A Stromal Hsp100 Protein Is Required for Normal Chloroplast Development and Function in Arabidopsis

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Molecular chaperones are required for the translocation of many proteins across organellar membranes, presumably by providing energy in the form of ATP hydrolysis for protein movement. In the chloroplast protein import system, a heat shock protein 100 (Hsp100), known as Hsp93, is hypothesized to be the chaperone providing energy for precursor translocation, although there is little direct evidence for this hypothesis. To learn more about the possible function of Hsp93 during protein import into chloroplasts, we isolated knockout mutant lines that contain T-DNA disruptions in either atHSP93-V or atHSP93-III, which encode the two Arabidopsis (Arabidopsis thaliana) homologs of Hsp93. atHsp93-V mutant plants are much smaller and paler than wild-type plants. In addition, mutant chloroplasts contain less thylakoid membrane when compared to the wild type. Plastid protein composition, however, seems to be largely unaffected in atHsp93-V knockout plants. Chloroplasts isolated from the atHsp93-V knockout mutant line are still able to import a variety of precursor proteins, but the rate of import of some of these precursors is significantly reduced. These results indicate that atHsp93-V has an important, but not essential, role in the biogenesis of Arabidopsis chloroplasts. In contrast, knockout mutant plants for atHsp93-III, the second Arabidopsis Hsp93 homolog, had a visible phenotype identical to the wild type, suggesting that atHsp93-III may not play as important a role as atHsp93-V in chloroplast development and/or function.

The vast majority of plastid proteins are encoded within the nucleus, rather than in the plastid genome. As a consequence, these proteins must be imported into the organelle following their synthesis on cytoplasmic ribosomes (Chen and Schnell, 1999; Keegstra and Cline, 1999; Schleiff and Soll, 2000; Jarvis and Soll, 2002). The process of plastid protein import has been studied extensively using isolated pea (Pisum sativum) chloroplasts as a model system. From these studies, it has been determined that protein import is mediated by a proteinaceous transport machinery located within the two membranes of the chloroplast envelope (Chen and Schnell, 1999; Keegstra and Cline, 1999; Schleiff and Soll, 2000; Jarvis and Soll, 2002).

Energy for the translocation of precursor proteins into plastids is provided by ATP hydrolysis within the stroma (Theg et al., 1989). ATP hydrolysis has also been implicated in providing the driving force for the post-translational import of proteins into the endoplasmic reticulum (ER) and mitochondria (Rapoport et al., 1999; Herrmann and Neupert, 2000). The major factors mediating ATP hydrolysis in the ER and mitochondrial protein transport systems are heat shock protein 70s (Hsp70s; Jensen and Johnson, 1999; Piløn and Schekman, 1999; Strub et al., 2000; Voos and Röttgers, 2002). Thus, it was believed that a stromatic Hsp70 would be found to drive protein translocation into chloroplasts as well. However, when isolated import complexes from pea chloroplasts were probed for the presence of stromal molecular chaperones, a member of the Hsp100 family of chaperones was found instead (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998).

Hsp100 proteins (also known as Clp [caseinolytic protease] proteins) are a diverse class of molecular chaperones involved in a wide variety of essential metabolic processes throughout prokaryotic and eukaryotic phylogenies (Schirmer et al., 1996). They were first identified as the regulatory component of the Clp proteolytic complex in Escherichia coli (Hwang et al., 1987; Katayama et al., 1988). Subsequently, they have been found to be involved in regulating the DNA-binding activity of several proteins (Mhammedi-Alaoui et al., 1994; Wickner et al., 1994; Lazazzera and Grossman, 1997) and in providing tolerance to a variety of environmental stresses such as heat (Sanchez and Lindquist, 1990; Squires et al., 1991) and salt (Krüger et al., 1994), among other functions. In general, Hsp100 proteins operate by disassembling protein aggregates or oligomers via the energy provided by ATP hydrolysis (Schirmer et al., 1996).
Two classes of Hsp100 proteins exist. Hsp100s of the ClpA, ClpB, ClpC, ClpD, and ClpE subfamilies are Class 1 proteins (Schirmer et al., 1996; Derré et al., 1999). These proteins have two ATP-binding domains, whereas class 2 proteins, which are shorter, have just a single nucleotide-binding domain (Schirmer et al., 1996). Higher plants contain homologs of the ClpB, ClpC, and ClpD subfamilies (Schirmer et al., 1996). The plant ClpB proteins are primarily cytoplasmic (Boston et al., 1996), while the plant ClpC and ClpD homologs are found within chloroplasts (Moore and Keegstra, 1993; Shanklin et al., 1995; Boston et al., 1996; Nakashima et al., 1997; Nakabayashi et al., 1999; Zheng et al., 2002). The molecular chaperone that was found in association with the pea chloroplastic protein import apparatus is a member of the ClpC subfamily and is known as Hsp93, reflecting its calculated molecular mass of 93 kD for the mature form of the protein (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). While the majority of Hsp93 molecules are present in soluble form in the chloroplast stroma, a significant proportion of Hsp93 proteins are found in association with the inner envelope membrane, presumably through their interaction with the import complex (Moore and Keegstra, 1993; Nielsen, 1997; M. Akita and K. Keegstra, unpublished data). A similar situation exists for the mitochondrial import-associated Hsp70 chaperone, which is mostly localized within the mitochondrial matrix but can be found in a membrane-bound form via an interaction with the inner membrane import complex component Tim44 (Pfanner et al., 1997; Herrmann and Neupert, 2000; Voos and Röttgers, 2002).

Hsp93 was found to be a component of chloroplastic import complexes regardless of whether precursor proteins were also present (Nielsen et al., 1997; Kouranov et al., 1998). Several lines of evidence indicate that this association of Hsp93 with isolated import complexes is relevant to the process of precursor transport. First, Hsp93 coimmunoprecipitates the precursor to the small subunit of Rubisco (prSS) only under conditions that support either binding or translocation of the preprotein (Nielsen et al., 1997). Secondly, Hsp93 is able to coimmunoprecipitate several precursor proteins that utilize the general import apparatus of the chloroplast envelope but not plastid proteins that do not use this import machinery (Nielsen et al., 1997). The association of Hsp93 with prSS is disrupted by the addition of ATP, but not GTP, to an import reaction (Nielsen et al., 1997). Because Hsp100 chaperones interact with their substrates in an ATP-sensitive manner (Wickner et al., 1994; Wawrzynow et al., 1995), this ATP dependence suggests that the association between Hsp93 and prSS is physiologically relevant (Nielsen et al., 1997). Finally, the interaction between Hsp93 and prSS decreases with time during an import reaction (Nielsen et al., 1997). This observation indicates that prSS proteins associated with Hsp93 are functional import intermediates (Nielsen et al., 1997).
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Madison (Krysan et al., 1999). A second mutant, in the Columbia (Col) background, was obtained from the Syngenta Arabidopsis Insertion Library, which contains approximately 100,000 T-DNA mutagenized Arabidopsis lines (Sessions et al., 2002). The T-DNA inserts in these knockout mutant lines were located within the seventh exon (Col background), out of nine total exons, and within the last exon (Ws background) of the atHSP93-V gene (Fig. 1A). Both mutants had identical visible phenotypes (see below); only the mutant in the Ws background was used for the detailed characterization described in this report.

The presence of the T-DNA insert within the last exon of atHSP93-V resulted in a truncated mRNA being produced. Primers specific to the 5' end of atHSP93-V were able to amplify a product from mRNA isolated from mutant plants, but primers specific to the 3' end of the gene were not (Fig. 1B). Thus, accumulation of the full-length mRNA for atHSP93-V was abolished in the mutant line. The gene encoding atHsp93-III, a homolog of atHsp93-V, was still expressed in atHsp93-V mutant plants (data not shown).

Because the mature forms of atHSP93-V and atHsp93-III are almost 90% identical to one another at the amino acid level (Supplemental Fig. 1), it is unlikely that antibodies generated against the whole protein, such as those used in this study, would be specific. Thus, when either total protein extract or chloroplast protein from atHSP93-V knockout plants was probed for the presence of Hsp93 proteins, an immunoreactive band of the expected size was detected (Fig. 1C). The overall amount of Hsp93 proteins appeared to be reduced in the mutant, however. Interestingly, a smaller immunoreactive band was detected in protein isolated from the mutant line but not in protein from the wild type (Fig. 1C). Thus, as is suggested by the reverse transcription (RT)-PCR results, it appears that a truncated version of atHSP93-V is in fact being produced in the knockout plants. This truncated version would likely not be functional, however, because the protein would lack several important regions of atHSP93-V, including the second nucleotide-binding domain. This hypothesis is supported by the fact that the observed phenotype (see below) of atHSP93-V knockout plants is inherited in a recessive manner, suggesting that any product being generated by the mutated gene is non-functional within the plant.

The same samples that were analyzed via immunoblotting were also examined by Coomassie staining. No significant differences could be observed when total protein extracts or chloroplast proteins from wild-type and mutant plants were compared, with one exception (data not shown). A band of approximately 90 to 100 kD was observed to be reduced in intensity in atHSP93-V knockout plants when compared to wild-type individuals (data not shown). This band was cut out and the proteins contained within it were identified by mass spectrometry. In the wild-type sample, peptide fragments identical to both atHSP93-V and atHsp93-III were

Figure 1. Characterization of the insertional mutations in atHSP93-V and atHSP93-III knockout lines. A, Schematic depicting the structure, from the start codon to the stop codon, of the atHSP93-V gene. Exons are represented by gray boxes; introns are symbolized by thin lines. The approximate locations of the T-DNA insertions in the two knockout mutant lines described in this report are indicated. The second T-DNA insertion shown (in the last exon) is found in the line used for all subsequent analyses. B, RT-PCR analysis for atHSP93-V. Primers specific to either the 5' end of the atHSP93-V gene, upstream of the T-DNA insertion, or the 3' end of the gene, downstream of the T-DNA insertion, were used on mRNA isolated from wild-type (WT) and atHSP93-V knockout (KO) mutant plants. C, Immunoblot analysis for Hsp93 proteins. Total leaf protein from 4-week-old soil-grown plants (wild-type [lane 1] and atHSP93-V mutant [lane 2] lines) was extracted by boiling tissue samples in SDS and β-mercaptoethanol. Protein extract equivalent to equal amounts of starting fresh weight was separated by SDS-PAGE and analyzed by immunoblotting with antibodies to Hsp93 proteins. Intact chloroplasts were isolated from 4-week-old wild-type (lane 3) and atHSP93-V mutant (lane 4) plants that had been grown on plates. Total chloroplasts equivalent to 10 μg of chlorophyll were separated by electrophoresis and immunoblotted with antisera against Hsp93 proteins. A possible truncated protein produced by the atHSP93-V gene in mutant plants is indicated (*). D, Schematic for the atHSP93-III gene (from the start codon to the stop codon), indicating the location of a T-DNA insertion in the knockout mutant line. E, RT-PCR analysis for the atHSP93-III gene. Primers specific to the region of the gene upstream of the T-DNA insert (5' primers) or to the region of atHSP93-III downstream of the insert (3' primers) were used to analyze mRNA isolated from either wild-type (WT) or atHsp93-III knockout (KO) plants.
detected (data not shown). However, in protein extracts from mutant plants, only fragments identical to atHsp93-III were found (data not shown). Thus, it appears that full-length atHsp93-V protein is not present in the knockout mutant line.

atHsp93-V mutant plants were much smaller and paler than wild-type plants of the same ecotype (Fig. 2). These differences could be observed throughout development, although the size difference in very young seedlings was minor (data not shown). As the plants grew, however, the disparity in size between wild-type and mutant seedlings became more pronounced and was obvious by 2 to 3 weeks after germination (compare Fig. 2, A and C). Despite the alterations in overall size and color, atHsp93-V mutant plants reached major developmental milestones, such as the emergence of flower buds and bolting, at approximately the same age as did wild-type individuals (compare Fig. 2, B and D).

We quantified the chlorophyll levels present in wild-type and atHsp93-V mutant plants at various ages (Fig. 3). Average chlorophyll levels for the wild type ranged from approximately 1.5 mg chlorophyll/g fresh weight during the first 10 d after germination to approximately 1.0 mg chlorophyll/g fresh weight at later times. Chlorophyll levels in the atHsp93-V knockout mutant line were significantly lower. Average values were between approximately 0.6 and approximately 0.8 mg chlorophyll/g fresh weight at all ages tested. Overall, the atHsp93-V mutant plants contained approximately 50% to 60% of the chlorophyll levels observed for wild-type plants throughout development.

In addition to the atHsp93-V knockout mutant described above, we also isolated an atHsp93-III knockout mutant line from the AFGC T-DNA mutagenized Arabidopsis population (Krysan et al., 1999). The T-DNA insert present in this line was located within the fifth exon (out of nine) of \textit{atHSP93-III} (Fig. 1D). RT-PCR analysis confirmed that no \textit{atHSP93-III} mRNA was produced in the mutant plants (Fig. 1E), although mRNA from \textit{atHSP93-V} was detected (data not shown). atHsp93-III knockout mutants appeared similar to wild-type plants at all stages of development (Fig. 2, E and F). Due to their lack of an obvious phenotype, no further molecular characterization was carried out on these plants at this time.

Chloroplast Structure and Composition in the atHsp93-V Knockout Mutant Line

Disruption of both copies of the \textit{atHSP93-V} gene by a T-DNA insert resulted in a significant reduction in overall chlorophyll levels in mutant plants (Fig. 3). These plants, therefore, could possibly have other alterations in chloroplast physiology. To examine this possibility in more detail, transmission electron microscopy was performed on leaf tissue isolated from wild-type and atHsp93-V knockout mutant plants (Fig. 4; data not shown). Tissue for these experiments was taken from 6-d-old, 2-week-old, and 4-week-old soil-grown individuals. At 6 d after germination, chloroplasts from the mutant line were slightly smaller than wild-type chloroplasts (compare Fig. 4, A and B). In addition, there appeared to be less thylakoid membrane present in mutant chloroplasts than in chloroplasts from wild-type tissue. A comparison of chloroplasts isolated from older tissues also showed differences between the wild type and the knockout mutant line (Fig. 4; data not shown). These results may explain the decrease in chlorophyll levels observed for the atHsp93-V mutant plants. A reduction in the amount of thylakoid membrane would mean less surface area for chlorophyll incorporation and thus, a paler phenotype.

Having determined that atHsp93-V knockout mutant plants have decreased amounts of thylakoid...
membrane within their chloroplasts, we wanted to determine whether endogenous plastid protein levels were also affected in the mutant line. To do this, we analyzed, by immunoblotting, total protein extracted from 2-week-old and 4-week-old soil-grown plants (compared on the basis of equal amounts of starting tissue fresh weight) and total chloroplast protein isolated from 4-week-old plate-grown plants (compared on the basis of equal amounts of chlorophyll). Figure 5 shows representative results from the protein samples derived from isolated chloroplasts; total protein extracts from soil-grown plants gave similar results. Overall, no significant differences were observed when samples from wild-type and atHsp93-V mutant plants were compared. The protein levels of various components of the chloroplastic protein import machinery appeared to be largely unaffected by the loss of atHsp93-V. In addition, several stromal enzymes, from a variety of metabolic pathways, appeared to be present in mutant chloroplasts at levels comparable to those seen for wild-type chloroplasts. The one exception to these results was S78, a stromal Hsp70, whose protein levels were slightly increased in samples from the mutant line. Thus, it is possible that the absence of atHsp93-V from mutant chloroplasts resulted in an up-regulation of the protein levels for this molecular chaperone.

It is interesting to note that no reduction in protein levels for plastocyanin (PC), a thylakoid lumen protein, and light-harvesting chlorophyll a/b-binding protein (LHCP), a protein localized to the thylakoid membrane, was observed in atHsp93-V knockout plants despite the fact that mutant chloroplasts had less thylakoid membrane (Fig. 5; data not shown). However, it is possible that if sample normalization had been performed differently (i.e. if gels were loaded with equal numbers of chloroplasts instead of equal amounts of chlorophyll [plate-grown plants] or equal fresh weight [soil-grown plants]), then different results may have been obtained.

Import into atHsp93-V Knockout Mutant Chloroplasts Is Impaired

In the pea chloroplast protein import system, Hsp93 is predicted to be the factor responsible for driving precursor translocation (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). Therefore, we analyzed atHsp93-V knockout plants, which lack one of the two Arabidopsis chloroplastic Hsp93 isoforms, to determine whether absence of the chaperone had any effect on import into mutant chloroplasts. First, we determined whether chloroplasts isolated from atHsp93-V mutant plants were able to import precursor proteins during an in vitro assay. The precursors that were used represent four distinct subcompartments within the chloroplast: prSS, a stromal protein; the precursor to LHCP (prLHCP), a thylakoid membrane protein; the precursor to PC (prPC), which is localized to the thylakoid lumen; and a truncated version of the...
precursor to Tic110 (tp110-110N), an integral protein of the inner envelope membrane. Mutant chloroplasts were able to import all of these precursors, as indicated by the appearance of the mature-sized protein, within 10 min during an import assay (data not shown). On average, the total amount of precursor import into chloroplasts isolated from atHsp93-V knockout plants was only 65% to 75% of that observed for chloroplasts isolated from the wild type, with the exception of prPC, which was imported at approximately the same levels as in wild-type chloroplasts (Fig. 6). Thus, mutant chloroplasts appear to be impaired in the transport of some, but not all, precursor proteins.

Next, to investigate the import of precursor proteins into chloroplasts isolated from atHsp93-V mutant plants in more detail, we compared the rate of prSS transport into either wild-type or mutant chloroplasts. prSS was very rapidly imported into chloroplasts isolated from wild-type plants. Conversion of prSS to SS, indicating that the precursor had been translocated across the chloroplast envelope, was observed even at the very earliest time points tested (Fig. 7A). The import of prSS into wild-type chloroplasts was saturated within 10 min (Fig. 7B). Import of prSS into chloroplasts isolated from atHsp93-V knockout mutant plants, however, proceeded at a significantly slower rate. Processing of prSS to SS was not apparent until 2 min after reaction initiation (Fig. 7A). As with wild-type chloroplasts, prSS import into mutant chloroplasts achieved maximal levels after 10 min, although the levels obtained were not as high as those seen for the wild type. On average, the rate of prSS import into chloroplasts isolated from atHsp93-V mutant plants was approximately 50% of that measured for chloroplasts isolated from wild-type plants (Fig. 7B).

**DISCUSSION**

At least two possibilities exist for the function of Hsp93, an Hsp100 protein of the ClpC subfamily, within chloroplasts. Hsp100s were first identified in *E. coli* as a component of an ATP-dependent protease complex (Hwang et al., 1987; Katayama et al., 1988). The Hsp100 identified, ClpA, in this two-subunit complex acts as a regulatory factor, controlling the...
action of the proteolytic component, ClpP (Hwang et al., 1987; Katayama et al., 1988). Chloroplasts of higher plants encode a homolog of the ClpP protein within the organellar genome (Shanklin et al., 1995; Boston et al., 1996). It is hypothesized that plastid-localized ClpC proteins, which are homologous to bacterial ClpA, may substitute for the function of ClpA in the plastid Clp proteolytic complex (Shanklin et al., 1995; Boston et al., 1996). Immunological experiments have demonstrated an interaction of ClpP and ClpC proteins within barley chloroplasts (Desimone et al., 1997). In addition, Hsp93 (a ClpC protein) isolated from pea chloroplasts has been found to stimulate the in vitro activity of bacterial ClpP, although it had no effect on the in vitro activity of recombinant pea ClpP (Shanklin et al., 1995).

Hsp93 has also been identified as a component of the protein import apparatus of pea chloroplasts (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998). It is known that molecular chaperones are essential components of the posttranslational protein import systems of mitochondria and the ER, providing the energy to pull the incoming precursor proteins into the organelle (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000; Voos and Röttgers, 2002). Thus, it is predicted that a similar role for a chaperone exists within the chloroplastic protein transport machinery. Recent experiments investigating the role of a stromal Hsp70 during chloroplastic protein import have found that when the transit peptide of a precursor protein is mutated such that it can no longer bind to stromal Hsp70, the import of the precursor is unaffected (Rial et al., 2003). These results, along with the observation that Hsp93 is the only chaperone consistently found to be present within import complexes isolated from chloroplasts (Akita et al., 1997; Nielsen et al., 1997; Kouranov et al., 1998), make Hsp93 currently the best candidate to provide the driving force needed during plastid precursor translocation.

To investigate the possible role of Hsp93 within chloroplasts in more detail, we isolated a homozygous Arabidopsis knockout line containing a disruption in \( atHSP93-V \), which encodes one of the two Arabidopsis chloroplast-localized Hsp93 homologs. The \( atHSP93-V \) mutant line was distinctly paler than wild-type plants (Fig. 2). Thus, some aspect of chloroplast development is affected in the mutant plants. This impairment may be related to thylakoid development as \( atHSP93-V \) mutant chloroplasts contained less chlorophyll (Fig. 3) and less thylakoid membrane (Fig. 4) than did wild-type chloroplasts. Two separate knockout mutants for \( atHSP93-V \), in two different ecotypes (Ws and Col) of Arabidopsis, display similar visible phenotypes (data not shown), confirming that the phenotype observed for the knockout mutant line is indeed due to the disruption in the \( atHSP93-V \) gene.

In addition to having a pale phenotype, \( atHSP93-V \) mutant plants were altered in their capacity to import precursor proteins into chloroplasts. When the rate of import of prSS, a stromal protein, was examined, a decrease of approximately 50% in the overall translocation rate into mutant chloroplasts was observed (Fig. 7), suggesting that \( atHSP93-V \) may indeed be important for the movement of precursors into the organelle. The translocation of two additional chloroplastic proteins, prLHCP and tp110-110N, which also utilize the general import machinery, was impaired in
the atHsp93-V knockout line as well (Fig. 6). However, the import of a fourth protein, prPC, into mutant chloroplasts was largely unaffected, indicating that atHsp93-V function may not be needed for the transport of all precursor proteins or, alternatively, that it is not always involved in the rate-limiting step of chloroplast protein import. Additional experiments addressing the rate of import of a variety of precursors will be necessary to determine the exact effect the loss of atHsp93-V function has on the chloroplast protein import process.

Although the experiments reported here provide new insights toward understanding the role of Hsp93 in chloroplasts, they also raise some interesting new questions. One is whether the reductions seen in the amounts of chlorophyll (Fig. 3) and thylakoid membrane (Fig. 4) in the atHsp93-V mutant are caused by the observed defect in chloroplast protein import (Figs. 6 and 7). Many proteins necessary for the development of thylakoids are encoded in the nucleus and must be imported into the organelle (Keegstra and Cline, 1999). Thus, when protein import into chloroplasts is impaired (as it is in atHsp93-V mutant plants), thylakoid biogenesis may be influenced. However, the endogenous levels of several plastid proteins were similar between wild-type and atHsp93-V mutant plants, as measured by immunoblotting and Coomassie staining (Fig. 5; data not shown), despite the fact that some of these proteins (i.e. LHCP, Tic110) were shown to be imported less efficiently into mutant chloroplasts in vitro (Fig. 6). One possible explanation of these results is that the precursors to these proteins are stable in the cytoplasm such that they can eventually be imported to the same level as in the wild type, although at a slower rate. On the other hand, it is possible that some precursor proteins are not stable in the cytoplasm and slower rates of import for these precursors could lead to a reduced accumulation of the mature proteins in mutant chloroplasts and ultimately to changes in the amounts of chlorophyll and thylakoid membranes. If this interpretation is correct, one important issue for future studies is to identify proteins that do not accumulate to wild-type abundance in atHsp93-V mutant chloroplasts and determine whether their reduced levels cause the observed developmental defects.

A second question that needs further investigation is whether the observed defects in chloroplast protein import (Fig. 6 and 7) are caused directly by the lack of atHsp93-V or whether the import defects are a secondary consequence of the absence of atHsp93-V. The simplest interpretation is that atHsp93-V is directly involved in protein import and that when this protein is absent, the import of chloroplast precursors is impaired. While we favor this interpretation, we cannot eliminate alternative explanations. For example, it is possible that the slower rate of import is caused indirectly by reduced turnover of certain proteins due to lowered Clp protease function. It is also possible that the loss of atHsp93-V chaperone activity in mutant plants leads to an impairment in the ability of imported proteins to achieve their native conformation and/or suborganellar location, which ultimately leads to reduced rates of protein import. Further experiments will be needed to evaluate these possibilities.

A third unresolved question is why the knockout mutants for the two Arabidopsis Hsp93 genes give such different phenotypes. In contrast to the dramatic phenotype of the atHsp93-V mutant, knocking out the atHSP93-III gene in Arabidopsis had no obvious effect on plants (Fig. 2). The visible phenotype of these mutants was identical to that of wild-type plants at all ages examined. One possible explanation is that atHsp93-III may play a different, less important role in Arabidopsis chloroplasts than does atHsp93-V. Alternatively, although the mature forms of atHsp93-V and atHsp93-III are almost 90% identical to one another at the amino acid level (Supplemental Fig. 1), it is possible that atHsp93-III cannot completely compensate for the loss of atHsp93-V. For example, even though atHSP93-V and atHSP93-III have been reported to be expressed constitutively under all conditions tested to date (Nakabayashi et al., 1999; Zheng et al., 2002), they may be in fact differentially expressed at a specific point in development that has not yet been looked at, such that atHsp93-III is not present at sufficient levels when the need for Hsp93 proteins in Arabidopsis cells is critical but that atHsp93-V is present at the necessary time. It is also possible that atHsp93-V and atHsp93-III, despite their overall similarity, are actually performing specialized, although perhaps partially overlapping, functions within Arabidopsis chloroplasts. Thus, atHsp93-III may be able to only partially compensate for the loss of functional atHsp93-V because it can only inefficiently accomplish the task normally done by atHsp93-V. Finally, it is possible that the total level of Hsp93 protein, rather than the specific Hsp93 isoform present, within Arabidopsis cells is what determines the overall phenotype. If atHsp93-V protein were normally present at much higher levels than atHsp93-III protein, then loss of atHsp93-V may indeed have a dramatic effect on phenotype because the levels of atHsp93-III remaining would not be high enough to compensate. Antibodies currently available in our lab for the Hsp93 proteins do not allow an evaluation of this possibility at this time, although the observation in this report that atHsp93-V mutant plants have a noticeable reduction in the levels of a band of approximately 90 to 100 kD on Coomassie-stained protein gels (see “Results” data not shown) may lend support to the hypothesis. It should be possible to distinguish among these proposed explanations through complementation studies of the atHsp93-V mutant, using the atHSP93-V and atHSP93-III genes expressed under various constitutive and native promoters; such studies are currently under way.

Recently, a splice-site mutation in the atHSP93-III gene (also known as ClpC2) has been described in a report looking for suppressors of the variegation
phenotype of the Arabidopsis var2 mutation (Park and Rodermel, 2004). Plants homozygous for this mutation in athSP93-III, which leads to an absence of atHsp93-III protein, have a phenotype that is very similar to wild-type plants, although they may be slightly darker and slower-growing than normal (Park and Rodermel, 2004). Antisense plants that partially suppress both athSP93-V (also known as ClpC1) and athSP93-III expression have a “yellow-heart” phenotype in which leaves are initially pale in color, similar to that observed in the athHsp93-V knockout mutant described above, but later in development are able to develop green chloroplasts, presumably due to the incomplete suppression of the two Hsp93 isoforms (Park and Rodermel, 2004). The phenotypes of both of these mutants are consistent with those of the athHsp93-V and athHsp93-III T-DNA insertion mutants analyzed in this report (Fig. 2). The different phenotypes of their athSP93-III splice-site mutant and antisense plants led the authors to speculate that athHsp93-V and athHsp93-III may have different, or perhaps partially overlapping, functions within Arabidopsis plastids (Park and Rodermel, 2004), a conclusion that our data supports.

Attempts to generate double mutants lacking both athHsp93-V and athHsp93-III have so far been unsuccessful (J. Froehlich and K. Keegstra, unpublished data), possibly suggesting that together these two proteins are essential in Arabidopsis. Further work to explore this possibility in more detail is under way. Interestingly, plants that are homozygous for an insertion in athSP93-V but heterozygous for an insertion in athSP93-III have a visible phenotype that is similar to the athHsp93-V single knockout mutant (J. Froehlich and K. Keegstra, unpublished data).

It is also possible that chaperones of other families, such as Hsp70s, could at least partially substitute for athHsp93-V and/or athHsp93-III in mutant plants. This may indeed be the case if the role of athHsp93-V and/or athHsp93-III is to drive precursor protein translocation as Hsp70 proteins are known to perform this function in other posttranslational import systems (Jensen and Johnson, 1999; Pilon and Schekman, 1999; Rapoport et al., 1999; Herrmann and Neupert, 2000; Strub et al., 2000; Voos and Röttgers, 2002). In fact, a stromal Hsp70, S78, has been found in import complexes isolated from pea chloroplasts, although not under all conditions as Hsp93 is (Nielsen et al., 1997). In athHsp93-V mutant plants, the protein levels of S78 appeared to be elevated (Fig. 5), suggesting there may be a greater need for S78 in the absence of a functional athHsp93-V protein. More experiments will be necessary to investigate this hypothesis.

Previous studies on the Arabidopsis chloroplast-localized Hsp100 proteins have indicated that these factors likely play a housekeeping role within the plastid, although the exact nature of this role has yet to be determined (Shanklin et al., 1995; Nakabayashi et al., 1999). We obtained athHsp93-V and athHsp93-III knockout mutant plants in order to learn more about the possible functions of this molecular chaperone. Based on our current results, it is apparent that athHsp93-V is important, but not essential, for normal chloroplast development and function. On the other hand, the presence of athHsp93-III appears to be much less important in Arabidopsis chloroplasts. Additional work on these mutant lines, as well as any double mutants that may be generated, will attempt to further address what the role of these chaperones within chloroplasts may be, especially whether they directly or indirectly impact the process of chloroplast protein import.

MATERIALS AND METHODS

Plant Material

Wild-type Arabidopsis (Arabidopsis thaliana) plants used in this study, as well as athHsp93-III knockout mutants, were of the Ws ecotype. athHsp9-V knockout mutants were obtained in both the Ws and the Col background; only the plants in the Ws background were characterized in detail. Seeds were surface-sterilized in 30% (v/v) bleach, 0.02% (v/v) Triton X-100 for 30 min, washed three times with water, and imbibed overnight at 4°C before being sown on soil or plated on 1X Murashige and Skoog salt and vitamin mixture (Gibco BRL, Grand Island, NY), 1% (w/v) Suc, and 0.8% (w/v) phytagar (Gibco BRL). Plants were then grown in either 12-h d (12 h light:12 h dark; approximately 70–80 µmol m⁻² s⁻¹) at 20°C (soil-grown plants) or in long days (16 h light:8 h dark; approximately 30–50 µmol m⁻² s⁻¹) at 22°C (plate-grown seedlings). Upon request, all novel materials described in the publication will be made available in a timely manner for noncommercial research purposes.

Identification of athHsp93-V and athHsp93-III Knockout Mutant Lines

A T-DNA mutagenized Arabidopsis population, containing a total of 60,480 mutagenized lines, was screened to obtain athHsp93-V and athHsp93-III knockout mutants in the Ws background. This population is available at the Arabidopsis Functional Genomics Consortium Arabidopsis Knockout Facility at the University of Wisconsin, Madison (Krysan et al., 1999). A PCR-based screening strategy was employed, as described previously (Krysan et al., 1996, 1999). The following PCR primers were utilized: athHsp93-V 5′, attcggattc- tctgccatactatcctctaaaagcctcat; athHsp93-V 3′, tctgccatactatcctctaaaagcctcat; athHsp93-III 5′, taacgcgtgtagcatcaaccttggtaggtg; athHsp93-III 3′, atggatgtggtaccttaatctgtc; T-DNA left border, cattttataataacgctgcggacatctac. The location of the T-DNA insert within the genes was determined by sequencing PCR products produced from positive hits detected in the screening reactions.

The Syngenta Arabidopsis Insertion Library, consisting of approximately 100,000 sequence-indexed T-DNA mutagenized Arabidopsis lines, was screened in silico to identify an athHsp93-V knockout mutant in the Col ecotype (Sessions et al., 2002). Once a putative knockout line was identified, seeds were obtained and plants homozygous for the T-DNA insertion were identified by PCR analysis. The following primers were used in the characterization of this line: athHsp93-V 5′ and athHsp93-V 3′ (see above); T-DNA left border, tagatctgaatcttaaactaataagtgct.

mRNA Isolation and RT-PCR

Total RNA was extracted from 4-week-old soil-grown wild-type and athHsp93-V mutant plants as described previously (Chang et al., 1993) or from 4-week-old soil-grown wild-type and athHsp93-III mutant plants using the RNeasy Plant Mini kit (Qiagen, Valencia, CA). Approximately 150 µg of each total RNA sample was used to isolate mRNA using the PolyATrac mRNA Isolation System (Promega, Madison, WI) or the Oligotex mRNA Isolation kit (Qiagen). Approximately one-twentieth of the resulting mRNA eluate was used as template in the Titan One Tube RT-PCR System (Roche Molecular Biochemicals, Mannheim, Germany) or the Access Quick RT-PCR System (Promega). The following primers specific for athHsp93-V were used: 5′ forward, gggatggagctgggttt; 5′ reverse, ggggaggtggtgtgtgtga; 3′ forward,
Chlorophyll Isolation and Quantitation

Whole wild-type or atHsp93-V knockout mutant plants at 4, 7, 11, 14, or 20 d after germination were weighed and then ground with sand in 80% acetone. Ground tissue was spun at approximately 2,000g for 5 min to remove the sand and other debris from the extracted chlorophyll. The supernatant was then measured spectrophotometrically at 645 nm and 663 nm. The amount of chlorophyll (µg/mL) in each sample was determined using the equation given in Arnon (1949).

Transmission Electron Microscopy

Leaf tissue isolated from soil-grown wild-type and atHsp93-V knockout mutant plants at 6 d, 2 weeks or 4 weeks after germination was fixed, under vacuum, for 90 min at room temperature in 2% paraformaldehyde, 2.5% glutaraldehyde, 0.1 M sodium phosphate, pH 7.4. Fixation was then continued for an additional 24 h at 4°C, followed by a second fixation in 1% osmium tetroxide, 0.1 M sodium phosphate, pH 7.4, for 2 h. After the second fixation, samples were dehydrated in acetone, embedded in Spurr resin, and sectioned. The thin sections (approximately 70 nm) were stained with uranium and lead prior to examination in a JEOL 100CX electron microscope (JEOL USA, Peabody, MA). This work was performed by the Center for Advanced Microscopy, Michigan State University.

Protein Extraction, Mass Spectroscopy, and Immunoblotting

Whole 2-week-old or 4-week-old wild-type or atHsp93-V knockout plants grown on soil were ground and then extracted in 0.15 M Tris-HCl, pH 8.0, 7.5% β-mercaptoethanol, 3% SDS, 0.2 mM phenylmethylsulfonyl fluoride for 5 min at 100°C. Following centrifugation at approximately 20,000g for 20 min to pellet insoluble material, the soluble extract was used for further study. SDS-PAGE was performed as described previously (Laemmli, 1970). Chloroplast protein samples were loaded according to equal amounts of total chlorophyll; total protein extracts were loaded on the basis of equal amounts of starting tissue fresh weight. After separation by SDS-PAGE, the proteins were either stained with Coomassie Brilliant Blue R250 or transferred overnight to Immobilon-P PVDF membranes (Millipore, Bedford, MA). A band of approximately 90 to 100 kD was excised from chloroplast protein samples stained by Coomassie. Excised samples were soaked in several changes of destain solution (30% methanol, 10% acetic acid) and then incubated overnight in distilled water. Mass spectroscopy was performed on the samples by the Proteinomes Core of the Genomic Technology Support Facility, Michigan State University.

Immobilon-P PVDF membranes were incubated in blocking buffer (0.1% TBS [20 mM Tris-HCl, pH 7.4, 150 mM NaCl], 0.1% Tween 20, 5% nonfat dry milk) for 30 min, followed by incubation in TBS, 0.1% Tween 20, 1% nonfat dry milk supplemented with antiserum. Washings were done in TBS, 0.1% Tween 20. Primary antibodies, against all proteins examined except biotin carboxyl carrier protein (BCCP) and PC, was detected with horseradish peroxidase-conjugated goat anti-rabbit antibodies (Kirkegaard and Perry Laboratories, Gaithersburg, MD). Secondary antibody was visualized with the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL). Primary antibody against PC was detected with alkaline phosphate-conjugated goat anti-chicken antibodies; anti-biotin antibodies directly conjugated to alkaline phosphatase (Kirkegaard and Perry Laboratories, Gaithersburg, MD) were used to detect BCCP. These antibodies were then visualized using nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

Antibodies to Tox75, Tic110, Tic40, and S78 were generated as described by Tranel et al. (1995), Akita et al. (1997), Chou et al. (2003), and Nielsen et al. (1997), respectively. Antiserum against Tic22 and IEP35 was a gift from D. Schnell (University of Massachusetts, Amherst, MA; Schnell et al., 1994; Kouranov et al., 1998). Antiserum to allene oxide synthase was a gift from C. Howe (Michigan State University, East Lansing, MI; Howe et al., 2000). Antibodies against FtsZ1 were a gift from K. Osteryoung (Michigan State University, East Lansing, MI; Stokes et al., 2000).

Antibodies to Hsp93 were prepared by injecting an Escherichia coli-produced version of pea (Pisum sativum) Hsp93, received as a gift from J. Shanklin (Brookhaven National Laboratory, Upton, NY; Shanklin et al., 1995), into rabbits. Antiserum obtained from rabbits was tested against total leaf protein extract and total chloroplast protein from both pea and Arabidopsis; it was found to react specifically with a single band of the expected size in all samples tested.

Isolation of Chloroplasts

Chloroplasts were isolated from 4-week-old Arabidopsis plants that had been grown on plates, as described previously (Fitzpatrick and Keegstra, 2001). Final resuspension of chloroplasts, at a concentration of 1 mg chlorophyll/mL, was in import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0).

In Vitro Import Assays

Precursor proteins used in this investigation were preSS from pea, prHCP from pea, prPC from Silence protein, and tp110-110N from pea (Bauerle and Keegstra, 1991; Lubeck et al., 1997). All precursors were made using the TNT-coupled transcription and translation system (rabbit reticulocyte; Promega) containing 35S-Met and either Sp6 RNA polymerase (prSS, prLHCII and prPC) or T7 RNA polymerase (tp110-110N).

Import reactions were performed essentially as described previously (Bruce et al., 1994). In brief, chloroplasts (25 µg chlorophyll) were incubated with radiolabeled precursor in 150 µl import buffer (330 mM sorbitol, 50 mM HEPES-KOH, pH 8.0) supplemented with 4 µM ATP. Import was halted, at the times indicated in the figures, by sedimenting intact chloroplasts through a 40% (v/v) Percoll cushion. Pellets were then resuspended in SDS-PAGE sample buffer and analyzed by electrophoresis and fluorography. Quantification of the amount of radiolabeled precursor imported was done using a phosphorimager (Molecular Imager FX; Bio-Rad, Hercules, CA).

ACKNOWLEDGMENTS

We thank Linda Danhof and Ana Kelly for experimental assistance. We thank Gregg Howe, Katherine Osteryoung, Danny Schnell, and John Shanklin for providing antibodies and recombinant protein used in this investigation.

Received September 3, 2004; revised for publication September 22, 2004; accepted September 22, 2004.

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