

## Article Addendum

# Mitochondria biogenesis via Lon1 selective proteolysis

Who dares to live for ever?

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Quality control of proteins in eukaryotic organelles is predominantly maintained by members of the ATP-dependent proteases. Even though numerous biological analyses have shed light on the functional implications of such proteases, their involvement in developmental processes of multicellular organisms has not been determined. We recently identified two *lon1* mutant alleles, both missing the carboxy terminal proteolytic domain, that show post-embryonic growth retardation resulting in delayed seedling establishment. In this addendum, we enlighten the role of Lon1 selective proteolysis in plant mitochondria biogenesis, a prerequisite for post-embryonic development and growth. In contrast to the weak *lon1-2* allele, the polypeptide encoded by the strong *lon1-1* allele carries the sensor- and substrate-discrimination domain allowing substrate recognition and binding. This type of molecular recognition hinders further degradation by the complementary Lon-independent proteolytic machineries resulting in an extra deleterious accumulation of protein aggregates into *lon1-1* mitochondria. The most challenging and informative task will be to identify the recognition motifs on the Lon protein substrates and elucidate the molecular events that control plant mitochondrial differentiation.

Maintenance of biological functions requires the constant cycling between protein synthesis and degradation. Selective proteolysis occurs in various cellular compartments to remove short-lived regulatory proteins and to prevent the potentially harmful accumulation of non-native polypeptides. The ubiquitin/26S proteasome pathway has evolved as the main highly selective proteolytic mechanism in the cytoplasm and nucleus modulating several aspects of plant development.<sup>1,2</sup> In plant organelles however, protein quality control is performed by ATP-dependent proteases that belong to the Clp,



Figure 1. The growth phenotype of *lon1* mutants. Growth retardation of *lon1-2* plants is milder than the *lon1-1* mutant allele. Bar: 1 cm.

FtsH and Lon families.<sup>3-6</sup> These proteases are members of the AAA<sup>+</sup> protein superfamily (ATPases associated with diverse cellular activities).<sup>7,8</sup>

Arabidopsis has four genes encoding for Lon proteases that contain a Serine (S)-Lysine (K) catalytic dyad<sup>4</sup> and, like their bacterial and eukaryotic homologs, may combine proteolytic and chaperone-like activities.<sup>5,6</sup> Presumably oxidative stress induces Lon-like proteolysis.<sup>9,10</sup> Nevertheless, the only designated function of Lon proteases to date is the control of cytoplasmic male sterility in common bean plants.<sup>11</sup> We recently characterized two mutant alleles of Arabidopsis *Lon1* gene, *lon1-1* caused by EMS mutagenesis resulting in a premature termination codon and *lon1-2* mutant allele caused by T-DNA insertion.<sup>12</sup> Both mutations occur at the 18<sup>th</sup> exon of *AtLon1* gene, which consists of 19 exons. The main phenotypic feature of *lon1* mutants is the post-embryonic growth retardation resulting in delayed seedling establishment. This growth retardation remains throughout the entire life cycle of the plant. Biometrics revealed that *lon1-2* allele corresponded to a weak mutant allele in comparison to the strong *lon1-1* mutant phenotype (Fig. 1).

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We postulate that ATP-dependent Lon1 protease is essential for controlling the quality of newly imported or synthesized mitochondrial polypeptides, comprising an essential regulatory mechanism towards mitochondria biogenesis. However, the possibility that Lon1 could function as a protease or chaperone on pre-existing protein moieties within undifferentiated mitochondria is not excluded. Approximately 95% of the mitochondrial proteome is encoded from the nucleus.<sup>13</sup> Hence, most of the polypeptides are imported into the organelle. Components of the mitochondrial protein import apparatus are highly abundant in promitochondrial structures and operational during the onset of dry seed embryos imbibition.<sup>14</sup> Nevertheless, these components are barely detected in mature mitochondria.<sup>14–16</sup> Moreover, the promitochondria proteome is significantly different from that identified in metabolic capable mature mitochondria.<sup>14</sup> The degradation of the mitochondrial import machinery components appears to occur with the shift in the mitochondrial population from promitochondria, to mature mitochondria. Increases in oxygen consumption and respiration are observed during this heterotrophic growth period. The activity of mitochondrial proteases may modulate the developmental transition between the two mitochondria types, through the quality control of the imported or newly synthesized polypeptides and the selective degradation of pre-existing proteins. Several lines of experimental evidence support this hypothesis. AtLon1 is targeted to plant mitochondria and complements the respiratory-deficient phenotype of the yeast *PIM1* gene homolog. Yeast *pim1* mutant mitochondria have irregular shape and accumulate protein aggregates in the matrix.<sup>17</sup> The activities of respiratory chain complexes and TCA cycle enzymes are decreased in *lon1* mutants, indicating malfunction of these pathways. Transmission electron micrographs indicated that *lon1-1* mitochondria morphology is reminiscent to maize<sup>18</sup> and rice<sup>14</sup> promitochondria from dry seed embryos. The germination efficiency of *lon1* mutants is dramatically diminished under heat-shock conditions (32°C), indicating the existence of a signaling mechanism that orchestrates mitochondria differentiation and seed germination. Ultimately, the mobilization of storage lipids for germination requires not only the peroxisomal biochemical pathways, such as  $\beta$ -oxidation and the glyoxylate cycle, but also specific enzymatic reactions performed in mature plant mitochondria. Mutations that compromise storage lipid mobilization result in post-embryonic growth retardation leading to impaired seedling establishment, as in the case of *lon1* mutants.<sup>19,20</sup>

We further propose a mechanism for the molecular recognition of protein substrates by Lon1 protease. The structural core of Lon1 protease is the AAA<sup>+</sup> module (residues 484–810) that consists of two fundamental domains: the nucleotide-binding ( $\alpha/\beta$ ) domain and the helical ( $\alpha$ ) domain. The  $\alpha/\beta$ -domain contains the conserved motifs sensor-1, Walker A and B. The  $\alpha$ -domain contains the sensor-2 motif that is characterized by a conserved Arginine residue. The AAA<sup>+</sup> module is involved in ATP hydrolysis and in protein substrate remodeling.<sup>7,8</sup> Adjacent to the AAA<sup>+</sup> module is the sensor- and substrate-discrimination (SSD) domain (Fig. 2A). This domain is mainly involved in modulating selective substrate recognition by Lon proteases so as the target protein to be either degraded or properly folded.<sup>21</sup> In line with its highly selective mode of action, the SSD domain is substantially diverse between Lon proteases (Table 1; Fig. 2B). The strong *lon1-1* mutant allele contains the entire SSD

domain, while the weak *lon1-2* allele carries only a minor segment (Fig. 2A and B). The truncated polypeptide encoded by *lon1-1* mutant allele potentially participates in protein substrate recognition and binding due to the activity of the SSD domain. Consequently, while it recognizes the substrate proteins, no further degradation is accomplished due to the absence of the proteolytic (P) domain (residues 811–985). This type of protein recognition most likely leads to delayed degradation of the misfolded or non-native polypeptides by the complementary Lon-independent proteolytic machineries, which are also abundant in the mitochondria matrix. This may result in the formation of high-molecular weight aggregates deleterious for mitochondria organization and function, causing the severe *lon1-1* growth phenotype. Experimental observations in yeast revealed the overlapping substrate specificity between the m-AAA high-molecular weight complex of mitochondrial FtsH proteases and the yeast Lon protease homolog PIM1.<sup>22,23</sup> Furthermore, the SSD domains of the bacterial Clp ATPases and Lon protease that interact with substrates have similar predicted structures.<sup>21</sup> The *lon1-2* allele lacks the SSD domain being unable to recognize target polypeptides and, plausibly does not lead to the formation of high-molecular weight aggregates. Nevertheless, the non-native polypeptides still accumulate resulting in the aberrant but rather weak *lon1-2* phenotype.

Elevated temperatures increase the fraction of high-molecular weight aggregates within cellular compartments. Under these conditions *lon1-1* germination efficiency was dramatically diminished, whereas *lon1-2* seeds exhibited relatively better germination.<sup>12</sup> Thus, loss of Lon1 proteolysis sustains the conditional formation of supramolecular protein structures with prolonged deleterious effects on seed germination indicating that mitochondria differentiation substantiates seedling establishment and development. The marginal nevertheless noticeable difference in germination rate between the *lon1* alleles further confirms that the Lon1-1 polypeptide forms auxiliary aggregates, due to the activity of the SSD domain.

In conclusion, our study has revealed the critical role for AtLon1 protease in organelle biogenesis and seedling establishment. The hypothesis we propose here should provide novel insights into the developmental mechanisms of selective proteolysis in plant mitochondria. Further analysis using a combinatorial approach, mutant analysis and proteomics, is required to decipher the molecular recognition of protein substrates by Lon1 protease and the events that control plant mitochondrial differentiation.

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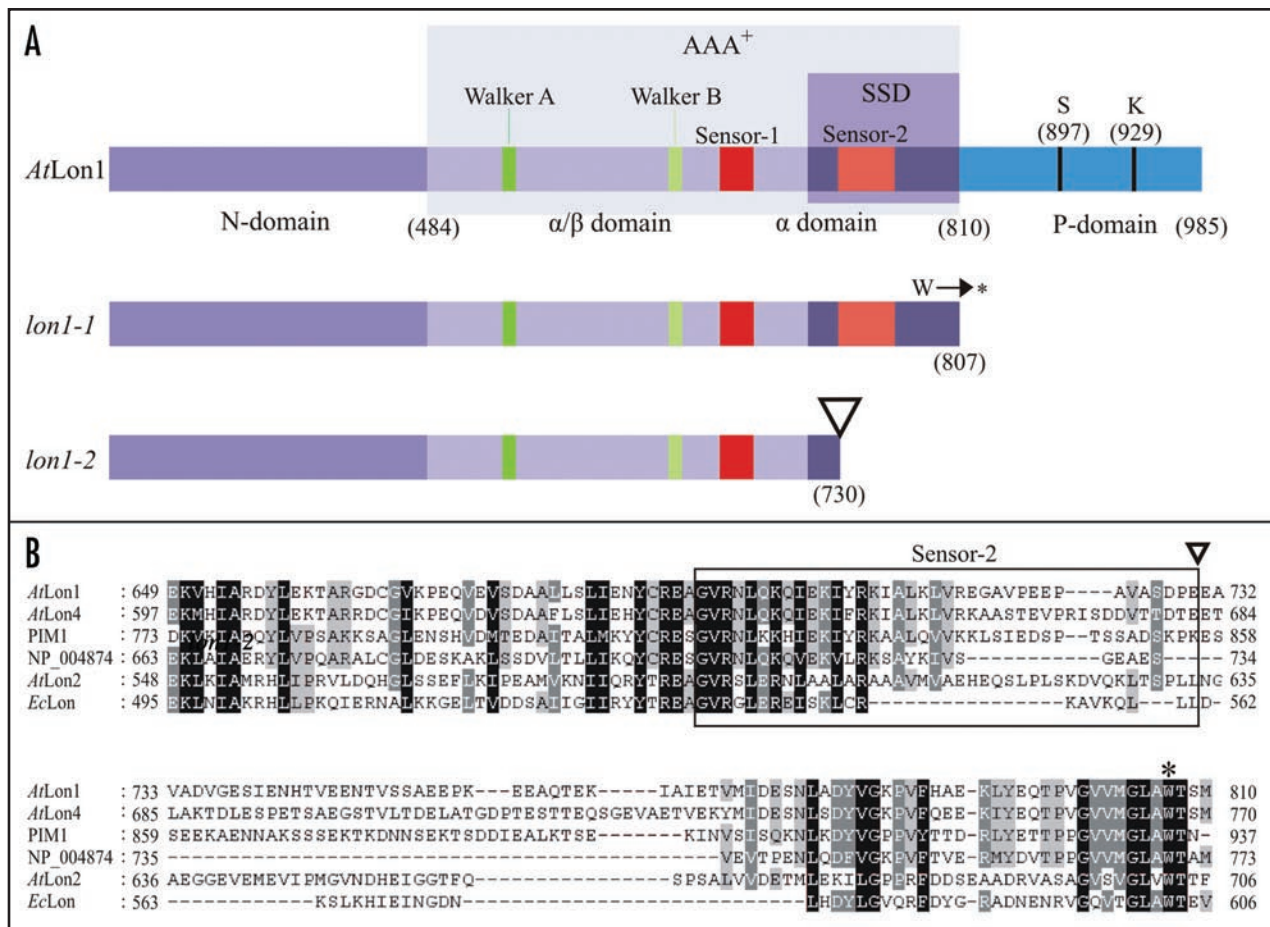


Figure 2. The structural features of AtLon1 protease and the locations of the identified mutations. (A) Schematic representation of AtLon1 protease outlining the domain structures with important consensus sequences in *lon1* mutant alleles. Numbers in parentheses represent amino acid residues defining the conserved domains. (B) Multiple sequence alignment reveals that the SSD is the most variable domain between evolutionary diverse Lon accessions. Box encompasses the amino acid residues of sensor-2 motif. In contrast to the strong *lon1-1* allele (asterisk), the weak *lon1-2* mutant allele (inverted triangle) bears part of the SSD domain.

**Table 1 Percentages of amino acid identity and similarity of the structural domains between AtLon1<sup>a</sup> and the arabidopsis AtLon2 and AtLon4 or the non-plant Lon homologs<sup>b</sup>**

	% Amino acid (identity/similarity)	
	AAA <sup>+</sup> domain (484-642aa)	SSD-domain (649-810aa)
AtLon4	91/94	63/74
PIM1	75/87	38/64
NP_004874	79/91	35/45
AtLon2	61/80	20/39
EcLon	61/77	21/34

<sup>a</sup>Coordinates of the AtLon1 domains in parentheses were defined by the comparative analysis with EcLon protease primary structure although there is no absolute definition and the domain boundaries are frequently subjected to revisions. <sup>b</sup>The Lon protein accessions are AtLon1 (At5g26860), AtLon2 (At5g47040) and AtLon4 (At3g05790) from *Arabidopsis thaliana*, the *Homo sapiens* Lon1 (NP\_004784), PIM1 (P36775) from *Saccharomyces cerevisiae* and EcLon (AAC36871) from *Escherichia coli*.

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