* L.) seeds were washed and seeded on a wet paper towel in a tray (120 × 120 × 60 mm) at a rate of 7 g per tray, and grown with 12 h/12 h light/dark at 25 °C. The tray was covered with aluminum foil, which was removed 2 days after seeding. The rye seedlings were grown under the above conditions until harvested.

3.2. Flavonoids and phenols

Luteolin (**3**), quercetin (**4**), apigenin (**5**), *o*-cresol (**6**), catechol (**7**), and guaiacol (**8**) were purchased from Wako Pure Chemicals (Osaka, Japan). L2GlcUA (**2**) and L3GlcUA (**1**) were purified from rye primary leaves based on the method of Strack et al. (1982). Freeze-dried rye primary leaves (20 g dry wt) were milled and extracted twice with a 20-fold volume of MeOH–H_2O (4:1, v/v). The extract was then filtered, and the filtrate was evaporated to dryness and dissolved in a minimal amount of MeOH–H_2O (4:1, v/v),

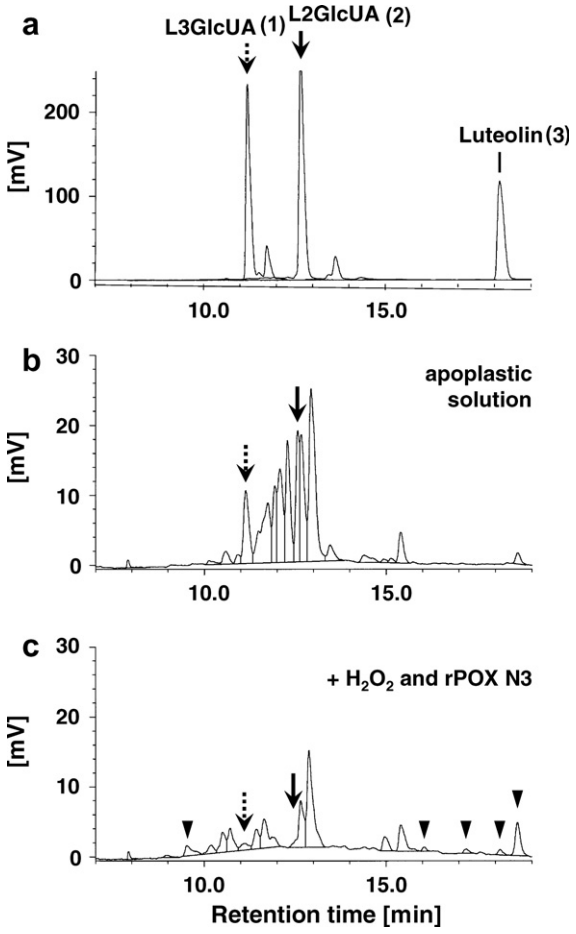

Fig. 6. Detection of endogenous hydrogen donors in apoplastic solution of rye primary leaves. (a) HPLC pattern of luteolin (**3**) and luteolin glucuronides. Commercial luteolin (**3**) and purified L2GlcUA (**2**) and L3GlcUA (**1**) were separated by reversed-phase HPLC. (b) HPLC pattern of apoplastic solution extracted from rye primary leaves. The apoplastic solution from rye primary leaves was readily deproteinized and separated under the same conditions as in a. (c) HPLC pattern of apoplastic solution to which H_2O_2 and rPOX N3 had been added. The concentration of the apoplastic solution was as in B. Note: rPOXs N1 and N2 showed the same peak pattern. *Solid arrows* and *dashed arrows* indicate the peaks corresponding to L2GlcUA and L3GlcUA, respectively. *Arrow heads* indicate the reaction products.

which was then applied to a polyamide column (Polyamide C-200, Wako, Osaka, Japan; 280 × 30 mm) equilibrated with MeOH–H_2O (4:1, v/v). After washing the column with the same solvent, flavonoids were eluted with 0.1% (v/v) NH_4OH in MeOH and fractionated. L2GlcUA (**2**) and L3GlcUA (**1**) fractions were pooled, evaporated to dryness, and dissolved in a minimal amount of Milli Q water. The L2GlcUA (**2**) and L3GlcUA (**1**) mixture was applied to a gel filtration column (Sephadex LH-20, GE Healthcare Bio-Sciences Corp., NJ, USA; 350 × 15 mm), equilibrated with Milli Q water and eluted with the same solvent and fractionated. The purified L2GlcUA (**2**) and L3GlcUA (**1**) were then individually freeze-dried until needed. L2GlcUA (**2**) and L3GlcUA (**1**) were analyzed by TLC (Silicagel 70 Plate-Wako, Wako, Osaka, Japan) with EtOAc–MeOH–HCO_2H–H_2O (5:2:1:1, by vol.). Spots were visualized by UV irradiation (302 nm) after spraying with 1% (w/v) $AlCl_3$ in aq. EtOH–H_2O (95:5). The R_f values of L2GlcUA (**2**) and L3GlcUA (**1**) were 0.65 and 0.35, respectively.

3.3. Extraction of an extracellular soluble fraction of rye

An extracellular soluble fraction (apoplastic solution) was extracted from the apoplast of rye primary leaves using the method of Hon et al. (1994) with modifications. Rye primary leaves (5 g fr. wt) were cut into 5-mm sections and rinsed three times with 10 mM K-phosphate buffer (pH 6.0). The leaves were then placed in a strainer made from a plastic funnel and cotton filter, and rinsed with 100 mM acetate buffer (15 ml, pH 5.0). After removing excess buffer, the strainer was placed in a centrifuge tube and vacuum infiltrated for 5 min. The tube was then centrifuged (1150 × g for 10 min) to recover the apoplastic solution. After adding 100 mM acetate buffer (3 ml, pH 5.0) to the leaf residue, the tube was vacuum infiltrated and centrifuged again.

3.4. Detection of H_2O_2 release in rye seedlings

Detection of H_2O_2 release in rye seedlings was performed according to the method of Rodriguez et al. (2002). Whole seedlings aged 3, 5, 7 and 9 days were embedded in medium containing 1% (w/v) agar and 50 μM DCFH (DCFH-diacetate; Sigma, MO, USA). A fluorescence image was then taken 1 h later under UV irradiation (302 nm).

3.5. Determination of L2GlcUA (2)and L3GlcUA (1)

Determination of L2GlcUA (**2**) and L3GlcUA (**1**) was performed with an HPLC system (CCP& 8010, Tosoh, Tokyo, Japan). Fifty microliters of sample was applied to a reversed-phase column (CAPCELLPAK C18 ACR S-5 μm; Shiseido, Tokyo, Japan) equilibrated with 0.85% phosphoric acid in H_2O and eluted with a linear gradient from 0.85% phosphoric acid in H_2O to CH_3CN for 30 min at a flow rate of 0.6 ml/min. The effluent was then monitored according to the absorbance at 340 nm. The amounts of L2GlcUA (**2**) and L3GlcUA (**1**) were calculated from the standard curves obtained with an authentic sample.

3.6. Enzyme assay

The standard reaction mixture (total volume 150 μl) for the enzyme assay consisted of 100 μM L2GlcUA (**2**), 15 μl of enzyme solution, and 100 mM K-phosphate buffer (pH 6.0). The reaction was initiated by adding 150 μl of 1 mM H_2O_2 in 100 mM K-phosphate buffer (pH 6.0) to the reaction mixture at room temperature (25 °C). The decrease in absorbance at 340 nm was then measured after 1 min. The

quantification of peroxidase activity was performed using HPLC under the conditions described above.

3.7. Separation and purification of rye peroxidase isoenzymes

All separation and purification procedures were carried out at 4 °C, and all chromatography steps were performed using a fast protein liquid chromatography (FPLC) system (ÄKTApurifier, GE Healthcare Bio-Sciences Corp.). Solid $(NH_4)_2SO_4$ was added to the extracellular soluble fraction extracted from 8-day-old rye primary leaves (150 g fr. wt) to reach 80% saturation. The precipitate was collected by centrifugation at 15,600*g* for 20 min and then dialyzed against 25 mM acetate buffer (pH 5.0). The enzyme solution was applied to an anion exchange column (HiTrap Q HP 1 ml, GE Healthcare Bio-Sciences Corp.) equilibrated with 25 mM acetate buffer (pH 5.0), and the proteins passed through the column were recovered before washing the column with the same buffer. The proteins bound to the column were eluted with a linear gradient of NaCl from 0 to 250 mM for 30 min at a flow rate of 1.0 ml/min with the fractions collected at 1 ml per tube. Three active peaks (peaks 1–3: fraction Nos. 8–10, 14–15, and 21–25, respectively) were obtained. The fractions of peak 3 were concentrated by ultrafiltration (Centriprep YM-50, Millipore, MA, USA) and applied to a gel filtration column (Superdex 200 HR 10/30, GE Healthcare Bio-Sciences Corp.) equilibrated in 25 mM acetate buffer (pH 5.0). Proteins were eluted with the same buffer at a flow rate of 0.2 ml/min. Fractions were collected at 0.4 ml per tube and the active fractions (fraction Nos. 16–18) were recovered as rPOX N1.

The proteins passed through the HiTrap Q HP column were concentrated, and the buffer was changed to 75 mM Tris-CH_3COOH buffer (pH 9.3). The enzyme solution was then applied to a chromatofocusing column (Mono P HR 5/5, GE Healthcare Bio-Sciences Corp.) equilibrated in 75 mM Tris–CH_3COOH buffer (pH 9.3). The proteins passed through the column were recovered, and the column was washed with the same buffer. The proteins bound to the column were then eluted with 10% Polybuffer 96-CH_3COOH (pH 6.0) at a flow rate of 0.5 ml/min. The fractions were collected at 0.5 ml per tube and two active peaks (peaks 4 and 5: fraction Nos. 8–10 and 12, respectively) were obtained. The fractions of peak 4 were concentrated and then applied to a Superdex 200 HR 10/30 column equilibrated with 25 mM Tris–HCl buffer (pH 7.5). Proteins were eluted with the same buffer and collected as described above. The active fractions (fraction Nos. 19–20) were recovered as rPOX N2.

The proteins passed through the Mono P HR 5/5 column were concentrated and applied to a Superdex 200 HR 10/30 column equilibrated with 25 mM Tris–HCl buffer (pH 7.5). Proteins were eluted with the same buffer and collected as described above. Two active peaks (peaks 6 and 7: fraction Nos. 12–14 and 18–25, respectively) were obtained and the fractions of peak 6 were recovered as rPOX N3.

3.8. Electrophoresis

IEF-PAGE was performed using 5% polyacrylamide gel containing 2% ampholytes (Bio-Lite 3/10 and 8/10, 1:1 or 3/10 and 3/5, 1:2 by vol.; Bio-Rad, CA, USA) and 10% sucrose. The samples and IEF standard marker (Bio-Rad) were focused by 200 V for 3.5 h at 4 °C. After focusing, the peroxidase isoenzymes were visualized by incubating the gel for 10 min in 100 mM K-phosphate buffer (pH 6.0) containing 1 mM H_2O_2 and 1 mM DAB or 1 mM guaiacol. SDS–PAGE was then performed using a 12% separating gel, according to the method of Laemmli (1970). The proteins were visualized by silver staining (Silver Stain II Kit; Wako).

3.9. Optimum pH and temperature of rPOXs N1, N2, and N3

The effect of pH on the enzyme activity of rPOXs N1, N2, and N3 were evaluated from pH 3.0 to 10.5 using the following buffers: 200 mM citrate buffer (pH 3.0–6.0), 200 mM Tris–maleate buffer (pH 5.5–8.0), 200 mM Tris–HCl buffer (pH 7.0–9.0), and 200 mM glycine–NaOH buffer (pH 8.5–10.0). The effect of temperature on the enzyme activity of rPOXs N1, N2, and N3 were evaluated from 20 to 80 °C.

3.10. Activities of rPOXs N1, N2, and N3 on different substrates

The reaction mixture (total volume 200 μl) used to determine the activities of each rPOX using 50 μM flavonoids or 250 μM phenols consisted of 500 μM H_2O_2, 10 μl peroxidase isoenzyme (1 μg/ml), and 100 mM K-phosphate buffer (pH 6.0). Each reaction mixture was incubated at 25 °C for 5 min then boiled for 3 min before being applied to HPLC as described above. The effluents were monitored by absorbance at the following wavelengths: L2GlcUA (**2**), L3GlcUA (**1**), luteolin (**3**), and apigenin (**5**), −340 nm; quercetin (**4**) −370 nm; *o*-cresol (**6**), catechol (**7**), and guaiacol (**8**) −277 nm. The amount of hydrogen donors decreased was calculated from standard curves.

3.11. Detection of the endogenous hydrogen donors for rye peroxidase isoenzymes

The apoplastic solution from 7-day-old rye primary leaves was readily deproteinized by ultrafiltration (Microcon YM-3, Millipore). One hundred microliters of deproteinized apoplastic solution was then added to 100 μl of 100 mM K-phosphate buffer (pH 6.0) and 100 μl of the same buffer containing 1 mM H_2O_2 and 10 μl peroxidase isoenzyme. Each mixture was incubated at 25 °C for 15 min then boiled for 3 min before applying to HPLC as

described above. The peak patterns were then recorded and peaks corresponding to L3GlcUA (**1**) and L2GlcUA (**2**) at retention times of 11.1 and 12.6 min, respectively, in the apoplastic solution were separated and concentrated. Each fraction was then applied to MS analysis and mass molecular ion peaks were compared with authentic samples (L3GlcUA (**1**): $m/z = 815.0$ $[M + H]^+$, L2GlcUA (**2**): $m/z = 639.0$ $[M + H]^+$). Mass spectroscopy was performed with a quadruple mass spectrometer (API 300 LC/MS/MS, TAKARA BIO INC., Seta, Japan).

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