Differential Distribution of Proteins Expressed in Companion Cells in the Sieve Element-Companion Cell Complex of Rice Plants

Akari Fukuda 1,4,*, Syu Fujimaki 1,5, Tomoko Mori 1,6, Nobuo Suzuki 1,5, Keiki Ishiyama 2,7, Toshihiko Hayakawa 2, Tomoyuki Yamaya 2, Toru Fujiwara 1,3,8, Tadakatsu Yoneyama 1 and Hiroaki Hayashi 1,9

1 Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan
2 Department of Applied Plant Science, Graduate School of Agricultural Science, Tohoku University, Aoba-ku, Sendai, 981-8555 Japan
3 PRESTO, JST, Honcho, Kawaguchi, Saitama, 332-0012 Japan
4 Present address: Takasaki Radiation Chemistry Research Establishment, Japan Atomic Energy Research Institute, Gunma, 370-1296 Japan
5 Present address: Fuji Photo Film Co. Senzui, Asaka, Saitama, 351-8585 Japan
6 Present address: Department of Paddy Farming, National Agricultural Research Center for Tohoku Region, Yotsuya, Daisen, Akita, 014-0102 Japan
7 Present address: Department of Applied Biological Chemistry, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan
8 Present address: Department of Paddy Farming, National Agricultural Research Center for Tohoku Region, Yotsuya, Daisen, Akita, 014-0102 Japan
9 Corresponding author: E-mail, akfukuda@affrc.go.jp; Fax, +81-187-66-2639.

Sieve tubes are comprised of sieve elements, enucleated cells that are incapable of RNA and protein synthesis. The proteins in sieve elements are supplied from the neighboring companion cells through plasmodesmata. In rice plants, it was unclear whether or not all proteins produced in companion cells had the same distribution pattern in the sieve element-companion cell complex. In this study, the distribution pattern of four proteins, β-glucuronidase (GUS), green fluorescent protein (GFP), thioredoxin h (TRXh) and glutathione S-transferase (GST) were analyzed. The foreign proteins GUS and GFP were expressed in transgenic rice plants under the control of the TRXh gene promoter (PTRXh), a companion cell-specific promoter. Analysis of leaf cross-sections of PTRXh-GUS and PTRXh-GFP plants indicated high accumulation of GUS and GFP, respectively, in companion cells rather than in sieve elements. GUS and GFP were also detected in phloem sap collected from leaf sheaths of the transgenic rice plants, suggesting these proteins could enter sieve elements. Relative amounts of GFP and endogenous phloem proteins, TRXh and GST, in phloem sap and total leaf extracts were compared. Compared to TRXh and GST, GFP content was higher in total leaf extracts, but lower in phloem sap, suggesting that GFP accumulated mainly in companion cells rather than in sieve elements. On the other hand, TRXh and GST appeared to accumulate in sieve elements rather than in companion cells. These results indicate the evidence for differential distribution of proteins between sieve elements and companion cells in rice plants.

Keywords: Companion cell — Oryza sativa — Phloem — Rice — Sieve element.

Introduction

In higher plants, phloem is the major route of long distance transport of metabolites and signals. Sieve elements, the individual cells that make up sieve tubes, are highly specialized for assimilate translocation, and lose most of their intracellular organelles, nuclei, vacuoles, Golgi bodies and most ribosomes during the course of differentiation (Cronshaw 1981). More than a hundred proteins were detected in phloem exudates of Cucurbita maxima, Triticum aestivum, Ricinus communis and Oryza sativa (Eschrich and Heyser 1975, Fisher et al. 1992, Nakamura et al. 1993, Sakuth et al. 1993). These phloem proteins were considered to maintain the physiological functions of sieve tubes, such as sieve plate occlusion, signal transduction, redox regulation, and phloem loading, among others (Hayashi et al. 2000).

Since the enucleated sieve elements are unable to synthesize proteins, the proteins in sieve tubes are thought to be supplied from the neighboring companion cells through plasmodesmata. Indeed, a high frequency of plasmodesmata between companion cells and sieve elements has been reported (Chonan et al. 1981, Lucas et al. 1993). Moreover, mRNAs encoding phloem proteins, such as C. maxima PP1, PP2 and CmPP16, and O. sativa thioredoxin h (TRXh), have been detected in companion cells by in situ hybridization analysis (Bostwick et al. 1992, Clark et al. 1997, Dannenhoffer et al. 1997, Xoconostle-Cázares et al. 1999, Ishiwatari et al. 2000), suggesting the possible synthesis of phloem proteins in companion cells. In addition, some phloem proteins, such as C. maxima PP2 and CmPP16, R. communis glutaredoxin and cystatin, and O. sativa TRXh, were reported to increase the size exclusion limit of plasmodesmata and modify their own cell-to-cell movement after being microinjected into mesophyll cells (Bafachandran et al. 1997, Ishiwatari et al. 1998, Xoconostle-Cázares et al. 1999). Furthermore, analysis of O. sativa TRXh
modesmata (Kragler et al. 1998). The plasmodesmata carrier wild-type sucked out by the aphids (Shi et al. 1994). GFP was detected in enter the sieve tubes from the companion cells and then be promoted. This result suggested that snowdrop lectin could selective mechanism in transgenic traffic into sieve elements from companion cells by a non-lectin and green fluorescent protein (GFP) were reported to sieve elements from companion cells by a selective process.

On the other hand, two non-phloem proteins, snowdrop lectin and green fluorescent protein (GFP) were reported to traffic into sieve elements from companion cells by a non-selective mechanism in transgenic N. tabacum plants. Snowdrop lectin was detected in the honeydew of aphids feeding on transgenic N. tabacum expressing the snowdrop lectin gene under the companion cell-specific O. sativa sucrose synthase1 promoter. This result suggested that snowdrop lectin could enter the sieve tubes from the companion cells and then be sucked out by the aphids (Shi et al. 1994). GFP was detected in wild-type N. tabacum shoots grafted onto transgenic plants expressing the GFP gene from the companion cell-specific AtSUC2 promoter, suggesting that GFP could enter the sieve tubes and be transported into the grafted shoots (Imlau et al. 1999).

It has been revealed that the proteins expressed in companion cells are able to enter the sieve elements. However, it is unclear whether or not all proteins have the same distribution pattern in the sieve element-companion cell complex. In the present study, we generated transgenic rice plants expressing foreign proteins, either β-glucuronidase (GUS) or GFP from the companion cell-specific TRXh promoter (PTRXh) (Ishiwatari et al. 1995, Ishiwatari et al. 1998), and asked if the abundance of these proteins, GUS and GFP, and two endogenous phloem proteins, TRXh and GST, in phloem sap was different from that in companion cells.

Results

Histochemical assay for GUS activity in the leaves of PTRXh-GUS transgenic rice plants

The localization of GUS protein was examined histochemically in transgenic rice plants expressing the GUS gene from the TRXh promoter (PTRXh-GUS) (Fig. 1A). Two independent lines were analyzed and there were no observable differences between them. The results for one of the lines are described below. In leaf cross-sections, GUS activity was detected in both large and small vascular bundle tissues, but not in mesophyll or epidermal cells (Fig. 2A). Strong GUS activity was detected only in phloem tissues (Fig. 2B). Weak GUS activity was also detected in xylem parenchyma cells and some bundle sheath cells (Fig. 2B). TRXh promoter induced weak expression in these cells (Ishiwatari et al. 2000). At higher magnification, companion cells and sieve elements were easily distinguishable in the large vascular bundle (Fig. 2C; CC and SE, respectively). The companion cells in particular showed strong GUS activity (Fig. 2C). Although the sieve elements also showed a significant level of GUS activity, the intensity of blue staining was much lower than that in companion cells (Fig. 2C).

Detection of GFP fluorescence in the leaves of PTRXh-GFP transgenic rice plants

GFP fluorescence was observed by fluorescence microscopy in transgenic rice expressing the GFP gene from the TRXh promoter (PTRXh-GFP) (Fig. 1B). Four independent PTRXh-GFP T0 plants were examined and found to have the same pattern of fluorescence. The results for one of the plants are described below. In leaf cross-sections, we detected red autofluorescence from chlorophyll in mesophyll cells and yellow autofluorescence in sclerenchyma cells in both PTRXh-GFP (Fig. 2D, E) and wild-type plants (Fig. 2F). In PTRXh-GFP plants, green fluorescence was detected in large and small vascular bundle tissues (Fig. 2D). Strong green fluorescence was detected in the phloem tissue (Fig. 2D), and at least five cells were observed to have especially strong green fluorescence (Fig. 2E). These cells were identified as companion cells by their size and position in the phloem region (Chonan et al. 1981). Weak green fluorescence was also detected in xylem parenchyma cells of PTRXh-GFP plants. The TRXh promoter induced weak GFP expression in xylem parenchyma cells (Ishiwatari et al. 2000). Wild type plants showed no green fluorescence in the leaves (Fig. 2F).
Detection of GUS in the phloem sap of PTRXh-GUS transgenic rice plants

The presence of GUS protein in the phloem sap of PTRXh-GUS plants was detected by immunoblotting. Phloem sap was collected from severed stylets of brown planthoppers by YAG laser (Kawabe et al. 1980). Protein extracts were prepared from whole leaf and phloem sap of wild-type plants and T1 individuals from two independent PTRXh-GUS transgenic lines that showed GUS activity in the phloem region by histochemical assay (Fig. 2A, B, C). Silver staining of the gel showed that almost the same amount of protein was loaded in each lane (Fig. 3B). Immunoblot analysis showed that the phloem sap and leaf extract of PTRXh-GUS plants contained bands of about 90 kDa (black arrowhead) and about 68 kDa (black diamond), respectively (lane 1 and lane 3 in Fig. 3A). Because phloem sap and leaf extract of wild-type plants did not have these bands, they were presumed to be GUS protein. Since the predicted molecular mass of GUS is 68 kDa, the band of 90 kDa in phloem sap might be modified GUS. Immunoblot analysis also showed smaller bands of 33 kDa and 13 kDa in phloem sap of transgenic plants (white arrowheads). Because wild type plants did not have these smaller bands, they might originate in GUS. Modified band and smaller bands were detected in the phloem sap of T1 plants of two independent pTRXh-GUS lines used in this study (data not shown).

Detection of GFP in the phloem sap of PTRXh-GFP transgenic rice plants

Phloem sap collected from severed stylets of brown planthoppers and leaf extract of PTRXh-GFP plants were analyzed by immunoblotting using GFP-specific monoclonal antibody. We examined four independent PTRXh-GFP T0 individuals that had shown GFP fluorescence in companion cells in the fluorescence microscopic analysis (Fig. 2D, E). In wild-type plants, no band was detected in leaf sheath or phloem sap by the GFP antibody (Fig. 4A). GFP was detected in both leaf extracts and phloem sap of the four independent PTRXh-GFP lines (Fig. 4B, C). The line which contained the highest amount of GFP in leaves showed the highest concentration of GFP in phloem sap (Fig. 4B, C).
Comparison of the contents of GFP, TRXh, and GST in phloem sap and leaves

We used immunoblot analysis to compare the amount of GFP with that of endogenous phloem proteins, TRXh and glutathione S-transferase (GST), in phloem sap collected from severed stylets of brown planthoppers and leaf sheath extract. The protein concentrations were calculated from the band intensities of the recombinant proteins. The intensity of bands detected in the immunoblot analysis was measured using NIH-image software (National Institute of Health, MD, USA). A line derived from one PTRXh-GFP T0 plant that showed GFP fluorescence in companion cells (Fig. 2D, E) was used for the analysis.

Fig. 3 Detection of GUS protein in phloem sap and leaf extract of PTRXh-GUS rice plants. (A) Immunoblot analysis of GUS protein in phloem sap and leaf extract; lane 1, 20 μl of phloem sap from a T1 PTRXh-GUS plant; lane 2, 20 μl of phloem sap from a wild-type plant; lane 3, 3 μg of soluble leaf extract protein from a T1 PTRXh-GUS plant; lane 4, 3 μg of soluble leaf extract protein from a wild-type plant. Lane 1 contains GUS protein with a molecular mass of about 90 kDa (black arrowhead) and degradation products of GUS protein (white arrowhead). Lane 3 contains GUS protein with a molecular mass of about 68 kDa (black diamond). (B) Silver-stained SDS-PAGE gel of the same fractions used in (A).

Fig. 4 Detection of GFP in phloem sap and leaf extract of PTRXh-GFP rice plants. (A) Immunoblot analysis of GFP protein in phloem sap and leaf extract; lane P, 10 ng of recombinant GFP as positive control; lane 1, the transgenic line of T0 generation; lane wt, wild type plant. Five μg of leaf soluble proteins and 2 μl of phloem sap were analyzed. (B) Immunoblot analysis of GFP in phloem sap. Lane P, 10 ng of recombinant GFP as positive control; lanes 1, 2, 3 and 4, independent transgenic lines of T0 generation. Three μl of phloem sap from PTRXh-GFP lines were analyzed. (C) Immunoblot analysis of GFP in leaf extract. Lane P, 10 ng of recombinant GFP as positive control; lanes 1, 2, 3, and 4, independent transgenic lines of T0 generation. Five μg of leaf soluble proteins from PTRXh-GFP lines were analyzed.

Fig. 5 shows a representative result from the immunoblot analysis. Though non-specific bands were detected in GFP and GST analysis, they were not used for a calculation of their amounts (Fig. 5). The reason why non-specific bands of GFP in leaf-sheath extract were not detected in Fig. 4B might be that
Comparison of GFP, TRXh, and GST contents in leaf sheath and phloem sap. (A) Immunoblot analysis of GFP in a T0 PTRXh-GFP plant. Three different amounts of recombinant GFP (15, 10, and 5 ng) were loaded as a positive control. GFP (27 kDa) bands were detected in the lanes containing recombinant protein, leaf sheath soluble proteins, and phloem sap. Non-specific bands of higher molecular mass were also detected in the lanes containing leaf sheath soluble proteins. Only the GFP bands (indicated by the arrow) were used for the intensity analysis. (B) Immunoblot analysis of TRXh in a T0 PTRXh-GFP plant. Recombinant TRXh was loaded at 15, 10, and 5 ng per lane as a positive control. TRXh (13 kDa) bands were detected in the lanes containing recombinant protein, leaf sheath soluble proteins, and phloem sap. (C) Immunoblot analysis of GST in a vector control plant. Recombinant GST was loaded at 30, 20, and 10 ng per lane as a positive control. As the recombinant proteins contained a His-tag, they had a higher molecular mass than the GST in leaf sheath and phloem sap. GST (31 kDa) bands in leaf sheath and phloem sap are indicated by an arrow. Two non-specific bands were also detected in phloem sap. Only the GST bands (indicated by the arrow) were used for the intensity analysis.

Because of the absence of positive control protein, GUS contents could not be calculated. But the intensity of the GUS band in leaf sheath (per gFW) was 1.4 times higher than in phloem sap (per liter). Only the major bands (indicated by the black arrowhead and black diamond in Fig. 3A) were used for the GUS intensity analysis.

Discussion

Immunoblot analysis revealed both GUS and GFP in the phloem sap of transgenic plants expressing these foreign proteins from the TRXh promoter (Fig. 3, 4). Moreover, histochemical analysis of PTRXh-GUS plants showed that sieve elements, as well as companion cells, had GUS activity (Fig. 2A, B, C). Since enucleated sieve elements are thought to be incapable of protein synthesis, and the TRXh promoter has been shown to drive transcription in companion cells (Ishiwatari et al. 1998), the results suggested that GUS and GFP are synthesized in companion cells and transported into sieve elements through plasmodesmata. Based on experiments using transgenic plants, snowdrop lectin and GFP have been reported to traffic into sieve elements in N. tabacum (Shi et al. 1994, Imlau et al. 1999). In the present study, GUS, which has a larger molecular mass (68 kDa) than snowdrop lectin (12 kDa) or GFP (27 kDa), was shown to enter sieve elements. Previous microinjection experiments demonstrated that 10 kDa, but not 40 kDa, fluorescent dextran could move through plasmodesmata between sieve elements and companion cells of Vicia faba (Kempers and van Bel 1997). In O. sativa, direct introduction experiments produced similar results: that 3 kDa dextran could move through the plasmodesmata between sieve elements and companion cells, but 42 kDa dextran could not (Fujimaki et al. 2000). Dextrans of 3, 10 and 40 kDa have Stokes’ radii of approximately 1.2, 2.0 and 4.3 nm, respectively (Fisher and Cash-Clark 2000), suggesting that the size exclusion limit of the sieve element-companion cell boundary is at least 1.2 nm in V. faba and 2.0 nm in O. sativa. In the present study, GUS, with a Stokes’ radius of 3.3 nm (Fisher and Cash-Clark 2000), could enter the sieve elements. Some phloem proteins, like C. maxima PP2 and CmPP16, R. communis glutaredoxin and cystatin, and O. sativa TRXh, were reported to increase the size exclusion limit of mesophyll plasmodesmata and facilitate their own cell-to-cell movement after microinjection (Balachandran et al. 1997, Ishiwatari et al. 1998, Xoconostle-Cázares et al. 1999). In contrast, it was reported that GUS protein alone did not show cell-to-cell movement after microinjection into trichomes of Nicotiana clevelandii (Wagmann and Zambraski
suggesting that GUS protein is unable to mediate its own cell-to-cell transport through plasmodesmata, at least in trichomes. To explain the cell-to-cell movement of proteins larger than the size exclusion limit of plasmodesmata, protein unfolding is proposed. Large proteins like GUS might be unfolded prior to moving through plasmodesmata and then refolded in the sieve elements. Indeed, homologues of two chaperones, rubisco-subunit-binding protein and cyclophilin, have been detected in sieve-tube exudates from T. aestivum, O. sativa, Yucca filamentosa, C. maxima, Robinia pseudoacacia and Tilia platyphyllos (Schobert et al. 1998). Moreover, a small heat-shock protein has been detected in phloem sap from O. sativa (Fukuda et al. 2004b).

Although GUS can enter sieve elements, histochemical analysis of PTRXh-GUS plants revealed that the companion cells had stronger GUS activity than the sieve elements (Fig. 2C). Furthermore, in leaf sections of PTRXh-GFP plants, strong GFP fluorescence was detected only in companion cells (Fig. 2E). The weaker GUS and GFP signals in sieve elements of the transgenic plants suggested that these proteins were less abundant in sieve elements than in companion cells. This distribution pattern differs from those of several phloem proteins of rice, GST (Fukuda et al. 2004a) and small heat-shock protein (Fukuda et al. 2004b). GST and small heat-shock protein were reported to be mainly in sieve elements. PP1 was reported to be mainly in sieve elements and only weakly detected in companion cells (Clark et al. 1997). CmPP16 (Xoconostle-Cázares et al. 1999) and SUT1 (Kühn et al. 1997) were detected only in sieve elements. On the other hand, C. maxima PP2 and trypsin inhibitor were mainly in companion cells and weakly detected in sieve elements (Dannenhoffer et al. 1997, Dannenhoffer et al. 2001). One of the reasons for differential distribution proteins might be efficiency of protein transport from companion cells to sieve elements. TRXh and GST might be efficiently transported from companion cells to sieve elements. In contrast, the rate of GUS and GFP trafficking might be low, and these proteins might accumulate in companion cells. Because GFP (27 kDa) and GST (31 kDa) had a similar molecular mass, the distribution pattern in sieve element-companion cells was not determined only by molecular mass. Microinjection experiments on N. tabacum mesophyll cells revealed that TRXh increased the size exclusion limit of plasmodesmata and modified their own cell-to-cell movement, and that certain structural motifs of TRXh are necessary for cell-to-cell movement of this protein (Ishiwatari et al. 1998). The efficient transport of proteins from companion cells to sieve elements might need specific structural motifs, which are recognized by plasmodesmata carriers, and modify cell-to-cell movements of proteins. Another reason for the differential distribution pattern of proteins in the sieve element-companion cell complex might be the degradation speed of proteins in sieve elements. The degradation speed of GUS and GFP in sieve elements might be faster than those of

![Fig. 6 Amount of GFP, TRXh and GST in leaf sheath and phloem sap. (A) Proteins in leaf sheath (µg g⁻¹ FW). (B) Proteins in phloem sap (µg ml⁻¹ phloem sap). The GFP bars represent the average of seven experiments with standard deviations. The GST columns represent the average of seven experiments with standard deviations.](https://academic.oup.com/pcp/article-abstract/46/11/1779/1894575)
TRXh and GST, causing the differential distribution pattern of these proteins in the sieve element-companion cell complex. The analysis of the phloem sap from PTRXh-GUS plants suggested the modification of GUS protein in phloem sap (Fig. 3A), although it was not clear whether the modification of GUS was correlated with the degradation of this protein or not. Further analysis of the transport efficiency and degradation speed of proteins is necessary for the understanding of the nature of phloem.

Materials and Methods

Construction of GUS expression vector and GFP expression vector for rice transformation

The TRXh promoter (between −1025 and −16 bp from the translation start site ATG) and GUS gene were transcriptionally fused as follows. The promoter region of the TRXh gene (Genbank accession No. D26547, Ishiwatari et al. 1997) was amplified by PCR using two primers, 5'-CTAGTGGAATCTCATGAAAATGGGAAATAGG-3' and 5'-CTCGGATCCCAATTCCTCGCGGCAAGAGATC-3'. The amplified TRXh promoter fragment (PTRXh) was subcloned into the HindIII-EcoRI sites of pGL121Hm (pBI121 containing a hygromycin-resistance gene; Ohta et al. 1990), replacing the cauliflower mosaic virus 35S RNA promoter. The resulting plasmid was termed PTRXh-GUS (Fig. 1).

The TRXh promoter (between −1025 and −3 bp from the translation start site ATG) and GFP gene were transcriptionally fused as follows. The promoter region of the TRXh gene (Genbank accession No. D26547, Ishiwatari et al. 1997) was amplified by PCR using two primers, 5'-CTAGTGGAATCTCATGAAAATGGGAAATAGG-3' and 5'-CGGAATCTCGCGGCAAGAGATC-3'. The amplified TRXh promoter fragment (PTRXh) was subcloned into the BamHI and NcoI sites of the 35S Omega-sGFP (S65T) plasmid (Chiu et al. 1996), containing the GFP gene and nopaline synthase terminator (TNOS). The fusion gene PTRXh-GFP-TNOS was digested with HindIII and EcoRI, and subcloned into the BamHI-NcoI sites of the 35S Omega-sGFP (S65T) plasmid (Chiu et al. 1996), containing the GFP gene and nopaline synthase terminator (TNOS). The fusion gene PTRXh-GFP-TNOS was digested with HindIII and EcoRI, and subcloned into the same sites of pBIN19 (Bevan 1984). The fragment containing PTRXh-GFP-TNOS was excised using HindIII and SpeI, and inserted into the corresponding sites of the pTF338 vector, which contained the hygro-mycin phosphotransferase gene in place of the kanamycin selectable marker of pBIN19 (Bevan 1984) (Fig. 1).

Transformation of rice plants

The transgenic rice plants were generated by the Agrobacterium tumefaciens-mediated method (Hei et al. 1994). Rice (O. sativa L.) cultivar Sasanishiki and A. tumefaciens strain EHA101 were used to produce GUS-expressing plants (Goto et al. 1997). Rice cultivar Tsukumihikari and A. tumefaciens strain C58RiR (pGV2260) (Deblaere et al. 1985) was used for the determination of total protein content. Protein Assay Reagent (BIO-RAD Co., CA, USA) was used for the determination of total protein content.

Production of recombinant proteins

The recombinant GFP used for a positive control in the immunoblot analysis was produced in Escherichia coli. A fragment containing the open reading frame encoding GFP was excised from 35S Omega-sGFP (S65T) (Chiu et al. 1996) with NcoI and EcoRI, and subcloned into the same sites of pET30b (Novagen Co., Darmstadt, Germany). The resulting plasmid was used to transform E. coli strain BL21 (degradation speed of 20°C to 30°C until analysis. Protein Assay Reagent (BIO-RAD Co., CA, USA) was used for the determination of total protein content.

Immunoblot analysis

Proteins were separated by SDS-PAGE (Nakamura et al. 1993) and transferred to polyvinylidene difluoride membranes. Immunoblotting was performed to detect GUS, GFP, TRXh, and GST. GUS was detected with rabbit anti-glucuronidase IgG (H+L) fraction (Molecular Probes Co., OR, USA) and goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate (BIO-RAD Co., CA, USA) as the secondary antibody. GFP was detected with mouse monoclonal anti-GFP IgG (Zymed Co., CA, USA) and anti-mouse IgG horseradish peroxidase-linked whole antibody from sheep (Amersham Co., Buckinghamshire, UK) as the secondary antibody. TRXh was detected with rabbit IgGs against recombinant rice TRXh and goat anti-rabbit IgG.
(H+L) horseradish peroxidase conjugate (Bio-RAD Co.) as the secondary antibody. GST was detected with rabbit IgGs against recombinant GST as described by Fukuda et al. (2004a). Diaminobenzidine was used as the substrate for detection. The intensity of bands detected in the immunoblot analysis was measured using NIH-image software (National Institute of Health, MD, USA).

References


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