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Molecular Characterization of a Chinese Cabbage cDNA Encoding Thioredoxin-h that is Predominantly Expressed in Flowers

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Even though three isotypes of thioredoxins (-f, -m and -h types) have been identified in a variety of plant cells, there are only a few reports on thioredoxin-h that were recently identified. In this study, a cDNA encoding a h-type of thioredoxin was isolated from a cDNA library of Chinese cabbage, and named here *CTrx-h*. An open reading frame of the gene contained a polypeptide of 133 amino acids with a conserved active center, WCGPC, which appeared in all of the thioredoxin proteins. A deduced amino acid sequence of the CTrx-h showed the highest sequence identity with those of *Arabidopsis* thioredoxin-h2 (75.2%) and thioredoxin-h5 (46.6%) proteins, but it shared a low sequence homology to other isotypes of plant thioredoxin-m and thioredoxin-f. The CTrx-h protein that is expressed in *E. coli* represented not only an insulin reduction activity, but also electron transferring activity from NADPH to thioredoxin-dependent peroxidase. A genomic Southern blot analysis using the cDNA insert of *CTrx-h* revealed that the gene consisted of a small multigene family in Chinese cabbage genome. On the contrary to other thioredoxin-h proteins that were widely distributed in most tissues of the plant, the *CTrx-h* was predominantly expressed in flowers. The expression was very low in other tissues. The data of the Northern blot analysis suggests that the CTrx-h may have other functions in flower development or differentiation, in addition to its defensive role.

Keywords: Chinese cabbage, Flower-specific expression, Thioredoxin-h, Trx isotypes

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Introduction

Thioredoxins are ubiquitous, low-molecular-mass proteins that are characterized by the presence of an exposed active center with the amino acid sequence of Trp-Cys-Gly-Pro-Cys (Holmgren, 1985). The two cysteine residues, which are in close proximity, can form an intermolecular disulfide bridge with its target molecules (Lee *et al.*, 2001b). Via the reversible disulfide/dithiol reduction reaction involving these cysteines, thioredoxins participate in numerous redox processes (Lee *et al.*, 2001a). Three major functions of thioredoxin are proposed as follows: (1) They act as structural components that are required for the activity and synthesis of some components of T7 DNA polymerase or for phage assembly (Wakasugi *et al.*, 1990). (2) They are intermediate energy donors to some enzymes like ribonucleotide reductase, PAPS reductase, methionine sulfoxide reductase, and hydrogen peroxide reductase that accept energy through a proton transfer on cysteines (Holmgren, 1979). (3) They regulate the function of enzymes or transcription factors by modifying a bridged conformation of their disulfide bonds, which have been described in mammals, such as NF-κB and AP-1 (Meyer *et al.*, 1993; Hayashi *et al.*, 1993).

Whereas most non-photosynthetic cells contain one or two thioredoxins, which are closely related to the well studied thioredoxin from *E. coli* (Miranda-Vizuete *et al.*, 1997), photosynthetic cells contain several types of thioredoxins, which are distinguished by their specific functions (Eklund *et al.*, 1991; Makino *et al.*, 1996). Two types of the thioredoxin family, thioredoxin-m and thioredoxin-f, have been identified from plant chloroplasts. They are reduced by the ferredoxin-dependent thioredoxin reductase and implicated in the light/dark regulation of CO_2 assimilation (Crawford *et al.*, 1989; Hartman *et al.*, 1990). More specifically, thioredoxin-f interacts with a number of target enzymes of the reductive pentose phosphate cycle. Through the interaction, thioredoxin-f activates them in the light and deactivates them

in the dark (Geck *et al.*, 1996). But the thioredoxin-m is efficient in modulating enzymes not related to the reductive pentose phosphate cycle, such as the chloroplast-specific, NADP-dependent malate dehydrogenase, which is part of a mechanism for the export of reducing equivalent (Wenderoth *et al.*, 1997). Recently, another isotype of thioredoxin, h-type, was isolated from various plant sources that have a close sequence similarity with the thioredoxins that exist in animals and are found in plant cytosol, endoplasmic reticulum, and mitochondria (Buchanan *et al.*, 1994). For example, at least five cytosolic thioredoxin-h proteins were identified from *Arabidopsis thaliana*, two from *Nicotiana tabacum*, one from spinach, and two from green algae, *Chlamydomonas reinhardtii* (Mittard *et al.*, 1995). It has been shown that the cytoplasmic thioredoxin in mammalian cells quickly translocates into the nucleus in response to phobol 12-myristate 13-acetate (PMA). It also activates AP-1 transcription activity by direct association with an intra nuclear redox factor, Ref-1 (Hirota *et al.*, 1997; Storz *et al.*, 1990). In response to reactive oxygen stresses (Scandalios, 1997), thioredoxin-h inhibited the nuclear translocation of NF-kB (Jin *et al.*, 1997). In addition, thioredoxins have been shown to participate in some developmental stages, such as meiosis, embryo development, and pregnancy (Tonissen *et al.*, 1993; Gallegos *et al.*, 1996). In these reactions, thioredoxin as a reducing molecule of NF-κB cannot be replaced by the glutathione system, another type of reducing molecules that contains glutathione, glutathione reductase, and NADPH. This suggests that the interaction between transcription factors and reducing enzymes are highly specific. Likewise, the plant thioredoxin-h proteins also utilize NADPH-dependent thioredoxin reductase in redox regulation and regulate the enzyme activities that are involved in diverse cellular signaling processes, such as in the self-incompatibility signaling cascade by forming a stable complex with a S locus receptor kinase (Bower *et al.*, 1996), in the regulation of cellular reducing enzymes through a thiol-mediated electron transfer (Verdoucq *et al.*, 1999), and in the regulation of sulfur assimilation or H_2O_2 tolerance (Mouahebe, 1998).

However, in spite of these studies, the exact function of plant cytosolic thioredoxin-h and its target proteins are poorly understood. In order to understand the specific role of the plant thioredoxin-h, in this paper we described the isolation of a cDNA encoding thioredoxin-h protein from a cDNA library of Chinese cabbage, molecular characterization of the gene, and analyzed its biochemical properties using the bacterially expressed recombinant protein.

Materials and Methods

Plant materials and other reagents Chinese cabbage (*Brassica campestris* L. ssp. pekinensis) was grown in a growth chamber with a 16/8 hr light/dark cycle, a temperature of 20°C, and a relative humidity of 70%. Different tissues of Chinese cabbage, grown in a greenhouse, were dissected and used for the preparation of genomic DNA and RNA. Yeast thioredoxin reductase was obtained from the *E. coli* transformant, as reported previously (Chae *et al.*, 1994). Radioisotopes were purchased from Amersham. Various enzymes were from Boehringer Mannheim, New England Biolabs, and Stratagene. The oligonucleotides were synthesized from Bioneer Co. (Daejeon, Korea) and purified by gel electrophoresis in 0.6% polyacrylamide gel containing 7 M urea.

Cloning of a thioredoxin-h cDNA, CTrx-h Previously, we prepared a phagemid-based flower bud-specific cDNA library and carried out random cDNA sequencing (Lim *et al.*, 1996) of the clones. From among our expressed sequence tag (EST) clones, we isolated a partial cDNA clone, EST-F1121, which exhibited a sequence homology to an arabidopsis thioredoxin-h2 (Rivera-Madrid *et al.*, 1995). Using the [^{32}P]-labeled EST-clone as a screening probe, we then isolated a full-length cDNA from the same cDNA library, designated CTrx-h. The deduced amino acid sequence of the clone was compared to the sequence data deposited in the public databases using the Blastx program that was provided by the NCBI e-mail server (Altshul *et al.*, 1996).

Expression of the CTrx-h protein in *E. coli* For expression of the CTrx-h protein in *E. coli*, an open reading frame of the CTrx-h sequence was subcloned into the pGEX expression vector. After transforming the *E. coli* strain BL21(DE3)pLysS with the DNA construct of pGEX-CTrx-h, the bacteria were grown in a LB-medium that contained 100 ug/ml ampicillin and 12.5 ug/ml chloramphenicol. The glutathione *S*-transferase (GST)-fusion protein was isolated and purified from the cytosolic fraction of *E. coli* extracts by glutathione-agarose affinity chromatography (Smith and Johnson, 1988; Jung *et al.*, 2001). The N-terminal GST portion of the protein was then removed with thrombin.

Assay of thioredoxin activity The protein disulfide oxidoreductase activity was assayed at 25°C by measuring the precipitation of the insulin β-chain that followed the reduction of the intercatenary disulfide (Holmgren, 1979). To this end, the reductive process was started by the addition of 0.5 mM DTT in 1 ml solution of 0.1 M potassium phosphate buffer, pH 7.0 containing 0.1 mM EDTA, and 0.5 mg/ml insulin. The rate of the A_{650} increase was the expression of the protein disulfide oxidoreductase activity.

Reduction of thioredoxin-dependent peroxidase activity by the CTrx-h Since a tight coupling between the thioredoxin-dependent peroxidase activity and the NADPH consumption rate was previously reported (Chae *et al.*, 1994), we measured the reduction activity of the CTrx-h by measuring the thioredoxin-dependent peroxidase activity of 2Cys-Prx isolated from the same plant (Cheong *et al.*, 1999). The peroxidase activity of 2Cys-Prx was monitored by the decrease in A_{340} in a 500 ul reaction mixture that contained 0.3 mM NADPH, 5 uM thioredoxin, 5 ug thioredoxin reductase, 1 mM H_2O_2, and 50 mM Hepes-NaOH, pH 7.0 (Park *et al.*, 2000).

Genomic Southern and Northern blot analysis Chinese cabbage genomic DNA was prepared from leaves and a Southern analysis was carried out under high stringency conditions (Kim *et al.*, 1996). For the Northern blot analysis, tissues from various parts

of Chinese cabbage were obtained by sectioning, and the total RNA was extracted from the sections, as described previously (Chomcznski and Sacchi, 1987). Twenty μg of total RNA from each sample were denatured, separated on a 2% formaldehyde agarose gel, and transferred onto a GeneScreen plus membrane (NEN). The *Eco*RI/*Xho*I excised cDNA insert of *CTrx-h* was labeled with [^{32}P]-labeled dATP by the random-priming method, and used as a probe in the Southern and Northern analyses (Choi *et al.*, 1999).

Results

Molecular cloning of a Chinese cabbage thioredoxin-h cDNA A partial cDNA clone, closely matching the thioredoxin-h2 gene of *Arabidopsis thaliana,* was isolated from a Chinese cabbage flower bud cDNA library (Lim *et al.*, 1996). Using the [^{32}P]-labeled probe under stringent conditions, a full-length cDNA, *CTrx-h*, was isolated and directly sequenced. We found that the cloned *CTrx-h* gene contained 618 bp with 399 bp open reading frame. It would encode 133 amino acids, resulting in a calculated molecular mass of 14,630 Da. It contained 29 bp of 5'-untranslated region and 190bp of 3'-untranslated sequence. The nucleotide sequence of the gene will appear in the GenBank database under the accession number of AF352030. An amino acid sequence comparison of the clone to entries in the EMBL and Swiss-Prot sequence database reveals that the gene belongs to the thioredoxin-h protein family.

Comparison of the deduced amino acid sequence of the CTrx-h with other thioredoxin proteins Fig. 1 displays the result of a comparison of the amino acid sequence of the CTrx-h protein with other typical thioredoxin-h proteins. The putative translation product of the CTrx-h shares the highest sequence identity with that of *Arabidopsis* thioredoxin-h2 (75.2%) and thioredoxin-h5 (46.6%). These homologies were found in the regions coding between V^{50} and P^{67}, including a strong similarity region over the 15bp stretch coding for the active site WCGPC. Also, the amino acid sequences of Gly^{60}, Pro^{61}, Pro^{102}, Gly^{118}, and Ala^{119} are highly conserved. Based on the X-ray crystal structure of *E. coli* thioredoxin, this region consists of a flat hydrophobic surface and is thought to

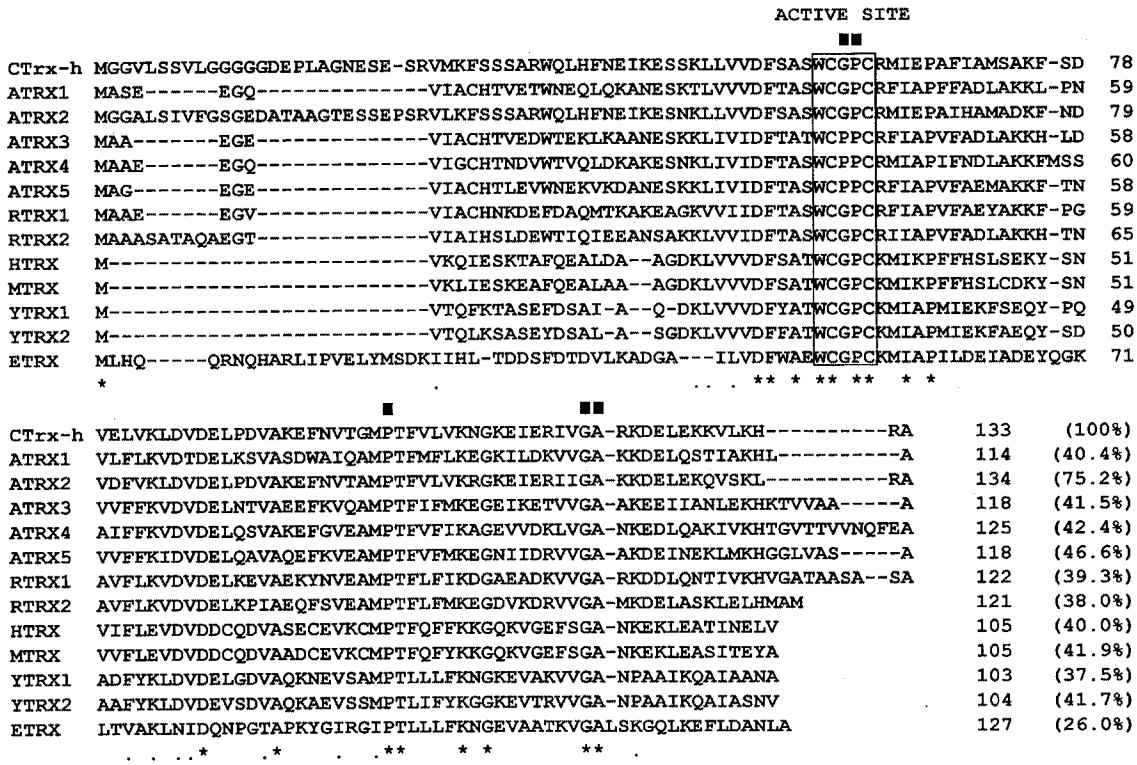

Fig. 1. Comparison of protein sequence of the CTrx-h with other protein members of the thioredoxin-h family. Open reading frames of the corresponding cDNA clones are translated and compared after alignment. Amino acid sequences of thioredoxin-h in Chinese cabbage (CTrx-h; this work), *A. thaliana* (ATRX1-5; Rivera-Madrid *et al.*, 1995), rice (RTRX1-2; GenBank accession #, BAA05546 and BAA20886), human (HTRX; accession #, 1827676), mouse (MTRX; accession #, S04107), yeast (YTRX1-2; accession #, NP013144 and NP011725), and *E. coli* (ETRX; accession #, AAC76786) are aligned for comparison. Asterisks (★) denote positions perfectly conserved and dots (·) mark well-conserved positions. Gaps are introduced to maximize the alignment. The conserved active site, WCGPC, found in all members of the thioredoxin-superfamily, is represented as a box. The putative binding sites of thioredoxin-h with other interacting proteins are marked as a rectangular (■) above the sequences (3). The alignment was computed with the CLUSTAL program of the PCGENE software package (Genofit SA, Geneva, Switzerland/IntelliGenetics Inc. Mountain View, CA). Amino acid sequence identity with the CTrx-h is shown in the last column (%).

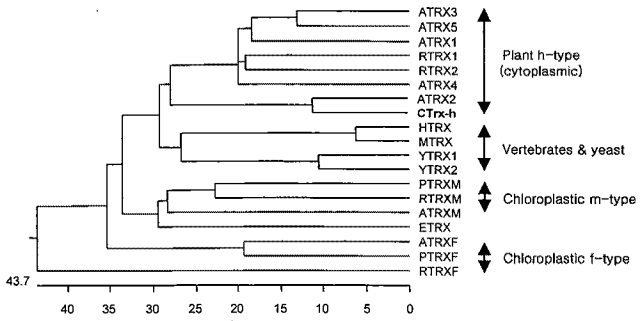

Fig. 2. A dendrogram of the CTrx-h protein and other types of thioredoxin families. The dendrogram is constructed by the PCGENE program. The sequences of thioredoxin-h proteins are used as in Fig.1; thioredoxin-m proteins isolated from *Pisum sativum* (PTRXM; GenBank accession #, AAC49358), rice (RTRXM; accession #, CAA06736), *A. thaliana* (ATRXM; accession #, 048737); thioredoxin-f proteins isolated from *A. thaliana* (ATRXF; accession #, Q9XFH8), *Pisum sativum* (PTRXF; accession #, P29450), and rice (RTRXF; accession #, AAB82144).

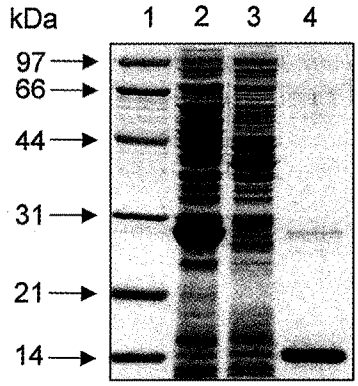

Fig. 3. SDS-PAGE of proteins isolated by affinity chromatography. Polyacrylamide gel was loaded with proteins obtained from the lysate of noninduced cells (lane 2), the lysate of cells induced for 4.5 h with IPTG (lane 3), and mature form of the CTrx-h purified by glutathione agarose column. A portion of GST was cleaved-off with thrombin treatment (lane 4). In lane 1, molecular standards are shown.

mediate the interaction of the thioredoxin with other interacting proteins (Katti *et al.*, 1990; Qin *et al.*, 1994). However, based on amino acid sequence determinations, it was observed that the similarity among the three isotypes of plant thioredoxin, -m, -f and h types, was less than 30%. Also, there were transit peptide sequences that are required for the protein targeting to the plant chloroplasts, except for the h-type of thiroedoxin (Karlin-Neumann and Tobin, 1986). The result suggests that the CTrx-h is a cytosolic protein, whereas the thioredoxin-m and thioredoxin-f are localized in chloroplast. To compare the evolutionary distance of thioredoxin-h, we constructed a phylogenic tree with the three isotypes of thioredoxin from various sources (Altshul *et al.*, 1990) (Fig. 2). All of the thioredoxin-h proteins in plants are grouped in different branches of the tree, together with the vicinal distance to vertebrate thioredoxins, but the chloroplast thioredoxin-m and thioredoxin-f form separate groups from the thioredoxin-h family and neighbors with those of prokaryotes. Even though all of the thioredoxin isotypes were found in plant sources, the protein members that belong to the thioredoxin-h family were diverged from thioredoxin-f and thioredoxin-m very early in their evolution. This result suggests that the function of each thioredoxin family may be quite different.

Biochemical properties of the bacterially expressed recombinant CTrx-h protein To analyze the function of the CTrx-h protein, an open reading frame of the CTrx-h sequence was expressed in *E. coli* by using the pGEX expression vector, which resulted in the majority of recombinant CTrx-h that were found in the soluble fraction of the transformed E. coli cells. The recombinant protein was purified to almost homogeneity by affinity chromatography with a glutathione-agarose affinity column. Samples from different steps in the purification process were analyzed by SDS-PAGE on a 15% gel. A single mature protein band of 14 kDa CTrx-h was detected by cleaving the fusion protein with thrombin after the affinity chromatography (Fig. 3). After purification of the protein, we examined the insulin β-chain reduction activity of CTrx-h, a classical insulin reduction activity assay by thioredoxin in the presence of DTT. As shown in Fig. 4, the CTrx-h efficiently reduced insulin β-chain with the help of DTT, whereas it could not use insulin as a substrate without supplying DTT. Furthermore, when DTT was replaced for ascorbate, it was unable to reduce the insulin, suggesting that the reduction activity of CTrx-h was absolutely dependent on the thiol-reducing groups (data not shown).

Since it was reported that multiple thioredoxin-h in *Arabidopsis* originated from a toti-potent ancestor with different target proteins, we analyzed whether the CTrx-h could transfer electrons of NADPH to 2Cys-Prx, a thioredoxin-dependent peroxidase that is isolated from the same plant (Cheong *et al.*, 1999). The 2Cys-Prx reducing activity of CTrx-h was measured by coupling the reduction of H_2O_2 to NADPH oxidation and monitoring the decrease in A_{340}. The velocity of CTrx-h linked NADPH oxidation exhibited characteristic first-order kinetics and a saturation pattern as the reaction time increased in the presence of the thioredoxin system that contained thioredoxin reductase, thioredoxin, and NADPH (Fig. 5). The result suggested that CTrx-h could deliver reducing power of NADPH to 2Cys-Prx. In the reduction of H_2O_2 by 2Cys-Prx, CTrx-h is an essential component, because the reaction was not processed without the CTrx-h protein. Even though CTrx-h contained the redox-sensitive conserved cysteines, WCGPC, it could not reduce H_2O_2 directly.

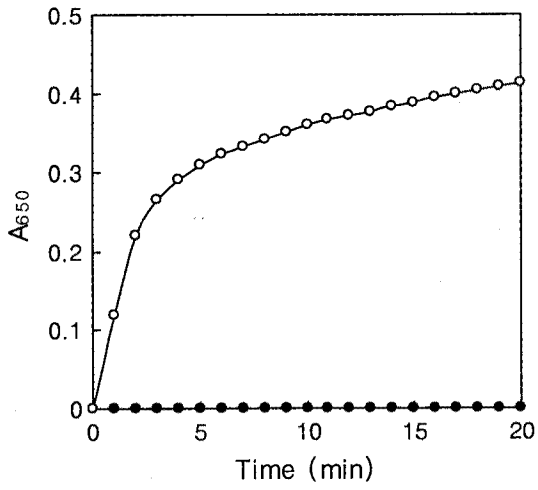

Fig. 4. Thiol-dependent insulin reduction activity of CTrx-h. The CTrx-h was assayed for its ability to reduce insulin β-chain in the presence (○-○) or absence of DTT (●-●).

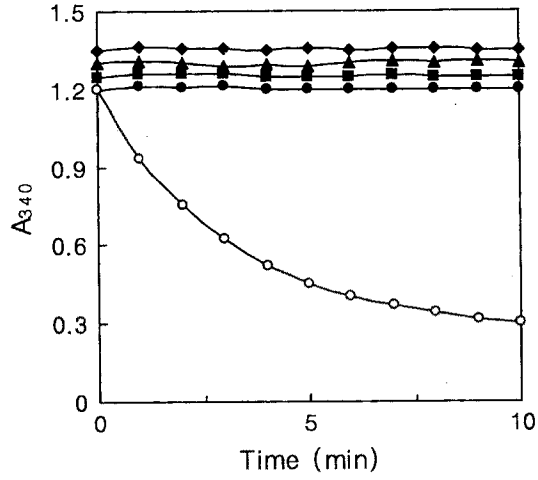

Fig. 5. NADPH oxidation coupled by thioredoxin reductase, thioredoxin-dependent peroxidase (2Cys-Prx, Cheong *et al.*, 1999), and CTrx-h to the reduction of H_2O_2. NADPH oxidation was monitored as the decrease in A_{340} in a 0.5 ml reaction mixture containing 50 mM Hepes-NaOH (pH 7.0), 5 ug thioredoxin reductase, 2.1 uM 2Cys-Prx, 5 uM CTrx-h, and 1 mM H_2O_2 (○-○). In other reaction mixtures, thioredoxin reductase (◆-◆), 2Cys-Prx (▲-▲), CTrx-h (■-■), or H_2O_2 (●-●) was omitted and the activity was measured by the same procedure.

Genomic complexity of the *CTrx-h* gene We examined the genomic complexity of the *CTrx-h* gene in Chinese cabbage genome by Southern blot analysis using the coding region of *CTrx-h* as a probe. When genomic DNA that was digested with *Eco*RI, *Hin*dIII, or *Bam*HI restriction enzymes that did not cleave within the coding sequence was hybridized with the probe, a few discrete restriction fragments were strongly recognized (Fig. 6). In addition to the strongly hybridizing fragments, there were also several weakly hybridizing

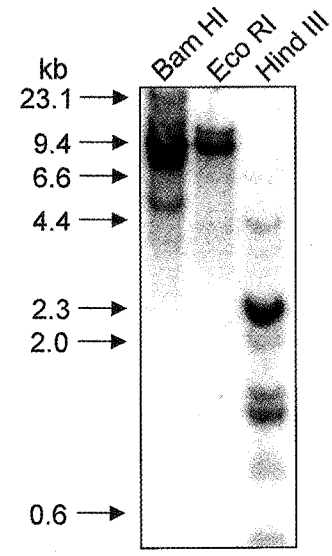

Fig. 6. Genomic Southern blot analysis of the *CTrx-h* gene. Genomic DNA was isolated from the leaves of Chinese cabbage. A hybond N+nylon membrane was prepared by using the purified genomic DNAs (10 μg, each) digested either with *Bam*HI, *Hin*dIII, or *Eco*RI and size fractionated on a 0.8% agarose gel. The filter was hybridized with [^{32}P]-labeled coding sequence of the CTrx-h and washed at a high stringency. Numbers indicate the sizes of standard DNA in kilobases (kb).

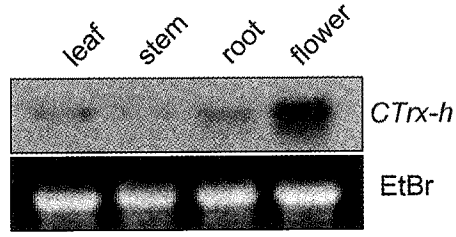

Fig. 7. Tissue-specific expression of the *CTrx-h* in various tissues of Chinese cabbage. RNA blot was prepared with 20 μg of total RNAs that was isolated from the leaf, stem, root, and flowers of Chinese cabbage. It was probed with [^{32}P]-labeled coding sequence of the CTrx-h. To show an equal loading of the RNAs in agarose gel, an EtBr stained gel was included.

fragments. The result of the Southern analysis, therefore, showed that the CTrx-h gene is a member of a small multigene family in Chinese cabbage genome. Considering the diverse function (Rivera-Madrid *et al.*, 1995) and multigenes of thioredoxin-h that is isolated from several plant sources, it might be reasonable to assume that the Chinese cabbage genome also contained a small gene family of the thioredoxin-h.

Flower specific expression of the *CTrx-h* To identify the possible physiological function of the *CTrx-h*, we examined the expression levels of mRNAs in various tissues by Northern blot analysis. We used total RNA that was prepared from leaf, flower, stem, and root tissues of Chinese cabbage.

We probed the RNAs with the [^{32}P]-labeled coding region of the *CTrx-h* under strong hybridization conditions. This resulted in a single transcript of approximately 0.7 kb in length that was hybridized in a tissue-specific manner. As shown in Fig. 7, the transcript level of *CTrx-h* was significantly higher in flowers and a relatively low expression was detected in roots and leaves. However, little transcript was found in the stem. Although its physiological significance is not understood, the specific expression of the *CTrx-h* in flowers suggests that the *CTrx-h* gene-product may play an important role in protecting the reproductive organ against oxidative damage.

Discussion

In this paper, we reported a cloning of the *CTrx-h* gene from Chinese cabbage and analyzed its molecular characterization, which could be helpful in achieving a better understanding of the role of thioredoxin-h in plant cells. The deduced amino acid sequence of the cDNA that contained the conserved active center of thioredoxin, Trp-Cys-Gly-Pro-Cys, and a high degree of sequence homology to the thioredoxin-h family, suggest that the clone we identified was indeed a member of a plant thioredoxin-h gene family. Unlike the chloroplastic thioredoxin-f and thioredoxin-m, the *CTrx-h* had no signal peptide, and was probably cytoplasmic. When the *CTrx-h* gene was expressed and purified from *E. coli*, the active recombinant CTrx-h was successfully produced and purified. The majority of the protein was found in a soluble fraction that facilitated the protein purification using standard procedures. The recombinant CTrx-h reduced insulin in the presence of DTT. The protein was capable of activating thioredoxin-dependent peroxidase with the help of the thioredoxin system that contained thioredoxin, thioredoxin reductase, and NADPH. This indicates that the protein might play a defensive role against oxidative stresses in plant cells. As the thioredoxin-related genes constituted a multigene family in plant cells, just as seen in *Arabidopsis thaliana* (Rivera-Madrid, 1995), spinach, and tobacco (Brugidou *et al.*, 1993), our data of the Southern analysis under high stringency conditions, represented that the *CTrx-h* was also a part of a small multigene family in Chinese cabbage. Furthermore, the hybridization analysis under low stringency conditions revealed a few more hybridizing bands (data not shown). These observations, together with the large number of thioredoxin-related genes in plants identified by PCR, clearly demonstrate that there exists quite a complexity in the thioredoxin-h protein family in plants. From the result of the five thioredoxin-h genes in *Arabidopsis thaliana*, which have been shown to have functional specificity against the sulfate assimilation and H_2O_2 resistance, the multiple thioredoxin-h proteins may have their specific function in plant cells. It was also verified by complementation experiments of thioredoxin-deficient mutant yeast (Rose and Broach, 1991; Brehelin *et al.*, 2000) that each member of the thioredoxin protein could have a specific role in the steps of cell growth, cell proliferation, and/or defense system.

Whereas the thioredoxin-m and thioredoxin-f were specifically localized in the chloroplasts, it was reported that thioredoxin-h proteins were mostly distributed in plant tissue (Marcus *et al.*, 1991). However, the transcript of *CTrx-h* is predominantly expressed in flower tissues. This suggests that the gene may have another important function in floral development or differentiation, as well as its defensive role against the environmental stress (Britt, 1996), which will be further studied. Indeed the exact physiological role of the numerous thioredoxin families in plant cells, especially for the thioredoxin-h subfamily, is still unclear. To solve these questions, if possible, transgenic plants that contain either the mutated or antisense construct of a certain type of thioredoxin has to be prepared. This could can elucidate the direct physiological function of each type of thioredoxin.

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