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Short communication

Molecular cloning and expression analysis of novel wheat cysteine protease☆

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Abstract

A cDNA clone encoding a novel papain-like cysteine protease was isolated from wheat germ (*Triticum aestivum*). This cDNA encoded a 371-residue protein, designated WCP2, composed of signal peptide followed by a propeptide and a mature protease containing active site residues that are highly conserved among the papain family. The mature WCP2 protein (26 kDa) was detected in the quiescent embryo and its level of expression in the germinating embryo was greatly increased.

Keywords: Cysteine protease; Embryo; Germ; Germination; Papain; Wheat

Seed germination is a complex, multi-stage process requiring the coordinated expression of numerous genes in different tissues [1,2]. Mature grain such as wheat, rice and barley is morphologically divided into an embryo and endosperm. The endosperm consists of an aleurone layer and a starchy endosperm. When germination starts with the uptake of water by the seed, hydrolytic enzymes such as amylase and proteases are secreted from the scutellar epithelial and aleurone layers, and these catalyze the degradation of starch and proteins stored in the endosperm. In contrast to monocot seeds, most dicot seeds contain storage proteins in the cotyledons that are part of the embryo. The proteins in cotyledons are also degraded by proteases during germination [1,3]. Papain-like cysteine proteases play a key role in this step [1]. Gibberellic acid is synthesized by the germinating embryo and secreted into the aleurone layer. In response to gibberellic acid, the aleurone cells synthesize cysteine proteases which are secreted into the endosperm [4–7]. Degradation products are absorbed by the scutellum and translocated to the developing seedling. Various wheat cysteine proteases have been identified in germinated [8–10] and dormant seeds [11]. In addition to papain-like cysteine protease, a novel cysteine protease (called legumain) with strict specificity for Asn in the cleavage site position also participates in breaking down the globulin stored in cotyledons of seedlings after germination [12]. However, it is still unclear how an embryo mobilizes its internal reserve of proteins during germination in monocot seeds.

To identify novel cysteine proteases in wheat germ, we designed degenerate PCR primers corresponding to conserved sequences (CGSCWAF and YWIVKNS, active site residues are underlined) in the catalytic domain of plant papain-like cysteine proteases. The sequences of the sense (P1) and antisense (P2) primers was 5′-TG(C/T)GG(A/C/G)TC(G/A/T/C)TG(C/T)TGGGC(G/A/T/C)TT(C/T)-3′ and 5′-(G/A/T/C)(G/C)(A/T)GTTCTT(A/C/G)AC(A/G)ATCCA(A/G)TA-3′, respectively. Wheat germ (quiescent embryo) was provided by Nissin Flour Milling (Tokyo) and total RNA was isolated. Following PCR of the total cDNA reverse-transcribed from wheat total RNA, DNA sequences of PCR products of the expected size were determined. Two products designated WCP1:522bp and WCP2:489bp and having amino acid sequences that are conserved in papain-like cysteine protease were identified. Both fragments contained the active site residue histidine as well as cysteine and asparagine residues that are important for the catalytic properties of cysteine protease. A comparison with other cysteine proteases revealed that WCP1 had

☆ The nucleotide sequence of WCP2 has been deposited in the GenBank under the accession number AB109216.

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99% amino acid identity to wheat cysteine protease (*War 5.2*) the expression of which is induced by aluminum [13]. In contrast, no genes with more than 50% amino acid identity to the WCP2 PCR product were found in database, suggesting WCP2 is a novel cysteine protease. To obtain the full-length WCP2 cDNA, the 5′ - and 3′ -end cDNA fragments overlapping the first PCR product were amplified by a second PCR from a wheat seed (cv. Minamino) cDNA library and

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                                                cgacaaaatcaagtgttcttgcatcaacac   -1

ATGGGCATGGCTCCCCTCTTCCGCTCGTTGGCTCTCCTCGTCCTCCTAGTCACCCTGTCC    60
M  G  M  A  P  L  F  R  S  L  A  L  L  V  L  L  V  T  L  S     20

AGCACCACATTGCCCTCCTCCCGTGCTACGTCCGGCGATGGAGACGACCATGACCTGCTG   120
S  T  T  L  P  S  S  R  A  T  S  G  D  G  D  D  H  D  L  L     40

ATGCTGGGTAGGTTCCACCGGTGGATGTCAGCGCATGGCCGGACGTACCATAGTGCCGCT   180
M  L  G  R  F  H  R  W  M  S  A  H  G  R  T  Y  H  S  A  A     60

GAGAAGTGCGGCGGTTTGAGGTGTACCGTCGCAACGTGGACCTCATCGACGCCTCCAACA   240
E  K  C  G  G  L  R  C  T  V  A  T  W  T  S  S  T  P  P  T     80

GGGACGCCGAGAGGCCTCGGCTATGAGCTCGGCGAGAACGAGTTCACCGACCTCACTAAC   300
G  T  P  R  G  L  G  Y  E  L  G  E  N  E  F  T  D  L  T  N    100

GAGGAGTTCATGGCGCGGTATGTCGGTGGGGCTTATGGTGGAGCCGGTGATGGTGGTGGT   360
E  E  F  M  A  R  Y  V  G  G  A  Y  G  G  A  G  D  G  G  G    120

CTCATCACTACTTTAGCTGGAGATGTTGTCGAGGGGGCGGCATCATCCAAGAACGCCATC   420
L  I  T  T  L  A  G  D  V  V  E  G  A  A  S  S  K  N  A  I    140

GAGGAGGATCGTAATTTGACGATGACTGCCTCTGACCCTCCCCGGCAGTTCGACTGGAGG   480
E  E  D  R  N* L  T  M  T  A  S  D  P  P  R  Q  F  D  W  R    160

GAACATGGTGTCGTCACGCCTGCTAAGCAACAAGGAGCATGTGGATGCTGCTGGGCATTT   540
E  H  G  V  V  T  P  A  K  Q  Q  G  A  C  G  C  C  W  A  F    180

GCTGCAGCGGCGACGGTGGAGAGCTTGAACAAGATAAATGGCGGGGAGCTGGTTGACCTG   600
A  A  A  A  T  V  E  S  L  N  K  I  N  G  G  E  L  V  D  L    200

TCCGTGCAGGAGCTGGTGGACTGCAGCACGGGCGTGTTCAGCTCGCCATGCGGCTACGGG   660
S  V  Q  E  L  V  D  C  S  T  G  V  F  S  S  P  C  G  Y  G    220

TGGCCCAAGAGCGCACTCGCATGGATCAAATCAAAAGGGGGCCTCCTCACGGAGGCCGAG   720
W  P  K  S  A  L  A  W  I  K  S  K  G  G  L  L  T  E  A  E    240

TACCCCTACATGGCCAAGCGAGGCAGATGCGCGGTGCACGACACAGCCCGCGTGTCGGCA   780
Y  P  Y  M  A  K  R  G  R  C  A  V  H  D  T  A  R  V  S  A    260

AAATCACCGGCGTCCAGGATGTACGGCCGGGCAGCAGCGAGAGCCCCTGGCGCTGGCGTG   840
K  S  P  A  S  R  M  Y  G  R  A  A  A  R  A  P  G  A  G  V    280

CTCGGGAGGCCGGTGACCGTCCAGATCGACGGGAGCGGCCCCGTCCTGCAGGACTACAAG   900
L  G  R  P  V  T  V  Q  I  D  G  S  G  P  V  L  Q  D  Y  K    300

TCCGGCGTGTACAGGGGGCCGTGCACCACCAGCCAGAACCACGTGGTGACGGTGGTCGGC   960
S  G  V  Y  R  G  P  C  T  T  S  Q  N  H  V  V  T  V  V  G    320

TACGGAGTCACCGGTGCCGGAGAAGAGTACTGGATCGCCAAGAACTCGTGGGGACAGACC  1020
Y  G  V  T  G  A  G  E  E  Y  W  I  A  K  N  S  W  G  Q  T    340

TGGGGTCAGAAGGGCTTCTTCTTCGTGCGCAGGGGAGCCGACGGGCCCCGCGGGCTGTGT  1080
W  G  Q  K  G  F  F  F  V  R  R  G  A  D  G  P  R  G  L  C    360

GGCATCGCCATGTACGGTGCCTACCCCGTCATGTAGttagtatatcggcatatagtctgc  1140
G  I  A  M  Y  G  A  Y  P  V  M  *                             371

cccctgggtatacatatgataatgtgcaaactaaataaactgcatccaattatgtgcgac  1200
gcacatgctggctacgcaaaaaaaaaaaaaa                               1231
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Fig. 1. Nucleotide and deduced amino acid sequence of WCP2 cDNA. Nucleotides and predicted amino acids are numbered on the right. The protein-coding region is shown in uppercase letters, while 5′ - and 3′ -untranslated regions are shown in lowercase letters. Amino acid 1–29 and 30–151 comprise a putative signal peptide and propeptide, respectively. The essential catalytic amino acids (cysteine^{177}, histidine^{314} and asparagine^{335}) are shown in bold. The polyadenylation signal (AATAAA) is underlined. Primers used for the first PCR correspond to nucleotides 520–540 (P1) and 988–1008 (P2). The positions of the primer used for 5′ - and 3′ -RACE are nucleotides 632–652 (P3) and 889–908 (P4), respectively. The full-length WCP2 cDNA was amplified by PCR using P5 and P6 primers corresponding to residue −30–(−10) and 1120–1142, respectively.

sequenced. The 5′ -WCP2 cDNA fragment was amplified by PCR using oligonucleotide primers corresponding to the sequence of the T3 promoter in the vector and the internal sequence of the first PCR product. Sequences for the sense and antisense primers were 5′ -ATTAACCCTCACTAAAG-3′ (T3 primer) and 5′ -CGCATGGCGAGCTGAACACAC-3′ (P3 primer), complementary to nucleotide residues 113–133, of the first PCR product, respectively. The 3′ -WCP2 cDNA fragment was amplified by PCR using oligonucleotide primers corresponding to the internal sequence of the first PCR product and T7 promoter in the vector. The sequence of the sense and antisense primers was 5′ -CAG-GACTACAAGTCCGGCGT-3′ (P4 primer), corresponding to residue 370–390 of the first PCR product and 5′ -GTAA-TACGACTCACTATAGGGC-3′ (T7 primer), respectively. The composite cDNA sequence of the three PCR products included 1231 nucleotides and all overlapping nucleotides were identical. We confirmed that full-length cDNA for WCP2 was amplified using 5′ - and 3′ -end primers (P5 and P6). No difference of nucleotide sequence between wheat germ and seed WCP2 cDNA was found, although the wheat germ we used was prepared from a mixture of various origins. Fig. 1 shows the nucleotide sequence and the deduced amino acid sequence of the full-length cDNA. WCP2 contains a hydrophobic region close to the initial methionine, which likely corresponds to the signal peptide. A short poly(A) sequence at the 3′ -end is 37 bp downstream from a consensus poly(A) addition site, AATAAA, and presumably derived from the poly(A) tail of the mRNA. The amino acid sequence alignment showed that the active site residues (cysteine^{177}, histidine^{314}, and asparagine^{335}) of cysteine protease and the sequences surrounding these three residues are well conserved as shown in Figs. 1 and 2. In addition, the cysteine residues involved in disulfide-bridge formation are also completely conserved (Cys^{174}/Cys^{217}, Cys^{208}/Cys^{250}, and Cys^{308}/Cys^{360}) as shown in Fig. 2. One striking difference in the sequence around the active cysteine residue between WCP2 and other cysteine proteases is that serine^{176} is replaced by cysteine in the former. Recently, tomato cysteine protease (*Rcr3* gene) was identified as a disease resistant gene by Krüger et al. [14]. As in WCP2, the serine residue adjacent to the active cysteine residue in this protease is replaced by cysteine. Krüger et al. also showed that *RCr 3* protease was an active enzyme. In the corn cysteine protease gene (*Mir1*), the expression of which is induced by insect feeding, the serine residue^{176} is replaced by glycine [15]. These findings suggest that the serine residue adjacent to the active cysteine is not required for enzyme activity but is highly conserved among papain-like cysteine proteases. The active, mature form of WCP2 starts at the aspartic acid^{152} residue, since it immediately precedes the proline residue that is always conserved at position 2′ in all papain-family members. To date, various wheat cysteine proteases have been identified by cDNA cloning. WCP2 shows 22%, 22%, 35% and 29% amino acid identities with the other wheat cysteine proteases, CP1 [6], CP2 [6], TP [16] and War 5.2 [13], respectively. A comparison of the entire amino acid sequence of WCP2 with sequences in the data base of *Oriza sativa* revealed that the highest degree of identity was with a putative cysteine protease (39% amino acid identity, accession no:

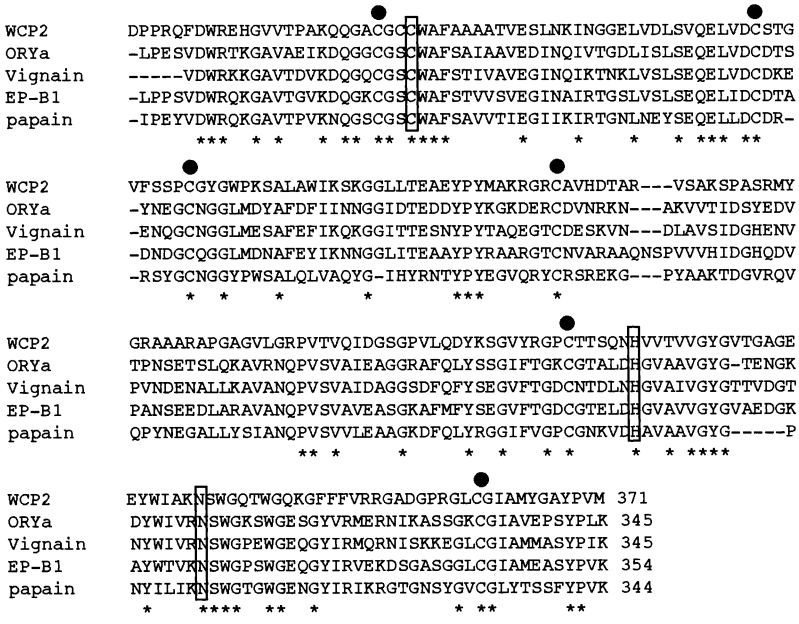

Fig. 2. Sequence similarities between WCP2 and other plant cysteine proteases. The five sequences were aligned for maximum homology. The proteases are: ORYα (GenBank accession no: D90406); Vignain (accession no: X15732); EP-B1 (accession no: U19359); papain (accession no: M15203). Active site residues (Cys, His and Asn) were boxed. Identical amino acid residues among all aligned sequences and Cys residues involved in disulfide bridges are marked with asterisks and closed circles, respectively.

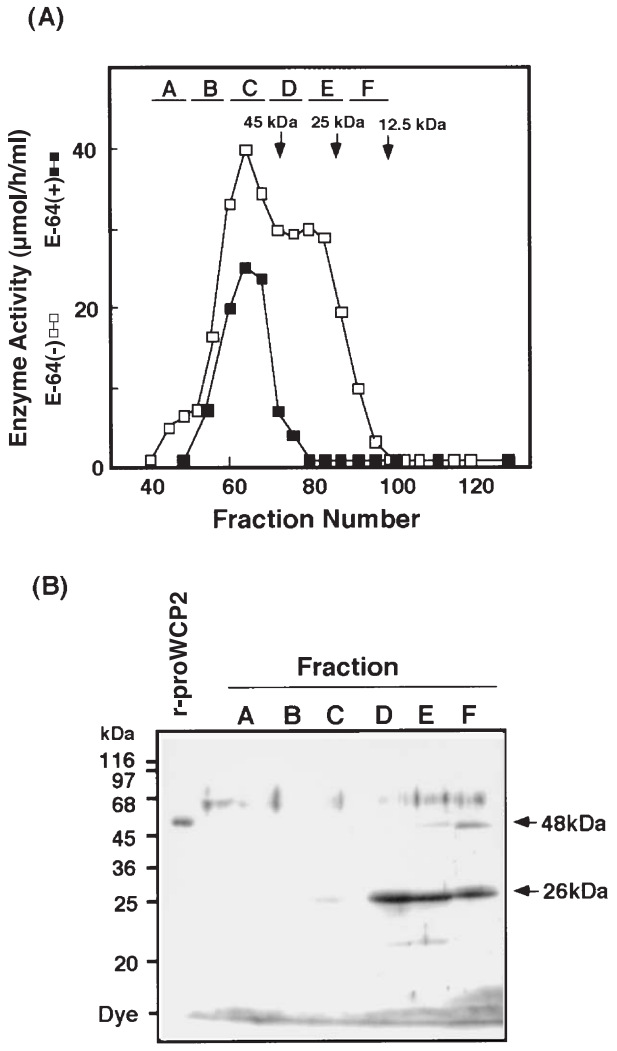

Fig. 3. Identification of WCP2 protein in cysteine protease fraction of wheat germ extract. Wheat germ (10 g) was extracted with 100 ml of ice-cold 50 mM acetate buffer, pH 6.0, containing 2 mM β-mercaptoethanol and 1 mM EDTA (buffer A), fractionated by ammonium sulfate precipitation (0–60% saturation) and then applied to a CM-cellulose column (1.5 × 3 cm) equilibrated with buffer A. The passed-through fraction was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, containing 1 mM β-mercaptoethanol and 1 mM EDTA (buffer B). The dialysate was applied to a DEAE-cellulose column (1.5 × 2.5 cm) equilibrated with the same buffer and eluted with a linear gradient of NaCl (0–0.25 M). The fraction containing WCP2 protein was applied to a Sephadex G-100 column (1.8 × 100 cm) equilibrated with B buffer containing 0.1 M NaCl. The protease activity was assayed using Z-Phe-Arg-MCA in the absence and presence of E-64 (50 μg/ml). In separate runs, the elution volume of the standard proteins; ovoalbumin (45 kDa), chymotrypsinogen (25 kDa) and cytochrome *c* (12.5 kDa) were determined. The fractions indicated by the horizontal bar were pooled (A), concentrated 40-fold by ultrafiltration and analyzed by Western blot using anti-WCP2 antiserum (B). r-ProWCP: recombinant proWCP2 lacking signal peptide.

AP003852–6) predicted from the analysis of rice chromosome 1. In the data base of *Arabidopsis thaliana*, F9P14.12 protein (accession no: AC02590–612) was most homologous to WCP2 (amino acid identity 37%). Thus no WCP2 homologue showing more than 50% identity was found in databases. On the other hand, the proteins showing more than 60% identity with WCP1 (*War 5.2*) have been found in various plants including corn seed (accession no: D45402; amino acid identity: 80%) [17], Chinese-lantern lily (accession no: AF411121, 69%) [18], tobacco leaves (accession no: AJ242994, 65%), and *A. thaliana* (AY133844–51, 66%) [19]. These results indicate that WCP2 cDNA codes for a novel member of the papain family.

To identify WCP2 at the protein level, specific anti-WCP2 antiserum was prepared by immunizing rabbits with thioredoxin-mature WCP2 fusion protein. The WCP2 cDNA coding mature WCP2 (amino acid 151–371) was amplified by PCR and ligated into pBAD/Thio-TOPO vector (Invitrogen). The fusion protein (54 kDa) was expressed in *Escherichia coli* strain BL-21 cells and purified

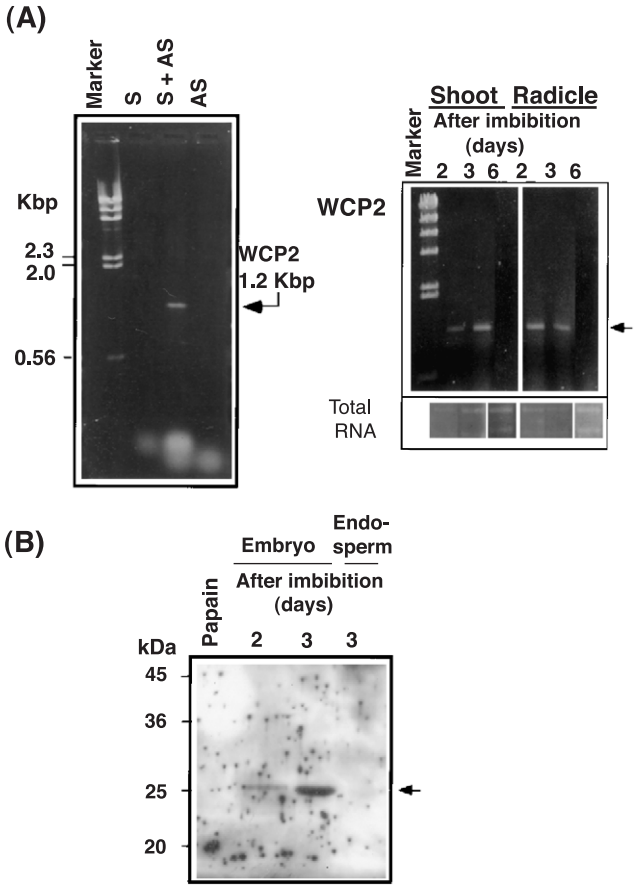

Fig. 4. Expression analysis of WCP2 at the mRNA and protein levels during germination. Wheat seeds (*Triticum aestivum* L. cv. Chikugoizumi) were allowed to imbibe water on filter paper at 20 °C in darkness until collection. (A) RT-PCR analysis of the WCP2 transcript in wheat quiescent (left) and germinating embryo (right). WCP2 cDNA was amplified using sense (S, P5) and antisense (As, P6) primers. Staining profiles of total RNA (2 μg) were also shown as a control. (B) Western blot analysis of WCP2 protein in germinating embryo and endosperm fraction after imbibition. Extracts from germinating embryo and endosperm fractions were applied to a Sephadex G-100 column. The fractions with E-64-sensitive protease activity were pooled, concentrated and analyzed by Western blot (embryo, 10 μg protein; endosperm, 30 μg protein). To confirm the specificity of anti-WCP2 antiserum, papain (2 μg) was also analyzed.

by Ni-nitrilotriacetic acid-agarose (Qiagen) affinity chromatography. The final preparation was used for the immunization of rabbits. The antiserum was absorbed with recombinant thioredoxin and used for immunodetection of WCP2. One nanogram of recombinant proWCP2 protein was detectable by Western blotting using this antibody. The WCP2 protein was not detected in crude quiescent embryo extract by Western blotting, suggesting that the expression level of WCP2 protein is very low. Therefore WCP2 protein was purified by CM-cellulose, DEAE-cellulose column chromatography and Sephadex G-100 gel filtration from crude quiescent embryo extract. As shown in Fig. 3, WCP2 protein was detected as a 26 kDa mature form in fractions D, E and F on Sephadex G-100 gel filtration. ProWCP2 (48 kDa) was also detected. The activity of fraction D and E was inhibited by E-64, a specific inhibitor of cysteine protease. Thus these results clearly showed the presence of WCP2 protein (both pro and mature enzyme) in the quiescent embryo fraction.

To understand the role of WCP2 during germination, the expression of WCP2 in the germinating embryo was analyzed at both the RNA and protein levels. Wheat (cv. Chikugoizumi) germinating embryo (shoot and radicle) was carefully dissected at 4 °C from seedlings 2 and 3 days after imbibition. The scutellum was removed from the embryo. WCP2 transcript was first analyzed by RT-PCR. As shown in Fig. 4A (left), full-length WCP2 cDNA (nucleotide −30 to 1142) was amplified by RT-PCR in quiescent embryo. When either sense or antisense primer was used, no DNA fragment was amplified, indicating the specificity of the PCR. Similarly, the DNA fragments with expected size were amplified in both shoot and radical parts of the embryo 2 and 3 days after imbibition (Fig. 4A, right). However, the band was not detected in either shoot or radicle 6 days after imbibition. The expression of WCP2 in germinating embryo and endosperm was further analyzed at the protein level by Western blotting. As shown in Fig. 4B, a 26-kDa band of mature WCP2 was detected in the germinating embryo. The expression level of WCP2 protein in germinating embryo was much higher 3 days after imbibition compared to 2 days. In contrast, WCP2 protein was not present in the endosperm fraction. Expression levels of WCP2 in the endosperm fraction, quiescent and germinating embryo after 3 days imbibition were further quantified by enzyme immunoassay using His tag-proWCP2 as a standard protein. One nanogram of WCP2 was able to be quantified by this method. The concentration of WCP2 (ng/mg protein) in the quiescent embryo, germinating embryo (3 days after imbibition) and endosperm fraction (3 days) were 2.7, 96.0, and below the detection level, respectively. These results clearly indicate that WCP2 is expressed in the embryo but not in the aleurone layer, in contrast to germination cysteine proteases such as proteinase A [9] and oryzain [5].

In this study, we identified two cysteine protease transcripts, WCP1 and WCP2, in quiescent embryo by PCR. Although further analysis is necessary to understand the function of WCP2, the present study strongly suggests that WCP2 has a unique role among the cysteine proteases identified in wheat seedlings.

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