

## Plant mitochondria contain proteolytic and regulatory subunits of the ATP-dependent Clp protease

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### Abstract

The proteolytic machinery of plant organelles is largely unknown, although indications so far point to several proteases of bacterial origin. In this study an *Arabidopsis thaliana* cDNA was isolated that encodes a homologue of bacterial ClpX, a molecular chaperone and regulatory subunit of the ATP-dependent, serine-type Clp protease. Computer analysis of the predicted plant ClpX revealed a putative mitochondrial transit peptide at the N-terminus, as well as overall sequence similarity to other eukaryotic ClpX homologues. Specific polyclonal antibodies were made to the *Arabidopsis* ClpX protein and used to confirm its localization in plant mitochondria. In addition to ClpX, a ClpP protein located in mitochondria was also identified from the numerous ClpP isomers in *Arabidopsis*. Localization of this nuclear-encoded protein, termed ClpP2, was determined first by its close sequence similarity to mitochondrial ClpP human, and later experimentally using ClpP2-specific antibodies with isolated plant organellar fractions. In *Arabidopsis*, transcripts for both *clpX* and *clpP2* genes were detected in various tissues and under different growth conditions, with no significant variation in mRNA level (i.e. 2-fold) for each gene between samples. Using  $\beta$ -casein as a substrate, plant mitochondria were found to possess an ATP-stimulated, serine-type proteolytic activity that could be strongly inhibited by antibodies specific for ClpX or ClpP2, suggesting an active ClpXP protease. The recent discovery of homologous mitochondrial ClpX and ClpP proteins in mammals suggests that this type of protease may be common to multicellular eukaryotes.

### Introduction

Protein degradation plays a vital role in regulating the level of many cellular enzymes and regulatory proteins in all organisms. It is also responsible for the targeting and efficient removal of damaged or otherwise abnormal polypeptides before they reach levels toxic to metabolic processes. Much of the selective proteolysis in bacteria is performed by ATP-dependent proteases, of which the Clp protease in *Escherichia coli* is one of the best characterized (Gottesman, 1996; Porankiewicz *et al.*, 1999). Clp proteases are multi-meric complexes consisting of proteolytic (ClpP) and

regulatory subunits (either ClpA or ClpX). The regulatory subunits contain either one (ClpX) or two (ClpA) highly conserved ATP-binding sites, and can function independently as molecular chaperones in addition to being regulatory components of the energy-dependent protease (Gottesman *et al.*, 1997).

Clp proteases have a barrel-like structure architecturally similar to that of the 26S proteasome in eukaryotes (Horwich *et al.*, 1999). Central to the Clp proteolytic complex are two adjacent heptameric rings of ClpP which together form a single chamber where the proteolytic active sites reside (Kessel *et al.*, 1995; Wang *et al.*, 1997). Both entrances to the inner cavity are relatively narrow, preventing the entry of most folded proteins, and thereby avoiding inadvertent degradation of non-targeted proteins. Capping

The nucleotide sequence data reported will appear in the GenBank Nucleotide Sequence Database under the accession number AF036328 (clpX).

the ClpP annuli are single hexameric rings of either ClpA or ClpX, which are essential for activating the proteolytic activity of the enzyme. Binding of ATP is sufficient for oligomerization of the regulatory subunits and their association to the ClpP annuli, whereas proteolytic activity of the entire complex is dependent on ATP hydrolysis (Gottesman *et al.*, 1997). Protein substrates are selectively bound by the ClpA or ClpX hexamers and presumably unfolded to enable efficient translocation into the ClpP proteolytic chamber. Once accessible to the proteolytic active sites, the targeted protein is quickly degraded into small fragments (Gottesman, 1996). Since ClpA and ClpX differ in their protein binding specificity, the ClpAP and ClpXP complexes can be considered as distinct proteases.

Since the discovery of Clp proteins in *E. coli*, many more varieties have been identified in a wide range of organisms. Plants in particular have a tremendous diversity of Clp-like proteins, of which the first discovered was a plastid-encoded homologue of ClpP (Gray *et al.*, 1990; Maurizi *et al.*, 1990), now designated ClpP1. In addition to the plastid-encoded ClpP, five nuclear-encoded isomers have now been identified in *Arabidopsis thaliana*, and have been designated ClpP2 to -P6 (Clarke, 1999; Adam *et al.*, 2001). As for ClpP1, all five nClpP proteins possess the catalytic triad motifs that constitute the proteolytic active site of ClpP. Four additional nuclear-encoded proteins with lower sequence homology to ClpP have also been found in *Arabidopsis*. However, these four proteins lack the proteolytic active site characteristic of ClpP and consequently have been distinguished as a separate group (i.e., ClpR) (Clarke, 1999; Porankiewicz *et al.*, 1999; Adam *et al.*, 2001). Of the five nuclear-encoded ClpP isomers, little is yet known about their intracellular distribution except that ClpP3 localizes to the chloroplast stroma along with ClpP1 (Sokolenko *et al.*, 1998). The other nuclear-encoded ClpP proteins also have N-terminal extensions that presumably function as organelle targeting sequences but no specific localization has yet been demonstrated.

In addition to ClpP, homologues of the bacterial ClpA protein also exist in higher plants. These ClpA-like proteins, termed ClpC and ClpD, are nuclear-encoded (Gottesman *et al.*, 1990; Kiyosue *et al.*, 1993) and imported into chloroplasts post-translationally (Moore and Keegstra, 1993; Weaver *et al.*, 1999). Like ClpP1 and ClpP3, both ClpC and -D are constitutively synthesized in most plant tissues (Shanklin *et al.*, 1995; Ostersetzer and Adam, 1996; Halperin and Adam, 1996). However, the constitutive level of

ClpD is relatively low, but it is strongly inducible during certain stress conditions such as desiccation (Kiyosue *et al.*, 1993). ClpC and -D are localized primarily in the chloroplast stroma (Moore and Keegstra, 1993; Weaver *et al.*, 1999), with some ClpC also attaches to the stromal surface of the inner envelope membrane, associated with the protein import machinery (Nielsen *et al.*, 1997). Structural association of ClpC with ClpP1 and ClpP3 (Desimone *et al.*, 1997; Sokolenko *et al.*, 1998) suggests they form active Clp proteases within the chloroplast stroma, although such an occurrence has yet to be shown *in vivo*.

Given the existence of plant counterparts to the ClpP and ClpA subunits of the bacterial Clp protease, we investigated whether higher plants also possess a homologue to ClpX. In this study we describe the cloning and sequencing of an *Arabidopsis* cDNA encoding a protein similar to ClpX from both bacteria and animals. In contrast to the chloroplastic localization of ClpC and -D, we show that the plant ClpX protein is instead located in mitochondria. Furthermore, we identify both theoretically and experimentally a nuclear-encoded ClpP isomer that also localizes to mitochondria with ClpX. We therefore demonstrate that plant mitochondria possess both proteolytic and regulatory subunits of a Clp protease, and that the ATP-stimulated serine proteolytic activity found in this organelle can be attributed to a ClpXP protease.

## Materials and methods

### *Plant growth and isolation of organelles*

Pea seedlings (*Pisum sativum* cv. Alaska) were germinated and grown at 25 °C for 10 days under a 12 h photoperiod, at an irradiance of 150  $\mu\text{E m}^{-2} \text{s}^{-1}$ . Intact chloroplasts were isolated on Percoll gradients as previously described (Levy and Adam, 1995). For isolation of mitochondria, pea seedlings were germinated and grown in the dark. After homogenization of the etiolated seedlings, intact mitochondria were purified on a 32% Percoll cushion as previously described (Hamasur *et al.*, 1990).

### *Cloning and sequencing of Arabidopsis clpX cDNA*

A computer search of the *Arabidopsis* expressed sequence tag (EST) database using the *E. coli* ClpX sequence identified a single cDNA clone (designated 207D12T7). The EST clone was obtained from the *Arabidopsis* Resource Center at Ohio State University,

and the cDNA sequenced using an Applied Biosystems model 373 DNA Sequencer. The 1500 bp cDNA was found to encode a protein with homology to known bacterial ClpX sequences, although the sequence was incomplete. To obtain a full-length clone, the 1500 bp cDNA fragment was used to screen an *Arabidopsis* cDNA library made in  $\lambda$ -Zap-Lox (obtained from the *Arabidopsis* Resource Center). Positive plaques detected by the radioactive probe were excised *in vivo*, and plasmid DNA was prepared. Clones containing the longest cDNA inserts were sequenced as described above. DNA and deduced amino acid sequences were analyzed with the Genetics Computer Group (GCG) programs. Phylogenetic comparisons were done with the CLUSTAL W (1.74) program.

#### *Expression of clpX and clpP2*

*Arabidopsis* seedlings were grown at 25 °C under an irradiance of 100  $\mu\text{E m}^{-2} \text{s}^{-1}$  for 4 weeks. To test the effect of temperature on expression of *clpX* and *clpP2*, plants were transferred to either 40 or 15 °C, and leaf samples were harvested after 2 or 4 h. To assess the effect of light intensity, plants were transferred to either 40 or 700  $\mu\text{E m}^{-2} \text{s}^{-1}$  for 3 d before leaves were harvested. Samples were homogenized in 0.1 M Tris-HCl (pH 7.5) and 0.5% sarcosyl. Total RNA was extracted with phenol/chloroform followed by precipitation with 2 M LiCl and ethanol. RT-PCR was performed with a commercially available kit (Promega), using 0.25 and 0.1  $\mu\text{g}$  RNA for *clpX* and *clpP2* reactions, respectively. These RNA concentrations were found in preliminary experiments to be within the linear response range. The forward primer used for detection of *clpX* mRNA matched nucleotides 666–683, and the reverse primer matched nucleotides 1609–1593. The forward and reverse primers used to detect the *clpP2* transcript matched nucleotides 59–84 and 538–513, respectively.

#### *Generation of specific antibodies*

For generation of specific polyclonal antibodies against *Arabidopsis* ClpX, a synthetic peptide was made to a unique 15 amino acid sequence (LTEPKNALGKQYKKM), conjugated to BSA and KLH, and injected into rabbits (AnaSpec, Inc.). Antibodies for *Arabidopsis* ClpP2 were also generated against a synthetic peptide sequence (MVIEHSSRGGERAYDIC), conjugated to KLH and prepared commercially in rabbits (AgriSera, Hällnäs). The peptide sequence for ClpP2 was chosen because of its absence in all

other *Arabidopsis* ClpP isomers and its uniqueness in the GenBank database.

#### *Polyacrylamide gel electrophoresis and immunoblot analysis*

Total protein extracts from chloroplast and mitochondrial fractions were solubilized, resolved on 15% polyacrylamide gels and then transferred to nitrocellulose membranes. ClpX and ClpP2 were detected with specific antibodies diluted to 1:1000. Antibodies against the chloroplast protein OE33, a subunit of the oxygen-evolving complex of photosystem II, were used at a dilution of 1:10 000. Antibodies against the serine hydroxy-methyl transferase (SHMT) from pea, a known mitochondrial protein of 53 kDa, were used at 1:1000 dilution. Prior to their use, ClpX- and ClpP2-specific antibodies were purified on a Protein A-Sepharose column (Harlow and Lane, 1988). Antigen-antibody complexes were visualized by enhanced chemiluminescence (ECL) using a commercially available kit (Pierce).

#### *Casein degradation assay*

The reaction mixture used to assay proteolytic activity contained 4  $\mu\text{g}$   $\beta$ -casein, 30  $\mu\text{g}$  protein of mitochondria extract, 0.3 M sucrose and 5 mM MOPS-KOH (pH 7.5). All reactions were carried out at 37 °C. For the proteolytic inhibition assay, 1 mM phenylmethylsulfonyl fluoride (PMSF) or 5 mM EDTA were included in the reaction mixture. To test the effect of specific antibodies on the caseinolytic activity, 15  $\mu\text{l}$  of purified ClpX or ClpP2 antibodies were added to the mitochondrial extract (30  $\mu\text{g}$  protein), and incubated at 25 °C for 30 min.  $\beta$ -casein was then added to the mixture under the same conditions as described above, and further incubated at 37 °C for 120 min. Proteins were then resolved by SDS-PAGE, and the relative amount of casein in each sample was quantified from the Coomassie blue-stained gel.

## **Results**

### *A ClpX homologue in Arabidopsis thaliana*

To ascertain whether higher plants possess a protein homologous to ClpX in bacteria, a computer search was done of the numerous EST sequences within the *Arabidopsis* database. The resulting search identified a single cDNA clone showing considerable homology

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95  SSQGDPPDLWQPPGDGVSVRVNGSSVNLGRGGGGGSSNPGGPGNGTGSNS
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
1  MTDKRDGSGKLLYCSFCGKSQHEVRKLIAGPSVYICDRCVLDLCNDIIRE
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
145 KEDCWGGSNLGSDFFTPKEICKGLNKFVIGQERAKKVLVSAVYNYHYKRIY
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
51  EIKEVAPHRRERSALPTPHEIRNHLDDYVIGQEQARKVLAVALVYNYHYKRL-
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
195 HESSQKRSAGETDSTAAPADDDMVELKSNILLMGGSGKLLAKTLA
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
100 -----RNGDTSNGVELGKSNILLGGSGKLLAKTLA
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
245 RFVNVFPFIADATTLTQAGYVGEDVESILYKLLTVADYNVAAAQQC
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
134 RLLDVFFTMADATTLTEAGYVGEDVENIIQKLLQKCDYDVQKAQRG
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
295 VDKITTKAESLYISRDVSGEGVQALLKMLEGTIVNVPEKGARKHPRG
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
184 VDKISRKSDNPSITRDVSGEGVQALLKMLEGTVAAVFPQGGGRKHHPQ
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
345 DNIQIDTKDILFICGGAFVDIEKTIERRH-DSSIGFGAPVRANMRAGGV
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
234 EFLQVDTSKILFICGGAFAGLDKVIISHRVETGSGICFGATV-----KAK
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
394 TTAAVASNLMETVETSLLIAYGLIPEFVGRFPVLVLSALTENQLMQVLT
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
278 SDKASEGELLAQVFPEDLIKFGIPEFIRGLPVVATLNELSEELIQILK
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
444 EPKNALGKQYKMYQMSVVKLHFTESALRLIARKAITKNTGARGLRALLE
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
328 EPKNALTKQYQALFNLEGVLDLEFRDEALDAIAKKAMARKTGARGLRSTVE
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
494 SILMDSMYEIPDEGTGSDMI EAVVVD EAVEGEGR
   : : : : : : : : : : : : : : : : : : : : : : : : : : : : :
378 AALLDTMYDLP----SMEDVEKVVIDESVIDGQSK

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Figure 1. Comparison of amino acid sequences of *Arabidopsis* and *E. coli* ClpX. Deduced *Arabidopsis* (upper line) and *E. coli* (lower line) amino acid sequences are presented. Indicated are the two Walker A and B regions of the ATP-binding domain (boxes), the four Cys residues of the putative Zn-finger in *E. coli* ClpX (triangles), and similar (:) and identical (|) amino acid residues.

to several bacterial *clpX* genes. Since this cDNA encoded only a partially complete protein, we used it as a probe to screen an *Arabidopsis* cDNA library for a full-length clone. The resulting 1956 bp cDNA (GenBank accession number AF036328) encoded a protein of 579 amino acids, with a predicted molecular mass of 67 kDa and pI of 9.6. BLAST comparison revealed that the deduced amino acid sequence was closely related to ClpX sequences from both bacteria and other eukaryotic organisms. In comparison to the best characterized ClpX, that from *E. coli*, the *Arabidopsis* protein also contains the highly conserved Walker A and B motifs within the single ATP-binding domain (Figure 1). Like the yeast form, however, the *Arabidopsis* ClpX lacks the putative C4 Zn-finger found in the amino termini of the *E. coli* and murine proteins (Santagata *et al.*, 1999). The amino terminus of the *Arabidopsis* ClpX is also longer, suggesting the presence of a possible targeting sequence to one of the plant organelles.

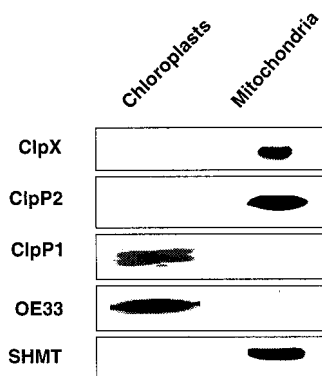
Sequence comparison with the *Arabidopsis* database reveals that the cloned cDNA is identical to the EST used as a probe for its isolation. Compari-

son with *Arabidopsis* genomic sequences revealed that the isolated cDNA is encoded by a gene on chromosome 5 (accession number AB013388) that contains 13 introns. Interestingly, another genomic sequence that may encode a similar protein has recently been found on the same chromosome (accession number AB025612). A corresponding partial cDNA, lacking its 5' end, was also identified (not shown, accession number T14143) implying that plants may contain two isomers of ClpX.

#### *Plant ClpX is localized to mitochondria*

Comparison of *Arabidopsis* ClpX to related proteins shows a high degree of similarity to bacterial homologues. Since plant chloroplasts and mitochondria are evolutionarily related to prokaryotes, and given the N-terminal extension of the *Arabidopsis* ClpX, we speculated that this protein would be targeted to either one of these organelles. Using cellular localization prediction programs (PSORT and ChloroP), *Arabidopsis* ClpX was predicted to be located in mitochondria and not in chloroplasts. To test this, polyclonal antibodies were made against the *Arabidopsis* ClpX protein and used in an immunoblot analysis of isolated mitochondria and chloroplasts. A single protein of the expected size for mature ClpX (ca. 55 kDa) was detected in isolated mitochondria, but not in chloroplasts (Figure 2). Purity of the organellar fractions was confirmed using antibodies against mitochondrial (SHMT) and chloroplastic (OE33) marker proteins. The immuno-localization of ClpX to mitochondria, therefore, confirmed the sequence prediction.

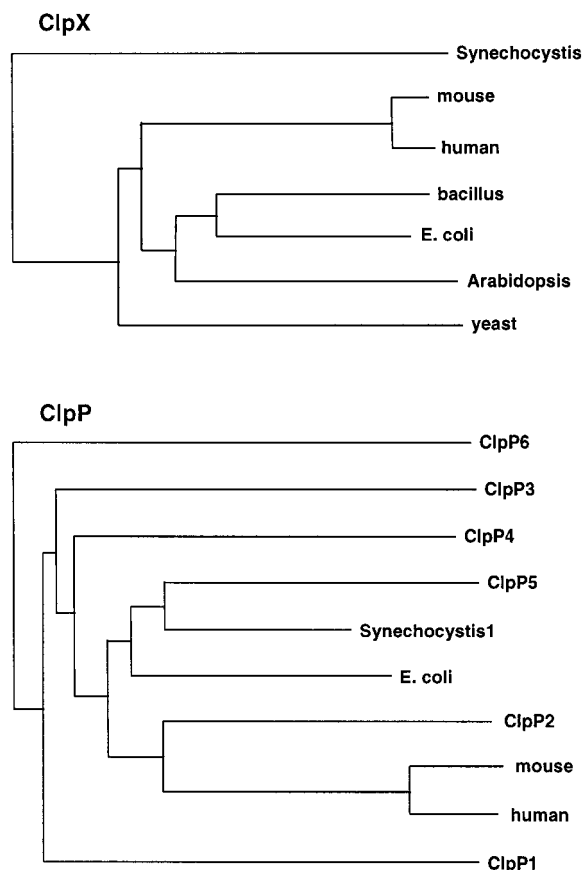
To further explore the relationship of *Arabidopsis* ClpX to other related proteins, a phylogenetic analysis was performed. As shown in Figure 3, the *Arabidopsis* ClpX protein is most closely related to bacterial ClpX sequences, represented here by *E. coli* and *B. subtilis*. Sequence similarity is also observed to the human and murine homologues, and to a lesser extent to the yeast ClpX. Interestingly, like the *Arabidopsis* ClpX in this study, the yeast and murine proteins have also recently been localized to mitochondria (van Dyck *et al.*, 1998; Santagata *et al.*, 1999). In contrast, the cyanobacterium *Synechocystis* contains a ClpX homologue that is only distantly related to *Arabidopsis* ClpX (Figure 3). Given that cyanobacteria are evolutionarily related to chloroplasts, this analysis overall supports the localization of the plant ClpX protein to mitochondria and not to chloroplasts.



**Figure 2.** Localization of ClpX and ClpP2 in plant mitochondria. Total protein extracts from intact chloroplasts and mitochondria were isolated from pea seedlings. Samples from each extract (30  $\mu$ g protein) were separated on 15% SDS-PAGE gels and then transferred to nitrocellulose filters for immunoblot analysis. Filters were incubated with polyclonal antibodies against either ClpX, ClpP2, plastid-encoded ClpP (ClpP1), a chloroplast protein (OE33), or a mitochondrial protein (SHMT). Immuno-complexes were visualized by ECL.

#### *Plant ClpP2 is localized to mitochondria*

In *E. coli*, ClpX can associate with the proteolytic subunit ClpP to serve as its regulatory subunit, forming an ATP-dependent ClpXP protease. At the time of this study, the single human ClpP had been reported in mitochondria (Corydon *et al.*, 1998), and so we sought to test whether a mitochondrial ClpP also exists in higher plants. In contrast to other eukaryotes, however, plants have multiple ClpP forms, with one plastid-encoded protein (ClpP1) and five nuclear-encoded isomers (ClpP2-6) identified so far in *Arabidopsis* (Clarke, 1999; Porankiewicz *et al.*, 1999). Sequence analysis of all five nuclear-encoded ClpP proteins revealed putative N-terminal transit peptides, with all but one (ClpP2, accession number AB006708) being predicted as chloroplastic. Sequence alignments of the *Arabidopsis* ClpP proteins with selected bacterial and mammalian homologues showed that ClpP2 was most closely related to the mitochondrial ClpP in mammals (Figure 3). To test for possible localization of ClpP2 in plant mitochondria, antibodies were prepared against a 15 amino acid region found only in plant ClpP2 proteins and used in an immunoblot analysis of isolated chloroplasts and mitochondria. As shown in Figure 2, the specific antibodies detected ClpP2 only in the mitochondrial fraction. Purity of the organelle fractions were again confirmed using antibodies to the marker proteins SHMT and OE33, with the addition of an antibody against plastid ClpP exclusively located

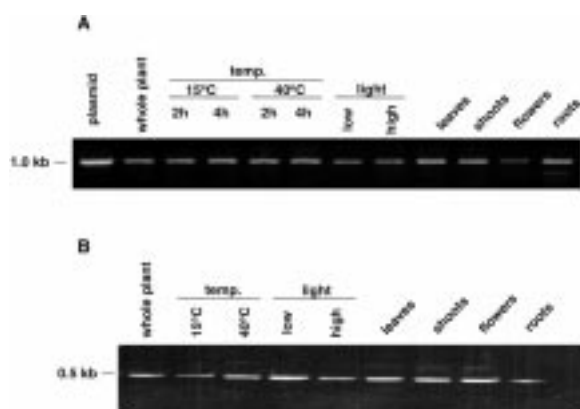


**Figure 3.** Phylogenetic trees of ClpX and ClpP proteins. Protein sequences of selected Clp proteins were aligned and phylogenetic trees were constructed using the CLUSTAL W (1.74) program. Accession numbers of the proteins used, from top to bottom, are as follows. ClpX: U92039; AF134983; NM006660; X95306; L18867; AF036328; Z36096. ClpP: AF016621; AF032123; AL021246; AJ012278; P54416; U55059; AB006708; AJ005253; Z50853; AP000423.

in chloroplasts (Ostersetzer *et al.*, 1996). This result demonstrates that both ClpX and ClpP2 are localized inside plant mitochondria, potentially forming a ClpXP protease.

#### *Expression of clpX and clpP2*

To study the expression characteristics of the *clpX* and *clpP2* genes, RT-PCR analysis of total RNA isolated from *Arabidopsis* plants was performed. As shown in Figure 4, transcripts for both genes could be detected in leaves, shoots, roots and flowers. The relative level of these transcripts was similar in each tissue, although the amount of *clpX* mRNA in flowers was slightly lower. Changes in environmental conditions also did not significantly alter the expression of *clpX*

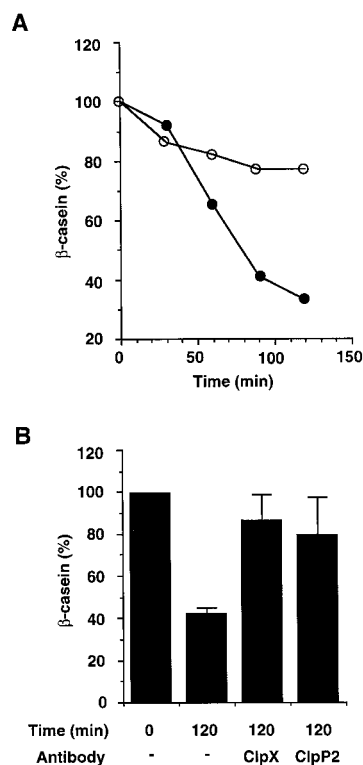


**Figure 4.** Expression of *clpX* and *clpP2* in *Arabidopsis*. *Arabidopsis* plants were grown at 25 °C, at light intensity of  $100 \mu\text{E m}^{-2} \text{s}^{-1}$  for 4 weeks, and then exposed to different environmental conditions, as described in Materials and methods. Total RNA was extracted from the plants and RT-PCR was performed using gene-specific primers for *clpX* (A) or *clpP2* (B).

or *clpP2*, as observed during changes in temperature or light intensity (Figure 4). The only exception to this was a slight reduction in *clpP2* mRNA levels in leaves shifted to 15 °C. Overall, the expression of the *clpX* and *clpP2* genes appears to be primarily constitutive in *Arabidopsis*, although changes in expression levels during other types of growth conditions cannot be excluded at this stage. Furthermore, because of the poor detection of ClpX and ClpP2 proteins in *Arabidopsis* whole leaf extracts using the specific polyclonal antibodies (possibly due to their relatively low cellular content), it remains unclear whether the transcript levels observed in this study correspond to similar protein contents.

#### Degradation of $\beta$ -casein in isolated mitochondria

$\beta$ -casein is a preferred substrate to assay proteolytic activity under *in vitro* conditions (Beynon and Bond, 1989). We thus tested whether a soluble extract from isolated pea mitochondria containing ClpX and ClpP2 exhibits  $\beta$ -casein degradation activity. When the mitochondrial extract was incubated with  $\beta$ -casein, only 20% of the substrate was degraded during the first 2 h (Figure 5A). However, supplementing the reaction mixture with MgATP stimulated the proteolytic rate significantly. Different serine-type protease inhibitors could inhibit this degradation, but not those for metalloproteases (data not shown). The stimulation of casein degradation by ATP and its inhibition by serine-type protease inhibitors is consistent with the known characteristics of Clp proteases. To test the possible



**Figure 5.** Casein-degrading activity in plant mitochondria. A. A representative course of  $\beta$ -casein degradation experiment. Mitochondrial extract was incubated with  $\beta$ -casein, in the presence (●) or absence (○) of 5 mM MgATP for the indicated times. B. Effect of Clp antibodies on casein-degrading activity in mitochondrial extracts. Specific antibodies were preincubated for 30 min with mitochondrial extracts prior to addition of  $\beta$ -casein. Reaction mixtures were then incubated for 2 h and SDS-PAGE was used to estimate the remaining amount of  $\beta$ -casein. Values presented are means of three independent experiments with SD.

activity of a ClpXP protease in the plant mitochondrial extract, we tested the effect of Clp-specific antibodies on the observed  $\beta$ -casein-degrading activity. Mitochondrial extracts were incubated with purified IgG fractions against ClpP2 or ClpX at 25 °C for 30 min. Casein was then added to the mixture, and the amount remaining in the extract was measured after 2 h. As shown in Figure 5B, both antibodies were effective in inhibiting  $\beta$ -casein degradation, preventing proteolysis of all but 10–20% of the substrate. Thus, the ATP-stimulated, serine-type proteolytic activity observed in the plant mitochondrial extract could be attributed to a ClpXP protease.

## Discussion

Higher plants are different from other eukaryotic organisms in possessing genes that code for many isomeric forms of Clp proteins. The underlying reasons for this complexity remain elusive, although one obvious explanation is their differential localization within the plant cell. Several of the Clp proteins first identified in plants have since been localized to chloroplasts, but the intracellular locales for many of the newly discovered isomers remain unsolved (Clarke, 1999). In some cases, the presence of putative N-terminal transit sequences suggest possible organelle targets for these proteins. However, predictions of such targeting sequences to chloroplasts and mitochondria are unreliable based solely on sequence analysis. The experimental work presented here demonstrates that plants, in addition to the previously described ClpCP protease located in chloroplasts, have a ClpXP protease located in mitochondria. In this study, we have also identified which of the numerous ClpP isomers in plants is located inside mitochondria (ClpP2) along with ClpX, with the remaining nuclear-encoded ClpP forms likely to be restricted to chloroplasts. Both the ClpX and ClpP2 proteins are responsible for much of the ATP-dependent proteolytic activity in isolated plant mitochondria, suggesting they participate together as a ClpXP proteolytic complex.

The ClpXP protease was first characterized in *E. coli*, where it functions as an ATP-dependent protease (Gottesman *et al.*, 1993; Wojtkowiak *et al.*, 1993). Chaperone activity of ClpX, independent of the proteolytic ClpP, was also documented (Levchenko *et al.*, 1995). Recently, homologues of ClpX were identified in yeast and murine mitochondria (van Dyck *et al.*, 1998; Santagata *et al.*, 1999), as was ClpP in human mitochondria (Corydon *et al.*, 1998; de Sagarra *et al.*, 1999). The existence of a human ClpX-like sequence in the database, its high degree of similarity to murine ClpX, and the localization of human ClpP to mitochondria all suggests that mammalian mitochondria might have ClpX not only as a potential chaperone, but also as a regulatory component of ClpXP protease. This suggestion is further supported by the findings of the current study that in plant cells both ClpX and one isomer of a nuclear-encoded ClpP co-exist in mitochondria. Thus, ClpXP appears to be a component of the proteolytic machinery in the mitochondria of multicellular organisms, whereas lower ones such as yeast might have ClpX functioning only as a chaperone.

Although only little is known about proteolytic mechanisms in plant mitochondria, the degradation of one specific protein was documented and characterized. The product of ORF239, a mitochondrial protein associated with male sterility, was found to be degraded by an ATP-dependent serine protease (Sarría *et al.*, 1998). *In vitro* assays suggested that this degradation can be attributed to Lon protease, another ATP-dependent serine protease found in bacteria and mitochondria of yeast. However, it was not clear whether Lon was solely responsible for this process. Thus, the ClpXP protease described in this study may also be instrumental in the degradation of this protein, as well as in the degradation of other plant mitochondrial proteins. Plant mitochondrial ClpXP is likely to play a housekeeping role, as its expression appears to be constitutive and relatively unchanged under different environmental conditions. In this respect, it is similar to chloroplast ClpCP protease that is also expressed constitutively (Clarke *et al.*, 1994; Shanklin *et al.*, 1995; Ostersetzer *et al.*, 1996; Ostersetzer and Adam, 1996). However, a regulatory role of ClpXP cannot be excluded, as it was shown to be essential for the control of cell cycle in bacteria (Jenal and Fuchs, 1998).

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