Chloroplast protein import across the inner envelope is facilitated by the translocon of the inner envelope of chloroplasts (Tic). Here we have identified Tic32 as a novel subunit of the Tic complex. Tic32 can be purified from solubilized inner envelope membranes by chromatography on Toc110 containing affinity matrix. Co-immunoprecipitation experiments using either Tic32 or Tic110 antisera indicated a tight association between these polypeptides as well as with other Tic subunits, e.g. Tic40, Tic22, or Tic62, whereas the outer envelope protein Toc75 was not found in this complex. Chemical cross-linking suggests that Tic32 is involved late in the overall translocation process, because both the precursor form as well as the mature form of Rubisco small subunit can be detected. We were unable to isolate Arabidopsis null mutants of the attic32 gene, indicating that Tic32 is essential for viability. Deletion of the attic32 gene resulted in early seed abortion because the embryo was unable to differentiate from the heart stage to the torpedo stage. The homology of Tic32 to short-chain dehydrogenases suggests a dual role of Tic32 in import, one as a regulatory component and one as an important subunit in the assembly of the entire complex.

Chloroplasts must import most of their protein constituents from the cytosol in a posttranslational process (1–4). Cytosolically synthesized precursor proteins are generally made with an N-terminal transit peptide that is both necessary and sufficient for chloroplast recognition and translocation initiation. Translocation across the outer and inner envelopes of chloroplasts is achieved by the Tic1 and Tic complexes, respectively (translocon at the outer/inner envelope of chloroplasts). Upon import, the targeting peptide is removed by a stromal processing peptidase, and the mature protein is further sorted and assembled into active structures (5).

Preproteins are recognized in a GTP-dependent manner by the Tic34 receptor at the chloroplast surface (6–8). The GTPase activity of Tic34 is greatly stimulated by precursor binding, and upon GTP hydrolysis the preprotein is released from Toc34GDP to Toc159 (9). Toc159, another GTP-dependent protein, interacts with the preprotein in a manner that also involves GTP hydrolysis (6, 10, 11). Toc159 facilitates the translocation of the preprotein through the import channel Toc75 (12, 13). The preprotein then engages the Tic complex to move across the inner envelope in an ATP-requiring fashion (14–16). Several subunits of the Tic complex have been identified. Tic110, which is the second most abundant protein in the inner envelope (17–20), can form an aqueous ion channel in vitro and is therefore thought to form the translocation pore (21). However, because of different proposals about the topology and structure of Tic110 (21, 22), the exact function of Tic110 is not resolved. In addition, Tic110 could interact with the molecular chaperones cpn60 and Hsp93 on the stromal face of the membrane. It could also act as a chaperone recruitment factor (17, 19), a role that could also be mediated by Tic40, a Tic subunit that might function as a co-chaperone because of the presence of a TPR domain and homology to heat shock interacting and heat shock organizing proteins (23, 24). The molecular role of the other Tic subunits is less well defined. Tic62 and Tic55 have redox properties and could be involved in regulation of translocation across the inner envelope membrane (25, 26). Tic62 is a peripheral subunit exposed to the intermembrane space and could therefore function as a link between the Tic and the Tic complexes (27). Tic20 is an integral protein of the inner envelope and shows homology to amino acid transporters (27, 28). This led to the assumption that Tic20 is involved in the formation of a translocation channel that might be formed independently or together with Tic110. Biochemical evidence for a distinct function of Tic20 is, however, still missing. Plastids isolated from an Arabidopsis Tic20 antisense line show reduced import yields, and translocation seems to be arrested at the level of the Tic complex (29).

Tic110 is the most abundant of all Tic subunits in the inner envelope of chloroplasts. The N-terminal domain of Tic110 (amino acids 1–180) contains 1–2 hydrophobic transmembrane α-helices that anchor the protein in the membrane and that are also required for targeting and insertion into the inner envelope (17, 18). In an attempt to find interaction partners of Tic110, we identified a novel 32-kDa protein localized in the inner envelope. This protein interacts tightly with other Tic subunits and forms a chemical cross-link product with a precursor protein. It is an essential gene in Arabidopsis and we propose to call it Tic32.

MATERIALS AND METHODS

Growth of Plants and Isolation of Chloroplast Membrane Fractions—Intact chloroplasts were isolated from 12–14-day-old plants (Pisum sativum L. var. Gold) as previously described (30, 31). Outer and inner envelope membrane vesicles were isolated from purified chloroplasts by sucrose density centrifugation (31).
Fig. 1. Tic32 is an interaction partner of Tic110. A, the N terminus of Tic110 was heterologously expressed with a His6 tag either with (left panel) or without (middle panel) a mSSU spacer arm. Overexpressed proteins were bound to a metal-chelating matrix. Purified inner envelope membranes were solubilized in 3% decylmaltoside, and solubilized proteins (A) were incubated with the Tic110N matrix. Unbound material (FT) and proteins, obtained by either washing (W) or elution with increasing salt concentrations, were analyzed by SDS-PAGE followed by silver staining. Right panel, an mSSU-His6-containing matrix was used as control and treated exactly as above. B, deduced amino acid sequence of a cDNA clone isolated from a pea leaf cDNA library. Conserved active side residues of short-chain dehydrogenases are shaded in gray. Peptide sequences of tryptic Tic32 fragments obtained by Edman degradation are underlined. C, sequence comparison of pea Tic32 (Tic32_Ps) with orthologs from A. thaliana (at4g23430 and at4g11410), Oryza sativa (WWOX_oryza), Drosophila (CG30491-Drosophila), human (RDH11, RDH13, CGI-82, WWOX1), and Pseudomonas testosteroni 3β/17β-HSD (3β/17βHSD). Active site motifs and conserved residues of the SDR family are marked with letters and highlighted by boxing (A, TOXXXGXG motif; B, NNAG motif; C1-C3 active site residues comprising tetrad of Asn, Ser, Tyr, and Lys residues; D and E, involved in cofactor binding, reaction mechanism).
Arabidopsis thaliana (var. Columbia) were grown under a 14-h light/10-h dark cycle at 20 °C on Murashige and Skoog medium (32) or soil. Mutants were selected with Basta.

**Production of Antibodies**—Full-length tic32 was cloned into the pET21d vector (Novagen, Schwabach, Germany) with a C-terminal His6 tag. The protein was expressed in *Escherichia coli* and purified by metal-chelating chromatography prior to immunization.

**Isolation of Tic32 Mutants**—Mutant line 117H08—Mutant line 117H08 was obtained from Syngenta (33). Mutant line 117H08 was generated in the context of the GABI-KAT (Max-Planck-Institute of Plant Breeding, Cologne, Germany) (34) program. When grown on medium containing Basta, no homozygous plants could be observed, but compared with wild-type seeds about 20% of mutant seeds did not germinate. In Garlic 861 the T-DNA insertion is located in the first intron; in 117H08 the T-DNA is located in exon IV as verified by DNA sequencing (see “Results”).

**Screening of Phage-based cDNA Library**—Screening of pea (*Pisum sativum* L.) Uni-Zap cDNA library was performed according to the manufacturer’s recommendation (Stratagene, La Jolla, CA) using degenerate oligonucleotides deduced from peptide sequences of the pea protein. Both strands of the obtained cDNA clone were sequenced (GenBank™ accession number AA88758).**

**Interaction Partners of Tic110**—To find interaction partners of the N terminus of Tic110, a hybrid protein of mature Tic110 (amino acids 39–289) and a C-terminal fusion with mSSU-His6 (35) or with a His6 tag alone were overexpressed in *E. coli* as inclusion bodies, dissolved in 20 mM Tris/HCl, pH 8.0, 250 mM NaCl, and 8 M urea, immobilized onto nickel-nitrilotriacetic acid matrix, and washed with the above buffer. After washing, the refolding of the protein on the matrix was achieved by creating refolding conditions on the matrix with a linear gradient of 8-0 M urea in 20 mM Tris/HCl, pH 8.0 (36). The column was then equilibrated in phosphate-buffered saline (157 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4, pH 7.2).

500 μl of inner envelope vesicles corresponding to 0.5–1 mg of protein were pelleted and resuspended in 100 μl of phosphate-buffered saline, pH 7.2, 3% decylmaltoside, incubated on ice for 10 min, and diluted to 2 ml with phosphate-buffered saline. Unresolved membrane fractions were removed by centrifugation, and the supernatant was passed three times over the matrix. Proteins bound to the matrix were eluted with a KCl step gradient (200, 400, 600, 800, and 1000 mM KCl in phosphate-buffered saline). Fractions were precipitated with 10% trichloroacetic acid and analyzed by SDS-PAGE followed by silver staining (37). For protein sequencing, the affinity purification was repeated three times. The fractions were pooled, precipitated with 10% trichloroacetic acid, separated by SDS-PAGE, and transferred onto polyvinyldene fluoride membrane. The membrane was briefly stained with Coomassie G250, and the protein band of interest was treated with trypsin and used for protein microsequencing (Top Lab, Munich, Germany).

**Immunoprecipitation and Cross-linking**—Isolated inner envelope vesicles were solubilized in 2% octylglucoside, 25 mM Hepes/NaOH, pH 7.6, 150 mM NaCl, and 0.05% egg albumin. After centrifugation, the supernatant was incubated with antisera against Tic110 and Tic32, incubated with protein A-Sepharose (Amersham Biosciences) for 1 h, supernatant was incubated with antisera against Tic110 and Tic32, washed with 25 mM glycerol for 15 min on ice. Chloroplasts were lysed (25 mM Hepes/NaOH, pH 7.6, 0.5 mM EDTA). After centrifugation, 30,000 g for 30 min, the membrane fractions were solubilized in 1% SDS in 25 mM Hepes/NaOH, pH 7.6, 150 mM NaCl, 0.05% egg albumin. Solubilized membranes were diluted to 0.1% KCl, immunoprecipitated with antisera against Tic75, Tic110, and Tic32 and preimmune serum and coupled onto protein A glass beads (ProSep-Va-High Capacity; Millipore, Eschborn, Germany). The matrix was washed extensively with 20 mM Hepes/NaOH, pH 7.6, 150 mM NaCl, 0.05% egg albumin, with ∼mercaptoethanol and washed with the above buffer. Cross-links were cleaved with β-mercaptoethanol in SDS-sample buffer. Radiolabeled proteins were analyzed by a phosphorimaging (Fuji BAS 3000) and quantified using the Aida software.

**Microscopy**—Seeds were cleared in Hoyer’s solution (39) and observed using Nomarski optics (Axioskop; Zeiss, Jena, Germany).

**RESULTS**

**Identification of Tic110 Interaction Partners**—To identify potential interaction partners of Tic110, we expressed the N-terminal amino acids 1–231 either with a C-terminal mSSU-His6 tag or with a C-terminal hexahistidine tag in *E. coli*. The proteins were bound separately to a nickel-chelating matrix and incubated with purified inner envelope proteins solubilized in 3% decylmaltoside. The matrices were washed, and bound protein was eluted by increasing salt concentrations. Although the flow-through from the affinity matrix contained numerous proteins, only one polypeptide was eluted from the Tic110N-mSSU-His6 matrix at salt concentrations around 600 mM KCl (Fig. 1A, left panel). This polypeptide migrated on SDS-PAGE with an apparent molecular mass of 32 kDa (subsequently named Tic32). A protein of similar size eluted from the Tic110N-His6 matrix though at slightly lower KCl concentrations (Fig. 1A, middle panel). This could be because of the missing spacer arm in this construct, which could result in steric hindrance of Tic32 binding. An affinity matrix containing only mSSU did not bind any envelope proteins (Fig. 1A, right panel). Peptide sequences were obtained from the trypsin-treated protein (Fig. 1B) and used for the generation of degenerate oligonucleotides. Screening of a pea cDNA library resulted in the isolation of a single clone, the deduced amino acid sequence of which contained all three sequenced peptides (Fig. 2).
Tic32 Is Essential in Chloroplast Biogenesis

Membranes were isolated and solubilized with 1% SDS. Immunoprecipitation was performed either with preimmune serum or Tic32 anti-serum. Cross-links were cleaved with β-mercaptoethanol in SDS-sample buffer. Samples were analyzed by SDS-PAGE. Autoradiograph of a gel showing pSSU and mSSU associated with Tic32 is presented. C, Tic32 plays a role in a late stage of import. Import reactions in the presence of 3 mM ATP were performed for the times and temperature indicated before cross-linking and immunoprecipitation experiments were done as outlined above using antisera against Toc75, Tic110, Tic32, Tic40, and OEP24. After 2 min, pSSU can be cross-linked to every component but mostly to Toc75. mSSU can be precipitated from all Tic components.

Next we investigated whether Tic32 is present in a protein complex together with other components of the translocon. Purified inner envelope membranes were treated with 2% octylglycoside, and solubilized proteins were incubated with either α-Tic110 or α-Tic32. Antigen-antibody complexes were purified by protein A-agarose, and co-fractionated protein was incubated with protein A-Sepharose. Aliquots of inner envelope (i.e. incubated with Tic32 and Tic110 antibodies, and the mixture was solubilized with 2% octylglycoside. The supernatant was subjected to SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antisera against Tic110, Tic32, Tic40, Tic22, and Tic23. As a control, antisera against Toc75 was used. The deduced amino acid sequence encodes a protein with a calculated molecular mass of 34.3 kDa and a pI of 9.47. Sequence comparison of the pea 32-kDa protein shows homologies to enzymes in monocotyledonous and dicotyledonous plants, bacteria, and mammals and reveals that Tic32 belongs to the enzyme superfamily of short-chain dehydrogenases/reductases (SDR) (Fig. 1C).

To determine the localization of Tic32 within chloroplasts, proteins purified from organelar subfractions were separated by SDS-PAGE and immunoblotted using different antisera against marker proteins. Tic32 was detected in chloroplasts and in purified inner envelope membranes such as the marker protein Tic110. Very little antigen was found in the outer envelope and none in the stroma or the thylakoids. Tic32 shows strong homologies to the gene product of at4g23430, which was found in a proteomic analysis from purified mixed envelope preparations from Arabidopsis (40). Tic32 behaves as an integral membrane protein because it is not extractable by either 1 mM NaCl, 0.1 mM Na2CO3, or 6 M urea (Fig. 2B). We conclude that Tic32 is a genuine inner envelope protein from chloroplasts.

Tic32 Is Involved in Protein Import—Chemical cross-linking can be used to identify proteins that are in close vicinity to each other. We incubated isolated chloroplasts with radiolabeled precursor pSSU under conditions that allow binding and partial translocation (i.e. 3 mM ATP, 4 °C) and the formation of import intermediates or complete translocation (i.e. 3 mM ATP, 25 °C) (31, 41, 42). Cross-linking was done using the thiol-cleavable cross-linkers dithiobis succinimidyl propionate (Fig. 3B) or sodium tetrathionate (data not shown). Cross-linkaged products were enriched by immunoprecipitation under denaturing conditions (0.1% SDS) and subsequently analyzed after cleavage of the cross-link products by β-mercaptoethanol by SDS-PAGE. Antisera against Toc75, Tic110, Tic40, and Tic32, but not against OEP24 or preimmune serum, could co-immunoprecipitate the radiolabeled precursor at 4 °C and 3 mM ATP (Fig. 3, B and C). These conditions allow the formation of translocation intermediates, i.e. precursor proteins are in contact with Toc and Tic subunits but do not allow complete translocation. At 25 °C in the presence of 3 mM ATP, the mature form (mSSU) also became detectable when α-Tic110 or α-Tic32 was used for immunoprecipitation. The mSSU form was less pronounced in α-Toc75 precipitates, indicating that Tic32 is in close proximity to the preprotein at a late stage of translocation. Processing of precursors occurs concomitant to translocation; thus the presence of mSSU as a cross-linked partner is not surprising and indicates that Tic32 is in close proximity to a preprotein late in the translocation process. A similar cross-link product ratio of pSSU and mSSU with Toc75, Tic110, and Tic40 was observed in import reactions that lasted up to 20 min (23).

Next we investigated whether Tic32 is present in a protein complex together with other components of the translocon. After import, chloroplasts were reisolated, cross-linked with 0.5 mM dithiobis succinimidyl propionate, and lysed hypertonically. The supernatant was used. Import reactions were performed for 20 min on ice in the presence of 3 mM ATP. After import, chloroplasts were solubilized, cross-linked with 0.5 mM dithiobis succinimidyl propionate, and lysed hypertonically.

Fig. 3. Tic32 is involved in protein import. A, inner envelope vesicles were solubilized with 2% octylglycoside. The supernatant was incubated with Tic32 and Tic110 antibodies, and the mixture was incubated with protein A-Sepharose. Aliquots of inner envelope (i.e., supernatant (A)), flow-through (FT), the last washing step (W), and all of the eluted protein (E) were subjected to SDS-PAGE, transferred to nitrocellulose, and immunodecorated with antisera against Tic110, Tic32, Tic40, Tic22, and Tic23. As a control, antisera against Toc75 was used. B, Tic32 is associated with importing precursor protein. Import reactions were performed for 20 min on ice in the presence of 3 mM ATP. After import, chloroplasts were reisolated, cross-linked with 0.5 mM dithiobis succinimidyl propionate, and lysed hypertonically.
determined by immunoblot analysis. α-Tic32-immunoprecipitated complex contained Tic110, Tic62, Tic 40, and Tic32, but no Tic75 and very little Tic22 (Fig. 3A). The complex composition obtained by α-Tic110 co-immunoprecipitation was similar, except that we constantly observed significant amounts of Tic22. Because Tic22 is a peripheral subunit of the Tic complex, it could be that Tic22 and Tic110 also interact independently of other Tic subunits.

**Tic32 Knock-out Lines**—In Arabidopsis two genes are present that show significant homologies to Tic32 over the entire coding region, namely at4g23430 and at4g11410. As shown by RT-PCR analysis (Fig. 4A), only at4g23430 is expressed in all examined tissues. Together with the data from the protomic analysis of envelope membranes from Arabidopsis chloroplasts, we anticipate that pea Tic32 is the orthologue of the at4g23430 gene product. Two independent T-DNA insertion lines for at4g23430 were available from different resource centers (Fig. 4B). Both Arabidopsis lines, att32syntrin1 and attic32KölnexIV, were cultivated and the F1 generation was analyzed for homozygous offspring. We were unable to obtain homoygous insertion lines, indicating that atTic32 is essential for viability. Support for this idea came from the observation that, in contrast to wild-type, seed pods from mutant lines contained numerous aborted seeds that seem to fail to develop properly (Fig. 4C). Embryos from wild-type seeds as well as from mutant seeds were analyzed by light microscopy using Normarski optics. In wild-type seeds we could see a clear development from globular (Fig. 4D, a) to heart stage (Fig. 4D, b and c) to torpedo stage (Fig. 4D, d). In mutant seeds the globular stage of embryo development was still normal (Fig. 4D, e and f); however, embryos in the heart stage looked deformed and not as symmetric as wild-type (Fig. 4D, g and h). In no case could we observe any torpedo stage in aborted seeds.

**DISCUSSION**

In this study a new component of the Tic complex was found while screening for interaction partners for the N terminus of Tic110. Only one protein with a mass of 32kDa could be observed in a silver stain after elution with increasing salt concentrations (Fig. 1A), indicating a strong interaction between Tic110 and this protein. Three peptide sequences for Tic32 were obtained. A degenerate oligonucleotide derived from one of the obtained amino acid sequences was used as a probe for a screen of a pea cDNA library, and a single cDNA clone was isolated. The primary structure deduced from the cDNA clone contained all three peptides, obtained from Tic32 by microsequencing, thus confirming that this cDNA corresponded to the purified protein. Blast searches of the protein data base revealed membership in SDR, an evolutionarily conserved, functionally heterogeneous protein superfamily found in all organisms, with a typical chain length of about 250 residues (43, 44). SDRs are defined by distinct sequence motifs, comprising an N-terminal TGXXXGXG motif that is responsible for NAD(P)− coenzyme binding, a highly conserved NNAG motif stabilizing the central β-sheet, and the active site tetrad consisting of Asn, Ser, Tyr, and Lys residues (Fig. 1C) (45). Short-chain dehydrogenases/reductases are involved in various processes in cellular homeostasis, e.g. in basic metabolic roles (fatty acid or sugar metabolism), control of hormone ligand levels, transcriptional regulation, and apoptosis (44, 46–50). SDR enzymes, such as several retinol dehydrogenases (RDH), are frequently membrane-bound, where they form complexes with other membrane proteins (51, 52). Tic32 is the Pisum ortholog of a previously defined SDR cluster found in a cross-species comparison between Homo sapiens, Caenorhabditis elegans, Drosophila melanogaster, and Arabidopsis thaliana (53), as shown in Fig. 1C. To this end, the molecular functions of this ortholog cluster have mostly been studied in humans, represented by the WW domain containing reductase WWOX; however, its substrate specificity has not yet been determined. WWOX is a proapoptotic oncogene and interacts with signaling molecules like JNK1 or p53 and is thereby involved in stress signaling. Tic32 displays several important structural features. Tic32 does not show the N-terminal WW interaction domain, as compared with human WWOX, but instead is highly similar to other members of the cluster, e.g. human retinol dehydrogenases RDH11 and RDH13. No obvious transmembrane domain

![Fig. 4. Analysis of Tic32 T-DNA insertion lines in A. thaliana.](image-url)
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Iron-sulfur center (25, 26). In addition, for some nuclear-encoded chloroplast proteins, regulation of gene expression by redox mechanism has been proposed (58–60). Recently, the influence of light, and therefore the redox state of the chloroplast, was suggested to affect protein import in vitro (61). In the presence of light, the non-photosynthetic ferredoxin III, which is usually localized in the stroma, was mis-sorted to the intermembrane space (61). Tic32 might be a subunit of a redox-sensing circuit that regulates the import of photosynthetic proteins into the organelle.

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