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Identification of novel *Mlo* family members in wheat and their genetic characterization

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Mlo is a plant-specific gene family, which is known to show stress responses in various plants. To reveal the genetic characteristics of the *Mlo* family in wheat, we isolated wheat *Mlo* members from a database and studied their expression in young shoots and roots under salt and osmotic stress conditions. In an *in silico* investigation, we identified seven *Mlo* members in wheat and named them *TaMlo-1*~*TaMlo-7*. None of the wheat *Mlo* showed significant induction or reduction of their expression under salt or osmotic stress, but organ-specific expression was observed in several *TaMlo* members. *TaMlo-1*, *TaMlo-2*, and *TaMlo-5* were constitutively expressed in both shoots and roots, but *TaMlo-3* and *TaMlo-4* showed root-specific expression, and *TaMlo-7* showed dominant expression in shoots. *TaMlo-6* was weakly expressed in both shoots and roots. Phylogenetic analysis classified the plant *Mlo* members into six classes; four of them were comprised of angiosperm *Mlo* members, and the remaining two consisted of fern and moss *Mlo* members. The seven wheat *Mlo* members were classified into four angiosperm *Mlo* classes, similar to those of *Arabidopsis* and rice, indicating that the formation of each of the *Mlo* classes preceded the divergence of dicots and monocots. The differentiation of the expressional patterns among the seven *TaMlo* members was not related to their phylogenetic classification. This result suggested that the organ specific expression of individual *Mlo* members occurred relatively recently in their evolution.

Key words: *Mlo*, wheat (*Triticum aestivum*), gene family, RT-PCR, stress response

INTRODUCTION

The *Mlo* genes encode a plant-specific and sequence-diversified class of seven transmembrane (7-TM) proteins that form a multigene family in both monocot and dicot plants (Büschges et al., 1997; Devoto et al., 1999). To date, many *Mlo* homologs have been identified in various plants. The families of two model plants, *Arabidopsis thaliana* (Devoto et al., 2003) and rice (Liu and Zhu, 2008), whose whole genome sequences have been determined, contain 15 and 12 members, respectively. Liu and Zhu (2008) further reported that *Mlo* members can be divided into four groups based on their phylogenetic relationships.

With respect to function, *Mlo* was first defined as the locus controlling disease resistance to powdery mildew in barley (Jørgensen, 1992; Wolter et al., 1993). Homozygotes for the recessive allele (*mlo*) show a wide spectrum of resistance to the powdery mildew fungus, *Blumeria graminis* f.sp. *hordei* (Jørgensen, 1992). Expressional analyses in barley found that *Mlo* transcripts accumulate in response to infection with the fungus and that overexpression of *Mlo* results in supersusceptibility to the fungus (Wolter et al., 1993; Kim et al., 2002; Piffanelli et al., 2002). The accumulation of the *mlo* transcript was also detected in rice infected with blast fungi and in wheat injected with powdery mildew-derived carbohydrate (Piffanelli et al., 2002). Recently, using *Mlo* mutant alleles in barley, Reinstädler et al. (2010) revealed the regions of the MLO protein that are functionally important for resistance to powdery mildew. However, microarray analyses found that not only the *Mlo* genes but also more than 300 other genes showed expressional changes after powdery mildew infection in barley, and some of them were

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induced or repressed by infection with other pathogens, such as rust and blast, suggesting that the powdery mildew resistance induced by the *Mlo* genes is part of a complex response to various pathogens (Zellerhoff et al., 2010). The expression of *Mlo* was also increased during leaf senescence, by wounding, and by Paraquat treatment ((Piffanelli et al., 2002). These findings suggest that *Mlo* is likely to have a functional role in cell death protection during periods of biotic and abiotic stress (Wolter et al., 1993; Peterhänsel et al., 1997; Piffanelli et al., 2002). Less information is available about the function of the *Mlo* genes under abiotic stress compared with the amount of data available about their role under biotic stress, but the involvement of the *Mlo* genes in abiotic stress has been investigated in previous reports. Microarray profiling of NaCl-treated *Arabidopsis* roots showed that five of the 14 *Mlo* genes were up- or downregulated together with several other signal transduction genes (Jiang and Deyholos, 2006). Although the multiple response functions of *Mlo* were indicated in previous studies, the correspondence between their functional differentiation and subfamily diversification has not been clarified.

In wheat, three homoeologous cDNA of *Mlo* (*TaMlo-1A*, *-1B* and *-1D*) were isolated as homologs of barley *Mlo* (Elliott et al., 2002). Recently, we found that a wheat partial cDNA (WESR3) induced by salt stress (Nemoto and Sasakuma, 2002) had 48% identity with barley *Mlo* in terms of its amino acid sequence but that its nucleotide sequence was not significantly similar to any member of *TaMlo-1*. This implies the presence of an additional *Mlo* locus in wheat. Considering the multiplicity of the *Mlo* genes in other plant species and the large genome size of wheat, additional members of the *Mlo* family should exist in wheat. Identification of more *Mlo* members in wheat would be helpful for understanding the evolution and functional differentiation of *Mlo*. Since *Mlo* is a key gene for resistance to powdery mildew, which is one of the most serious wheat and barley diseases, the finding of new *Mlo* members would contribute to wheat and barley breeding. In addition, although various sequences of *Mlo* have been identified in barley, they were allelic to each other; in other words, the barley *Mlo* sequences reported belonged to one class of the *Mlo* gene family (Piffanelli et al., 2004, Tacconi et al., 2006, Liu and Zhu, 2008). Therefore, the discovery of new *Mlo* members in wheat would contribute to the study of *Mlo* in barley.

In this study, we performed an *in silico* search for *Mlo* members in wheat followed by PCR amplification using sequence information for the *Mlo* members of other plants. For all *Mlo* candidates, we conducted expressional analysis in various tissues under abiotic stress. Based on these results, the structural and functional differentiation of the *Mlo* gene family in wheat was discussed.

MATERIALS AND METHODS

***in silico* search for *Mlo* homologs** We searched for wheat *Mlo* homologs in two DNA databases, Wheat Gene Index ver. 11.0 (http://www.tigr.org) and GenBank (http://ncbi.nlm.nih), with the computer programs BLASTN and TBLASTN using published rice and *Arabidopsis Mlo* sequences (Devoto et al., 1999, Chen et al., 2006, Liu and Zhu, 2008) as queries. The BLAST search was performed with the following criterion: E-value < 1e-10. The obtained wheat *Mlo* homolog candidates were aligned and assembled into unified sequences with the software CAP3 (Huang and Madan, 1999). Then, the transmembrane domains of the protein sequences deduced from the unified sequences were predicted by HMMTOP (Tusnády and Simon, 2001) and Pepwindowall (Kyte and Doolittle, 1982). The presence of the *in silico* deduced unified sequences in the wheat transcriptome was verified by sequencing of the RT-PCR products amplified using spe-

Table 1. Information about the seven wheat *Mlo* members classified in this study

Member	Number of EST hits	Nucleotide length of unified sequence obtained *in silico*			Nucleotide length of the sequence determined by RT-PCR			Accession number
		5'-UTR	CDS	3'-UTR	5'-UTR	CDS	3'-UTR	
TaMlo-1[a]	26	197	1605	84	n.d.	n.d.	n.d.	AX063298
TaMlo-2	12	165	1497	198	18	1497	148	AB581575
TaMlo-3[b]	10	43	787	–	–	615	–	AB581579
TaMlo-4	11	–	1497	120	–	1497	56	AB581576
TaMlo-5	28	25	1476	165	3	1476	58	AB581580
TaMlo-6	4	169	1683	296	33	1683	112	AB581577
TaMlo-7	11	127	1494	328	91	1494	220	AB581578

[a] The nucleotide length and accession number of *TaMlo-1* indicate those of *TaMlo-1A* reported in Elliott et al. (2002). n.d.: not determined in this study.
[b] *TaMlo-3* was a partial sequence containing only the upstream half of the coding region.

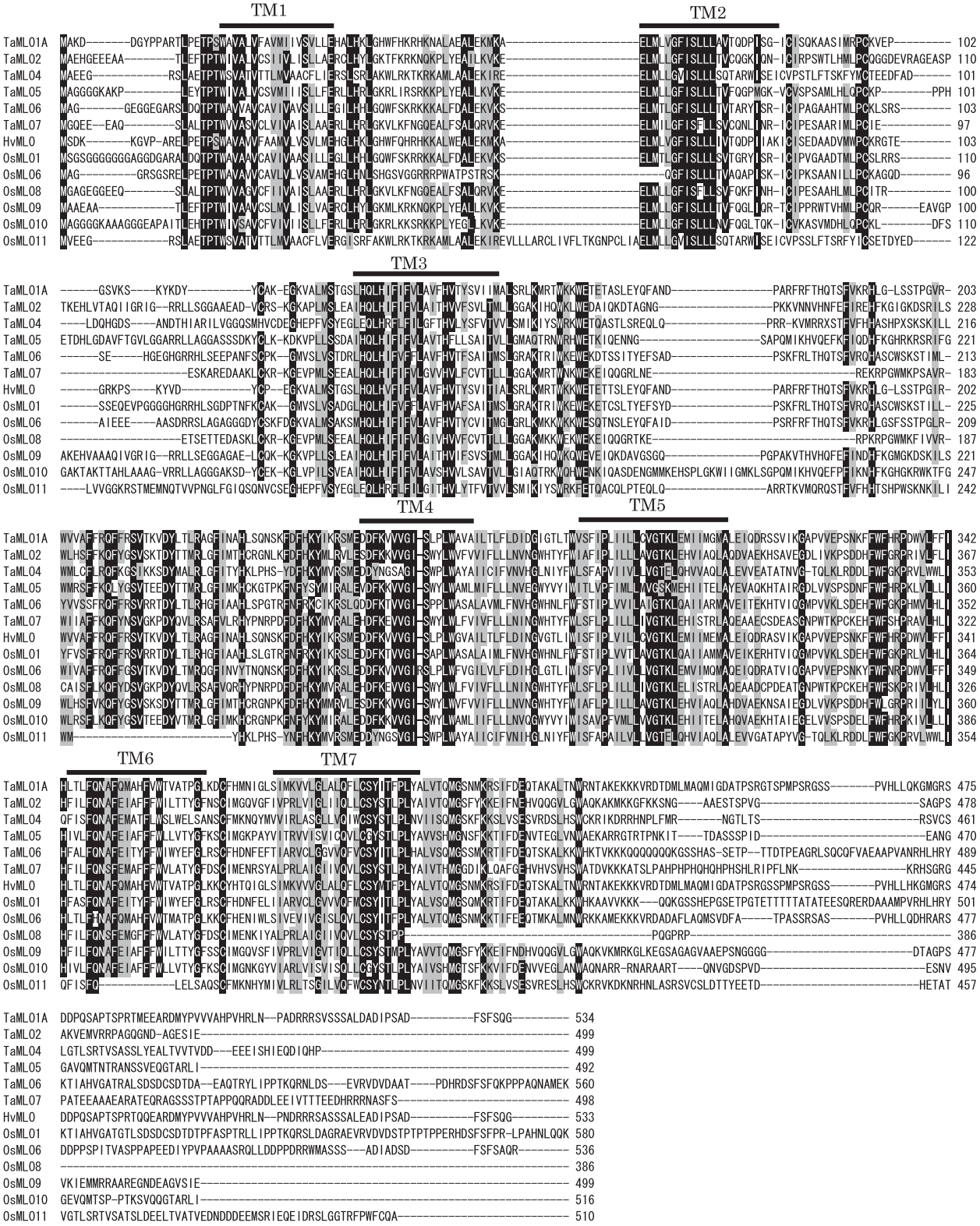


Fig. 1. Multiple alignment of the deduced amino acid sequences of the 13 *Mlo* members from wheat, barley, and rice. Although rice has 12 *Mlo* members (Liu and Zhu, 2008), five members were selected to represent the phylogenetically divergent groups of the *Mlo* gene family. The alignment was conducted by ClustalW. Consensus matches are indicated by darker shading for identities and light shading for conservative substitutions. The seven putative transmembrane domains of TaMLO-1A (TM1~TM7) predicted by HMMTOP are indicated by black bars above the sequences.

cific primers for each *Mlo* member.

Expressional analysis Seeds of *Triticum aestivum* L. cv. Chinese Spring (accession number KT020-003) provided by National BioResource Project (NBRP) KOMUGI were sterilized in NaClO solution (2.0% effective chloride) and sown on Murashige-Skoog (MS) medium (1% agar, supplemented with 150 mM NaCl or 2.5% mannitol) in sterilized glass bottles. The seedlings in the bottles were grown in a growth chamber under the following conditions: 20°C, 50% humidity, and 18 h illumination with fluorescent lights (170–240 $mmolm^{-2}s^{-1}$). Total RNA was extracted from two-week-old roots and shoots with TRIzol reagent. The first-strand cDNA was synthesized from 10 μg total RNA using oligo dT15 primers and the reverse transcriptase SuperScript III (Invitrogen). RT-PCR was carried out using 10 pmol of each of the primers for the wheat *Mlo* members and the α-tubulin gene in a 20 μl reaction mixture containing 0.5 units of a Takara *Taq* polymerase. The sequences of the member-specific primers used for the expressional analysis were AAGTTCTTCTGGTTCCACCG and TGGCTGAAGGAAAAATCTGC for *TaMlo-1*, CCATCGGATGACCACTTCTG and TTGCCCCCAATGTTTTACGG for *TaMlo-2*, CTGCTCCTGCTGGGCTTTGG and CTCCACCTGCCAGGAGTCTC for *TaMlo-3*, TCCAGAATGCCTTTGAGATG and CAGTCAGCGCCTCATACAG for *TaMlo-4*, GGATCACCTTGGTTCCATTC and ATCATGGGGAAACCAGCATA for *TaMlo-5*, GTCACGGCGAGGTACATATC and GCTGCCTCACGAAAGAAGTC for *TaMlo-6*, and CGGCACGAGGCTAAGATGAG and CTCATCTTAGCCTCGTGCC for *TaMlo-7*. The PCR conditions were as follows: 5 min at 94°C; 24 cycles of 30 sec at 94°C, 30 sec at 60°C, and 30 sec at 72°C; and 5 min at 72°C. The amplified products were stained in a gel with SYBR® GOLD (Molecular probe) and were quantified based on their fluorescence intensity using an FLA5000 and the Image Gauge software (Fujifilm). The amount of α-tubulin was used as an internal control to calculate the relative amounts of *Mlo* expression. Quantification by RT-PCR was conducted using three separate plants for each treatment, and the measurements were averaged.

Phylogenetic analysis Multiple alignment between

Table 2. Amino acid sequence identity matrix of *TaMlo* genes and their homologs in rice and barley

Gene	*TaMlo-1*[a]	*TaMlo-2*	*TaMlo-4*	*TaMlo-5*	*TaMlo-6*	*TaMlo-7*
within wheat						
TaMlo-1	–					
TaMlo-2	0.328	–				
TaMlo-4	0.260	0.317	–			
TaMlo-5	0.321	0.516	0.278	–		
TaMlo-6	0.405	0.342	0.295	0.304	–	
TaMlo-7	0.304	0.413	0.285	0.360	0.304	–
between wheat and barley						
HvMlo	0.851	0.346	0.268	0.323	0.415	0.310
HvMlo2	0.662	0.345	0.261	0.323	0.395	0.296
between wheat and rice						
OsMlo-1	0.413	0.342	0.296	0.308	0.780	0.309
OsMlo-2	0.319	0.708	0.334	0.525	0.344	0.409
OsMlo-3	0.634	0.340	0.259	0.317	0.399	0.299
OsMlo-4	0.245	0.287	0.349	0.235	0.275	0.268
OsMlo-5	0.291	0.507	0.293	0.539	0.294	0.379
OsMlo-6	0.600	0.338	0.265	0.316	0.383	0.313
OsMlo-7	0.287	0.400	0.252	0.360	0.286	0.478
OsMlo-8	0.271	0.410	0.282	0.353	0.286	0.663
OsMlo-9	0.196	0.472	0.209	0.295	0.198	0.266
OsMlo-10	0.305	0.502	0.285	0.684	0.315	0.370
OsMlo-11	0.214	0.257	0.670	0.227	0.254	0.241
OsMlo-12	0.282	0.232	0.188	0.206	0.323	0.212

[a] The values for *TaMlo-1* are means of those for the three homoeologous genes.

the deduced amino acid sequences of the wheat *Mlo* members identified in this study and those of 2 barley (Devote et al., 2003), 15 *Arabidopsis* (Chen et al., 2006), 12 rice (Liu and Zhu, 2008), 8 fern (*Selaginella moellendorffii*), and 10 moss (*Physcomitrella patens*) *Mlo* members was performed using CLUSTALW (Thompson et al., 1994). The fern and moss *Mlo* members were obtained from the files "Selmo1_GeneModels_AllModels_20071019_aa.fasta" and "proteins.Phypa1_1.FilteredModels.fasta.gz", which were downloaded from the JGI ftp site (ftp://ftp.jgi-psf.org/pub/JGI_data/). They were not annotated, but we identified them as *Mlo* members by a BLAST search, as described above. The amino acid sequence similarity between each pair of *Mlo* members was calculated by MEGA4 (Tamura et al., 2007). Based on the amino acid sequence similarity, a neighbor-joining (NJ) phylogenetic tree of the plant *Mlo* members was constructed using MEGA4 (Tamura et al., 2007). Bootstrap tests were conducted using 1,000 replicates.

RESULTS

Identification of additional members of the *Mlo* family in wheat Our *in silico* search of public databases using rice and *Arabidopsis Mlo* family members as queries detected a total of 102 wheat sequences that showed significant similarity to the *Mlo* members of the other plants. However, none of them, except three sequences known as *TaMlo-1*, had been annotated as *Mlo* genes. Among the 102 sequences, 26 were identified to be partial sequences of *TaMlo-1* (Table 1). Based on their sequence homology, the remaining 76 sequences were assembled into six unified sequences, and we tentatively named them *TaMlo-2, 3, 4, 5, 6,* and *7*. All of the unified sequences coded for almost full-length sequences with partial UTR sequences, except for *TaMlo-3* (Table 1). Based on sequence alignment, specific primers for each of the *TaMlo* genes were designed. To verify the presence of the unified sequences obtained *in silico*, we conducted RT-PCR using specific primers and determined the (almost) full sequences of the cDNA for all *TaMlo* genes, except for *TaMlo-3*. The comparisons with the barley and rice *Mlo* members are shown in Fig. 1 and Table 2. The determined cDNA sequences of the *TaMlo* genes were deposited in DDBJ, and the accession numbers are listed in Table 1.

We deduced the amino acid sequences of the *TaMlo* genes except *TaMlo-3* and revealed that all sequences contained seven transmembrane domains, which is a characteristic feature of MLO proteins (Figs. 1 and 2). The amino-acid sequence similarity between the *TaMlo* members varied from 26.0% (between *TaMlo-1* and

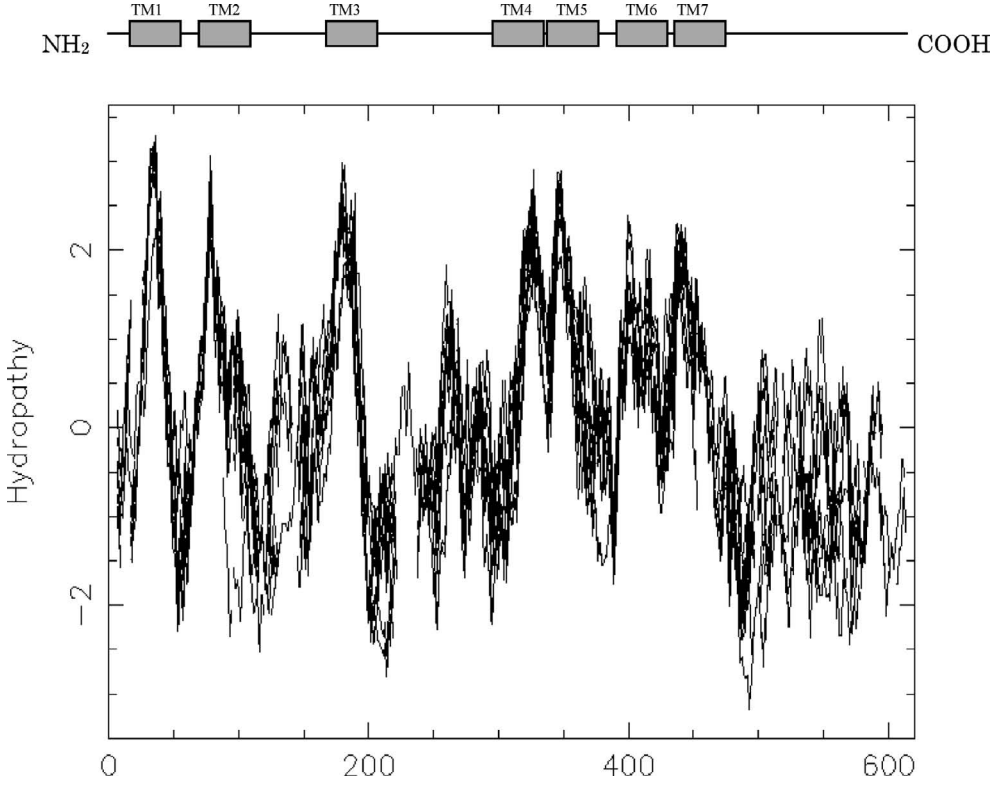


Fig. 2. Hydropathy plotting of the 13 Gramineae *Mlo* members shown in Fig. 1 obtained using Pepwindowall. The lines almost overlapped, showing the same pattern of hydropathy change. The seven hydropathy peaks in the panel accord with the positions of the seven putative transmembrane regions in TaMLO-1A predicted by HMMTOP shown above.

TaMlo-4) to 51.6% (between *TaMlo-2* and *TaMlo-5*) (Table 2). As shown in Fig. 1, the transmembrane domains were relatively conserved among the *Mlo* members. The level of similarity between *TaMlo* members was comparable to that between the *Mlo* members of rice and between those of *Arabidopsis*. A sequence comparison revealed that WESR3, the gene isolated as a salt-responding gene by Nemoto and Sasakuma (2002), was a partial sequence of *TaMlo-2*.

Expressional analysis of the *TaMlo* genes The expression profiles of the *TaMlo* genes under salt and osmotic stress were investigated by RT-PCR (Fig. 3). *TaMlo-1* and *TaMlo-2* showed constitutive expression in roots and shoots under both stress and control conditions. *TaMlo-3* and *TaMlo-4* exhibited root-specific expression under both stress and control conditions. Among the *TaMlo* members, *TaMlo-5* showed the highest expression in roots and shoots under both stress and control conditions. *TaMlo-6* showed the weakest expression among the *TaMlo* members, and its expression increased in roots under salt stress and in shoots under osmotic stress (Fig.

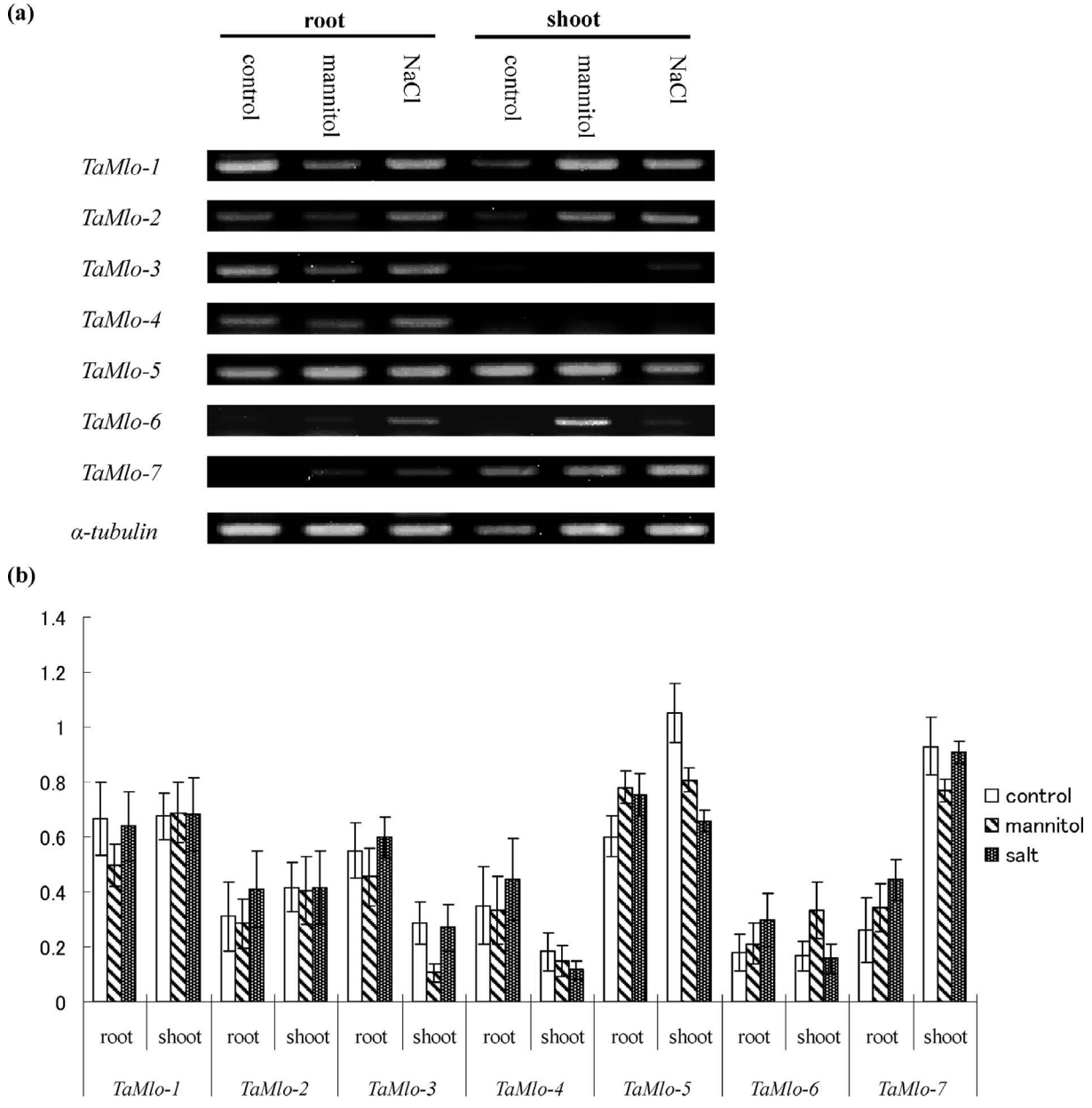
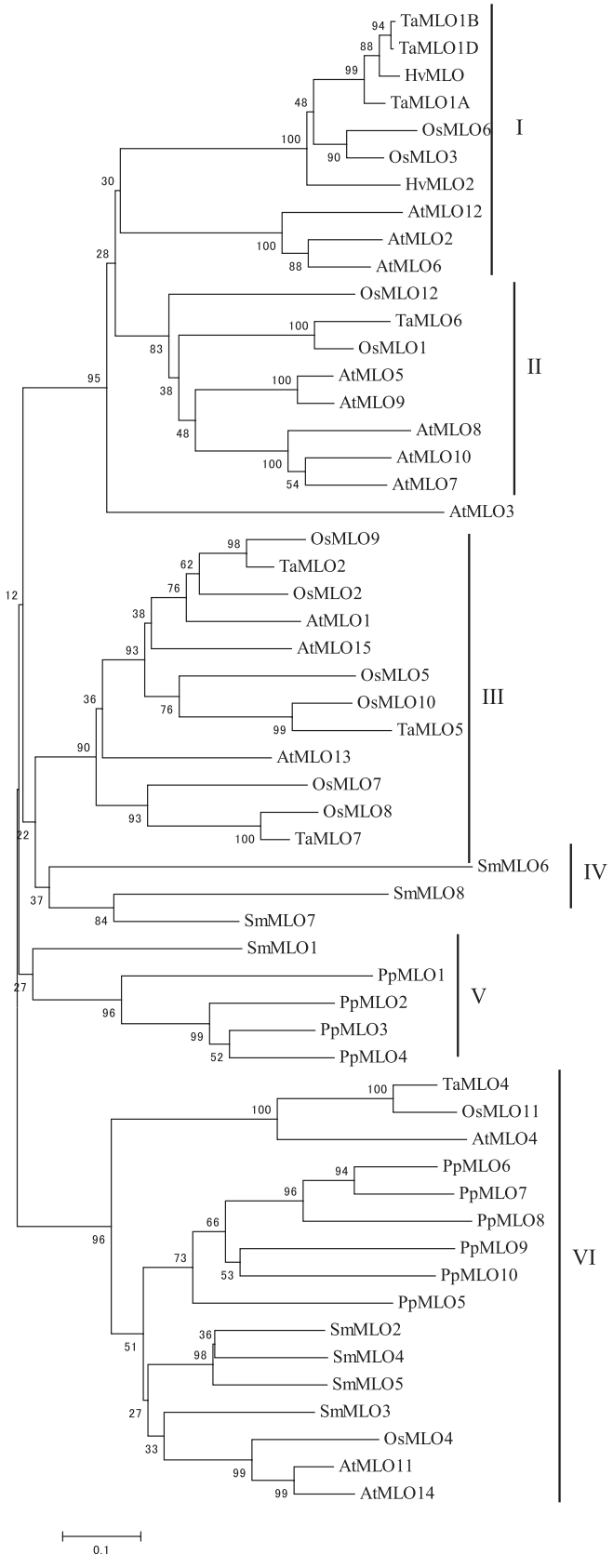


Fig. 3. Expression profiles of the seven *TaMlo* members in roots and shoots under salt and osmotic stress conditions. **(a)** Electrophoresis profiles of the RT-PCR products of the *TaMlo* members and **(b)** quantification of the expression of *TaMlo* members by RT-PCR shown as a bar graph with error bars.



3), although the increase was not significant. *TaMlo-7* was predominantly expressed in shoots and demonstrated slightly inducible expression in roots under both salt and osmotic stresses, although the induction level was not statistically significant (Fig. 3). Thus, none of the *TaMlo* members showed significant increases or decreases in response to stress conditions.

Phylogenetic analysis To clarify the phylogenetic relationships among the *Mlo* family members of wheat (except *TaMlo-3*) and other plants, a phylogenetic tree consisting of 55 *Mlo* members of dicots, monocots, a fern, and a moss was constructed (Fig. 4). In the phylogenetic tree, the members were mainly divided into six major clusters, Classes I - VI. Classes I, II and III consisted of only angiosperm *Mlo*. Classes IV and V consisted of only fern and moss *Mlo*. Class VI was composed of various *Mlo* from all plant species. The four major *Mlo* clusters reported by Liu and Zhu (2008) correspond to Classes I, II, III, and VI in this study, and each of the major clusters includes *Arabidopsis* and rice *Mlo* members.

The seven *TaMlo* members were classified into four clusters. *TaMlo-1*, *TaMlo-4*, and *TaMlo-6* belonged to Class I, Class VI, and Class II, respectively. *TaMlo-2*, *TaMlo-5,* and *TaMlo-7* were included in Class III, and each had rice orthologs (Fig. 4). Although a partial *TaMlo-3* sequence was excluded from the phylogenetic analysis, its sequence homology indicated that *TaMlo-3* should be placed in Class III.

DISCUSSION

Characteristics of wheat *Mlo* The level of sequence similarity (Table 2) and phylogenetic relationships between the plant *Mlo* members demonstrated that the seven identified wheat *Mlo* members were not homoeologous to one another, in spite of the hexaploid nature of wheat. Since *Arabidopsis* and rice, which are diploid species, possess 15 and 12 *Mlo* members, respectively, wheat, which has a larger genome than these species, should have more *Mlo* members. The reason we only identified seven *Mlo* members in this study is probably because not all *Mlo* members are expressed constitutively in wheat. Chen et al. (2006) revealed that some of the *Mlo* members in *Arabidopsis* are only expressed in single specific organs such as the inflorescence. In this study,

Fig. 4. A phylogenetic tree based on the amino acid sequences of the *Mlo* members of 55 land plants constructed by the neighbor-joining method. The numbers next to the nodes represent the bootstrap percentages after 1,000 replications. Scale bars are shown below the tree. *AtMlo*s, *OsMlo*s, *SmMlo*s, and *PpMlo*s are the *Mlo* members obtained from *Arabidopsis thaliana*, rice (*Oryza sativa*), fern (*Selaginella moellendorffiif*), and moss (*Physcomitrella patens*), respectively.

we also found organ specific expression of *Mlo*. It is noteworthy that the seven wheat *Mlo* members covered the four major groups of angiosperm *Mlo*. Presumably the *Mlo* member-specific primers we designed in this study amplified the three homoeologous loci simultaneously.

Evolution of plant *Mlo* In the phylogenetic tree we produced, the plant *Mlo* members were divided into six classes; four of them included members of both monocot and dicot plants. This indicated that the formation of *Mlo* classes preceded the divergence of monocot and dicot plants. In each class, the genetic relationships between the sequences were generally consistent with the phylogeny of the plant species. For example, the wheat *Mlo* members were closer to the rice *Mlo* members than to the *Arabidopsis* members in each cluster. In Class VI, the members of three angiosperm species formed a cluster and joined with the cluster of the fern *Mlo* members, and the moss *Mlo* members were located as an outgroup of the *Mlo* members of vascular plants. These results indicated that the *Mlo* members of each class were generally conserved during the evolution of land plants. However, in view of the relationships of the Gramineae family, there are inconsistencies. In Class I, the barley *Mlo* gene was placed in a cluster with three homoeologous wheat *Mlo* members. This result might have been caused by differences in the evolutionary rate among the homoeologous loci. Since the Class VI *Mlo* were found in the angiosperm, fern, and moss species, they may be regarded as the most conserved group of *Mlo* with functional importance in land plants. Classes IV and V are specific to ferns and mosses. Ferns and mosses are not a monophyletic group, and therefore, the loss of these classes from the angiosperm lineage is more likely to have occurred than their gain in the fern and moss lineage.

Each class contained various numbers of *Mlo* members from a single species. For example, Class II had five members from *Arabidopsis* and two from rice, and Class III included six members from rice and three from *Arabidopsis*. Four of the seven wheat *Mlo* members, including the partial sequence of *TaMlo-3*, belonged to Class III. The relatively larger number of Class III members in wheat, as was found in rice and maize (Liu and Zhu, 2008), suggests the occurrence of an increase in Class III members in the monocot or Gramineae lineage.

The barley *Mlo* members, which are the key genes responsible for powdery mildew resistance (Jørgensen, 1992; Wolter et al., 1993), were included in Class I together with the wheat homologs *TaMlo-1*, as reported by a previous study (Liu and Zhu, 2008). All of the other wheat *Mlo* members were newly identified in this study and were classified into different classes from the barley *Mlo*.

Expression of wheat *Mlo* Based on their expression patterns, the *TaMlo* members were classified into four types. The first type comprised *TaMlo-1*, *TaMlo-2*, and *TaMlo-5*, which were expressed constitutively in both roots and shoots. The second and the third types consisted of *TaMlo-3* and *TaMlo-4*, which displayed root-specific expression, and *TaMlo-7*, which displayed shoot-specific expression, respectively. The fourth type comprised *TaMlo-6* and displayed weak expression. In the phylogenetic tree, the seven wheat *Mlo* members were also classified into four classes, but their expression profiles did not correspond to their phylogenetic relationships. Three Class III *TaMlo* members, *TaMlo-2*, *TaMlo-5*, and *TaMlo-7*, showed different expression patterns from each other, and the two members with root-specific expression, *TaMlo-3* and *TaMlo-4*, were classified into Classes III and V, respectively. Also, in *Arabidopsis*, the organ-specific expression patterns of the *Mlo* genes did not correspond to the phylogenetic relationships among the *Mlo* members (Chen et al., 2006). These facts suggest that differentiation of the expression patterns of *Mlo* classes occurred after they had diverged and that organ-specific expression developed independently in the respective species.

As for salt and osmotic stress, neither significant induction nor a significant reduction in the expression of the *TaMlo* genes was observed in this study. Even *TaMlo-2*, a homolog of WESR3 that was isolated as an early salt-responding gene in wheat (Nemoto and Sasakuma, 2002), showed no significant expressional change in response to salt or osmotic stress. This suggests that *TaMlo*(s) responds to salt stress quickly but not continuously, as reported for the salt and osmotic responses of the *Mlo* genes in *Arabidopsis* (Chen et al., 2006). In fact, the responses of the *Mlo* genes to biotic and abiotic stresses are various (early, late, or continuous) and depend on the type of stress; for example, wounding causes a rapid increase in the expression of the *Mlo* genes in *Arabidopsis*, whereas fungal infection induces the expression of the *Mlo* genes five days after infection in *Arabidopsis* and barley (Jarosch et al., 2003; Chen et al., 2006). These diverse *Mlo* responses make it difficult to understand the mechanism and functional role of the *Mlo* genes.

In this study, we demonstrated that at least seven members of the *Mlo* family are present in wheat (actually at least 21 if the hexaploidy of bread wheat is considered), and some of them showed organ-specific expression. The *Mlo* gene is a key gene for resistance to powdery mildew in barley (Jørgensen, 1992; Wolter et al., 1993). The finding of novel *Mlo* loci and clarification of their expression profiles will aid future wheat breeding aimed at powdery mildew resistance.

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