4. Discussion
In the recent past many components of the TOC (Translocons at the outer envelope of chloroplast) and TIC (Translocons at the inner envelope of the chloroplast) complexes has been discovered. The TOC complex consists of the Toc159, Toc34, Toc33, Toc75, Toc64 and Toc12 whereas the TIC complex consists of Tic110, Tic20, Tic22, Tic40, Tic55, Tic62 and Tic32 (Stengel et al., 2007). Although a clear concept have evolved regarding the characterization of the Toc components, further studies are needed to be addressed to positively identify the components of Tic complex. Tic 110 along with Tic20 are proposed to function as part of channel for the Tic complex (Chen et al., 2002; Inaba et al., 2005; Kovacheva et al., 2005; Teng et al, 2006). On the other hand, Tic40 is thought to act as a co-chaperon along with hsp93, whose presence is essential for the efficient import of the chloroplast precursor proteins (Chou et al., 2003; Kovacheva et al., 2005). Tic55, Tic62 along with Tic32 are demonstrated to be components associated with the Tic complex and are proposed to be redox regulator of chloroplast protein import in pea (Caliebe et al., 1997; Kuchler et al., 2002; Bartsch et al., 2008; Chigri et al., 2006). Tic32 has also been characterized as an essential component of the chloroplast protein import essential for embryo development in *Arabidopsis* (Hormann et al., 2004). Tic22 the other component is proposed to mediate the TOC and TIC interaction during the import process (Akita et al., 1997; Nielsen et al., 1997). Most of the components were initially discovered in *Pisum sativum* and later characterized in *Arabidopsis* (Bedard and Jarvis, 2005).

The association of Tic62 and Tic55 with Tic110 and other components of TIC complex are shown by protein-protein interaction. However to positively identify the essentiality of Tic62 and Tic55 as components playing a vital role in protein import, it is essential to study protein import after partially or completely silencing their gene expression. Therefore the reverse genetic approach is taken to understand the function of Tic62 and Tic55 in protein import.
Functional genomics of *Arabidopsis AtTic62*, a putative component of chloroplast protein import apparatus

Tic62 was discovered in pea as an interacting partner in the stable core TIC complex along with Tic110 and Tic55 (Kuchler et al., 2002). An examination of the available *Arabidopsis* genomic sequence data revealed the existence of a single gene, designated *AtTic62* with an accession no. At3G18890, exhibiting a high degree of identity to psTic62. The deduced amino acid sequences of the proteins from the two species exhibit 60% identity in the sequences corresponding to the mature polypeptides (Fig 1). In contrast to the known psTic62 transit sequence of 64 amino acids, the *AtTic62* protein is predicted to contain an N-terminal transit sequence of 63 amino acid. Both the mature sequences carry highly conserved pyridine nucleotide binding site at their N-terminal (Fig.1) which was recently demonstrated to carry dehydrogenase activity by using the artificial electron acceptor NBT (Stengel et al., 2008). Analysis of the *AtTic62* through DNAsis MAX v2.9 software revealed its hydrophilic property. Kyte and Doolittle Plot for the *AtTic62* protein shows an average of -0.36 there by showing slight inclination towards hydrophilicity (Fig 2A). However 6 peaks raised above 2 unit suggesting its strong candidature for a transmembrane protein. There is a pretty even stretch of peaks past the 1 along y axis through out the amino acid sequence which seems to indicate pretty hydrophobic regions. All of this data suggests that this protein is a putative membrane protein. The Hopp and Woods antigenicity plot for *AtTic62* shows a pretty evenly distributed hydrophilic peaks throughout the amino acid stretch (Fig 2B). A more hydrophilic region is a better place for an antigen to bind. Thus an antigenic plot helps to determine where on a protein a monoclonal antibody would bind well. There are relatively high peaks at approximately amino acids 174 and 378 which are at about 2 with as many as 6 more peaks approaching the 2 unit. These are the most hydrophilic sites which would be best for antigen binding.

Cloning for the heterologous expression of *AtTic62* and subsequent production of polyclonal antibodies was also simultaneously carried out (Fig. 4-5). Since the full length construct failed to express, the highly conserved C-terminal with comparatively
Discussion

less hydrophobic property was used for the purpose of producing polyclonal antibodies in

rabbit. C62 (amino acid residues 541-641) was PCR amplified using *A. thaliana* cDNA

library, cloned in pET30a expression vector and was expressed in *E.coli*. Polyclonal

antibodies were raised in New Zealand white rabbit (Fig. 6).

Studies for the different components of the protein import apparatus have shown

that their function and levels of their expression correlate with plastid development,

peaking during periods of rapid growth and plastid proliferation (Dahlin and Cline, 1991;

Constan et al, 2004). As a first step in the in-vivo analysis of *AtTic62*, the tissue specific

and developmental expression profiles of the *AtTic62* gene was examined. A Real-Time

PCR analysis of the mRNA abundance of the *AtTic62* revealed presence of mRNA in

both the green and non-green parts of the plants. The expression was comparatively less

in the roots and high in mature parts of the plants i.e. leaves and developing flowers

suggesting its functional role in both photosynthetic as well as non-photosynthetic parts

of the plants (Fig. 7). As a next step in the in-vivo analysis of the *AtTic62*, developmental

expression pattern was studied. The mRNA abundance was high at 5 days

after germination, when the cotyledons were expanding, and decreased to a lower level at

10 days, when the cotyledons stopped expanding and primary leaves began to develop.

The expression increased with the expanding of primary leaves. The expression lowered

at the onset of rosette leaves at day 20. The mRNA abundance increased and reached a

peak as rosette leaves and cauline leaves developed and plant matured and began to bolt

(Fig. 8). These results clearly indicate that the expression of *AtTic62* mRNA correlates

with developmental stages involving cell differentiation and increases in chloroplast size

and numbers.

The process of chloroplast biogenesis involves light triggered expression of many

new nuclear encoded chloroplast proteins and their simultaneous import into the

chloroplast through the TOC/TIC complex. The expression of few components of this

complex is itself governed by the light. Therefore, light exerts a tight regulation of the

chloroplast protein composition both at transcriptional and translational levels (Hirohashi

et al., 2001). Tic62 is a component of the TIC complex (Kuchler et al., 2002; Stengel et
Thus mRNA abundance of \textit{AtTic62} was studied in etioplast and chloroplast (Fig. 9). \textit{Arabidopsis} Gene Regulatory Information Server (AGRIS), is widely used as an information resource of \textit{Arabidopsis} cis-regulatory elements and transcription factors (Davuluri et al., 2003). Analysis of the proximal and distal promoter revealed no major Light Responsive Elements associated with it. Experimental validation of the above observed characteristic was carried out using Real-Time PCR analysis. The \textit{AtTic62} showed abundant mRNA expression throughout light treatment from etioplast to chloroplast. There was also no change in the expression pattern following different quality of light treatment (Fig. 10). Thus the equivalent level of gene abundance in both etiolated and green seedling suggests that it is required in early developmental processes.

Redox regulated differential localization of Tic62 protein in stroma and membrane fraction was demonstrated by Stengel et al. in pea. Therefore it was imperative to study the redox regulated distribution of its \textit{Arabidopsis} homolog \textit{AtTic62} protein. Same approach of that of Stengel et al was followed to poised the intact chloroplast in oxidized state by pre-incubating them with HAR or in reduced state by pre-incubation with ascorbic acid or reduced glutathione. In order to confirm the work in pea, the chloroplast was poised in oxidized state by pre-incubating in different concentration of HAR for 10min and were fractioned to stromal and membrane fraction. Each fraction was analyzed by Western blot for Tic62. Results demonstrate that treatment of different concentration of HAR up to 15mM leads to migration of almost all stromal Tic62 into membrane fraction (Fig. 12A). To ascertain the redox regulated dynamic localization of Tic62, chloroplast were treated with 10mM of HAR, followed by reisolation and incubation in isolation buffer containing different concentration of ascorbic acid (0-10mM) for 15min to poised the chloroplast back in reduced state. Subsequent fractionation of intact chloroplast to stromal and membrane fraction and their western blot analysis reveal the redox regulated migration of \textit{AtTic62} from membrane to stroma in a reduced environment (Fig. 12B). This confirms the redox regulated dynamic localization of Tic62 in stroma and envelope membrane in pea.
To evaluate the redox regulated dynamic localization of AtTic62 in Arabidopsis, intact chloroplast were isolated and fractioned to stromal and membrane fractions. In contrast to pea, AtTic62 protein was exclusively localized in membrane fraction and no trace of it was found in stromal fraction by western blot analysis (Fig. 11). It is possible that chloroplast isolated from Arabidopsis were already in highly oxidized state leading to migration of all the stromal Tic62 to the membrane fraction. In such a situation addition of reducing agents like ascorbic acid or reduced glutathion should result in movement of AtTic62 from membrane to the stroma. Neither ascorbic acid nor reduced glutathione could induce such a migration from membrane to stroma (Fig. 13). This clearly proves the redox-independent exclusive localization of AtTic62 in the envelope membrane of Arabidopsis chloroplast.

Transgenic approach and mutant analysis has become a major tool in functional characterization of different components of protein import apparatus (Jarvis, 2008). In all the cases Arabidopsis was chosen as a model plant and the Arabidopsis homolog of corresponding pea Toc/Tic components was characterized. Mutants of Toc159 (ppi2), Toc33 (ppi1) and Tic40 were either albino or pale yellow with severe growth retardation (Bauer et al., 2000; Jarvis et al., 1998; Chou et al., 2003). Mutants of other essential components of import apparatus i.e. Toc75, Tic110 and Tic32 were shown to be embryo lethal (Baldwin et al., 2005; Kovacheva et al., 2005; Hormann et al., 2004). Antisense plants for Tic20 and stromal processing peptidase (SPP) exhibited pale phenotype with severe defect in chloroplast protein import (Chen et al., 2002; Zhong et al., 2003). Contrary to those mutants, Toc34 and Toc64 mutants were shown to develop normally as that of wild type with no defect in chloroplast protein import (Constan et al., 2004; Hoffman and Theg, 2005; Aronson et al., 2007). The only difference was stunted root in case of Toc34 mutants (Constan et al., 2004).

In the present investigation the functional significance of Tic62 was studied by post transcriptional gene silencing in Arabidopsis. Post-transcriptional gene silencing using constructs encoding self-complimentary ‘hairpin’ RNA (hpRNA) has been demonstrated to efficiently silence genes of interest in plants (Wasley et al., 2001).
this study we used pHANNIBAL as a PTGS vector to silence endogenous Tic62 in *Arabidopsis thaliana*. Through BLAST search for *AtTic62* (At3G18890) gene, the nucleotide fragment of 590bp (nt 961 to nt 1557) was found to be best fit to target the endogenous *AtTic62* gene without affecting the expression of other genes. This *AtTic62ptgs* fragment was PCR amplified using *A. thaliana* cDNA library and initially cloned in the pGEMT-EASY cloning vector (Fig. 14). The RNAi transgenic plants for *AtTic62* were raised in *Arabidopsis*. Several kanamycin resistant plants were selected and confirmed by PCR. The degree of mRNA degradation was confirmed by RT-PCR and Northern Analysis (Fig. 17). Simultaneous downregulation in the protein content of Tic62 was confirmed by western blot analysis using chloroplast membrane protein (Fig. 18). Despite the downregulation of the Tic62 protein import components, transgenic RNAi plants were visually green similar to that of WT (Fig. 21). As *AtTic62* is now proposed to be a component of chloroplast protein import apparatus, its downregulation may effect the mRNA expression and/or protein abundance of other protein import components. sqRT-PCR and western blot analysis of most of the major components of protein import apparatus revealed no change in their expression pattern, suggesting that other components of the import machinery are not affected as a result of silencing of *AtTic62* (Fig. 19-20).

Interestingly phenotypic analysis of the *AtTic62ptgs* plants exhibited slow stunted vegetative growth with retarded root length (Fig. 22A-B). It has been demonstrated that the expression of *AtTic62* is universal in all parts of the plant although low in root tissue. On the other hand it has been shown to express abundantly in dark. Bearing this in mind it appears that *AtTic62ptgs* may perturb root plastid biogenesis sufficiently to interfere with metabolism, and so affect root growth, but insufficient to give rise to gross structural defects. As the root growth phenotype was the only other phenotype observed in the *AtTic62ptgs* transgenics, the data suggest that *AtTic62* is relatively more important for the biogenesis of non-green plastids. Corroborating observed phenotype, there was no difference in the total chlorophyll and carotenoid content in *AtTic62ptgs* and wild type plants (Fig. 23). Chl a fluorescence is often used as a signature of photosynthetic efficiency (for reviews, see Krause and Weis, 1991, Govindjee, 1995, 2005).
AtTic62ptgs transgenic plants showed no deviation in chlorophyll a fluorescence as compared to the wild type (Fig. 24-25).

With the availability of Salk_087903.56.00.x line with T-DNA insertion (Alonso et al., 2003), the homozygous mutant line for the AtTic62 was selected which was named AtTic62-I (Fig. 26-27). Ultrastructure analysis of the intact chloroplast from AtTic62ptgs transgenic, AtTic62-I and wild type plants revealed no difference in the overall structure. Moreover all of them had well developed grana with stacked thylakoids further confirming that it is not essential for retaining the structure and function of chloroplast (Fig. 28).

Since Tic62 is proposed to play its role in regulating the chloroplast protein import (Kuchler et al., 2002), it is possible that the AtTic62ptgs transgenic and AtTic62-I mutant line may have link to impaired import of nuclear encoded chloroplast proteins. Tic62 supposedly functions in chloroplast import by interacting with channel protein Tic110. Tic62 has a N-terminal pyridine nucleotide binding domain with dehydrogenase activity and a C-terminal FNR binding domain (Kuchler et al., 2002; Stangel et al., 2008). Redox state of the chloroplast regulates the import of chloroplast protein (Kuchler et al., 2002; Bartsch et al., 2008). Several potassium channels comprise pore forming α-subunits and auxiliary β-subunits (Bahring et al., 2001). The β-subunits, is proposed to utilize pyridine nucleotides as a cofactor thereby regulating the activity of a potassium channel by a redox mechanism (Bahring et al., 2001; Zhou et al., 2001). Therefore, a function of Tic62 in regulating the translocation pore Tic110 dependent on its redox state has been postulated. Tic110 is proposed to import both photosynthetic and non-photosynthetic proteins and absence of this component is demonstrated to exhibit embryo lethality (Kovacheva et al., 2005; Inaba et al., 2005). The in-vitro biochemical experiments suggested elaborate models for the activity of the Tic62 protein in chloroplast protein import pathway (Kuchler et al., 2002; Stangel et al., 2008). These models incorporate two redox properties to the Tic62 protein: it regulates the movement of the import channel protein Tic110 by sensing the redox state of the chloroplast, and it has a dynamic localization in pea chloroplast which is dependent on the redox state of the...
chloroplast. The present study revealed that absence of the Tic62 does not have any serious effect on chloroplast biogenesis or plant development. The severe downregulation or complete loss with wide-ranging activities in the import mechanism would be expected to have severe consequences, as numerous other studies have demonstrated that defect in translocon components either cause pale/ albino phenotype or embryo ; lethal (Bauer et al., 2000; Jarvis et al., 1998; Chou et al., 2003; Baldwin et al., 2005; Kovacheva et al., 2005; Hormann et al., 2004). If AtTic62 indeed regulate Tic110, then a significant decrease in the AtTic62 protein content would be expected to impair the import process to some extent. However no such impairment was observed in the import efficiency of both SSU of Rubisco and FNR I in the AtTic62ptgs transgenic and AtTic62-I mutant line (Fig. 29). Further the proposed regulatory effect of AtTic62, a homolog of psTic62, under oxidized state was not established in the both wild type and Tic62 silenced plants (Fig. 30). Therefore it is concluded that AtTic62 does not play a vital role in the chloroplast preprotein import of photosynthetic proteins (e.g. smaller subunit of Rubisco) nor in its redox regulation.

Although AtTic62 has a significant homology with psTic62, its subplastidic localization is not redox regulated. Unlike its homolog psTic62 that is distributed both in the envelope membrane and stroma, AtTic62 is exclusively localized in the envelope membrane. This study suggest that AtTic62 does not play a vital role in the redox regulated preprotein import into chloroplast. Due to its exclusive localization in the envelope membrane, AtTic62 may not be able to sense the redox environment of the stroma and send the appropriate signal to the TIC complex in the inner envelope membrane. This could explain the non-responsiveness of AtTic62 to the oxidized or reduced environment of the chloroplast.
Functional genomics of *Arabidopsis* AtTic55, a putative component of chloroplast protein import apparatus

Tic55 was initially discovered in pea as a component of chloroplast protein import apparatus carrying Rieske-type iron–sulfur cluster and a mononuclear iron-binding protein (Caliebe et al., 1997). It was later demonstrated that it forms a part of the stable core complex of the translocon at the inner envelope of the chloroplast (Kuchler et al., 2002). This protein is proposed to function as a sensor protein whose possible role is to regulate protein import into chloroplasts by sensing and reacting to the redox state of the organelle. So far the only Tic55 protein studied in detail is that from *Pisum sativum* (Caliebe et al., 1997; Kuchler et al., 2002). This protein was found to have two functional modules: the conserved functional Rieske-type iron–sulfur cluster and a mononuclear iron-binding site. The blast search against the protein databases with psTic55 as a template resulted in several sequences of which *Arabidopsis thaliana* (Gene Bank: NP_180055.1) was one of them. Interestingly the Tic55 protein was found to have homologs in wide range of species in all groups of plant kingdom. A multiple sequence alignment of these proteins was done and a phylogenetic tree was built based on the alignment (Fig. 31). Based on the bootstrap value the tree can be classified roughly into 6 groups with *Arabidopsis thaliana* (NP_180055.1), *Pisum sativum* (CAA04157.1), *Oryza sativa* (NP_001048363.1) and *Physcomitrella patens* subsp. Patens (XP_001772643.1) placed close to each other in a group. An electronic search of the available *Arabidopsis* genomic sequence data revealed the existence of a single gene, designated AtTic55 with an accession no. At2g24820, exhibiting a high degree of identity to psTic55. The deduced amino acid sequences of the proteins from the two species exhibit 60% identity in the sequences corresponding to the mature polypeptides (Fig. 32). In contrast to the known psTic55 transit sequence of 60 amino acids, the AtTic55 protein is predicted to contain an N-terminal transit peptide sequence of 48 amino acids. Both the sequences carry highly conserved functional Rieske-type iron–sulfur cluster and a mononuclear iron-binding site (Fig. 2).

DNAsis MAX v2.9 analysis of the mature peptide sequence for AtTic55 was performed to understand its hydrophobicity and antigenecity. Kyte and Doolittle Plot for
the \textit{AtTic55} protein shows an average of -0.31 thereby revealing its inclination towards hydrophilicity (Fig 33A). Its N-terminal half is highly hydrophilic whereas its C-terminal half has five peaks above +2 suggesting its hydrophobicity. There was a pretty even stretch of peaks past the +1 along y axis through out the amino acid sequence which seems to indicate hydrophobic regions. The Hopp and Woods antigenicity plot for the protein seems to have pretty even distributions of hydrophilic and hydrophobic regions but there are several hydrophilic +peaks (Fig. 33B). A more hydrophilic region is a better place for an antigen to bind. Thus an antigenic plot helps to determine where on a protein a monoclonal antibody would bind well. There are relatively high peaks at approximately amino acids 28 and 335 which are at about 2 with as many as 6 more peaks approaching the 2 unit. These are the most hydrophilic sites which would be best for antigen binding.

Although the different components of protein import apparatus are primarily localized in the chloroplast envelope, few of them are also proposed to be found in soluble state. Among them are Toc159 whose dual localization in envelope and cytosol has attributed its functional role in "target hypothesis" and Tic62, whose dynamic localization in envelope and stroma is a response to the redox state of chloroplast (Hiltbrunner et al., 2001; Stengel et al, 2008). Interestingly in this study there was no stromal form of \textit{AtTic62} protein found even in reduced state. Thus it was necessary to study the localization pattern of \textit{AtTic55} protein, the \textit{Arabidopsis} homolog of psTic55. Polyclonal antibodies for \textit{AtTic55} were generated by use of immunogenic peptide for \textit{AtTic55} protein, C55 (position from aa274 to aa293) conjugated to Keyhole Limpet Hemocyanin ( 20AA-KLH) obtained from Link Biotech (India) (Fig. 34). Immunoblot analysis of the isolated fractions using antibodies against \textit{AtTic55} shows Tic55 to be an exclusive membrane protein which is completely absent in stromal fraction in \textit{Arabidopsis} behaving like its homolog in \textit{Pisum} (Fig.35).

Photosynthetic and non-photosynthetic proteins are proposed to use different isoforms of protein import components for their entry into the chloroplast (Gutensohn et al., 2000). It was therefore of interest to know whether differential gene expression in
photosynthetically active and inactive tissues could be observed for the AtTic55. Tissue specific expression of AtTic55 demonstrate it's preferential localization in both rosette and cauline leaves and to reduced extent in flower and stem. It has minimal expression in non-photosynthetic root tissue (Fig. 36).

As stated earlier studies for the different components of the protein import apparatus have shown that their function and levels of their expression correlate with plastid development, peaking during periods of rapid growth and plastid proliferation (Dahlin and Cline, 1991; Constan et al, 2004). AtTic55 mRNA levels were low at 5 days and 10 days after germination when the cotyledons were expanding and the primary leaves began to develop. At this stage active chloroplast biogenesis takes place. The expression of AtTic55 increased as the primary leaves expanded. The expression was again downregulated during the rosette leaves stage. Additionally the AtTic55 mRNA levels increased after 25 days as the cauline leaves developed and plants matured and began to bolt (Fig. 37). These results clearly indicate that the expression of AtTic55 mRNA as that of AtTic62 correlates with active growth phase and developmental stages involving cell growth and increases in chloroplast size and numbers.

Although most of the components of protein import apparatus are not light regulated, AtTic55 had light induced expression that increased on 4-36h of light exposure of etiolated seedlings. Real-Time analysis of mRNA abundance of AtTic55 revealed minimal expression in dark-grown seedlings (Fig. 39). Bioinformatics studies by the use of AGRIS server of the putative promoter suggested Tic55 expression to be light inducible. There are eleven GATA promoter motif (Light Regulation Elements) distributed evenly throughout the proximal and distal promoter region and two SORLIP 1 and one I Box distributed evenly at the distal promoter region suggesting its tight light regulated expression (Fig. 38). The expression is also dependent on the light quality with maximum expression in white light followed by blue and red (Fig. 40). This postulates a light-dependent function of AtTic55. Trans-acting proteins that bind to GATA motif were first identified as proteins that interact with conserved WGATAR (W = T or A; R = G or A) motifs involved in erythroid-specific gene expression in vertebrates (Evans et al.,
Numerous sequence comparisons between plant light-responsive genes have revealed the presence of conserved GATA motifs within their promoters (Dean et al., 1985; Grob and Stuber, 1987; Castresana et al., 1988; Giuliano et al., 1988; Manzara and Gruissem, 1988; Gidoni et al., 1989; Manzara and Gruissem, 1988; Gilmartin et al., 1990; Arguello-Astorga and Herrera-Estrella, 1996). In vitro studies on DNA-protein interactions have also identified a range of distinct nuclear GATA-binding proteins from a number of plant species (Lam and Chua, 1989; Buszby et al., 1990; Gilmartin et al., 1990; Lam et al., 1990; Schindler and Cashmore, 1990; Sarokin and Chua, 1992; Borello et al., 1993). Some of these factors are characterised by differential binding in response to either light or circadian rhythms (Buzby et al., 1990; Gilmartin et al., 1990; Borello et al., 1993). The significance of GATA factor to blue light response was supported by the molecular characterization of the *N. crassa* blue light photo-response mutants, wc-1 (Ballario et al., 1996) and wc-2 (Linden and Macino, 1997). Thus the presence of GATA promoter motif may account for the active expression of the *AtTic55* gene under blue light as compared to red light. I Box having a consenses sequence of "GATAAG" have been suggested to be an evolutionarily conserved protein binding sequence in the promoter of light-regulated genes (Giuliano et al., 1988). SORLIP1 promoter motif is strong conservation and abundant in the phyA regulated promoters (Hudson and Quail, 2003). Thus the strong presences of these light regulatory elements in the promoter of *AtTic55* are responsible for its light dependent expression pattern. The light dependent expression pattern of Tic55 could be proposed to have some regulatory function in regulating the import of smaller subunit of Rubisco under light-dark condition in barley, *tigrina d12* seedlings (Bartsch et al., 2008).

As described earlier transgenic approach and mutant analysis has become a major tool in functional characterization of different components of protein import apparatus (Jarvis, 2008). In all the cases *Arabidopsis* was chosen as a model plant and the *Arabidopsis* homolog of corresponding pea Toc/Tic components was characterized. Mutants of Toc159, Toc33 and Tic40 show albino or pale phenotype were as mutants of Toc75 and Tic32 were embryo lethal (Bauer et al., 2000; Jarvis et al., 1998; Chou et al., 2003; Baldwin et al., 2005; Kovacheva et al., 2005; Hormann et al., 2004). Antisense
Discussion

plants of Tic20 and stromal processing peptidase (SPP) exhibited pale phenotype with severe defect in chloroplast protein import (Chen et al., 2002; Zhong et al., 2003). Toc34 and Toc64 were shown to develop normally as that of wild type with no defect in chloroplast protein import (Constan et al., 2004; Aronson et al., 2007). As AtTic55 is a putative component of Tic complex it was of interest to study the effect of Tic55 silencing on the SSU import and overall development of plant. Antisense transgenic for AtTic55 gene was developed to study its functional role.

Antisense expression of AtTic55 led to downregulation of its gene expression and protein abundance as revealed by Real-Time PCR and Western blot analysis (Fig. 45-46). Despite the downregulation of the Tic55 protein import components, antiTic55 transgenic plants were visually green like that of wild type thereby suggesting that there is no defect in photosynthetic chloroplast proteins accumulation in antiTic55 plants as compared to wild type (Fig. 49). sqRT-PCR and western blot analysis of most of the major components of protein import apparatus revealed no change in their expression pattern in antisense plants (Fig. 47-48). These data suggest that other components of the import machinery are not altered as a complimentary effect for the absence of Tic55. This is in contrast to ClpC mutants of Arabidopsis where several components of protein import apparatus are upregulated (Sjogren et al., 2004). Careful analyses of plant growth, chlorophyll accumulation and photosynthetic performance of the antiTic55 transgenic plants reveal normal photosynthetic function, optimum growth and development (Fig. 50-53). With the availability of Salk_086048.33.10.x line with T-DNA insertion (Alonso et al., 2003), the homozygous mutant line for the AtTic55 was isolated and named, AtTic55-I (Fig. 54) Ultrastructure analysis of the intact chloroplast from antiTic55 transgenic, AtTic55-I and wild type plants revealed no difference in the overall structure. As expected all of them had well developed granum with stacked thylakoids again suggesting that Tic55 in Arabidopsis is least essential for the structural organization in chloroplast (Fig. 56).

Tic55 is a bona fide subunit of inner envelope translocon which forms a stable core complex with Tic110. It has a Rieske-type iron–sulfur cluster with a functional histidine residue which is proposed to regulate the import of Rubisco smaller subunit
Discussion

(Taliebe et al., 1997). Tic55 is also a potential target for thioredoxins (Trxs) which are powerful regulators of enzyme activity through two successive one-electron transfer reactions. If AtTic55 indeed perform such a vital role, then a significant decrease in the AtTic55 protein content would be expected to impair the import process. However, no such impairment was observed in binding of precursor protein (pRSS) or its subsequent import into chloroplast in antiTic55 transgenic plants (Fig. 57).

In conclusion, results demonstrate that AtTic55, the Arabidopsis homolog of psTic55 is a typical chloroplast membrane protein which is not essential for the general chloroplast import pathway. In the light of present investigation it is not clear whether the reported interactions between Tic55 and the other Tic components are physiologically relevant. Its wide distribution throughout the plant kingdom with its functional Rieske-type iron–sulfur cluster and its role in chloroplast protein import may be restricted to redox regulation of protein import into chloroplast. Tic55 is proposed to be regulated by Trx in dark and light (Bartsch et al., 2008). Trx is reduced by Fd in light and therefore Trx (reduced) concentration increase in light. The reduced Trx produced in light is proposed to regulate Tic55 that could interact with channel protein Tic110 to modulate protein import via TOC-TIC complex. This need further study.

Thermal Stability of Protein Import into Chloroplasts

The chloroplast biogenesis is regulated by several environmental factors, required for plant growth and development. Temperature has a profound effect on plant development (Xin and Browse, 1998; Guy, 1999; Browse and Xin, 2001, Allen and Ort, 2001). Plants exposed to low and high temperature, have impaired Chl biosynthesis due to down regulation of gene expression and protein abundance of several enzymes involved in tetrapyrrole metabolism (Tewari and Tripathy, 1998, 1999; Mohanty et al., 2006). Expression of the Rubisco is sensitive to both high- and low- temperature stress (Gesch et al., 2003; Zhou et al., 2006). It was therefore of interest to understand the effect of both low- and high temperature on the downstream post-translational events i.e. import of SSU into chloroplast.
Pea plants exposed to higher temperature had more extensive damage to their photosynthetic apparatus than those exposed to low temperature (Fig. 58-63). The increase in $F_0$ and decrease in $Fm$ in higher temperature could be due to inactivation of PSII and separation of LHCII from PSII and inhibition of electron flow from QA to QB (Schreiber and Armond, 1978; Bilger et al., 1984; Ducruet and Lemoine, 1985; Bukhov et al., 1990; Cao and Govindjee, 1990; Havaux, 1993; Yamane, 1997). The decline in $Fv/Fm$, $ETR$, $\Delta F_{PSII}$ and $qP$ in temperature-stressed plants was substantially restored when temperature-stressed plants were transferred to room temperature in light demonstrating that low or high temperature did not irreversibly inactivate the photosynthetic apparatus. The reversal of temperature-stress-induced damage to the photosynthetic apparatus of pea plants upon their transfer to room temperature may be accompanied by the recovery of impaired SSU gene expression as observed in soybean suspension cultures (Verling and Key, 1985). The increase in $qN$ in heat-stressed plants, while not a direct measure of $\Delta pH$, suggests that the proton gradient is increased or maintained to generate ATP and RuBP owing to increased electron flow through PSI (Bukhov et al., 1999, 2000). Although RuBP is probably maintained in temperature-stressed plant the enzyme Rubisco that converts it to phosphoglyceric acid is inactivated in temperature-stressed plants due to imbalance between rates of Rubisco inactivation and reactivation by activase (Crafts-Brandner and Salvucci, 2000). Maintenance of Rubisco in active state at high temperature requires faster activase reaction to overcome the high-temperature-induced Rubisco inactivation. As thermal stability (Salvucci and Crafts-Brandner, 2004) and gene expression (Law and Crafts-Brandner, 2001) of Rubisco activase decline at high temperature, Rubisco becomes mostly non-functional. An inhibited Rubisco activity is the basis for inhibition of photosynthesis in temperature-stressed plants (Kim and Portis, 2005). Besides reduced Rubisco activase, the protein abundance and gene expression of SSU is down-regulated by temperature stress leading to net loss of photosynthetic functions (Vierling and Key, 1985; Gesch et al., 2003; Zhou et al., 2006). The reduced gene expression and protein abundance of nuclear coded pRSS may require the
stressed plants to down regulate its protein import efficiency into chloroplasts. Our results demonstrate that ATP-driven import of pRSS into darkened plastid is indeed down-regulated both in low and high temperature treated pea plants.

Toc159 (Perry and Keegstra, 1994; Keegstra and Froehlich, 1999), Toc34 (Schnell et al., 1994; Becker et al., 2004a) and Toc64 (Sohrt and Soll, 2000; Qbadou et al., 2006) are proposed to act as primary receptors for binding of preproteins to the chloroplast envelope membrane. Reduction in binding of the pRSS at higher temperature (Fig. 65 B) could be partially due to the downregulation of Toc159 expression following 48h of high temperature treatment (Fig. 70). In fact impaired Toc 159 expression in Arabidopsis severely limits the protein transport into chloroplasts (Bauer et al., 2000). We did not observe any change in the gene expression or protein abundance of Toc34 in heat-stressed plants; rather it had constitutive expression in control, chill- as well as heat-stressed plants (Fig. 70). Although the gene expression of the channel protein, Toc75 was quite reduced in heat-stressed samples, its protein abundance was not affected and almost remained similar to that of control (Fig. 73). This suggests that the channel protein Toc75 is a thermal-stable protein having a low turnover rate. Among Tic complexes, Tic20 was downregulated in heat-stressed plants suggesting that protein trafficking through the inner envelope membrane could be severely affected (Fig. 71). It was previously shown that reduced Tic20 expression in antisense Arabidopsis plants (Chen et al., 2002) or mutant of Tic21 (Teng et al., 2006) that may function similar to Tic20, a potential chloroplast inner membrane protein-conducting channel, resulted in a defect in protein translocation across the inner envelope membrane. Other proposed components of the Tic complex i.e., Tic62 and Tic32 that may function as redox sensors (Kuchler et al., 2002; Hormann et al., 2004) had reduced gene expression in high temperature which might have resulted in reduced protein translocation across inner envelope membrane. In the same vein, deletion of Tic32 in Arabidopsis is reported to result in early seed abortion (Hormann et al., 2004).

Stromal Hsp93, a molecular chaperone that functions in close association with
the import apparatus of inner envelope membrane, is proposed to bind the precursor protein as they enter the chloroplast stroma preventing their backward movement, and to act as the translocation motor for the precursor protein translocation, through hydrolysis of stromal ATP (Theg et al., 1989). Double mutant of Hsp93 isoforms in Arabidopsis were chlorophyll-deficient, contained under-developed chloroplasts, and exhibited stunted growth with a severe defect in precursor import into chloroplast (Kovacheva et al, 2007). The impairment of protein import in at high temperature could be due to down-regulation of Hsp93 gene expression and protein abundance at high temperature. For proper targeting and attaining a conformation for optimal functional activity of nuclear encoded stromal protein, its transit peptide needs to be readily cleaved and any defect in this process may result in the loss of biochemical function of that protein (Grossman et al., 1980). The same is also true for all components involved in chloroplast protein import that are nuclear encoded and post-translationally targeted to the chloroplast (Jackson-Constan et al., 2001). Previous studies on tobacco and Arabidopsis antisense lines expressing reduced level of the processing enzyme showed severe defect in chloroplast protein import efficiency (Zhong et al., 2003). In the same vein it is propose that reduced protein import at high temperature could be partially attributed to down-regulation of stromal processing enzyme.

Exposure of plants to 24h of high temperature results in impairment of the pRSS import by 57% whereas the binding of the pRSS to the envelope membrane remained unaffected. This suggest that the net loss in the import efficiency under 24h of high temperature is due to defect in the import process only and not due to reduced binding of the precursore protein to the chloroplast envelope. Similarly the binding of the pRSS to the TOC complex is not altered where as its subsequent import is impaired after 48h of low temperature treatment to the plants. This suggest that the reduced import efficiency of pRSS under chill- stress is a result of defect in the import through the TOC-TIC complex solely.

Preprotein binding and protein import efficiency of intact chloroplast heated
at $35^0C$ or $40^0C$ for 10min prior to import, was reduced by 75% and 100% respectively (Fig. 66-67). However, when leaves excised from pea plants are directly heated at different temperatures for 10 min, precursor protein binding to the receptor or protein import into chloroplast was not affected (Fig. 68). This suggests that there may be a heat-labile component involved in the protein import process and a profound role of cytoplasmic proteins especially that of molecular chaperones in preventing the heat-induced structural alteration of protein import apparatus at high temperature. This protection was rather short-term i.e., 10min. Both binding as well as import were affected if excised leaves are heated at $40^0C$ for 40 min suggesting that protein import may not be viable if excised leaves are subjected to prolong period of heat stress (Fig. 69). This suggests that cytoplasmic factors that could provide short-term protection to import reaction are no more effective in prolonged treatment of high temperature.

Although Leheny and Theg in 1994 observed a similar temperature-dependent \textit{in vitro} import profile into intact chloroplast isolated from both warm-grown (control) and cold-grown (chill stress for 6 weeks) pea plants, in the present study the chill-stressed plants had reduced protein import. However, the binding of preproteins to the envelope membranes was not affected. In the same vein the gene and/or protein expression of Toc159 and Toc34 that act as receptors to preproteins were not down regulated at low temperature. The gene expression of most of the other components of Toc and Tic complexes responsible for protein import and the stromal molecular chaperone Hsp93 as well as stromal processing enzyme were not affected at low temperature treatment (Fig. 70-71). Only important exceptions were Tic110 and Tic40 whose gene expression and protein abundance were severely down regulated at low temperature (Fig. 71,73). This clearly demonstrates a central role of Tic110 and Tic40 in inhibition of protein import at low temperature. Tic110 is suggested to act as a molecular scaffold during the import across the inner envelope involving in the formation of the contact site to the Toc complex and recruiting stromal chaperons i.e., Hsp93 to drive preprotein translocation (for a
Discussion

binding of Hsp93 to Tic110 is presumed to maintain solubility of the associated pRSS, preventing their misfolding and aggregation prior to being transferred to Hsp70 and/or Cpn60 chaperons (Nelson et al., 1997) and to provide the driving force for complete translocation into stroma (Jackson-Constan et al., 2001b). Arabidopsis mutants of Tic110 showed pale/albino phenotype with severe down regulation in pRSS import into chloroplast (Inaba et al., 2005). Given its importance in protein translocation it is quite likely that down regulation of Tic110 would lead to impaired protein import at lower temperature. Tic40 is proposed to function as a co-chaperone in the stromal chaperone complex that facilitates protein translocation across the inner membrane and its null mutation in Arabidopsis results in pale phenotype and retarded growth with severe defect in import of pRSS (Chou et al., 2003). Tic40 functions at the same stage of import as Tic110 (Kovacheva et al., 2005) and its reduced gene and protein expression in low temperature might contribute to reduced protein import efficiency in chill-stressed plants.

The inhibition of photosynthetic function and CO₂ fixation in temperature-stressed plants could be due to reduced pRSS expression and impaired pRSS import into chloroplasts. In the same vein down-regulation of the greening process and plastid biogenesis in temperature stress could be attributed to the impaired gene and/or protein expression of nuclear coded plastidic proteins (Mohanty et al., 2006) and their reduced import into the chloroplast. As compared to chill-stress, exposure of plants to high temperature causes rapid proteolytic degradation of the chloroplast proteins. In order to retain their function, these proteins need to be replenished by newly synthesized preproteins that are imported into chloroplast. Thus under high temperature stress, the net loss in photosynthetic function could be due degradation of chloroplast proteins and impaired post-translational targeting of their precursor proteins into the chloroplast. In chill-stress (48h), the chloroplast proteins are not as rapidly degraded as that in high-temperature. Therefore, although the gene expression, protein synthesis and their post-translational import are impaired in plants exposed to chill-stress (48h), their photosynthetic functions are substantially retained. However prolonged exposure of
plants to chill-stress would affect their photosynthetic functions due to non-replenishment of slowly degrading chloroplast proteins (Yu et al., 2002).

To conclude downregulation or absence of the *Arabidopsis* homologs of *psTic62* and *psTic55* ie AtTic62 and AtTic55 respectively does not alter the chloroplast structural and functional integrity. Further it also demonstrates their dispensable role in the import of nuclear encoded chloroplast proteins. In the present investigation it also concluded that although post-translational import of pRSS is retained, it is severely down-regulated in plants exposed to high and low temperatures stress due to decreased gene/protein expression of different components of protein import apparatus.