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Preferential expression of a *HLP* homolog encoding a mitochondiral L14 ribosomal protein in stamens of common wheat

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Abbreviations: cDNA, DNA complementary to RNA; CaMV, cauliflower mosaic virus; CMS, cytoplasmic male sterility; CS, Chinese Spring; *cr*, *Aegilops crassa*; CSdt7BS, ditelosomic7BS line of CS; GFP, green fluorescent protein; HLL, HUELLENLOS; HLP, HUELLENLOS PARALOG; WHLP, wheat HLP homolog; mt, mitochondria; MTS, mt-targeting signal; ORF, open reading frame; RT-PCR, reverse transcriptase-PCR; *Rfd1*, fertility restorer gene against the *Ae. crassa* cytoplasm; 7BS, short arm of chromosome 7B; 7BL, long arm of chromosome 7B

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Abstract

Interaction between nucleus and cytoplasm has essential roles in plant development including that of floral organs. We isolated a wheat homolog *Whlp* of *Arabidopsis HUELLENLOS PARALOG* (*HLP*) gene encoding a mitochondrial (mt) ribosomal protein L14 that plays a role in ovule development. Transient expression analysis using the GFP fusion protein showed that 50 amino residues located on the N-terminal of the WHLP protein acted as a mt-targeting signal. Expression patterns of the *Whlp* gene were compared among floral organs of alloplasmic lines of wheat, in which intrinsic cytoplasms were replaced by the cytoplasm of a wild relative, *Aegilops crassa*. In these alloplasmic lines, pistillody (homeotic transformation of stamens into pistil-like organs) is induced by the alien cytoplasm in the absence of nuclear restorer genes. The *Whlp* transcripts preferentially accumulated in stamens compared with pistils, leaves and roots. The expression level of *Whlp* in the pistillate stamens of the alloplasmic lines was similar to that in genuine pistils of both euplasmic lines and fertile alloplasmic lines. The result suggested that the elevated expression of the *Whlp* gene plays a role in aiding the development of male reproductive organ but not in the determination of its whorl identity. A comparable expression pattern was observed in another nuclear encoded mt ribosomal protein gene but not in a mt-encoded gene. The different expression patterns of different mt ribosomal protein genes suggest that abundance of mt ribosomal proteins is differentially regulated in the organ/tissue development in wheat.

1. Introduction

Information on the roles of mitochondrial (mt) genes in the plant development is limited. This is mainly due to the fact that most mutations of mt genes result in serious respiratory defects and thus lethality in plants. One exceptional case is found in a maize mutant *Non-Chromosomal Stripe3*, which lacks functional mt *rps3* and *rpl16* genes and shows abnormal development of leaves and kernels (Hunt and Newton, 1991). Cytoplasmic male sterility (CMS) lines are another examples that show abnormalities only in the anther and pollen development. CMS phenotypes were reported in more than 150 plant species (Kaul, 1988), and they have provided powerful experimental tools for understanding the relationship between mt function and male reproductive organ development. In CMS lines, abnormal transcripts of mt genes are often derived from inter- or intra-molecular recombinations in the mt genome, and the expression of novel recombinant mt genes and accumulation of mt proteins leads to the abnormal anther and pollen development (Conley and Hanson, 1995; Levings, 1993; Laver et al., 1991).

Unusual types of the CMS lines have been reported in wheat, tobacco and carrot, in which severe pistillody (homeotic transformation of stamens into pistil-like organs) is induced by the alien cytoplasms (Murai et al., 2002; Zubko et al., 1996; Zubko, 2004; Linke et al., 2003). An alloplasmic line of common wheat (*Triticum aestivum* L.) cultivar 'Norin26' (N26) with cytoplasm of its wild relative *Aegilops crassa* (abbreviated as (*cr*)-N26) shows pistillody under long day conditions (>15 h day length) (Murai and Tsunewaki, 1993). On the other

hand, an alloplasmic line of cultivar ‘Chinese Spring’ (CS) with the same *Ae. crassa* cytoplasm [(*cr*)-CS] does not show such developmental defect, because this line has the fertility restorer gene (*Rfd1*) against the *Ae. crassa* cytoplasm locating on the long arm of chromosome 7B (Murai and Tsunewaki, 1994). The pistillody is observed in an alloplasmic line of ditelodisomic-7BS of CS [(*cr*)-CSdt7BS], which lacks both long arms of chromosome 7B (Murai et al., 2002). Furthermore, not only pistil development but also ovule development becomes defective and even female fertility is lost in this line. Normal integuments, which develop into seed coat, are not formed in the pistils in the (*cr*)-CSdt7BS line. All these abnormalities observed in the (*cr*)-CSdt7BS line should be ascribed to the incompatibility between nuclear and cytoplasmic genomes, because the euplasmic CSdt7BS line with the intrinsic CS cytoplasm shows normal flower development. A recent report has indicated that expression of nuclear genes involved in plant morphogenesis can be affected by the nuclear-cytoplasmic interaction in wheat, tobacco and carrot (Zubko, 2004). *In situ* hybridization study of class-B MADS-box genes has clearly showed that the pistillody in the alloplasmic wheat lines is caused by alterations in their expression patterns (Hama et al., 2004).

Arabidopsis HUELLENLOS (*HLL*) nuclear gene encodes mt ribosomal protein L14 (Skinner et al., 2001). In the *hll* mutant, ovule integuments fail to initiate and the female gametogenesis ceases soon after formation of tetrad megaspores (Schneitz et al., 1997; Schneitz et al., 1998; Skinner et al., 2001). A distal domain of ovule primordia is severely malformed in the *hll* mutant and it frequently exhibits cell collapse and degeneration. The

predominant and specific defect in ovule development in the *hll* mutant indicates that mitochondria have pivotal roles in the female reproductive organ development in *Arabidopsis*. The female sterile *hll* mutant, however, shows normal male fertility, plant vigor and morphology, presumably because of the presence of its paralog (*HUELLENLOS PARALOG; HLP*) in the *Arabidopsis* genome (Skinner et al., 2001). *HLL* and *HLP* are the only candidates to encode the mt L14 proteins in *Arabidopsis*. Furthermore, female fertility can be restored by the over-expression of *HLP* in the *hll* mutant. The functional redundancy indicates that *HLL* and *HLP* have similar or identical functions although their expression patterns are well differentiated (Skinner et al., 2001). The redundancy and differential expression patterns of the two genes allow male fertility in the *hll* mutant.

In this study, we isolated a wheat *HLP* homolog *Whlp* encoding a mt ribosomal protein L14. Comparative expression analysis of the *Whlp* gene in the alloplasmic and euplasmic lines revealed preferential expression of the *Whlp* gene in stamens and suggested its role in supporting the male organ development. Comparison of the expression patterns of the *Whlp* gene and another nuclear- and mt-encoded mt ribosomal protein genes suggested that mt ribosome activity during the organ/tissue development is differentially regulated by both nuclear- and mt-encoded genes in wheat.

2. Materials and methods

2.1. Plant materials

Three common wheat (*Triticum aestivum* L.) cultivars, ‘Chinese Spring’ (CS), ‘Norin26’ (N26) and ‘Norin61’ (N61), and a ditelosomic7BS line of CS (CSdt7BS) were used in this study. All these euplasmic lines with intrinsic cytoplasms develop normal and fertile reproductive organs. Alloplasmic lines with the cytoplasm of a wild relative, *Aegilops crassa*, used in the study were respectively abbreviated as (*cr*)-CS, (*cr*)-N26, (*cr*)-N61 and (*cr*)-CSdt7BS. The (*cr*)-N26 shows pistillody when grown under the long-day condition, and exhibits partial pistillody under the short-day condition (Murai and Tsunewaki, 1993). Both (*cr*)-CS and (*cr*)-N61 show normal male fertility because of their possession of fertility restorer gene(s) (*Rf*) against the *Ae. crassa* cytoplasm in their nuclear genome (Murai, 1997). CS has a single dominant *Rfd1* on the long arm of chromosome 7B (7BL) and N61 has multiple *Rf* genes. A CS monotelodisomic line of chromosome 7BS with the *Ae. crassa* cytoplasm [(*cr*)-CSmt7BS] is hemizygous for *Rfd1* and partially fertile. The (*cr*)-CSdt7BS, which is generated from a cross of (*cr*)-CSmt7BS with CSdt7BS, shows not only pistillody but also female sterility, independently of the day-length (Murai et al., 2002). A *Fr* mutant of (*cr*)-N26, which was obtained after EMS mutagenesis of (*cr*)-N26 and shows no pistillody even under the long-day condition (Murai et al., 1995), was used in the expression analysis.

Nulli-tetrasomic lines of CS produced by Sears (1966), three accessions of ancestral diploid species (*Ae. squarrosa*, *T. urartu* and *Ae. speltoides*) and tetraploid wheat (*T. durum* cv. ‘Langdon’) were used in PCR and DNA gel blot analysis. Single plants were grown in the

green house, and their total DNA was extracted.

2.2. Isolation and sequencing of Whlp *gene*

A following primer set was designed for amplification of the *Whlp* gene based on a nucleotide sequence of EST clones: 5'-TAGGCAGAGAGCAGGGTGAA-3' as an upper primer and 5'-CCTTGAGATGTCCATTTTCC-3' as a lower primer. DNA of CS and cDNA synthesized from CS pistils were used as templates to amplify the genomic and cDNA sequences of the *Whlp* gene, respectively. PCR and reverse transcriptase (RT)-PCR products were cloned into pGEM-T vector (Promega, USA). Nucleotide sequences of the PCR products were determined by the automated fluorescent dye deoxy terminator cycle sequencing system with ABI PRISM^{TM} 310 Genetic Analyzer (PE Applied Biosystems, USA). The nucleotide and deduced amino acid sequences were analysed using DNASIS (Hitachi, Japan) and the sequence homology was searched with the BLAST algorithm (Karlin and Altschul, 1993). A multiple alignment was calculated by Waterman's algorithm (Waterman, 1986) and a phylogenetic tree was constructed by the unweighted pair group method with arithmetic mean (UPGMA method) (Sneath and Sokal, 1973).

2.3. RT-PCR, Southern and northern blot analyses

Total RNA was extracted from floral tissues (pistils, stamens and lodicules) and vegetative

tissues (seedling leaves and roots) from the euplasmic and alloplasmic wheat lines. cDNA was synthesized from DNaseI-treated RNA with Rever-Tra Ace α (TOYOBO, Japan) and subjected to RT-PCR analysis. The gene-specific primer set for *Whlp* was already described. Primer sets for amplification of two wheat mt ribosomal protein genes, *TaMRPL5* and *TaRPL11*, and a ubiquitin gene were designed as follows: 5'-AAACTGAATCACGCCAATGT-3' and 5'-GCCGCTCCACAGTAGTAAAG-3' for *TaMRPL5*, 5'-ACAATCCGCCTCCTAGTTCC-3' and 5'-GCCGTGCCGATGATGGACTT-3' for *TaMRPL11*, and 5'-GCATGCAGATATTTGTGAA-3' and 5'-GGAGCTTACTGGCCAC for the ubiquitin gene. The PCR-amplified products were separated by electrophoresis through 2% agarose gel and stained with ethidium-bromide. The relative transcript abundance of different RNA samples was assessed based on their signal strengths compared with those of the pistil samples of CS or N61 using NIH IMAGE 1.61 software.

Total DNA was digested with *Eco*RI for Southern blot analysis, resolved through 0.8% agarose gel and blotted onto nylon membranes. The membranes were hybridized with ^{32}P-labeled gene-specific probes. For northern blot analysis, 20 μg of total RNA was resolved on denaturing gel containing 5% of formaldehyde and blotted onto nylon membranes. The protocols for Southern and northern blot analyses were as described (Takumi et al., 2000).

2.4. Transient GFP assay

To confirm the mt localization of the WHLP protein, the *Sal*I and *Nco*I linkers were added to the entire ORF of the *Whlp* gene without the stop codon and also to the first 50-amino acid region corresponding to the putative leader sequence. The high-fidelity DNA polymerase KOD-plus (TOYOBO, Japan) was used for the PCR amplification and the PCR products were cloned into *Sal*I and *Nco*I site of the sGFP(S65T) vector (Chiu et al., 1995) to produce fusion protein constructs with the green fluorescent protein (GFP). An anchor sequence of six glycine residues was inserted only between the *Whlp* ORF and the *gfp* gene. The two constructs were introduced into onion epidermal cells via particle bombardment. Bombardment conditions were described in Takumi et al. (1994). Mitochondria of the epidermal cells were stained by MitoTracker red (Molecular Probe, USA). The fluorescence of the MitoTracker and the GFP images of mitochondria were observed under fluorescence light microscopy (Olympus, Japan).

3. Results and discussion

3.1. Cloning of Whlp *gene*

To obtain wheat homolog of *HLL* or *HLP*, we searched the wheat EST database using the *Arabidopsis HLL* sequence. Two wheat EST clones (whdl26n11, whr6k11) were selected, which showed respectively 36.8% and 36.4% sequence identities to the *HLL* gene at the

nucleotide level and contained a single open reading frame (ORF) putatively encoding a polypeptide with 100% identity. A cDNA sequence and genomic sequence were obtained by PCR-amplification with the gene-specific primers designed according to the two EST sequences. We named this gene *Whlp* (wheat *HUELLENLOS PARALOG*). Comparison of the genomic sequence with the cDNA sequence showed that the *Whlp* gene contained three exons interrupted by two introns (Fig. 1A). The *Whlp* gene encodes a polypeptide of 170 amino acid residues. Carboxy-terminal 120 residues showed the highest similarity to plant mt L14 protein families (Fig. 1B). The identity of this amino acid sequence of the L14 protein region was 62.5%, 68.3%, 95.8% and 98.3% to *Arabidopsis* HLL and HLP, and HLL homologs of rice and barley, respectively. Wheat WHLP was a bit more similar to *Arabidopsis* HLP than HLL in the overall structure, suggesting that the *Whlp* gene is an ortholog of *Arabidopsis HLP* rather than *HLL*. A phylogenetic tree based on the amino acid sequence revealed that WHLP was grouped into the other plant mt and eubacterial L14 family and highly diverged from the chloroplast and cytosolic L14 families (Fig. 2). Amino-terminal 50 residues of the WHLP protein showed no homology to any known sequences except for the same N-terminal region of the rice and barley HLL homologs (68% and 90% sequence identity, respectively). The homology within the N-terminal 50 residues was much lower than that in the L14 region among the cereal HLL homologs.

To study copy number of the *Whlp* gene in the wheat genome, Southern blot analysis was conducted using total DNA extracted from a single accession each of three diploid relatives and tetraploid and hexaploid wheat. Southern blot probed with the *Whlp* cDNA showed one,

two and three bands in the genomes of diploid, tetraploid and hexaploid wheat, respectively (Fig. 3A). The result shows that the common wheat genome possesses three homoeologous loci of the *Whlp* gene, one in each of the three component A, B and D genomes. The wheat genome, therefore, seems not to have other *Whlp* paralogs. Two bands observed in *T. urartu* was likely due to the presence of one *Eco*RI site within its genomic sequence. To assign the three *Whlp* loci to homoeologous wheat chromosomes, aneuploid analysis was performed using a series of nulli-tetrasomic lines. In nulli2B-tetra2A, a middle fragment was missing and an upper fragment was thicker than the other two fragments (Fig. 3B). A lower fragment was missing in nulli2D lines. These results indicated that the three bands respectively represented the three homoeologous loci on chromosomes 2A, 2B and 2D in the common wheat genome. PCR analysis with the *Whlp*-specific primer set using the nulli-tetrasomic lines showed no amplification of the sequence in the lines lacking chromosome 2D and a higher level of amplification in nulli2B-tetra2D than in nulli2B-tetra2A (Fig. 3C). The result suggests that the *Whlp* gene we isolated represents a locus on wheat chromosome 2D.

3.2. WHLP protein was localized in the mitochondria

The C-terminal 120 residues of the WHLP protein showed considerable homology with the corresponding region of other plant mt L14 proteins, strongly suggesting that the *Whlp* gene encodes a mt-targeted ribosomal protein L14. Therefore, a mt-targeting signal should

be located at the N-terminal region of the WHLP protein. However, this region of WHLP showed a little sequence identity to that of *Arabidopsis* HLL or HLP (Fig. 1B). To confirm the mt localization of the WHLP protein, we constructed a chimeric gene consisting of the full length *Whlp* ORF fused to the *gfp* gene under control of the CaMV 35S promoter. The *CaMV35S::WHLP-GFP* construct was introduced into epidermal cells of onion by particle bombardment. The transient expression of the GFP fusion protein clearly demonstrated mt-targeting of the WHLP protein (Fig. 4A). The GFP fluorescence from the *CaMV35S::WHLP-GFP* construct, however, was weaker compared with a positive control containing a mt-signal peptide from *Arabidopsis* γ-ATPase. We therefore fused the predicted N-terminal signal peptide of 50 amino acid residues directly to the GFP protein. The chimeric construct (*CaMV35S::MTS-GFP*) was similarly introduced into the onion epidermal cells, and the transient expression of GFP was observed. Strong GFP signals were now observed in the mitochondria, which corresponded to the red fluorescence signals of the MitoTracker (Fig. 4B). The result demonstrates that the 50 residues of the N-terminal region of WHLP functions as a mt-targeting signal and that this region is sufficient to transport the fused protein into mitochondria.

In plant evolution, large fractions of mt genome have been transferred into nuclear genomes, and many of such mt-derived nuclear genes have acquired the mt-targeting signals by recombination and duplication events (Kadowaki et al., 1996; Kubo et al., 2000; Notsu et al., 2002). An insertion site of the second intron in the *Whlp* gene is located just after the mt transit peptide (Fig. 1B). The two intron insertion sites of the wheat *Whlp* gene was nearly

conserved in *Arabidopsis HLL* and *HLP* (Fig. 1B). However, no sequence homology in the mt-targeting signals was observed between monocots and dicots, suggesting that the translocation event of plant L14 gene from the mt genome to the nuclear genome might have independently occurred after the diversification of monocot and dicot species.

3.3. Expression analysis of Whlp *and other wheat ribosomal protein genes*

Nuclear-cytoplasmic interaction can affect expression patterns of some nuclear-encoded genes and alter floral organ identity. In the alloplasmic wheat lines with *Ae. crassa* cytoplasm, expression patterns of some nuclear genes including the class-B MADS-box genes show marked changes, which cause the induction of pistillody (Murai et al., 2002; Hama et al., 2004). If the *Whlp* gene plays a role in floral organ development similar to the *Arabidopsis HLL* gene (Skinner et al., 2001), modification of the *Whlp* gene expression should be expected in floral organs of the alloplasmic wheat lines showing pistillody. We thus compared the expression patterns of the *Whlp* gene in the several alloplasmic lines with those in the euplasmic lines by northern blot and RT-PCR analyses. Total RNA was extracted from floral organs of the euplasmic and alloplasmic lines including ones showing homeotic transformation of stamens (Fig. 5A). The northern blot showed that the *Whlp* transcript accumulated more in the stamens than in the pistils (Fig. 5B). In the transformed stamens of (*cr*)-CSdt7BS, however, the transcript level was similar to the levels in the pistils of both euplasmic and alloplasmic lines. RT-PCR was next conducted using the gene-specific

primer set of *Whlp,* which could amplify the transcript derived only from the homoeoallele on chromosome 2D. The RT-PCR analysis gave equivalent results to those of the northern blot analysis, suggesting the conservation of the organ-specific expression patterns of the three *Whlp* homoeoalleles in the common wheat. The *Whlp* transcripts accumulated more in the floral organs and roots than in the seedling leaves (Fig. 5C). Preferential accumulation of the *Whlp* transcripts was observed particularly in the stamens of all male-fertile lines such as CS, CSdt7BS and (*cr*)-CS. Microsporogenesis is a high-energy-demanding process (Warmke and Lee, 1978). During the anther development, the number of mitochondria increases dramatically in the anther cells, which is correlated with the increase in the steady state transcript levels of nuclear genes encoding mt respiratory proteins (Lee and Warmke, 1979; Huang et al., 1994). The preferential accumulation of the *Whlp* transcripts in the stamens may be associated with the increase of the number of mitochondria in wheat stamens, which may reflect a high level of protein synthesis in this male reproductive organ. In both the pistillate stamens and sterile pistils of (*cr*)-CSdt7BS, the *Whlp* transcript levels were comparable to that in the fertile pistils of CS. This observation indicates that the *Whlp* gene is involved in determining neither of pistillody nor of female fertility in (*cr*)-CSdt7BS. The observed expression pattern rather suggests that the elevated level of the *Whlp* gene expression in the stamens is required to support the development of male reproductive organ. In *Arabidopsis,* the *HLL* transcript was preferentially detected in inflorescences and carpels, and a little amount of its transcript was detected in the leaf tissue. The *HLP* expression, however, was observed not only in inflorescences but also in leaves, and less abundantly in

carpels (Skinner et al., 2001). The expression pattern of *Whlp* thus was more similar to that of *HLP* than that of *HLL*. These expression patterns of *Whlp* together with its structural homology suggest that a functional *Arabidopsis* ortholog of *Whlp* is *HLP* but not *HLL*. Furthermore, the *Whlp* gene showed a comparable expression level in the sterile pistils of (*cr*)-CSdt7BS to that in the fertile pistils, suggesting that it is not involved in the integument abnormality in ovule development of (*cr*)-CSdt7BS.

To investigate whether the organ-specific abundance of the *Whlp* transcript is a common feature among mt ribosomal protein genes or specific to *Whlp*, we analyzed the expression levels of two other members of the wheat mt ribosomal protein genes, *TaMRPL11* and *TaMRPL5*, in the wheat floral organs. The *TaMRPL11* gene is encoded in the nuclear genome (Handa et al., 2001), whereas the *TaMRPL5* gene is encoded both in nuclear and mt genomes and the both copies seem to be functional (Sandoval et al., 2004). To monitor expression only of the mt-encoded copy of the *TaMRPL5* gene, the gene-specific primer set was designed based on the nucleotide sequence polymorphisms between the two copies. The nuclear-encoded *TaMRPL11* gene showed quite similar expression patterns to those of the *Whlp* gene, and the *TaMRPL11* transcripts accumulated more in the stamens than in the pistils and lodicules (Fig. 5C). In contrast, the mt-encoded *TaMRPL5* transcripts showed a uniform accumulation in all examined floral organs. In sunflower, the mt-encoded genes such as *atpA*, *atp9*, *cob* and *rrn26* showed marked expression in young meiotic cells, but the transcripts of two nuclear genes (*atpB* and *ANT*) encoding mt proteins were not detected in these cells (Smart et al., 1994). The two distinguishable expression patterns between the

nuclear- and mt-encoded ribosomal protein genes in wheat thus disagree with the result reported in the anther development of sunflower. In the vegetative organs, both *MtRPL5* and *MtRPL11* transcripts were detected at the higher levels in the roots than in the seedling leaves similar to the *Whlp* transcript, suggesting their differential expression not only in the reproductive organs but also in the vegetative tissues.

To confirm the preferential expression in the stamens, the expression of these three ribosomal protein genes was also analyzed using the other series of euplasmic and alloplasmic wheat lines including fertile (*cr*)-N61 and (*cr*)-N26 and an EMS-induced sterile *Fr* mutant of (*cr*)-N26. RT-PCR analysis showed quite similar expression patterns in these alloplasmic lines to those in the euplasmic and alloplasmic lines with the nuclear genome of CS (Fig. 5D). The expression patterns of *Whlp* and *TaMRPL11* reflected the floral organ identity but not the floral whorl in all examined lines. But the transcript accumulation of *TaMRPL5* was uniform in all floral organs. Differential expressions of nuclear genes encoding cytosolic ribosomal proteins in different tissues have been reported in the human (Bortoluzzi et al., 2001). In *Drosophila minutes* mutants, mutations in the ribosomal protein genes lead to the characteristic phenotypes such as growth reduction, short and thin bristles and recessive lethality (Lambertsson, 1998). These *minute* phenotypes are correlated with the mRNA abundance of ribosomal protein genes, and the strength of the mutant phenotype differs among the mutations in different ribosomal protein genes, suggesting that each ribosomal protein component has its unique threshold and the expression pattern of each gene is different in different tissues (Saeboe-Larssen et al., 1998). The differential expression

patterns of mt ribosomal protein genes in wheat reproductive organs and vegetative tissues indicate that the threshold requirement of each component of ribosomal proteins also exist in wheat mitochondria as observed in the cytosolic ribosomal protein genes in human and *Drosophila*. In addition, the organ-associated expression patterns were observed only in the nuclear-encoded mt ribosomal protein genes, i.e. *Whlp* and *TaMRPL11,* but not in the mt-encoded gene *TaMRPL5*. The observation might suggest that the threshold levels of mt ribosome protein components may be different between the nuclear- and mt-encoded components and that the nuclear-encoded ones have higher thresholds at least in the development of wheat stamens. Comprehensive expression analysis of all nuclear- and mt-encoded components of mt ribosomal proteins will provide us with more information on their role and control mechanism in the organ/tissue development in wheat.

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Legends of figures

Figure 1. Structural features of the *Whlp* gene and its deduced amino acids. (A) A genomic structure of the *Whlp* gene. Boxes and lines indicate exons and introns, respectively. Nucleotide numbers of the genomic sequence are indicated under the boxes. Dotted, stripe and open boxes indicate the 5'- and 3'-untranslated region, a mt-targeting signal (MTS) and a L14 protein-coding region, respectively. (B) Sequence alignment of the deduced WHLP protein and its rice homolog and *Arabidopsis* HLL and HLP. Putative MTSs were boxed. Closed and open triangles indicate intron-inserted sites of the cereal and *Arabidopsis* L14 protein genes, respectively. Amino acid residues identical to the WHLP protein are indicated by dots. Deletions are shown by minus signs.

Figure 2. A phylogenetic tree based on the genetic distances among amino acid sequences of the WHLP protein and its homologous ribosomal L14 proteins. The tree was constructed by the UPGMA method based on the Nei's genetic distances of the L14 protein region. Accession numbers of the clones are indicated. *At*; *Arabidopsis thaliana*, *Zm*; *Zea mays*, *Agro*; *Agrobacterium tumefaciens.*

Figure 3. Copy number and chromosome assignment of the *Whlp* gene. (A) Southern blot of *Eco*RI-digested total DNA from three diploid ancestral species, *T. urartu* (genome

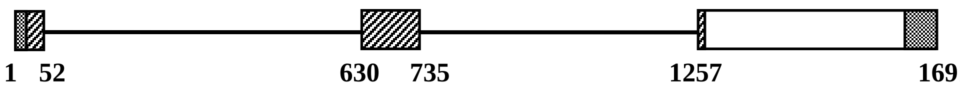
constitution; AA), *Ae. squarrosa* (DD) and *Ae. speltoides* (SS), and tetraploid wheat *T. durum* cv. Langdon (AABB) and common wheat CS (AABBDD). The blot was probed with the ^{32}P-labeled *Whlp* cDNA. (B) Assignment of the *Whlp* gene to the homoeolgous group 2 chromosomes. Southern blot was probed with the ^{32}P-labeled *Whlp* cDNA. A nulli2B-tetra2A line, for example, represents a line nullisomic for chromosome 2B and tetrasomic for chromosome 2A. (C) PCR amplification using the primers specific to the *Whlp* gene.

Figure 4. Mt-targeting of the WHLP protein in onion epidermal cells. (A) Transient expression after bombardment of a chimeric construct of GFP fused with a mt-signal peptide from an *Arabidopsis* γ -ATPase as a positive control (a) and with the full-length *Whlp* cDNA (c). The corresponding epidermal cell layers are shown in (b) and (d). (B) Transient expression of a chimeric constrcut of GFP fused with a predicted signal peptide from the WHLP protein. The GFP influorescences after bombardment are shown in (a) and (d). Mitochondria in the corresponding epidermal cell layers are seen by red influorescences via Mito-Tracker in (b) and (e) and the epidermal cell layers bombarded are shown in (c) and (f). Schmatic structures of the introduced fusion proteins are illustrated above the photographs.

Figure 5. Expression patterns of the *Whlp* gene and two other mt ribosomal protein genes, *TaMRPL11* and *TaMRPL5*, in the euplasmic and alloplasmic wheat lines. (A) Floret

structures of the esplasmic CS (left) and (*cr*)-CSdt7BS (right). Stamens are homeotically transformed into pistil-like organs in the (*cr*)-CSdt7BS. (B) Northern blot analysis of the *Whlp* gene in the pistils and stamens of euplasmic CS and its alloplasmic lines. The northern blots were probed with the ^{32}P-labeled *Whlp* cDNA. Loading patterns of rRNA are shown as internal controls. (C) RT-PCR analysis in floral organs, leaves and roots of the euplasmic and alloplasmic lines of CS. The ubiquitin gene was amplified as a control for normalization. The numbers below the electrophoresis patterns represent the relative accumulation levels compared with that of the CS pistil after normalization with the signals of the ubiquitin gene. The numbers of PCR cycles are indicated at the right side of the electrophoresis patterns. (D) RT-PCR analysis in floral organs, leaves and roots of the alloplasmic wheat lines. N61 carries multiple *Rfd* genes against the *Ae. crassa* cytoplasm, and no pistillody is observed in (*cr*)-N61. Flowers of (*cr*)-N26 showed partial and weak pistillody under short-day conditions. A *Fr* mutant line of (*cr*)-N26, recovered from the progeny of EMS-treated (*cr*)-N26, does not induce pistillody under the long-day conditions. P, pistil; S, stamen; L, lodicules; pTs, partially transformed stamens; tSt, transformed stamens.

(A)



(B)

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WHLP      MAAFLRSKCS SVGRAMMGSL GNNLYGGATS SIETVARPSR SDAVC-QQIR   50
rice HLL  ********** ****TL**G* ****F*AVN* *V***S***H C*PIF-****   50
HLL       **TA*A**L* K-**SLL*G* C*AFS*LMN* *SNGMMNG*I --LSQ-**H*   50
HLP       ***AFA*RLT RG**SLL*G* NNG--*SMN* *-NGMMNE*I --LSQQ**R*   50

WHLP      TFIQMRTNLK VVDNSGAKRV MCIQSLRGKK GARLGDMIIG SVKEAQP---  100
rice HLL  ********** ********** ********** ******T*** *******---  100
HLL       *****G*I** C****C**E* ********** ******I*** *****N*IVQ  100
HLP       *****G*V** ********K* ****A*K*** ******T*** *****M*---  100

WHLP      ---------R GKVKKGDVVY GVVVRAAMKK GRSDGSEVQF DDNAIVIVNN  150
rice HLL  ---------* ********** *********R **N****I** ******L***  150
HLL       KKVKKDAVPK ******M*** ********P* **A***Q*K* ******V*GI  150
HLP       ---------N ******A*** ********QR **V*****R* ****V*L*DS  150

WHLP      KG-------- ---------E LIGTRVFGPV PHELRKKKHL KILALAEHIV  200
rice HLL  **-------- ---------* ********** ********** **********  200
HLL       *EKKGQNNSH GSKRKMEYNQ PT******** ***M*LR*Q* ***S**Q***  200
HLP       *DKNTKTDR- ---------Q P********* ********** ******Q*VA  200
```

Fig. 1 (Mizumoto et al.)

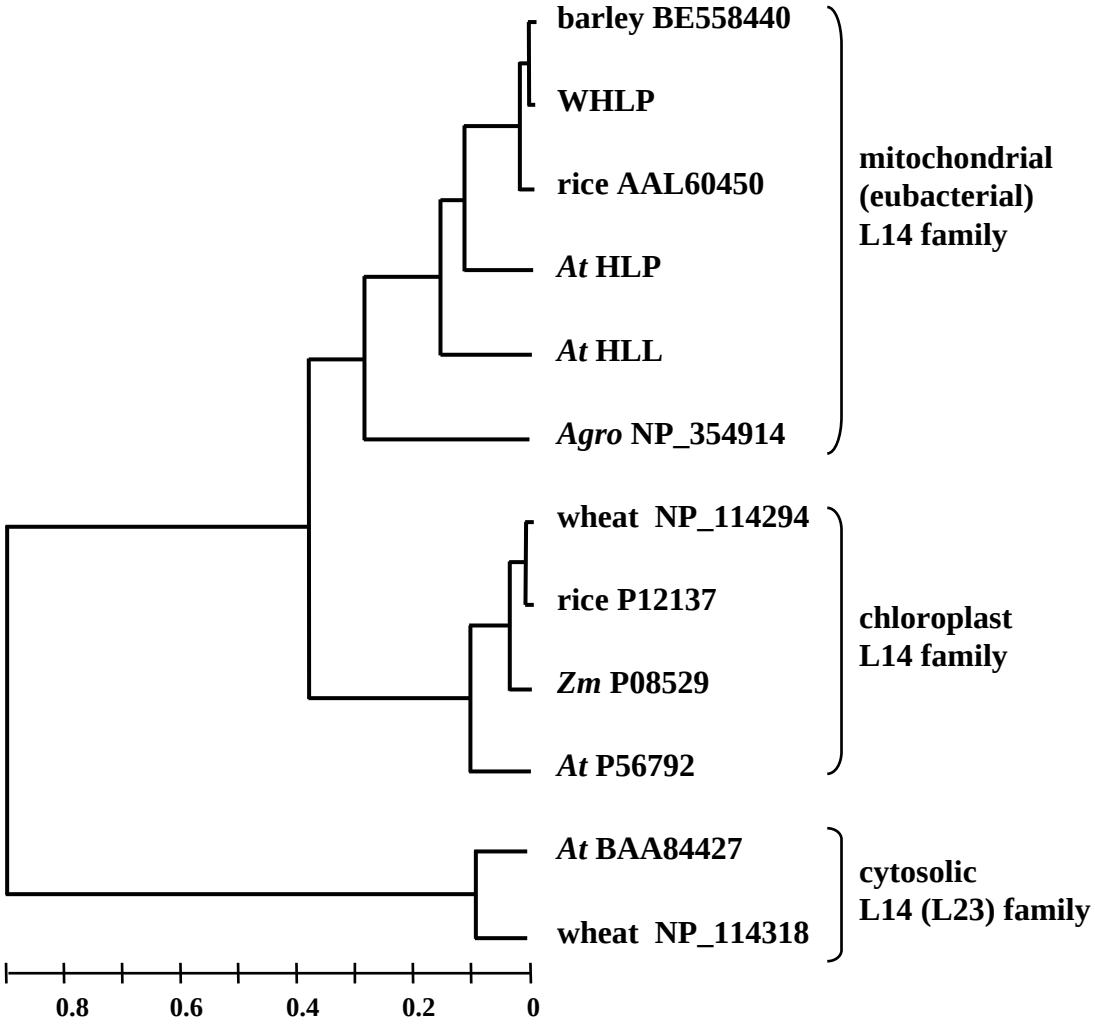

Fig. 2 (Mizumoto et al.)

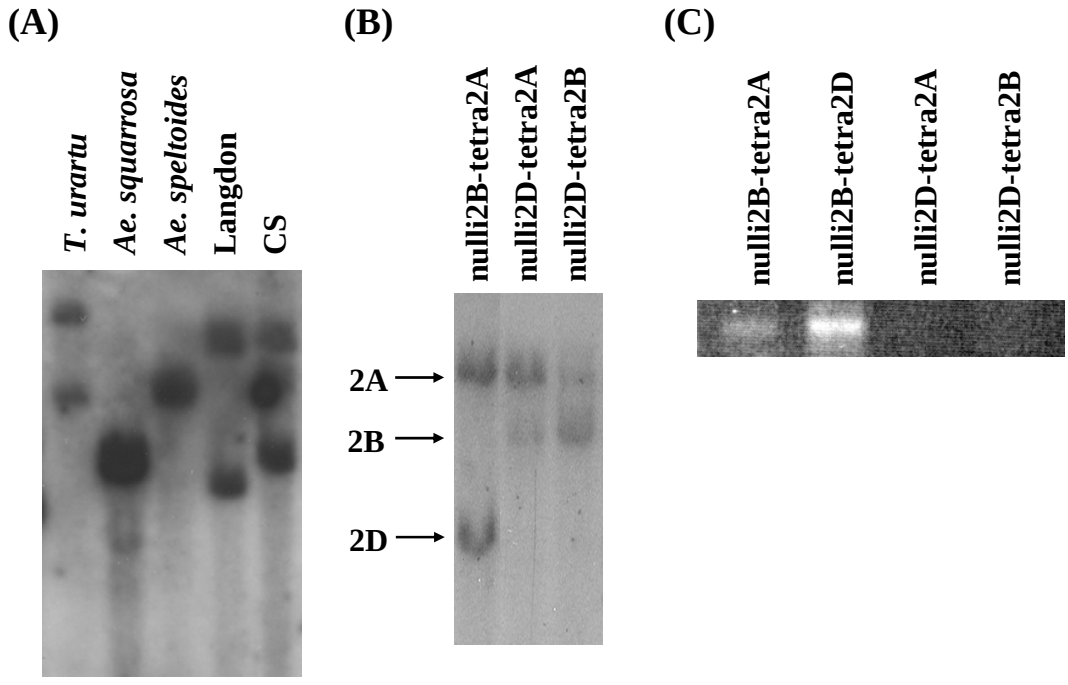

Fig. 3 (Mizumoto et al.)

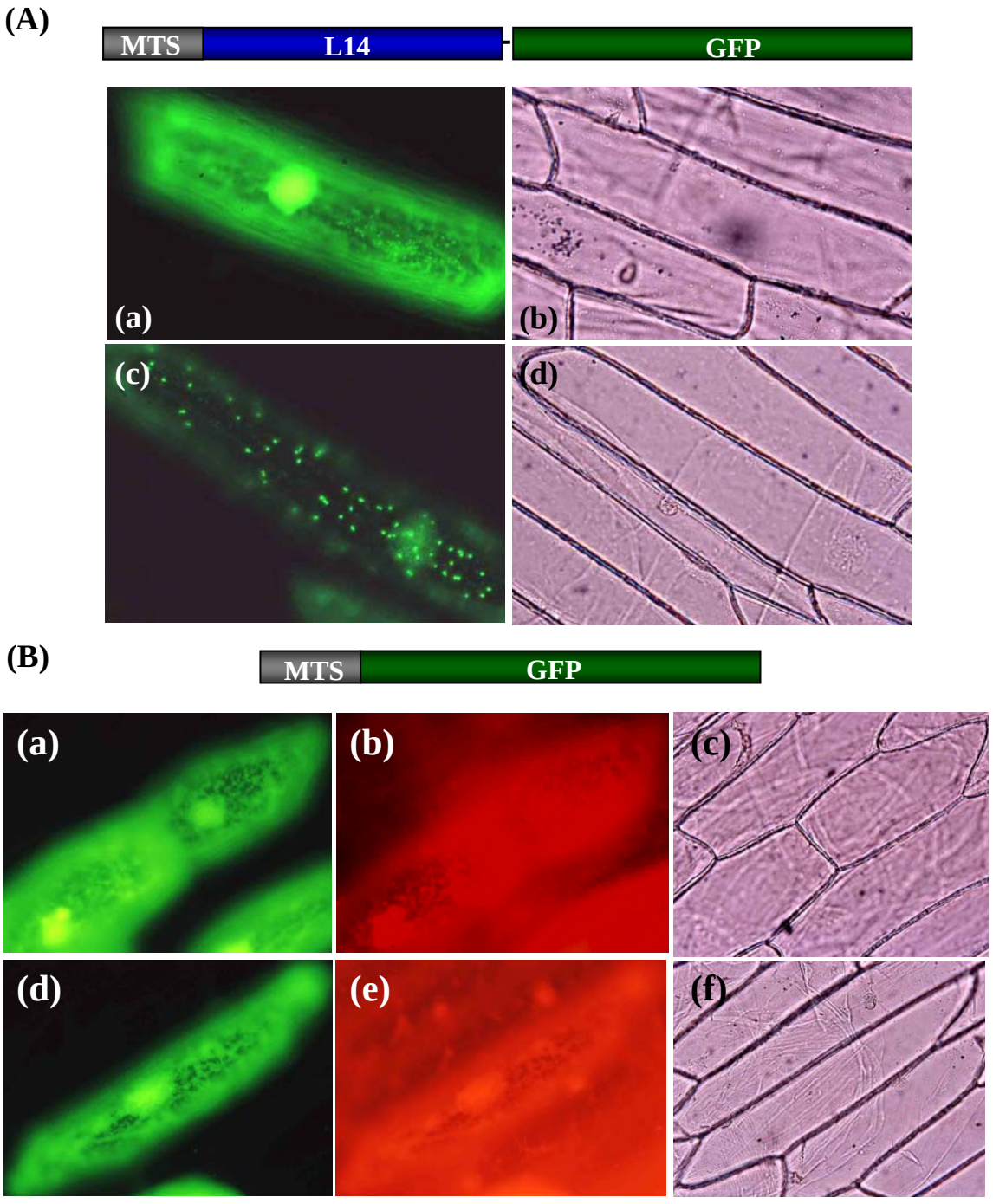

Fig. 4 (Mizumoto et al.)

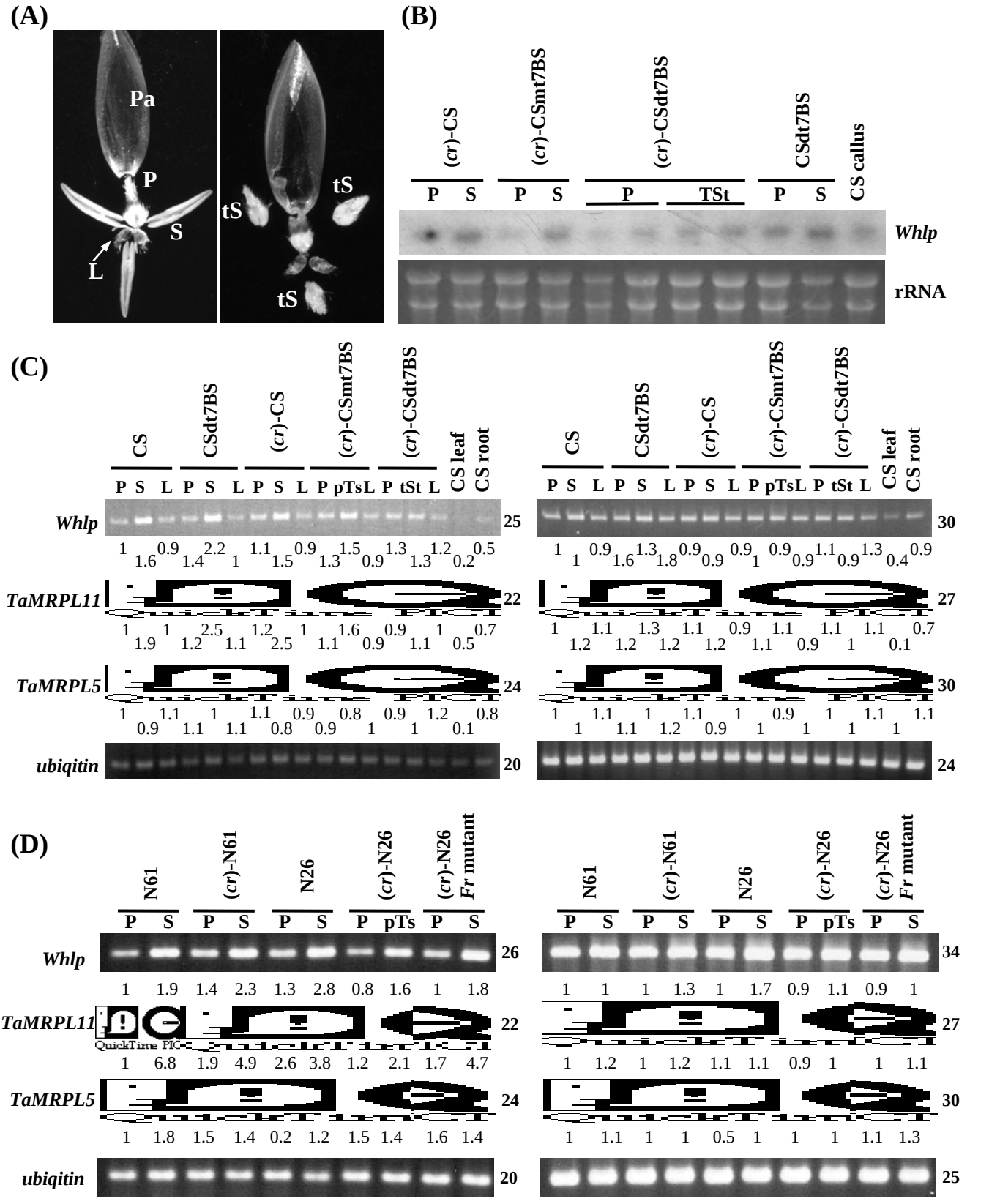

Fig. 5 (Mizumoto et al.)