A peroxidase homologue and novel plastocyanin located by proteomics to the *Arabidopsis* chloroplast thylakoid lumen

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Abstract A study by two-dimensional electrophoresis showed that the soluble, lumenal fraction of *Arabidopsis thaliana* thylakoids can be resolved into 300 protein spots. After subtraction of low-intensity spots and accounting for low-level stromal contamination, the number of more abundant, lumenal proteins was estimated to be between 30 and 60. Two of these proteins have been identified: a novel plastocyanin that also was the predominant component of the total plastocyanin pool, and a putative ascorbate peroxidase. Import studies showed that these proteins are routed to the thylakoid lumen by the Sec- and delta pH-dependent translocation pathways, respectively. In addition, novel isoforms of PsbO and PsbQ were identified.

Key words: Photosystem II; Oxygen evolution; Protein import; pH translocation pathway; Twin-arginine translocase

1. Introduction

The chloroplast is the site of oxygenic photosynthesis in green algae and higher plants. A double-layer envelope membrane surrounds the soluble stroma and the highly specialized thylakoid membrane, which contains photosystems I and II, the cytochrome b_6f complex and the ATP synthase that together carry out the light reactions of photosynthesis. Most of the known photosynthetic proteins are located in the stromal phase or the thylakoid membrane, but several lumenal proteins have also been shown to be involved. These include plastocyanin, which is essentially freely soluble in the lumen, and the 33, 23 and 16 kDa polypeptides of the oxygen-evolving complex (also known as PsbO, PsbP and PsbQ, respectively), which are loosely attached to the lumenal side of photosystem II. More recently, it has become clear that the thylakoid lumen performs other important functions, and other proteins have been identified including, for example, a violaxanthin de-epoxidase [1] and a polyphenol oxidase [2,3].

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Abbreviations: AC, accession number; MALDI-TOF-MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis

To perform a more systematic characterization of the lumenal compartment, we designed a new method to isolate a highly pure fraction of soluble lumen content from spinach thylakoids. A concise analysis showed that the thylakoid lumen had a high protein concentration and contained more than 25 distinct polypeptides, among which four novel lumenal proteins were identified [4]. The large number of unknown lumenal proteins raised the question as to their role in the thylakoids. Recently, a lumenal immunophilin was discovered, and it was suggested that it participates in a signal transduction chain over the thylakoid membrane [5]. HCF136, a key protein for the assembly of photosystem II, was also located to the thylakoid lumen [6]. However, most lumenal proteins have yet to be characterized.

To apply the fast growing knowledge about the genome of *Arabidopsis thaliana* to an investigation into the lumenal proteins, we have modified the method for isolation of lumenal content from spinach thylakoids, and we have started a systematic analysis of the soluble lumen proteins from *Arabidopsis*. In this work, we present the first data from a study of *Arabidopsis* lumenal proteins by two-dimensional (2D) electrophoresis. In addition, we have characterized the cDNAs of a novel plastocyanin and a putative ascorbate peroxidase (TL29), and we have analyzed the pathways used for the targeting of these proteins across the thylakoid membrane.

2. Materials and methods

2.1. Isolation of a lumenal fraction from A. thaliana

A. thaliana ecotype *Colombia* was grown hydroponically in a nutrient solution according to [7]. To obtain large leaves that were suitable for biochemical studies, the day time was set to 8 h and the light intensity to approximately 100 μmol photons/m^2/s. After 10 weeks, healthy *Arabidopsis* leaves were harvested in 50–100 g batches, and the thylakoid lumen was isolated essentially as described in [4]. Only the two washing steps of the thylakoids with 2 mM Tricine (pH 7.8), 300 mM sucrose were omitted to increase the yield of lumen. No major change in the purity of the lumenal fraction from *Arabidopsis* was observed due to this modification. Protein quantification was carried out according to [8].

2.2. 2D electrophoresis and image analysis

For analytical gels, 100 μg of total lumenal proteins were separated by isoelectric focusing in the first dimension using the IPG-Phor system (Amersham Pharmacia Biotech). The proteins were solubilized in 5 M urea, 2 M thiourea, 4% w/v CHAPS, 20 mM dithiothreitol (DTT) and 0.8% v/v carrier ampholytes (IPG-strip buffer pH 3–10 NL, Amersham Pharmacia Biotech), and applied to a non-linear IPG-strip pH 3–10 (Amersham Pharmacia Biotech) during rehydration. Proteins were focused for 15 min at 300 V followed by a linear

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Table 1
Abundant lumenal proteins in *Arabidopsis*

Protein	AC	Method of identification
major plastocyanin (six spots)	(AJ271355)	microsequencing
plastocyanin (two spots)	(P11490)	microsequencing
PsbO	(P23321, O81917)	MALDI-TOF-MS and microsequencing
PsbO isoform	(AL049862)	MALDI-TOF-MS and microsequencing
PsbP	(Q42029)	MALDI-TOF-MS and microsequencing
putative PsbP-like	(AAC00624, O49292)	MALDI-TOF-MS and microsequencing
PsbQ isoform	(O49568, CAA17547)	MALDI-TOF-MS and microsequencing
TL29	(P82281)	microsequencing

increase in voltage from 300 V to 3500 V in 3 h. The isoelectric focusing was then continued at a constant voltage of 8000 V until a total focus time of 80 000 Vh was reached. The strips were equilibrated for 15 min in 50 mM Tris–HCl (pH 6.8), 6 M urea, 30% v/v glycerol, 2% w/v sodium dodecyl sulfate (SDS) and 1% w/v DTT followed by a 10 min incubation in the same buffer without DTT but with 2.5% w/v iodoacetamide and a trace of bromophenol blue. In the second dimension, a vertical gradient slab gel $T = 9–16\%$ with a modified Laemmli-SDS system was used [9,10]. Following silver-staining according to [11], the gels were scanned using an image scanner and evaluated with the image master 2D software (both from Amersham Pharmacia Biotech).

2.3. MALDI-TOF-MS and microsequencing

MALDI-TOF analysis of in-gel-digested proteins was carried out with a Reflex III mass spectrometer from Bruker. The samples were prepared using sequencing-grade modified trypsin (from Promega) and analyzed as described by [12]. Database searches were done with the MS BioTools software from Bruker using the *Mascot* search engine[1]. Amino-terminal microsequencing was carried out with a Procise sequencer from Applied Biosystems. Proteins were sequenced from polyvinylidene difluoride membrane following resolution by 2D electrophoresis essentially as described [13].

2.4. Import studies

Precursor forms of lumenal proteins were synthesized by transcription of cDNA clones in vitro followed by translation in a weatgerm lysate in the presence of ^{35}S-labeled methionine. After synthesis, the proteins were imported into pea chloroplasts as described [14].

3. Results

3.1. Characterization of a lumenal fraction from Arabidopsis thylakoids

The isolation method for a lumenal fraction from *Arabidopsis* thylakoids takes into account the low amounts of starting material and the poor mechanical stability of *Arabidopsis* thylakoids. To overcome these restrictions, the original method for the isolation of lumenal fraction from spinach [4] was scaled down, and the second washing step that removed peripheral proteins from the stromal surface of the thylakoids was omitted. Characterization of the lumenal fraction showed that it did not contain any detectable amounts of the major integral thylakoid D1-protein and was free from contaminations by thylakoid membrane particles. Regarding stromal contamination, ribulose bisphosphate carboxylase/oxygenase accounts for the vast majority (probably over 80%) of total stromal protein but was found to account for less than 4% of the total protein content of the lumenal fraction (data not shown).

The analysis of the lumenal fraction from spinach thylakoids by one-dimensional (1D) SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and Coomassie-staining had made it possible to identify approximately 20 unknown proteins beside the well-established plastocyanin and PsbO, PsbP and PsbQ. To perform a more detailed study of the resident proteins of the thylakoid lumen, we applied 2D electrophoresis to investigate the lumenal proteins of *Arabidopsis*. Fig. 1 shows a silver-stained 2D gel of the soluble, lumenal fraction from *Arabidopsis* thylakoids. The average protein pattern reveals approximately 300 spots that could be reproducibly detected in seven independent experiments. To obtain a more detailed picture of the lumenal proteins in *Arabidopsis*, we analyzed them by MALDI-TOF-MS and amino-terminal microsequencing. The first results of this study are shown in Fig. 1 and Table 1: the most abundant proteins of the lumenal fraction were a novel plastocyanin and the well-known proteins PsbO and PsbP. In addition, we found an isoform of PsbO and of PsbQ, respectively. We also identified a novel lumenal protein (accession number (AC) AAC00624) in the sequence of which a Pfam[2] search detected a PsbP domain. Hence, this protein could be an unknown PsbP-like protein. Furthermore, we found a novel lumenal protein, termed TL29, that revealed a strong similarity to ascorbate peroxidases (see below).

The fact that some proteins, like the novel plastocyanin, occur in more than one spot makes it difficult to estimate the number of lumenal proteins with precision. To determine an approximate number of the more abundant lumenal proteins, three gels with a similar silver stain strength were analyzed. Of a total number of 318 protein spots that could be detected in all three gels, 241 were present in the acidic range and 71 in the basic range of the pH gradient. To minimize the error that could be caused by contaminants among the low-abundance proteins, we excluded all spots with a volume lower than 30 000. This threshold corresponds to the strength of the smallest spot of TL29 in the middle of the gel in Fig. 1. The total number of protein spots above this threshold was 72, of which 59 were detected in the acidic range and 13 in the basic range of the pH gradient. Considering that some proteins, like the major form of plastocyanin, occur in more than one spot, the number of the more abundant, soluble proteins in the thylakoid lumen of *Arabidopsis* is estimated to lie between 30 and 60. No stromal proteins were identified during the characterization of the lumenal proteins shown in Fig. 1, and we therefore believe that this represents a reasonable minimal estimate of the lumen protein complement.

Table 1 lists some of the lumenal proteins identified in this study. One corresponds to the known PsbO protein (the 33 kDa protein of the oxygen-evolving complex) whereas another

[1] Available on-line: http://www.matrixscience.com/home.html.

[2] Available on-line: http://www.cgr.ki.se/Pfam.

is a novel isoform of this protein. The gene encoding this protein has been sequenced as part of the *Arabidopsis* sequencing project and the mature PsbO proteins differ in 13 positions, with the presequences showing much greater variability (data not shown). PsbP has also been identified in this study, together with another protein (termed putative PsbP-like in Table 1) whose function is currently unclear. This protein exhibits very limited homology with PsbP and may not be a true isoform. A novel isoform of the third, 16 kDa protein of the oxygen-evolving complex, PsbQ, has also been identified in this study although the known form was not detected. This isoform is 96% identical to the published genomic sequence encoding PsbQ (data not shown).

3.2. A novel plastocyanin from A. thaliana

The strongest spots in the 2D gel shown in Fig. 1 were identified to be plastocyanin. Surprisingly, this plastocyanin is a novel form that represents the major component of the lumenal plastocyanin pool, and the known plastocyanin of *Arabidopsis* [15] was only present in a minor amount. Six different spots were found to represent the novel plastocyanin and all had the same amino-terminal sequence; it is currently unclear why these spots exhibit such differing electrophoretic mobilities (we have found that plastocyanin runs as 2–3 bands even in 1D gels). We used the amino-terminal sequence of this novel plastocyanin (AC AJ271355) in searches of the expressed sequence tag database and identified a full-length cDNA clone, K3F10TP, that encodes this protein. The cDNA K3F10TP was fully sequenced and submitted to the EMBL database (AC AJ271355). Fig. 2A shows the predicted primary structure of this novel plastocyanin aligned with that of the known plastocyanin of *Arabidopsis*. The precursors of these two *Arabidopsis* plastocyanins are very similar: their primary structures of the precursor and the mature proteins are 73% and 84% identical, respectively. As with all known lumenal proteins, the novel plastocyanin is synthesized with a bipartite presequence comprising a hydrophilic envelope transit peptide followed by a more hydrophobic signal peptide. The processing site for the thylakoidal processing peptidase follows the sequence Ala-Met-Ala as shown.

Two distinct pathways are used for the translocation of lumenal proteins across the thylakoid membrane [16]. One of the pathways relies on the Sec apparatus whereas the other uses a translocation system that requires the thylakoidal delta pH for activity. Substrates for the latter pathway contain an invariant twin-arginine motif prior to the hydrophobic region of the signal peptide [17]. Plastocyanin has been shown to be targeted by the Sec pathway in other plant species [16], and Fig. 3A shows that the same applies to the novel plastocyanin identified in this study. The in vitro-synthesized precursor protein is imported into intact pea chloroplasts, efficiently transported into the thylakoid lumen and processed to mature size. The delta pH-dependent pathway can be effectively inhibited by the inclusion of nigericin in the import assay, but this compound does not inhibit translocation to any substantial effect. Azide on the other hand partially inhibits the Sec pathway [18,16] and leads to an increase in the level of intermediate-size protein (int) found in the stromal fraction (lane S).

The import data also serve to reinforce the location of this protein, and this is important because the same protein has been reported to reside in the stroma. The novel *Arabidopsis*

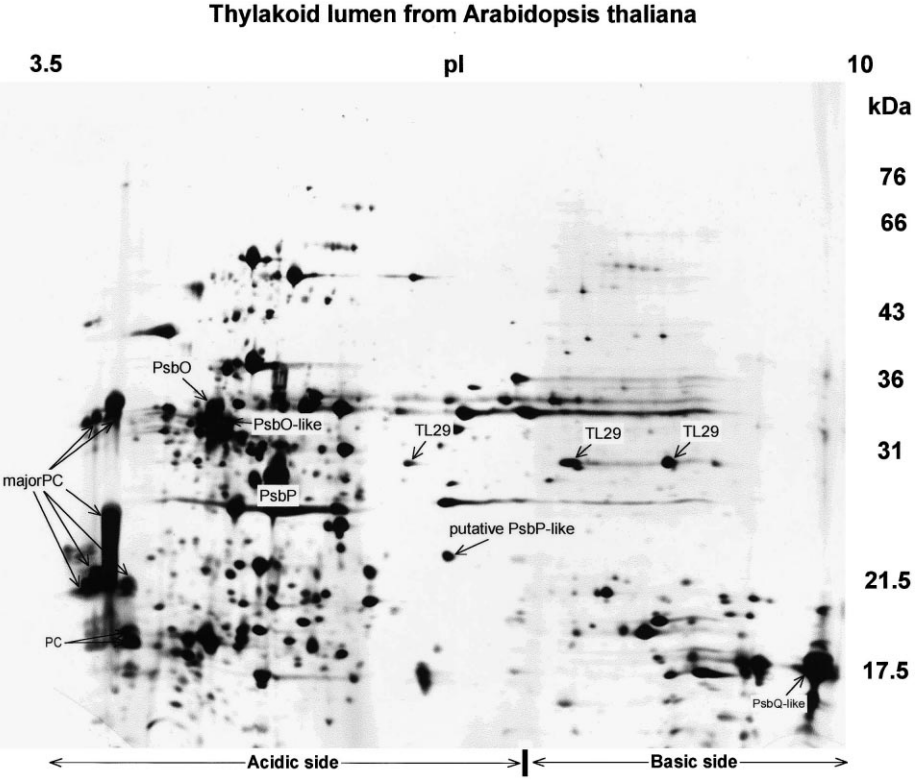

Fig. 1. Silver-stained 2D gel of the soluble, lumenal fraction of *Arabidopsis* thylakoids. The amount of protein applied to the first dimension (isoelectric focusing) was 100 μg. In seven independent experiments, the 2D gels revealed a protein pattern with a very good reproducibility and showed that the lumenal fraction can be resolved into approximately 300 protein spots.

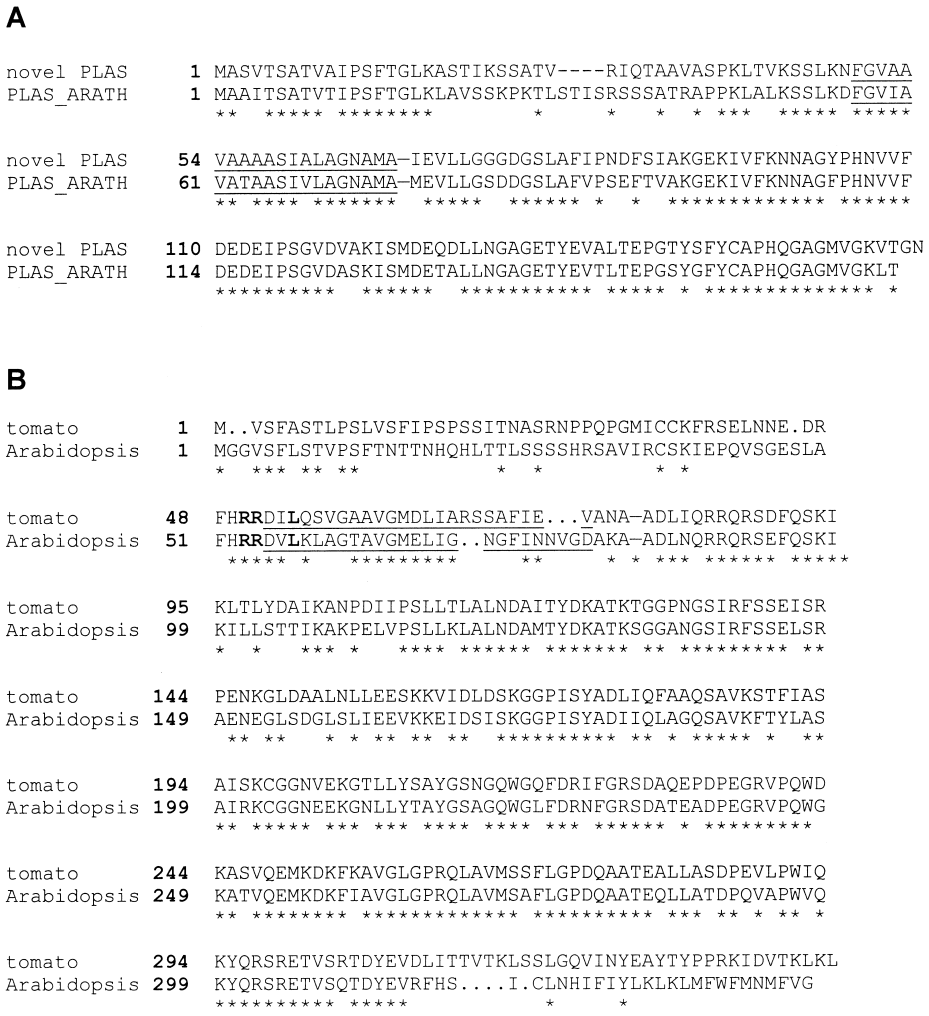

Fig. 2. A: Predicted primary sequences of a novel *Arabidopsis* plastocyanin precursor and maize/tomato TL29. The figure shows the novel plastocyanin identified in this study (AJ271355) aligned against that of the known *Arabidopsis* plastocyanin (P11490). The primary structure of the novel plastocyanin was predicted from the *Arabidopsis* cDNA K3F10TP (AA728654), and its 30 amino-terminal residues were confirmed by microsequencing. Preceding the processing site (dash) is a bipartite transit peptide with a hydrophobic core domain that is a typical feature of lumenal proteins. B: The precursor of TL29 from tomato was translated from the cDNA CLEBJ9, and the sequence of TL29 from *Arabidopsis* (p82281) was predicted from the DNA ac005359.em_pln using the program Genscan[3]. In addition, the 30 amino-terminal residues of TL29 from *Arabidopsis* were confirmed by microsequencing. As with all known lumenal proteins, the mature TL29 proteins are preceded by bipartite transit peptide: a basic, hydrophilic envelope transit peptide (first ca. 30 residues) is followed by a signal peptide containing a core hydrophobic domain (underlined). The processing site is denoted by a dash and twin-arginine motifs (suggestive of targeting by the delta pH pathway) are shown in bold.

plastocyanin identified in this study is identical to a protein, termed DRT112 (AC P42699), reported to function in DNA damage repair [19]. As DRT112 is a resident protein of the thylakoid lumen, and there is no DNA present in this compartment, we can only conclude that DRT112 was assigned an incorrect function. Our data show that this protein is, in fact, the major plastocyanin of *Arabidopsis*.

3.3. A putative lumenal ascorbate peroxidase

Amino-terminal sequence data (AC P82281) were obtained for a second protein (TL29) with a molecular mass of 29 kDa as judged by SDS–PAGE. Three different spots were found to represent this protein and, as with plastocyanin, all three had the same amino-terminal sequence (not shown). The sequence is homologous to a predicted sequence in the *Arabidopsis* genomic clone ac005359.em_pln, and the full-length protein sequence was predicted using the program Genscan[3] (Fig. 2B). A full-length *Arabidopsis* cDNA clone was not found for this protein, but we sequenced the homologous tomato cDNA CLEBJ9 that does encode the full-length precursor of TL29 (AC AJ251882). In Fig. 2B, the sequence of this precursor is shown aligned against that of TL29 from *Arabidopsis*. TL29 reveals a strong homology to ascorbate peroxidases from higher plants, but it does not have a distinct motif for heme binding and the catalytical center that are typical of peroxidases (not shown). Hence, its function needs to be clarified by additional experiments.

As expected for a lumenal protein, TL29 is synthesized with

[3] Available on-line: http://genius.embnet.dkfz-heidelberg.de:8080/menu.

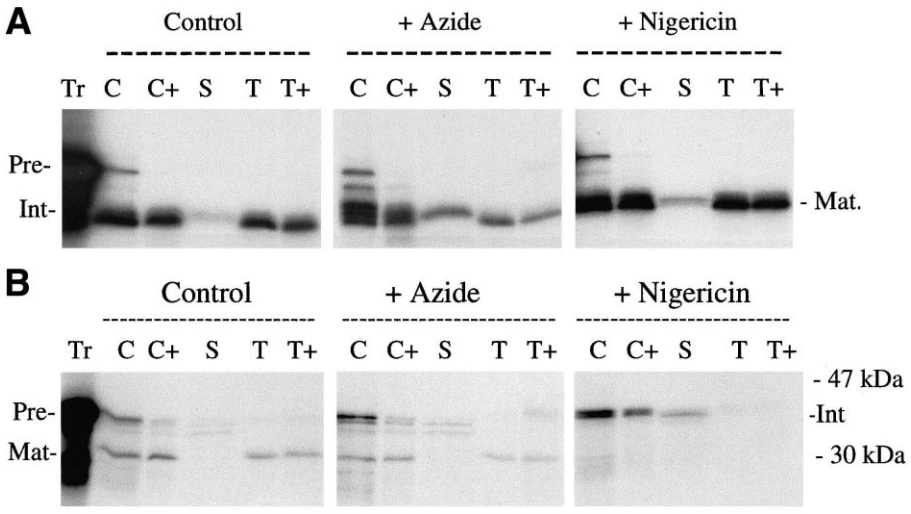

Fig. 3. The novel *Arabidopsis* plastocyanin and tomato TL29 are transported into the thylakoid lumen by the Sec- and delta pH-dependent pathways, respectively. Precursor forms of the *Arabidopsis* plastocyanin (A) and tomato TL29 (B) were synthesized in vitro by transcription–translation of cDNA clones, and the precursor proteins (lanes Tr) were incubated with isolated intact pea chloroplasts. After incubation, samples were analyzed of the chloroplasts (C), protease-treated chloroplasts (C+) and the stromal (S) and thylakoid (T) fractions after lysis of the organelles and pelleting of the membranes. Lane T+: thylakoids were treated with thermolysin before analysis. Lanes Pre, Int and Mat denote precursor proteins, stromal intermediates and mature forms, respectively. The mobilities of 47 and 30 kDa molecular weight markers are shown in B.

a bipartite presequence, and we again carried out import assays to identify the targeting pathway for this protein. Fig. 3B shows that tomato TL29 is synthesized as a precursor protein, imported into chloroplasts and converted to two forms: an intermediate-size protein in the stroma (lane S) and a mature form in the thylakoids (lane T). The latter protein is resistant to proteolysis of thylakoids (lane T+), confirming a lumenal location for TL29. The presence of the Sec pathway inhibitor, azide, has essentially no effect on import but nigericin totally blocks transport across the thylakoid membrane and leads to an accumulation of the stromal intermediate. This result indicates that TL29 is targeted by the delta pH-dependent pathway, and this supposition has been confirmed by competition assays using over-expressed precursors of known delta pH substrates (data not shown). Consistent with these data, a twin-arginine motif is present prior to the hydrophobic regions of both the *Arabidopsis* and tomato precursor proteins.

4. Discussion

We have designed a method to isolate a lumenal fraction from *A. thaliana* thylakoids that is sufficiently pure to study lumenal proteins. The first results of a proteomic study of the thylakoid lumen show that it contains between 30 and 60 soluble proteins. A detailed analysis of the lumenal proteins in *Arabidopsis* will be presented elsewhere, but this study has succeeded in identifying two major lumenal proteins. One of these is a novel isoform of plastocyanin in this species. Polymorphism of plastocyanin was already reported for some other higher plants such as the black poplar [20], parsley [21] and tobacco [22]. However, it remains to be determined whether the isoforms of plastocyanin have distinct functions in these species or in *Arabidopsis*. Isoforms were also detected of PsbO and TL29, and this indicates that polymorphism is a feature of a larger group of lumenal proteins in *Arabidopsis*. Multiple isoforms of PsbO have similarly been demonstrated in other plants including pea [23].

The second protein is highly homologous to peroxidases, in particular to ascorbate peroxidases. According to [24], there is no ascorbate peroxidase resident in the thylakoid lumen. Instead, hydrogen peroxide produced in the lumen has been considered to diffuse out to the stroma-located peroxidases for decomposition (reviewed in [25]). However, several reports have shown that photosystem II can produce hydrogen peroxide [26–28], and thus the presence of peroxidase in the lumen seems essential to protect the lumenal proteins and the membrane proteins exposed on the inner side of the thylakoid membrane. An earlier screening of a lumenal fraction from spinach for enzymatic activities showed that it contained a considerable amount of peroxidase activity [4]; TL29 could be a candidate for an enzyme responsible for this peroxidase activity. Despite the similarity of TL29 to other ascorbate peroxidases, its primary structure does not have a known peroxidase motif. In addition, we have not yet been able to experimentally show that TL29 is functionally active in the lumen of *Arabidopsis*.

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