Molecular Cloning and Characterization of the *TaLon1* in wheat

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Abstract

In yeast and human, the ATP-dependent Lon protease plays an important role in removal of the abnormal proteins and maintaining mtDNA integrity. In this paper, using RT-PCR and RACE techniques, we isolated a gene in wheat that encodes a product belonging to the Lon protease family. This gene, designated as *TaLon1*, is predicted to encode an 886 amino acid protein. *TaLon1* shows a constitutive expression pattern in wheat, which indicates that the TaLon1 plays a housekeeping role in wheat. Like its counterpart in yeast, it may degrade abnormal proteins in mitochondria and maintain the mtDNA integrity. Unlike the *lon* gene in yeast and *E. coli,* the *TaLon1* does not respond to heat-shock at 42 ?C. Under salt stress, the *TaLon1* expressions decline after treatment at both 150 m mol/L and 250 m mol/L NaCl for 24h. Given its important roles in yeast and *E. coli*, the salt stress damages to plant may be partly interpreted by the decrease of the *TaLon1* expression. It has been demonstrated that the Lon protease has some effects on cytoplasmic male sterility (CMS) in common bean. But in wheat, there is no difference in *TaLon1* expression between K-CMS line and normal lines.

Keyword

Lon protease, protein degradation, *Triticum aestivum* L, cytoplasmic male sterility

Protein degradation plays a variety of roles in all organisms. Protein degradation is an important mechanism of regulating gene expression and responsible for removal of damaged or abnormal proteins. Much of the protein degradation is performed by ATP-dependent proteases in bacteria, of which the Lon (also called La) in *E. coli* is one of the best characterized (Gottesman, 1996). The Lon protein is made up of four identical subunits of 87kDa(Goldberg *et al*, 1994). Each subunit carries a typical ATP-binding motif and a proteolytic domain with a catalytically active serine residue (Chin *et al*, 1988; Amerik *et al*, 1991; Fisher and Glockshuber, 1993) and plays a primary role in the degradation of many abnormal proteins as well as unstable regulatory proteins in bacteria (Mizusawa and Gottesman, 1983; Maurizi , 1987; Sonezaki *et al*, 1995; Wright *et al*, 1996; Liu *et al*, 1999).

In yeast a *lon* counterpart, *PIM1,* was cloned (Van Dyck *et al*, 1994; Suzuki *et al*, 1994) which was essential for removing abnormal proteins, selective proteolysis in matrix, maintaining mtDNA integrity and respiration-dependent growth.

Sarria *et al*(1998) and Barakat *et al*(1998) firstly isolated the *lon* homologous genes in *Arabidopsis and Zea mays* respectivly, the first ones in plant species. Similar to the counterpart in yeast, the *AtLon* in *Arabidopsis* could degrade the abnormal protein encoded by *pvs-orf239* in common bean mitochondria *in vitro*, which is considered the cause of the cytoplasmic male sterility (CMS)(Sarria *et al,* 1998). This indicates that the *lon* gene in plant mitochondria may play an important role in CMS.

In this paper, we cloned a *lon* homologous gene in common wheat (*Triticum aestivum* L.) and characterized its expression pattern in different tissues and the responses to heat-shock and salt stress. Furthermore, the possible relationship of Lon protease and *Aegilops kotschyi* Bioss cytoplasm male sterility (K-CMS) in wheat was discussed.

Materials and methods

Material preparation

The anthers of common wheat K-CMS line 5418A, maintainer line 5418B, restorer line Shan 229 and hybrid (5418A?Shan 229) were harvested on ice and frozen in liquid nitrogen, then stored at -80℃.

Seeds of 5418B were surface sterilized with NaClO solution (effective chloride concentration 2.0%). After germinated in Petri dish, the seeds were grown hydroponically in full strength Hoagland medium in greenhouse (16/8 h daily light period, 25 ?C temperature (night/day) and 60 to 70 % relative humidity). Two-week-old seedlings were transferred to fresh Hoagland medium supplemented with 150m mol/L NaCl and 250m mol/L NaCl. After exposure to the salt stress treatments for 7h and 24h, roots were collected and frozen with liquid nitrogen and stored at –80?C until further processed. At the same time, the heat-shock was carried out by transferring the two-week-old seedlings to 42 ?C fresh Hoagland medium and keeping for 90min in a 42 ?C oven. The roots and shoots were collected and treated as above.

RNA extraction

Total RNA was extracted with Trizol reagent (Invitrogen) according to the manufacturer's instructions. Reverse transcription was carried out in a 25μl reaction containing 2μg total RNA, 0.5μg M13APN(5'gtt ttc cca gtc acg act ttt ttt ttt ttt tt nn3'), 0.5m mol/L each dATP, dCTP, dTTP and dGTP(each to 0.5m mol/L final concentration), 20U RNase Inhibitor (Takara), 200U M-MLV reverse transcriptase (Promega). The reaction was performed at 42℃for 1h and subsequently diluted to 100ul.

Homologous cloning of the lon homologous gene in wheat

With the nucleotide sequence of maize *Lon1* gene, BLASTN against wheat EST database was performed on http://wheat.pw.usda.gov/west. The primers 5'upper-1 (5'ttccgccagccgtacacagcacac3') and 5'lower-1 (?5'accagcaggaaaatgtatgtg(a/g)a3') were designed based on the putative nucleotide sequence of wheat *lon* 5' part for RT-PCR. According to the sequence of *lon* 5'part, two *lon*3' nested upper primers (*lon* 3'upper1 5'gtggtgaagttgaaatggaagtta3' *lon*3'upper2 5'tcgcctatgattgttgatgag3') were designed. With the M13PM4 (5'gtt ttc cca gtc acg ac3') as the lower primer, the 3' Rapid Amplification of cDNA Ends (RACE) PCR was used to obtain the *lon* 3' part. A 1.1kb fragment was harvested and sequenced. Assembly of the two fragments was performed and a complete coding sequence of *lon* homolog in wheat was obtained.

Results

Cloning TaLon1 complete coding sequence

Using RT-PCR and RACE technique, we got the full coding sequence of *lon* homologous gene in wheat, designated as *TaLon1* (GenBank accession number: AY494984). Sequence analysis shows that *TaLon1* encodes an 886 amino acid protein with a predicted molecular weight of 97.6kDa. A very conserved ATP binding domain, so called Walker A sequence, in both prokaryote and eukaryote (Walker *et al*, 1982) was located at 410-418 residues.

To further explore the relationship of *TaLon1* to other related proteins, a phylogenetic analysis was carried out with CLUSTAL W on http://www.ebi.ac.uk/, the *TaLon1* is closer to those of eukaryotic organisms than to those of bacteria.

Interestingly, there are more than one *lon* homologs in some species. Two *lon* homologs, *Lon1* and *Lon2*, exist in maize (Barakat *et al*, 1998). While high similarity (94%) is present between TaLon1 and maize Lon1, the similarity between TaLon1 and maize Lon2 is only 40%. The same results happened when TaLon1 was compared to Lon1 and Lon2 in *Arabidopsis*.

TaLon1 expression pattern in anthers

With the tublin expression as internal control, we examined *TaLon1* expression pattern in anthers of different materials at different developmental stages.

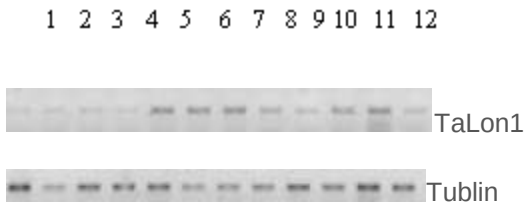

Figure1 *TaLon1* expression pattern in anthers. 1-4: the anthers at tetrad stage; 5-8: the anthers at mononucleate stage; 9-12: the anthers at binucleate stage.1,5,9: the anthers of 5418A; 2,6,10:the anthers of 5418B; 3,7,11: the anthers of 5418F$_1$;4,8,12: the anthers of shaan229

The *TaLon1* expression shows no significant difference in different materials and at different developmental stages (Figure 1). As a whole, the *TaLon1* expression in tetrad stage was slightly lower than those of other two stages. This may be a result of the more active metabolism existing at the mononucleate and binucleate stages.

Expression of *TaLon1* in response to heat-shock

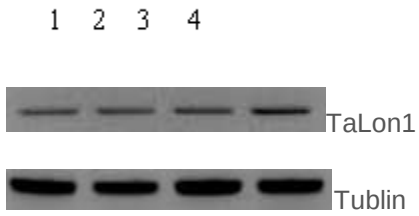

Figure 2 Expression of *TaLon1* in response to heat-shock. 1, treated roots; 2, treated shoots; 3, control roots; 4, control shoots.

In yeast and *E. coli*, the *lon* gene can be induced by heat-shock (Goff *et al*, 1984; Van Dyck *et al*, 1994). To determine whether the *TaLon1* is induced by heat-shock, 5418B seedlings were treated at 42℃for 90min. As above, the RT PCR was performed. The results indicated that *TaLon1* expressions were at comparable levels in all of these tissues (Figure 2). Taken together with the results of *TaLon1* expressions in anthers, we can conclude that the *TaLon1* is constitutively expressed in all tissues and developmental stages.

TaLon1 **expression under salt stress**

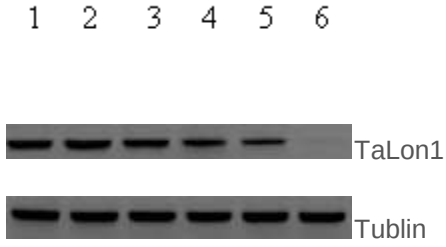

Figure 3 *TaLon1* expressions under salt stress. 1, control, treated for 7h; 2, treated for 7h at 150 m mol/L NaC l; 3, treated for 7h at 250 m mol/L NaCl; 4, control, treated for 24h; 5, treated for 24h at 150 m mol/L NaCl; 6, treated for 24h at 250 m mol/L NaCl

In *Bacillus subtilis,* the amount of *lon*-specific mRNA is increased after salt stress (Riethdorf *et al*, 1994). To explore whether *TaLon1*can also respond to the salt stress, the treatment at 150 m mol/L and 250 m mol/L NaCl for 7 and 24h respectively was carried out. RNA extraction and RT PCR were done as above.

As shown in Figure 3, the expressions of *TaLon1* show no difference between the control and those treated for 7h at both NaCl concentrations. But after treated for 24h, *TaLon1* expression decreased at both NaCl concentrations compared to the controls, especially at 250m mol/L.

Discussion

Based on the important roles of Lon protease in yeast mitochondria, we isolated a *lon* homologous gene in wheat designated as *TaLon1*. Unlike the *lon* gene in yeast (Van Dyck *et al*, 1994; Suzuki *et al*, 1994) and human (Wang *et al*, 1993), no typical mitochondrial targeting presequence was found at the N terminus of TaLon1. For the location of the TaLon1 in cell, there are three possibilities. Firstly, TaLon1 is really a mitochondrial protein, but it does not contain a common mitochondrial targeting presequence, as has been demonstrated for some mitochondrial proteins (Braun *et al*, 1994; Braun and Schmitz, 1995a, 1995b). Secondly, the TaLon1 contains a new type of mitochondrial presequence, since the mitochondrial targeting presequences are greatly various among the mitochondrial targeting proteins (Whelan and Glaser, 1997). In *Arabidopsis*, immunoblot analysis demonstrates that the *lon* homologs exist in both chloroplast and mitochondria (Adam *et al*, 2001). Furthermore, Olsen demonstrated that AtLon2 (GenBank accession number: At5g47040) was localized to peroxisome in *Arabidopsis*(Olsen, personal communication). Then, thirdly, the TaLon1 may not be targeted to mitochondria but to other organelles. For further research, a transient expression system may be able to help us to determine its location in wheat cell.

It is interesting that more than one homolog were found in maize (Barakat *et al*, 1998) and *Arabidopsis* (Adam *et al*, 2001). This may indicate that during evolution, along with the organelle evolution, the *lon* gene also evolved into different homologs localized to different organelles to function as a protease in eukaryote.

Like the *Lon1* in maize (Barakat *et al*, 1998), *TaLon1* shows a constitutive expression pattern in roots, leaves and anthers. This indicates that the *TaLon1* is an important gene playing a housekeeping-like role in wheat, i.e. similar to PIM1 in yeast, whose product may degrade abnormal proteins in mitochondria and maintain the mtDNA integrity.

Under salt stress, the *TaLon1* transcripts levels declined after treated for 24h at both 150 m mol/L and 250m mol/L NaCl. Given the important roles of *lon* gene in *E. coli* and yeast, the salt stress damages to plant may partly due to the decrease of the *lon* gene expression.

In this paper, no expression difference of *TaLon1* was present between K-CMS line and normal lines. But we can't exclude that there are some other *TaLon1* homologs in wheat that possess a different expression pattern between K-CMS line and normal lines in wheat.

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References

Adam Z, Adamska I, Nakabayashi K, Ostersetzer O, Haussuhl K, Manuell A, Zheng B, Vallon O, Rodermel S R, Shinozaki K, Clarke A K. 2001. Chloroplast and mitochondrial proteases in *Arabidopsis*. A proposed nomenclature. *Plant Physiol,* **125**(4): 1912-8

Amerik A Y, Antonov V K, Gorbalenya A E, Kotova S A, Rotanova T V, Shimbarevich E V. 1991. Site-directied mutagenesis of La protease. *FEBS Lett*, **287**:211-214

Barakat S, Pearce D A, Sherman F, Rapp W D. 1998. Maize contains a Lon protease gene that can partially complement a yeast pim1-deletion mutant. *Plant Mol Biol,* **37**(1): 141-154.

Braun H P, J?nsch L. Kruft V,Schmitz U K. 1994. The ‘Hinge’protein of cytochrome c reductase from potato lacks the acidic domain and has no cleavable presequence. *FEBS Lett*, **347**:90-94.

Braun H P, Schmitz U K. 1995a. Molecular structure of the 8.0 kDa subunit of cytochrome-c reductase and its ΔΨ-dependent import into isolated mitochondria. *Biochim Biophys Acta*, 1229:181-186

Braun H P, Schmitz U K. 1995b. Molecular features and mitochondrial import pathway of the 14 kilodalton subunit of cytochrome c reductase from potato. *Plant Physiol*, **107**:1217-1223

Chin D T, Goff S A, .Webster T, Smith T, Goldberg A L. 1988. Sequence of the lon gene in Escherichia coli. A heat-shock gene which encodes the ATP-dependent protease La. *J Biol Chem*, **263**(24): 11718-11728

Fisher H, Glockshuber R. 1993. ATP hydrolysis is not stoichiometrically linked with proteolysis in the ATP-dependent protease La from *Escherichia coli. J Biol Chem,* **268**:22502-22507

Goff S A, Casson L P, Goldberg A L. 1984. Heat shock regulatory gene htpR influences rates of protein degradation and expression of the lon gene in *Escherichia coli*. *Proc Natl Acad Sci U S A*, **81**(21): 6647-6651.

Goldberg A L, Moerschell R P, Chung C H, Maurizi M R. 1994. ATP-dependent protease La (lon) from *Escherichia coli. Methods Enzymol*, **244**:350-375

Gottesman S. 1996. Proteases and their targets in Escherichia coli. *Annu. Rev. Genet*, **30**:465-506

Liu J, Cosby W M, Zuber P. 1999. Role of lon and ClpX in the post-translational regulation of a sigma subunit of RNA polymerase required for cellular differentiation in *Bacillus subtilis*. *Mol Microbiol*, **33**(2): 415-28.

Maurizi, M. R. 1987. Degradation *in vitro* of bacteriophage lambda N protein by Lon protease from *Escherichia coli. J Biol Chem*, **262**(6): 2696-703

Mizusawa S, Gottesman S. 1983. Protein degradation in *Escherichia coli*: the lon gene controls the stability of sulA protein. *Proc Natl Acad Sci U S A*, **80**(2): 358-362

Riethdorf S, Volker U, Gerth U, Winkler A, Engelmann S, Hecker M. 1994. Cloning, nucleotide sequence, and expression of the Bacillus subtilis lon gene. *J Bacteriol*, **176**(21): 6518-6527.

Sarria R, Lyznik A, Vallejos C E, Mackenzie S A. 1998. A cytoplasmic male sterility-associated mitochondrial peptide in common bean is post-translationally regulated. *Plant Cell*, **10**(7) :1217-1228

Sonezaki S, Ishii Y, Okita K, Sugino T, Kondo A, Kato Y. 1995. Overproduction and purification of SulA fusion protein in Escherichia coli and its degradation by Lon protease *in vitro. Appl Microbiol Biotechnol* ,**43**(2): 304-309

Suzuki CK, Suda K, Wang N, Schatz G. 1994. Requirement for the yeast gene LON in intramitochondrial proteolysis and maintenance of respiration. *Science*, **264**:273-276

Van Dyck L, Pearce DA, Sherman F. 1994. PIM1 encodes a mitochondrial ATP-dependent protease that is required for mitochondrial function in the yeast *Saccharomyces cerevisiae*. *J Biol Chem.*, **269**:238-242

Walker J E, Saraste M, Runswick M J, Gay N J. 1982. Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J*, **1**(8): 945-951.

Wang N, Gottesman S, Willingham M C, Gottesman M M, Maurizi M R. 1993. A human mitochondrial ATP-dependent protease that is highly homologous to bacterial Lon protease. *Proc Natl Acad Sci U S A*, **90**(23): 11247-11251.

Whelan J and Glaser E. 1997. Protein import into plant mitochondria. *Plant Mol. Biol*, **33**:771-789

Wright R, Stephens C, Zweiger G, Shapiro L, Alley M R. 1996. Caulobacter Lon protease has a critical role in cell-cycle control of DNA methylation. *Genes Dev*, **10**(12): 1532-42.