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# The novel chloroplast outer membrane kinase KOC1 is a required component of the plastid protein import machinery

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Titre:

## "The novel chloroplast outer membrane kinase KOC1 is a required component of the plastid protein import machinery"

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# Abstract

The chloroplast constitutes the site of photosynthesis and is an essential organelle in plant cells. An endosymbiotic event was at the origin of the chloroplast, an ancestral eukaryotic cell engulfing a photosynthetic cyanobacterium. During evolution, the majority of ancestral chloroplast genes were lost or transferred to the nucleus. The protein products of the successfully transferred genes are now synthesized by cytosolic ribosomes and imported into the chloroplast. The chloroplast destined proteins (preproteins) acquired an additional sequence that encodes a cleavable N-terminal targeting peptide (transit peptides). The transit peptide is recognized by the chloroplast import machinery, which initiates import. The import machinery consists of translocon complexes at the outer (TOC) and inner membrane of the chloroplast (TIC). The import of hundreds of different chloroplast proteins depends on TOC and TIC complexes. The TOC complex core contains three proteins, the GTPase receptors: Toc159, Toc34 and the channel Toc75, together they recognize and transfer the pre-proteins across the outer membrane of the chloroplast. Both Toc34 and Toc159 are exposed at the surface of the chloroplast, consistent with a receptor function, and have homologous GTP-binding domains (G-domain). In addition to the G-domain, Toc159 has a N-terminal A- (acidic) domain that extends into the cytosol and controls receptor specificity and a C-terminal membrane anchoring M-domain. Toc75 belongs to the OMP85 family that serves to integrate proteins into the outer membrane of gram negative bacteria, in chloroplasts it has evolved to provide a protein translocation channel across the outer membrane.

Toc159 plays an essential role in chloroplast biogenesis. Phosphoproteomics databases show that Toc159 is highly phosphorylated at the A domain. Cytosolic casein kinase II phosphorylates the A-domain *in vitro*, however other A-domain kinases have been predicted.

While phosphorylation controls assembly and activity of protein import complexes in both mitochondria and chloroplasts no organelle-specific kinases have been identified so far. By co-purification with Toc159, we discovered "Kinase at the Outer Chloroplast membrane 1" (KOC1). KOC1 is an integral membrane protein facing the cytosol and stably associating with

TOC. KOC1 phosphorylated the A-domain of Toc159 family members *in vitro*. In mutant *koc1* chloroplasts preprotein import efficiency was diminished. Moreover, *koc1* seedlings had reduced survival rates when moved from the dark to the light when protein import is required to rapidly complete chloroplast biogenesis. Our data indicate that KOC1 is a functional component of the TOC machinery phosphorylating import receptors, supporting preprotein import and contributing to efficient chloroplast biogenesis.

**Keywords**: Arabidopsis, chloroplast, protein import, TOC complex, receptor, TOC159, integral membrane kinase, phosphorylation, regulation.

# Résumé

Le chloroplaste est un organite essentiel de la cellule végétale, il est le siège de la photosynthèse. Un événement d'endosymbiose est à l'origine du chloroplaste : une cellule eucaryote primitive a ingéré une cyanobactérie photosynthétique. Pendant l'évolution, la majorité des gènes du chloroplaste primitif ont été transférés vers le noyau. Les protéines issues des gènes transférés avec succès, sont maintenant synthétisées par des ribosomes dans le cytosol et importées dans les chloroplastes. Les protéines destinées au chloroplaste (pré-protéines) acquièrent une séquence additionnelle clivable codant pour un peptide à l'extrémité N-terminal (séquence d'adressage). La séquence d'adressage est reconnue par la machinerie d'importation du chloroplaste qui initie le transport des pré-protéines. La machinerie d'importation consiste en un translocon situé dans la membrane externe/interne du chloroplaste (TOC/TIC) ( Translocon at the Outer/Inner membrane of Chloroplast). L'importation de centaines de différentes protéines dépend des complexes TOC et TIC. Le noyau du complexe TOC est composé de trois protéines, les récepteurs GTPase Toc159 et Toc34 ainsi que le canal Toc75. Ensemble ils reconnaissent et transfèrent les pré-protéines à travers la membrane externe du chloroplaste. Toc34 et Toc159 qui sont exposés à la surface du chloroplaste, fonctionnent en tant que récepteurs et ont des domaines G (GTP-binding) homologues. En plus du domaine G, Toc159 possède le domaine A (acide) à l'extrémité N-terminal qui s'étend dans le cytosol et contrôle la spécificité du récepteur, et le domaine M à l'extrémité C-terminal qui ancre la protéine à la membrane. Toc75 appartient à la famille OMP85, protéines de la membrane externe des bactéries gram négatives. Dans les chloroplastes elles ont évolué pour fournir un canal de translocation de protéines à travers la membrane externe.

Toc159 joue un rôle essentiel dans la biogenèse du chloroplaste. Les bases de données de phosphoprotéomique montrent que le domaine A de Toc159 est fortement phosphorylé. La protéine cytosolique caséine kinase II phosphoryle le domaine A *in vitro*. Toutefois d'autres kinases ayant la même fonction ont aussi été prédites. Tandis que la phosphorylation contrôle l'assemblage et l'activité des complexes d'importation de protéines dans les chloroplastes et les mitochondries, aucune kinase organite-spécifique n'a été identifiée

jusqu'à présent. Par co-purification avec Toc159, nous avons découvert une protéine kinase dans la membrane externe du chloroplaste (KOC1 « Kinase at the Outer Chloroplast membrane 1 »). KOC1 est une protéine intégrale de membrane orientée vers le cytosol et associée de manière stable avec le complexe TOC. KOC1 phosphoryle le domaine A chez les membres de la famille Toc159 *in vitro*. Dans les chloroplastes des mutants *koc1*, l'efficience de l'importation des protéines a été réduite. Par ailleurs, les plantules *koc1* ont un taux de survie réduit quand elles sont déplacées de l'obscurité à la lumière, quand une importation rapide des pré-protéines est nécessaire pour une biogenèse de chloroplastes complète. Nos résultats indiquent que KOC1 est un composant de la machinerie d'importation TOC en phosphorylant les récepteurs, en soutenant l'importation de pré-protéines et en contribuant à une biogenèse de chloroplastes efficiente.

**Mots clef:** Arabidopsis, chloroplaste, importation des protéines, complexe TOC, récepteur, TOC159, kinase intégrale de membrane, phosphorylation, régulation.

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# Introduction

Plastids are a diverse group of double membrane bound organelles present in plants and implicated in essential metabolic and signaling processes. The chloroplast is the most studied member of plastid family present in green aerial tissues, constitutes the site of photosynthesis and is implicated in metabolic functions including amino acids or lipid synthesis.

## Chloroplast origin

Chloroplasts are derived from endosymbiotic photosynthetic bacteria. The endosymbiotic ancestor of the chloroplast originated from a free-living pigmented group of prokaryotes: Cyanobacteria. The acquisition of a photosynthetic cyanobacterium by a eukaryotic cell through the process of endosymbiosis is one of the most important events in plant cell history. According to molecular clock approaches plastid endosymbiosis dated between 1.4 and 1.7 billion years ago (McFadden, 2014). During evolution, the plastid progenitor retained its own genome (around 100 genes) but the majority of its genes were transferred to the host nucleus. The permanent endosymbiosis requires that the endosymbiont have to divide within the host cells (McFadden, 1999). The division of plastid is achieved by binary fission. The genes implicated in this process derived from the endosymbiont were identified in the host nucleus. This is the case for many indispensable endosymbiotic genes that during evolution have relocated from the ancestral genomes to the plant cell nucleus. These genes are functionally competent and at present drive the biogenesis of chloroplast as well as some others essential cellular processes (Timmis et al., 2004).

Chloroplast genes that were successfully transferred to the nucleus must be expressed, and the protein product located in the cytosol returned to the chloroplast. To mediate chloroplast targeting, transferred genes acquired additional sequences that encode a cleavable N-terminal targeting peptide, known as transit peptide (Bruce, 2000). Transit peptides contain the information to facilitate the targeting of proteins to the chloroplasts. The number of predicted chloroplast-targeted precursors identified by genomic sequencing is increasing. Databases now contains sequences of thousand of different transit peptides,

allowing the prediction of transit peptides in the unknown protein sequences (Bruce, 2000). Transit peptides are divergent in composition and length (~20-150 residues) but have a similar characteristics such as high content of alanine and hydroxylated amino acids, less acidic amino acids and the presence of short motifs (Lee et al., 2013). Recent efforts to identify conserved motifs in transit peptides, resulted in a model proposed by Chotewutmontri et al. The model proposed that transit peptides have an N-terminal domain able to interact with one or more Hsp70 molecular chaperones, followed by a region that interacts with the TOC components (Chotewutmontri et al., 2012).

## Chloroplast biogenesis

Chloroplast biogenesis implicates the development of undifferentiated proplastids or etioplasts into mature chloroplasts (Paila et al., 2015). Light has an important role as a stimulus for chloroplasts biogenesis. Once light is perceived by seedlings, proplastids, which contain no differentiated internal structures, are remodeled into developed chloroplasts and the components of the photosynthetic machinery are assembled. (Rudowska et al., 2012). The process give rise to the chloroplast composed of three membrane systems (outer/ inner envelope and thylakoid membranes) and three aqueous subcompartments (envelope intermembrane space, stroma and thylakoid lumen). Chloroplast development requires coordinated processes in different cell compartments. Upon light, the expression of nuclear and plastid genes is activated, lipids and pigments are synthesized within the plastids, nucleus-encoded preproteins are imported from the cytosol and the functional complexes are assembled (Rudowska et al., 2012).

The synthesis of proteins during the biogenesis of chloroplast occurs in two locations, the cytosol and within the organelle itself. This process requires coordination of two genomes (Jarvis and Lopez-Juez, 2013). Chloroplast biogenesis requires the synthesis of photosynthetic complex components, Calvin cycle enzymes and envelope transporters. The expression of photosynthetic plastid-encoded core components such as the products of psaA and psaB for photosystem I (PSI) and psbA and psbD for PSII depends on nucleus-encoded factors. The plastid genome in *Arabidopsis thaliana* encodes 54 core plastid photosynthetic proteins, 31 proteins related with plastid DNA expression and 45 encode tRNAs or rRNAs (Jarvis and Lopez-Juez, 2013). The synthesis of photosynthetic pigments, chlorophylls and

carotenoids, is also essential for chloroplast biogenesis. Eukaryotic carotenoids are synthesized in the cytosol and the synthesis of plastid carotenoids occurs in the organelle.

## Import pathways in chloroplasts

Chloroplast biogenesis requires the import of proteins. In Arabidopsis, proteomic and genomic analysis revealed that 2500-4000 plastid proteins encoded by the nuclear genome are imported from the cytosol to the chloroplast (Kessler and Schnell, 2006). The targeting of chloroplast proteins is mediated by several sorting and import systems at the chloroplast envelope depending on their final destinations within the organelle (Fig.1). In the first one, the preproteins (chloroplast protein with a transit peptide) are recognized by their transit peptides and imported via sequential interactions with the translocon complexes at the *O*uter and *I*nner membrane of the chloroplast (TOC-TIC import machinery) (Schnell et al., 1994; Paila et al., 2015; Richardson et al., 2014). The TOC-TIC import pathway constitutes the major protein import route in plastids, mediating the import of up to 4000 different proteins into the intermembrane space, the inner membrane or the stroma. A second cytosolic system recognizes integral membrane proteins and facilitates their insertion into the outer membrane (Hofmann and Theg, 2005). In a third pathway, the proteins are delivered to the chloroplast via vesicle trafficking through the endoplasmic reticulum and Golgi (Paila et al., 2015). Only very few proteins are transported through the secretory pathway, among them, the α-carbonic anhydrase reported by Villarejo et al (Villarejo et al., 2005). In the fourth pathway, an alternative route mediates the targeting for proteins lacking recognizable transit peptides or with non-cleavable internal sequences (Fig.1). (Schnell et al., 1994; Paila et al., 2015; Richardson et al., 2014; Andrès et al., 2010). Even though some imported proteins may use others pathways, the vast majority of chloroplast preproteins engage the TOC-TIC pathway.

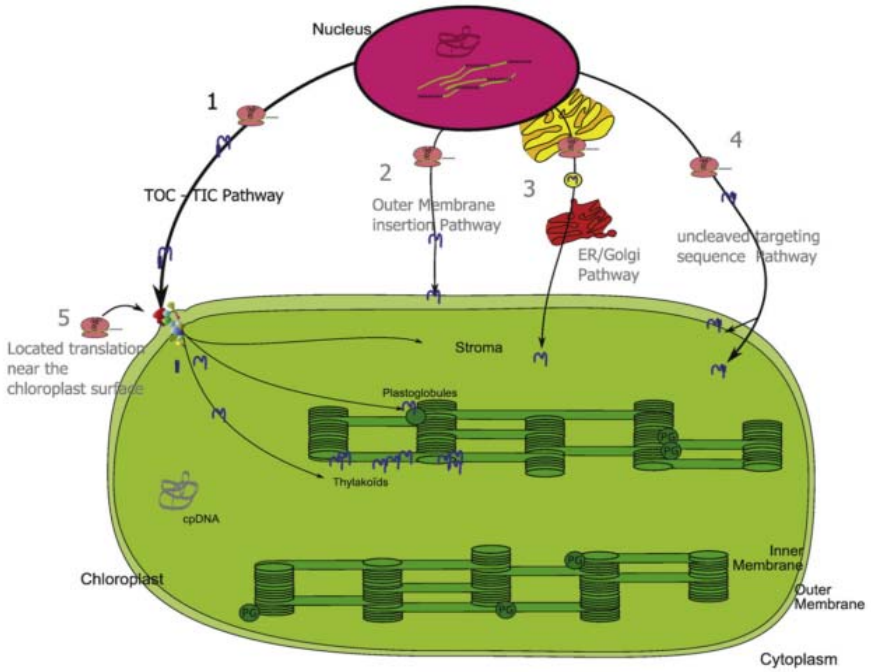


**Figure 1. Summary of the different pathways for the targeting of nuclear encoded chloroplastic proteins**. **1**. The TOC-TIC complex is the major route for preproteins. **2**. Proteins localized at the outer membrane are inserted directly or by receptor mediated pathways. **3**. Some proteins take the endoplasmic reticulum and Golgi pathway. **4**. Alternative pathway for some proteins lacking recognizable transit peptides. Figure taken from (Andrès et al., 2010).

## TOC-TIC pathway

The main route of protein import into the chloroplast implicates the *T*ranslocon complexes at the *O*uter (TOC) and *I*nner membrane of the *C*hloroplast (TIC) (Schnell et al., 1994; Jarvis and Soll, 2001; Kessler et al., 1994). The TOC-TIC pathway is the major protein import system in plastids, around 3500 proteins are imported via this system (Richardson et al., 2014). The core components of the TOC-TIC complex are well conserved in plant lineages that evolved from the endosymbiotic ancestor, indicating the central role of this machinery in chloroplast biogenesis (Paila et al., 2015).

## Identification of the TOC and TIC complexes

In the early 1990s, Schnell and Blobel (Schnell and Blobel, 1993) used a hybrid preprotein (pS/protA) consisting of the precursor of the small subunit of Rubisco fused to the IgG binding domains of Staphylococcal protein A to produce translocation intermediates arrested in the outer and outer and inner envelope membrane of the chloroplast, respectively. They identified two intermediates, the first one consisted of pS/protA inserted

across the outer membrane of chloroplast and was termed the early intermediate. The second one was partially translocated spanning both envelope membranes and termed the late intermediate. It was partially processed by the stromal processing peptidase (SPP) while remaining accessible to the exogenous protease thermolysin. Using immunoelectron microscopy, the intermediates were localized at specific regions of the envelope where the outer membrane was in proximity to the inner one. This indicated that the translocation process occurs at the contact sites between the outer and inner membrane of the chloroplast.

Components of the chloroplast machinery were isolated by IgG-affinity purification of the Protein A tagged early and late translocation intermediates followed by Edman sequencing of the protein bands. The identified proteins were termed Import intermediate Associated Proteins. Other groups identified the same proteins using chemical crosslinking and named the components or Outer (or Inner) Envelope Proteins. (Schnell et al., 1994; Kessler et al., 1994; Hirsch et al., 1994; Perry and Keegstra, 1994; Waegemann and Soll, 1991). Later the components were renamed according to TOC-TIC nomenclature (Schnell and Blobel, 1997) as ( *T*ranslocon at the *O*uter/*I*nner membrane of the *C*hloroplast followed by the molecular mass in Kilodaltons). The proteins initially identified were Toc86, Toc34 and Toc75 that were associated with both the early and the late intermediate followed by Tic110 that was exclusively associated with the late intermediate (Schnell et al., 1994).

## TOC core complex

Even in the absence of a translocating protein chain the three components, Toc159, Toc34 and Toc75 associate in a stable fashion and form the TOC core complex (Fig. 2). Toc159 and Toc34 are exposed to the cytoplasm and function as preprotein co-receptors (Kessler et al., 1994; Schleiff et al., 2003b). Toc75 embedded in the outer membrane functions as a protein conducting channel (Fig. 2). Toc159 was originally identified as 86 kDa chloroplast outer membrane protein (Kessler et al., 1994; Hirsch et al., 1994). Only the sequencing of the Arabidopsis genome revealed that Toc159 was a much larger protein (Bölter et al., 1998) and had an additional N-terminal A-domain that was later also confirmed in pea (Chen et al., 2000).

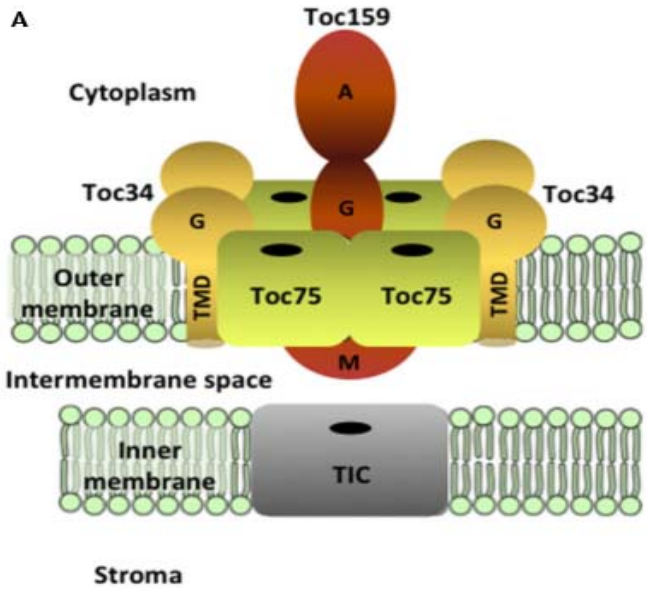


**Figure 2. Structural organization of TOC complex.** The TOC complex core consists of two GTPases receptors Toc159 and Toc34 together with Toc75 the protein-conducting channel. The stoichiometry of the complex was estimated at 1:4:4-5 (Schleiff et al., 2003b) or 1:3:3 (Kikuchi et al., 2006) (Toc159: Toc75: Toc34). Figure taken from (Richardson et al., 2014).

The molecular organization and architecture of the TOC complex has been the object of many studies. Schleiff et al (Schleiff et al., 2003b) suggested by size exclusion chromatography, that the core of the TOC complex in pea has a molecular mass of around 500 kDa and a molecular stoichiometry of 1:4:4-5 between Toc159, Toc75 and Toc34 (Fig.2). In contrast, Kikuchi et al (Kikuchi et al., 2006) using blue native PAGE of detergent-solubilized pea chloroplasts followed by immunoblotting estimated the mass of the core complex at 800-1000 kDa. This complex was purified in presence of protease inhibitors to avoid degradation of its components. The stoichiometry of the three proteins was calculated as 1:3:3 between Toc159, Toc75 and Toc34. Kikuchi et al suggested that the difference in the molecular mass compared to the Schleiff's study was probably due to the absence of protease inhibitors during membrane preparation in that study and Toc159 had been present as the 86 kDa fragment. TOC and TOC/TIC complexes were also identified in association with importing precursors. Chen et al (Chen and Li, 2007) found that during active import, the importing precursor associated with a 880-kDa TOC complex before forming a higher molecular mass TOC/TIC supercomplex. The 880-kDa complex is most likely the same complex identified before by Kikuchi (Kikuchi et al., 2006), and the interaction of precursors with this TOC complex takes place at the earliest steps during active import and

may therefore correspond to early import intermediated. Chen et al also reported that the processing of transit peptides takes place while precursors were associated with the higher molecular mass complex corresponding to TOC/TIC supercomplex and possibly the late translocation intermediate.

## Import of pre-proteins by the TOC Complex

The import of preproteins in chloroplasts requires energy and three stages of import have been defined according to energy requirements. Initially, the binding of the preprotein to the GTPase receptors is energy-independent and reversible (Perry and Keegstra, 1994) (Fig. 3). In the second step, the insertion across the outer membrane leads to the formation of the "early import intermediate" which is irreversible and requires GTP and low concentrations of ATP (≤100 μM) (Olsen et al., 1989; Olsen and Keegstra, 1992; Young et al., 1999). Finally, preproteins are translocated across the envelope membranes into the stroma followed by the removal of the transit peptide. This step requires high concentrations of ATP (~1mM) (Theg et al., 1989). At this stage the late import intermediate can be formed by placing translocating chloroplast on ice.

The newly synthesized preproteins are recognized by Toc34 and Toc159 at the chloroplast surface and the import is initiated by the binding of the transit peptide to the G domains of GTPase receptors (Fig. 3b). In contact with the transit peptide at the G domain, GTP to GDP exchange occurs and induces the separation of Toc34 dimer followed by heterodimerization with Toc159 (Fig. 3c). Hydrolysis of GTP allows the insertion of the preprotein into the Toc75 channel and translocation across the outer envelope (Fig. 3d) (Richardson et al., 2014; Kessler and Schnell, 2002; Paila et al., 2015).

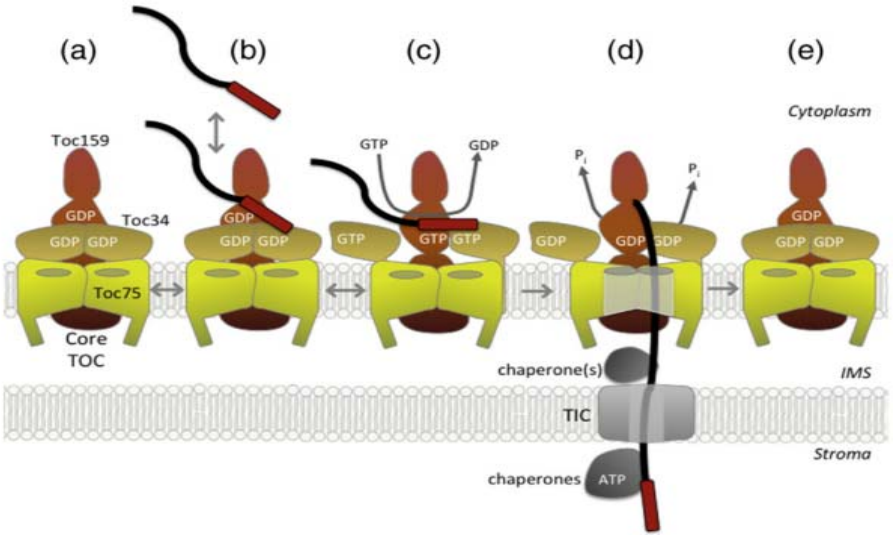


**Figure 3. Preprotein import at the TOC complex. (a)** The core of the TOC complex consists of two GTPase receptors Toc159 and Toc34 and a channel protein Toc75. **(b)** The GTPase receptors Toc159 and Toc34 recognize and bind the transit peptide of the preprotein at their G-domains, **(c)** binding of the transit peptide to the receptors triggers exchange of GDP for GTP and promotes Toc159-Toc34 heterodimerization. **(d)** GTP hydrolysis initiates preprotein translocation via Toc75 and across the inner membrane via the TIC complex. **(e)** The TOC complex ultimately resets to its initial GDP-bound state. Figure taken from (Paila et al., 2015).

## TOC complex composition

### *GTPase receptors*

Toc159 and Toc34 are integral GTPases and share homology in their GTP-bindings domains (G domain). The two proteins are protease sensitive in isolated chloroplasts indicating exposure at the chloroplast surface (Seedorf et al., 1995; Kessler et al., 1994; Hirsch et al., 1994). GTP-dependence of early intermediate formation suggested that Toc159 and Toc34 are implicated in the early stages of protein import, and are thus considered primary receptors of cytosolic preproteins. Arabidopsis genome sequencing has revealed homologs of all components of the import machinery including two of Toc34 and four of Toc159. They all share homology in the G domain (Fig. 4), forming a six member GTPase family with Toc34 and Toc159 subgroups (Fig. 4) (Hiltbrunner et al., 2001a).

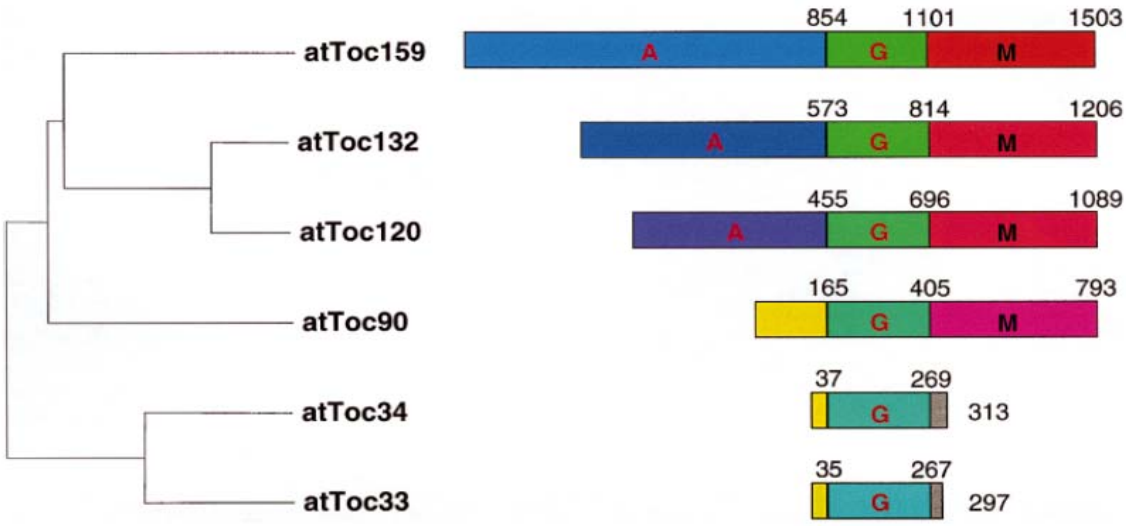


**Figure 4. Toc GTPase family of *Arabidopsis thaliana*.** Phylogenetic tree and schematic representation including the position of the acidic domains (A), GTPase domains (G) and membrane anchoring domains (M). The members of the family are in two subgroups, homologs of Toc159 (atToc159, atToc132, atToc120, and atToc90) and Toc34 (atToc33 and atToc34). Figure taken from (Hiltbrunner et al., 2001a).

## *Toc34 family*

Toc34 was first identified in pea associated with the early and late import intermediates (Kessler et al., 1994). Toc34 consists of a G domain homologous to that of Toc159 at the N-terminus followed by a single transmembrane domain (TMD) anchoring the protein in the outer membrane with G domain facing the cytoplasm. Toc34 is an integral membrane protein and is recognized by the ankyrin cytosolic factor, AKR2A, in the cytosol. AKR2A binds to the hydrophobic TMD of Toc34 and together with Hsp17.8 mediates the targeting and insertion of Toc34 at the outer membrane of chloroplasts. (Lee et al., 2014, 2013). The information for chloroplast targeting in Toc34 resides near the TMD region. The insertion of PsToc34 (Toc34 from pea) depends of NTP's. Absence of ATP did not affect its binding to the chloroplast surface, but insertion was reduced to around 50%. When its GTP binding capacity was reduced its insertion was also reduced (Hofmann and Theg, 2005). Toc34 is unique protein in pea, but in Arabidopsis there are two genes atToc33 and atToc34. atToc33 is considered the ortholog of Toc34 in pea (Jarvis, 1998). Null mutants of Toc34 and Toc33 has been reported in Arabidopsis (Constan et al., 2004; Jarvis, 1998). The green tissues of the atToc34 mutant, *ppi3* (plastid protein import 3) appeared similar to wild type plants, chloroplasts were structurally normal and able to efficiently import the Rubisco small

subunit precursor *in vitro*. In *ppi3* a significant growth reduction (20-30%) were observed in roots, compared to wild type (Constan et al., 2004).

Toc33 mutant, *ppi1* has a pale-green phenotype and contains reduced level of chlorophyll at all developmental stages (Jarvis, 1998). *ppi1* plants are uniformly pale during the first 2 weeks of its life cycle, but the appearance of old leaves is closer to wild type. *ppi1* chloroplasts are smaller than normal and contain fewer thylakoid membranes with smaller granal stacks (Jarvis, 1998). Concerning the role of Toc33 in protein import, Kubis et al (Kubis et al., 2003) used immunoblot analysis to compare the abundance of different photosynthetic and nonphotosynthetic proteins in chloroplasts isolated from 10-day-old *ppi1* and wild type plants. They observed that in *ppi1* chloroplasts, the abundance of photosynthetic proteins was reduced by 50% or more and nonphotosynthetic proteins were detected at the same levels as the wild type. The data suggested that atToc33 is involved preferentially in the import of photosynthetic protein and atToc34 in the import of nonphotosynthetic proteins in chloroplasts (Kubis et al., 2003). The activity of Toc33 and Toc34 are partially overlapping, in *ppi3* mutants the absence of phenotype in aerial tissues, reflects the ability of Toc33 to substitute for Toc34. However, the double mutant *ppi1-ppi3* has an embryo-lethal phenotype indicating that their overlapping function is essential (Constan et al., 2004).

### *Toc159 family*

Toc159 has three domains, the N-terminal acidic domain (A domain), followed by the central G domain and the C-terminal membrane anchoring domain (M domain). Due to the protease sensitivity of the A domain, pea Toc159 (psToc159) was originally identified as a fragment of 86 kDa (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994). The 86 kDa form contained only the G and M domains (Bölter et al., 1998).

The Toc159 family in *Arabidopsis thaliana* was investigated and four receptor homologs were found (Bauer et al., 2000; Hiltbrunner et al., 2001a; Infanger et al., 2011). These proteins were named atToc159, atToc132, atToc120 and atToc90. All contained G domains as well as a M domains. In addition, atToc159, -132 and -120, but not atToc90, had N-terminal A domains. The G and M domain are highly conserved, but the A domains vary

significantly in sequence and length. atToc159 and psToc159 share homology (48% overall identity, 74% identity at G-M domains) and are considered functional orthologs (Bauer et al., 2000).

The localization of Toc159 family members was tested by insertion experiments of radiolabeled proteins into intact isolated chloroplasts. The three Toc159 proteins tested in the study (atToc159, -132, -120) were resistant to alkaline extraction, and thermolysin treatment resulted in a fragment of 52kDa corresponding to the M domain. These data suggested that atToc159 -132 and -120 are integral proteins located at the outer chloroplast membrane with their A-G domains exposed to the cytoplasm (Bauer et al., 2000).

Differences between the mutant phenotypes of the Arabidopsis Toc159 family suggested distinct import receptor functions. *ppi2* that lacks Toc159 results in an albino phenotype and is lethal when grown on soil (Bauer et al., 2000). In *ppi2,* plastids lack normal thylakoid membranes and starch granules. Furthermore, they lacked the prolamellar body (rudimentary membrane structure) that is characteristic of etioplasts when grown on sucrose in the dark (Bauer et al., 2000). Molecular analysis reveled that expression of a number of photosynthetic proteins was strongly reduced in *ppi2* plants compared to wild type; in contrast the expression and import of a number of nonphotosynthetic proteins was not affected (Bauer et al., 2000). Interestingly, *ppi2* survived when grown on media containing sucrose, indicating that many constitutive plastid functions remained intact while photosynthetic activity was blocked (Smith et al., 2004).

Toc120 and Toc132 Arabidopsis mutants were studied by Ivanova (Ivanova et al., 2004) and Kubis (Kubis et al., 2004). They noticed that the Toc120 and Toc132 double mutant (*toc132/toc120*) exhibited an albino (Kubis et al., 2004) or even embryo lethal phenotype (Ivanova et al., 2004). Concerning the individual knockout mutants, both concluded that the *toc120* mutant displayed a normal visible phenotype. Kubis et al reported a chlorophyll deficient phenotype in *toc132* mutants, whereas Ivanova et al did not detect a visible phenotype. Overexpression of either Toc120 or Toc132 (but not of Toc159) in the *toc132/toc120* double mutant complemented the phenotype indicating a functional overlap between the two proteins and a distinct function of Toc159 (Kubis et al., 2004). In

Arabidopsis, the Toc complex association of Toc159 family receptors was examined by immunoaffinity chromatography. Toc120 and Toc132 were identified in complexes that excluded Toc159. Toc34 was present in complexes containing Toc120/Toc132 whereas Toc33 was specific for Toc159 complexes (Ivanova et al., 2004). These data are consistent with the proposal that there is a functional specialization in Arabidopsis Toc GTPase receptors. Toc120 and Toc132 were proposed to have a role in the import of house-keeping substrates in nonphotosynthetic tissues while Toc159 was preferentially associated with the import of photosynthetic proteins. A study reported by Bischof et al (Bischof et al., 2011) using transcriptome and proteome analysis, concluded that the substrate specificity of Toc159 is not restricted to photosynthetic proteins. They quantitatively analyzed the accumulation of plastid proteins in *ppi2* and WT plants and found that many photosynthetic proteins also accumulated in *ppi2* plants and several proteins from distinct metabolic pathways were negatively affected.

The receptor Toc90 has conserved G and M domains but lacks an A domain (Hiltbrunner et al., 2001b). The knockout of Toc90 termed *ppi4* had no visible phenotype (Hiltbrunner et al., 2004). Overexpression of atToc90 partially complemented the albino phenotype of *ppi2* and restored photoautotrophic growth, this suggested that atToc90 was able to support the import of photosynthetic proteins and had an overlapping function with atToc159 (Infanger et al., 2011; Hiltbrunner et al., 2004).

### *Toc75: The channel*

Toc75 was identified in association with the envelope-bound import intermediates (Schnell et al., 1994; Perry and Keegstra, 1994) and specifically with the transit peptide of the precursors (Ma et al., 1996). Toc75 is a β-barrel membrane channel protein and is considered an essential component of the import machinery at the outer membrane of chloroplast. In Arabidopsis, Toc75 is encoded by a single gene (TOC75-III) and null mutants are embryo lethal (Paila et al., 2016).

The GTPase receptors, Toc159 and Toc34 targeting the preproteins in the cytosol and drive to the Toc75 channel (Kessler and Schnell, 2009). Studies concerning the capacity of Toc75 to differentiate the transit peptides, show that Toc75 recognizes transit peptides based on both

conformational and electrostatic interactions (Hinnah et al., 2002). Toc75 has a ion conducting channel properties (Hinnah et al., 1997). The pore size of the reconstituted Toc75 was estimated, the narrow restriction zone corresponding to the selectivity filter with 14 Å and a wider pore vestibule with 26 Å (Hinnah et al., 2002). Importantly, these dimensions are sufficient to allow the passage of an unfolded protein across the Toc75 channel.

Toc75 is a member of the Omp85 family. Omp85 proteins are exclusively localized at the outer membrane of Gram-negative bacteria, mitochondria and plastids (Paila et al., 2016); characterized by N-terminal region consisting of three repeats of POTRA (POlypeptide-TRansport Associated) domains that extends into a soluble space, and a C-terminal region constituting the membrane-integrated β-barrels (Richardson et al., 2014). The POTRA domains in Omp85 family are multifunctional, and in Toc75 they are essential for the interaction with preproteins and mediate the recruitment of chaperones to facilitate membrane transport (Paila et al., 2016). In addition to the role of a protein-conducting channel, Toc75 may be implicated in the insertion of Toc159; Toc75 together with Toc34 act to mediate docking and insertion of Toc159 to the functional translocon (Wallas et al., 2003).

The targeting of Toc75 is unique among the outer envelope membrane proteins. pre-Toc75 (89 kDa precursor) has a N-terminal transit peptide followed by a glycine-rich region. pre-Toc75 is imported via TOC complex, processed to an intermediate form and arrested in the intermembrane space where it is cleaved by a peptidase; resulting in mature Toc75. Finally, Toc75 is imported by the TIC complex and directed to the outer envelope (Richardson et al., 2014).

### *Toc64 and Toc12*

In addition to the TOC core components, Toc64 and Toc12 were identified as TOC members. Toc64 has a large domain, containing three tetratricopeptide (TRP) repeats exposed at the chloroplast surface (Sohrt and Soll, 2000). The TRP domain of Toc64 recognizes the preproteins by interaction with Hsp90 that are subsequently transferred to Toc34. This transfer is mediated by GTP-dependent association of Toc64 and Toc34 (Qbadou et al., 2006). In Arabidopsis, three genes (atTOC64-III, atTOC64-V and atTOC64-I) encode for Toc64, and knockout *toc64* mutants are indistinguishable from wild type (Aronsson et al., 2007).

Recently, the relation of Toc64 (Toc64-III) and Toc33 was studied using a double mutant. In this double mutant (*ppi1* and *toc64-III*), the protein level of Toc75 is significantly reduced, indicating that Toc64 and Toc33 maybe cooperate in the insertion or stabilization of Toc75 (Sommer et al., 2013). The functional significance of Toc64 in the protein import remains unclear and much debated.

Toc12 was identified in association with the translocation complex at the intermembrane space. Toc12 is an integral protein of the outer membrane exposing a soluble domain (J domain) into the intermembrane space. The J domain stimulates ATP hydrolysis by DnaK and interacts directly with Hsp70 proteins. Toc12 stability depends on a disulfide bridge within the loop region of the J-domain, suggesting a redox-regulated activation of the chaperone (Becker et al., 2004a). Toc12 interacted with Toc64 and Tic22 and may therefore represent a link between Toc and Tic complexes (Becker et al., 2004a)

## Chaperones in the import of proteins

Import of preproteins requires cytosolic factors to facilitate preprotein targeting to chloroplasts. Several cytosolic factors such as a 14-3-3 protein, Hsp70, Hsp90 were identified. Chloroplast preproteins synthesized in a wheat germ extract interacted *in vitro* with a 14-3-3 dimer and Hsp70 to form a complex named the “guidance complex” (Fig. 5b) (May and Soll, 2000). The preproteins bound to the complex were more efficiently imported into isolated chloroplast compared to unbound preproteins (May and Soll, 2000). Hsp90 was identified in a complex with Toc64 (Qbadou et al., 2006). The presence of Hsp90 stimulated import *in vitro*, Hsp90 interacted with the transit peptide and mature regions of a number of preproteins (Fig 5a). Toc64 participated in the formation of Hsp70-Hsp90 chaperone complexes in the cytosol and facilitated transfer of preproteins from Hsp90 to the TOC complex (Paila et al., 2015). The chaperones, ClpC and cpHsp70 were reported to provide the driving force for preprotein import into the chloroplast (Theg et al., 1989; Nielsen et al., 1997; Akita et al., 1997; Su and Li, 2010; Shi and Theg, 2010)

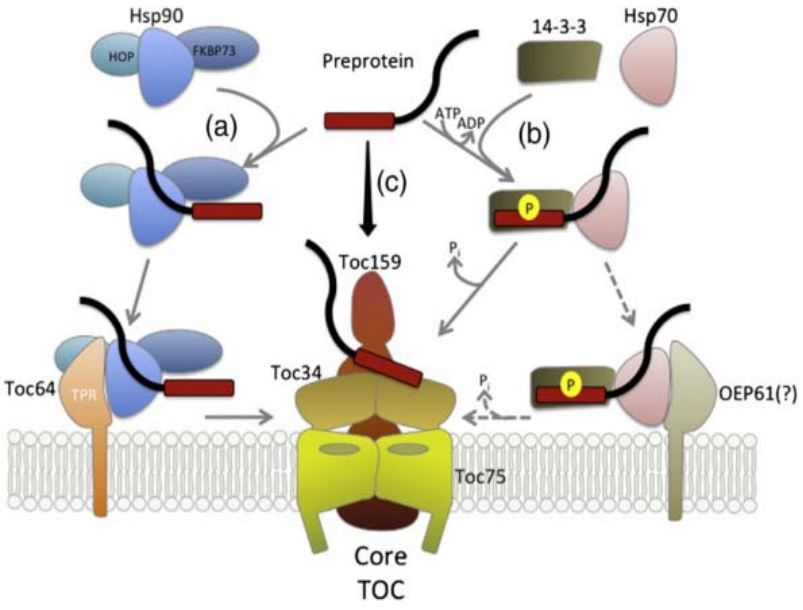


**Figure 5. Role of the chaperone system. (a)** Hsp90 together with the co-chaperones (Hop and FKB73) binds preproteins and docks at the outer membrane via an interaction with the TRP domain of Toc64. Toc64 delivers preproteins to the TOC complex by interaction with the receptors Toc159 and Toc34. **(b)** The transit peptide of preproteins binds to the guidance complex formed by Hsp70 and 14-3-3 proteins, which facilitates targeting to the TOC receptors. **(c)** Preproteins can also bind directly to the Toc159 and Toc34 receptors. Figure taken from (Paila et al., 2015).

## TIC Complex

Preproteins emerged from the intermembrane space-side of the TOC complex must subsequently interact with the TIC translocon at the intermembrane space and inner envelope membrane. The information about preprotein transfer between TOC and TIC complex it is unclear, but the analysis of the early intermediates in the import suggest that the preproteins are recognize by the TIC components. The preprotein translocation involves many components including Tic110, Tic20/Tic21, Tic22 and Tic40. This components were shown to associated with preprotein intermediates and is known as a Tic110 complex (Paila et al., 2015). Tic22 a soluble protein is located in the intermembrane space associated with the outer face of the inner membrane and was detected bound to the preproteins (Kouranov et al., 1998).

In summary, there are many candidate Tic proteins identified and characterized, which include Tic20, Tic21, Tic32, Tic40, Tic55, Tic56, Tic62, Tic100, Tic110 and Tic214 (Jarvis and Lopez-Juez, 2013). Tic110 is one of the most studied. Tic110 is proposed as central

component in the translocation channel (van den Wijngaard and Vredenberg, 1999). It contains two hydrophobic transmembrane-helices at its N-terminus to anchor the protein into the membrane, and four amphipathic a-helices in the C-terminal region implicate in the channel formation. In others studies, Tic110 is hypothesized to act as a scaffold to coordinate the stromal events of protein translocation into chloroplast and is inconsistent with the role as a β-barrel (Inaba et al., 2003). Tic110 interacts with chaperones such as Cpn60, Hsp93 and Hsp70 (Kovács-Bogdán et al., 2011). Tic20 is predicted to have four a-helical transmembrane domains and was also proposed to constitute a protein translocation channel.

Tic110 and Tic20 have a channel activity *in vitro* (Heins et al., 2002; Balsera et al., 2009; Kovács-Bogdán et al., 2011). Interestingly, the transcript and protein levels of Tic20 were generally less abundant compared to Tic110 and particularly in *Arabidopsis thaliana* plants compared to *Pisum sativum* (Kovács-Bogdán et al., 2011). The low abundance of Tic20, suggested that it cannot form a major protein translocation channel. However, Tic20 could be responsible for translocation of special subset of proteins and Tic110 could be considerate as a general translocation channel (Kovács-Bogdán et al., 2011).

The organization of the Tic proteins to form a stable TIC core complex remains controversial. Kikuchi et al (Kikuchi et al., 2009) identified by BN-PAGE a 1-MD (1-Megadalton) complex as an intermediate during protein translocation across the inner membrane, in Arabidopsis. Tic20 and Tic21 were detected in the 1-MD complex but not Tic110. In 2013, a new 1-MD complex was identified (Kikuchi et al., 2013). Transgenic Arabidopsis plants expressing a protein A-tagged Tic20-I (Tic20 isoform in Arabidopsis) were used to purified tagged Tic20-I-containing complexes. In this stable 1-MD complex, three proteins co-purified with Tic20-I were identified and termed Tic214, Tic100 and Tic56 (Fig. 6b) (Kikuchi et al., 2013). The 1-MD complex has been proposed to be a general TIC translocon because it has a channel activity when reconstituted *in vitro* into planar lipid bilayers. Tic56, Tic110 and Tic214 are missing in grasses, which raise doubts about the broader relevance of the complex (Jarvis and Lopez-Juez, 2013).

The coordinate function of TIC components in the import across the inner membrane was summarized by Paila et al (Paila et al., 2015) in the Fig. 6. In the complex including Tic110, Tic40 and Tic20 associates with molecular chaperones Tic22 and cpHsp70, Hsp90C and Hsp93/ ClpC, Tic22 is proposed to bind the preproteins from TOC complex and facilitate the transport to the inner membrane space. Tic40 and Tic110 are proposed to coordinate the assembly of the stromal chaperones to form the import motor (Fig.6a). The 1 MD complex consisting of Tic20, Tic56, Tic214and Tic100 (Kikuchi et al., 2013) has been shown to associate with preproteins (Fig. 6b). Another alternative model proposed that both complexes described in the Fig. 6 a and b would associate dynamically to form the translocation channel (Fig. 6c) (Paila et al., 2015).

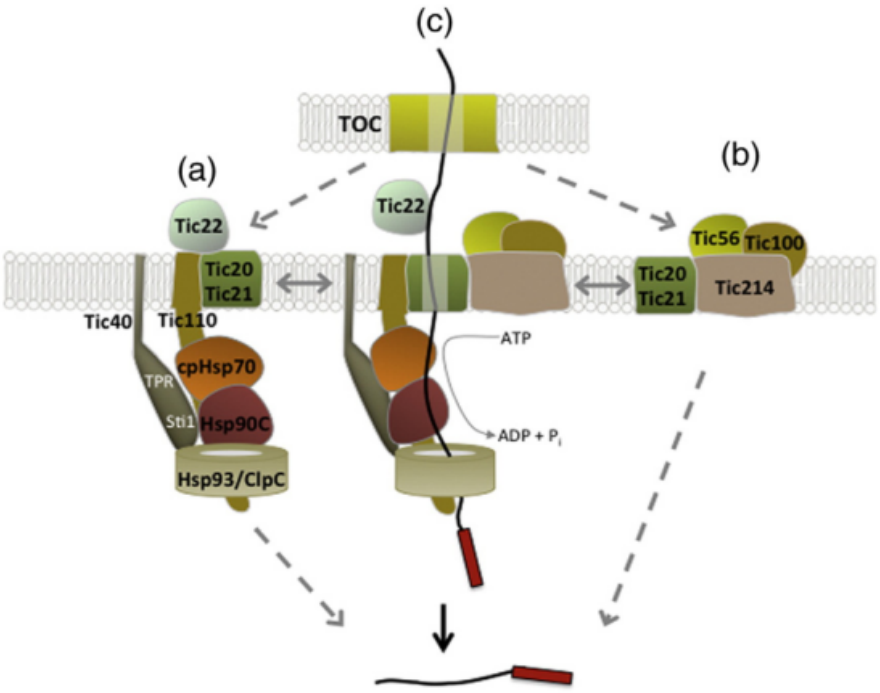


**Figure 6. Model of the import of preproteins for the TIC complex. (a)** Tic22 bound the preproteins from the TOC complex, Tic40 and Tic110 participate to the assembly of stromal chaperones to form the import motor. **(b)** The 1MD complex consisting of Tic20, Tic56, Tic214and Tic100 was also detected in the import of preproteins. **(c)** In the alternative model both complex (a and b) would associate to form the translocation channel. Figure taken from (Paila et al., 2015).

## Regulation of preprotein import

### *GTP-binding and hydrolysis*

GTP-binding and hydrolysis by the Toc GTPases constitute a regulatory mechanism in the import of preproteins. Preprotein import is inhibited by nonhydrolyzable GTP analogues, suggesting that the hydrolysis of GTP by the Toc GTPases is required for translocation (Ma et al., 1996; Young et al., 1999). More specifically, GTP is necessary for the generation of the early-import intermediates by Toc34 and Toc159, but probably is not required during the later stages of translocation (Young et al., 1999).

Toc159 and Toc34 share homology in their G domains with Ras-like GTPases (Kessler et al., 1994). However, the analysis of the crystal structure of pea Toc34 revealed a unique organization of motifs of the GTP/GDP binding pocket (Kessler and Schnell, 2002; Sun et al., 2002). The motifs G1-G5 are involved in GTP binding and hydrolysis in Ras proteins, in contrast, in Toc34 the corresponding motifs (with the exception of G1) diverge significantly from Ras, this suggests that Toc GTPases utilize a novel mechanism (Sun et al., 2002). The structural data showed that pea Toc34 forms GDP-bound dimers, suggesting a model in which GTP-regulated dimerization of Toc34 controls the targeting and translocation of preproteins in the chloroplasts (Kessler and Schnell, 2002). Studies in Arabidopsis showed that Toc33 not only forms homodimers but is also able to form heterodimers with Toc159 via their G domains (Weibel et al., 2003), thereby providing evidence that dimerization plays a central role in preprotein recognition (Paila et al., 2015).

The physiological role of dimerization was studied using Arabidopsis mutants. Mutations predicted to affect dimer formation in atTOC33 were analyzed. In these mutants the dimerization and the rate of protein import was reduced even though the GTPase activity remained stable (Lee et al., 2009). The interaction of Toc33 with Toc159 was also reduced, but the initial preprotein binding at the receptors did not decrease. All these results indicate that dimerization is important for receptor-receptor and receptor-preprotein interactions and plays a direct role in protein import as a part of GTP-regulated mechanism (Lee et al., 2009).

Toc159 and Toc33 are regulated by a GTPase cycle (Kessler and Schnell, 2004; Soll and Schleiff, 2004). To study the precise roles of Toc159 and Toc34 in the recognition of transit peptides and the function of their G domains, two models have been proposed. The first model, the "Targeting hypothesis" (Kessler and Schnell, 2004; Wang et al., 2008) (Fig. 7), proposes that Toc159 and Toc34 work together to form a GTP regulated gate and control the access of preproteins to Toc75. In this model, Toc159 (attached to the outer membrane or in a soluble form) is considered as a primary receptor binding to the transit peptide and Toc34 is proposed to function downstream in conjunction with Toc159 in the transfer to the Toc75 channel (Kessler and Schnell, 2004; Wang et al., 2008).

The second model "The motor hypothesis" (Fig. 8) (Schleiff et al., 2003a; Kessler and Schnell, 2004; Becker et al., 2004b) proposes that Toc34 acts as an initial receptor of preproteins and Toc159 is supposed to function downstream as a GTP-driven motor for translocation of preproteins by multiple rounds of GTP hydrolysis. In the two models the interaction between the Toc GTPases as well as their GTPase functions are critical in preprotein recognition. Biochemical studies suggested that the nucleotide state of Toc159 regulates the formation of a stable preprotein-binding intermediate, and favors a model in which Toc159 acts as a part of a GTP-regulated switch for preprotein recognition (Wang et al., 2008).

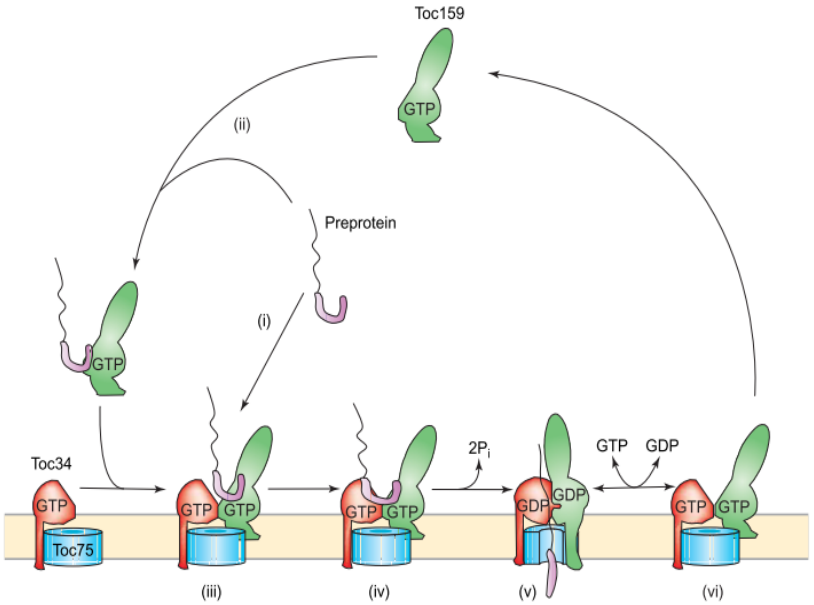


**Figure 7. The targeting hypothesis.** GTP-bound Toc159 attached to the outer membrane **(i)** or in a soluble form **(ii)** recognizes and binds the preprotein. Toc159 together with the preprotein docks at

Toc34 via the GTPase domains **(iii)**. The preprotein is then handed down to Toc34 **(iv)**.**T**he translocation of the preprotein across Toc75 is triggered by GTP hydrolysis **(v)**. GDP–GTP exchange resets the TOC complex **(vi).** Figure taken from (Kessler and Schnell, 2004).

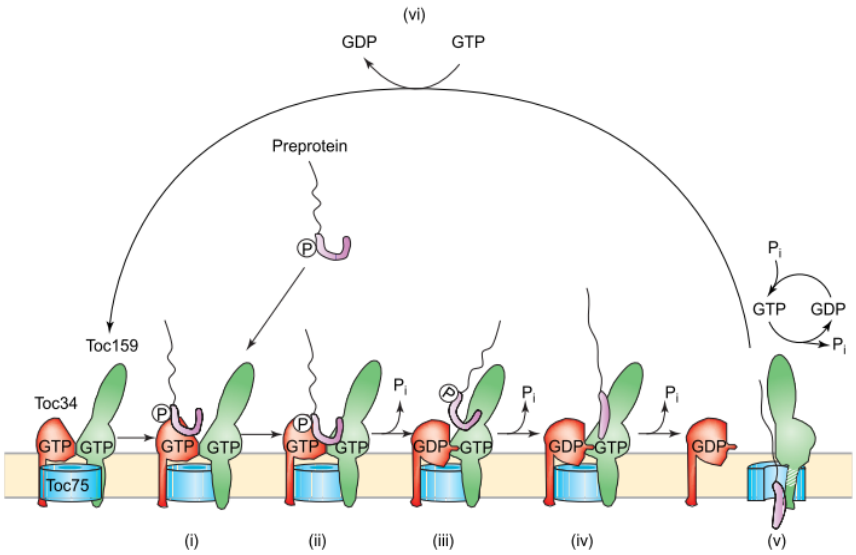


**Figure 8. The motor hypothesis.** GTP-bound Toc34 recognizes and binds the phosphorylated C-terminal part of the transit peptide of the preprotein **(i)** then GTP-bound Toc159 binds to the N-terminal region of the transit peptide **(ii)**. The preprotein binding stimulates the GTPase activity of Toc34 and release the preprotein **(iii)**. The transit peptide is dephosphorylated leading to the association with Toc159 at the G domain **(iv)** stimulating the GTPase activity of Toc159, Toc34 dissociates and the preprotein is threaded across the Toc75 channel via multiple round of GTP hydrolysis **(v)**. After translocation, the GTPases are regenerated by GDP–GTP exchange **(vi).** Figure taken from (Kessler and Schnell, 2004).

## *Phosphorylation of preproteins*

The phosphorylation of preproteins has been suggested as a regulatory mechanism in protein import. Phosphorylation on either serine or threonine residues of the transit peptides of the chloroplast-destined preproteins was detected in cytosol (Waegemann and Soll, 1996). Phosphorylation of pSSU (Preprotein of Small Subunit of Rubisco) permits the binding of 14-3-3 proteins together with Hsp70 in a “guidance complex” and enhances targeting to the chloroplast surface (May and Soll, 2000). Moreover, phosphorylation of pSSU modified the affinity between the precursor and Toc receptors. Phosphorylated pSSU interacted strongly with Toc34 (Sveshnikova et al., 2000) whereas Toc159 bound only the non-phosphorylated form (Becker et al., 2004b). The N-terminal part of the non-phosphorylated pSSU transit peptide is strongly bound by Toc159 and the C-terminal part induces GTP hydrolysis of Toc159 (Becker et al., 2004b). The interaction between Toc34 and

phosphorylated preproteins is also regulated by nucleotide binding. The phosphorylated preprotein binds to the GTP-bound form of Toc34 but not to the GDP-bound form. The data suggested that the interaction is regulated by nucleotide exchange in conjunction with protein phosphorylation (Sveshnikova et al., 2000).

### *Phosphorylation of GTPase receptors*

The regulation of preprotein import by phosphorylation of the Toc receptors has been proposed. Sveshnikova et al (Sveshnikova et al., 2000) found that when Toc34 is phosphorylated, GTP binding was inhibited and the binding of preproteins blocked. After dephosphorylation, Toc34 bound GTP and then formed a complex with the phosphorylated precursor. Toc34 is therefore supposed to act as a phosphorylation and GDP/GTP-regulated receptor (Sveshnikova et al., 2000). Fulgosi et al (Fulgosi and Soll, 2002) using kinase renaturation assays, detected two proteins kinases phosphorylating Toc34 and Toc159. psToc34 was phosphorylated by an 98 kDa ATP-dependent protein kinase at the outer envelope (OEK98). OEK98 is an integral membrane protein and is able to phosphorylate Toc34 at a specific site. Toc159 was phosphorylated by a 70-kDa protein kinase, which did not phosphorylate Toc34 (Fulgosi and Soll, 2002). The identity of the two kinases at the outer membrane is still unknown.

Both receptors, psToc159 and psToc34 are phosphorylated within their G-domains at the cytosol-facing side of the chloroplast outer membrane (Oreb et al., 2008), by kinases present in the chloroplast membrane (Fulgosi and Soll, 2002). In Arabidopsis, Toc33 can be phosphorylated at S181 (Aronsson et al., 2006). However, mutation of this residue to either non-phosphorylable alanine or to phospho-mimick aspartate or glutamate had no effect on the ability of Toc33 to complement the *ppi1* mutant (Aronsson et al., 2006). Using sucrose density centrifugation, it was demonstrated that phosphorylated Toc34 is no longer assembled in the Toc complex in endogenous membranes (Oreb et al., 2008). Phosphorylation of components of the Toc complex was suggested as a regulatory mechanism to switch between different functionalities of receptors, that change the state of association of the GTPases (Oreb et al., 2008).

## *Import regulation by the A domain*

In Arabidopsis, Toc159, Toc120 and Toc132 are considered as selective receptors of preproteins *in vitro* (Smith et al., 2004; Inoue et al., 2010). Toc159 is essential for chloroplast biogenesis and plants that lack Toc159 (*ppi2*) have a albino phenotype and diminished levels of prominent photosynthesis-associated proteins (Bauer et al., 2000). Interestingly, the Arabidopsis double mutant deficient in Toc120 and Toc132 with an albino or lethal phenotype could not be rescued by expression of Toc159 (Kubis et al., 2004; Ivanova et al., 2004), indicating that the receptors preferentially bind to different types of preproteins. The sequence divergence between the receptors is situated mostly within the A domains. Domain swapping experiments between Toc159 and Toc132 demonstrate that exchanging the A-domains of the two receptors can largely interconvert their functions *in vitro* and *in vivo* (Inoue et al., 2010). The A domains are therefore considered major determinants of the specificities of the TOC159 receptors and their associated complexes (Paila et al., 2015).

## *Phosphorylation of the Toc159A-domain*

Large-scale phosphoproteomics projects in Arabidopsis indicated that the A-domain is phosphorylated at multiple sites (PhosphAT 4.0). In total 43 sites were mapped (Demarsy et al., 2014). Twelve *in vivo* phosphorylation sites in the A domain of Toc159 were identified (S71, S210, S281, S288, S448, S589, S609, S630, S632, S684, S686, T692) (Agne et al., 2010; De La Fuente Van Bentem et al., 2008; Reiland et al., 2009), seven of them were predicted CKII (Casein Kinase II) targets, the remaining five could be phosphorylated by CKII and/or distinct kinases (Agne et al., 2010; Agne and Kessler, 2010). Recombinants A domains of Toc159, Toc120 and Toc132 purified from bacteria were phosphorylated by CKII *in vitro* (Agne et al., 2010). Using quantitative phosphoproteomics analysis, Wang et al identified Toc159 as a substrate of SnRK2 (Sucrose nonfermenting 1 (SNF1)-related protein kinase 2), the phosphorylation was up-regulated by ABA (abscisic acid). The phosphorylation of Toc159 was enhanced by ABA treatment in WT seedlings. The A domain of Toc159 was phosphorylated at T692 (Wang et al., 2013). The ABA-dependent phosphorylation of Toc120 and Toc132 was found in *snrk2* mutants. The phosphorylation of Toc159 and homologs by SnRK2, suggests that the import of preproteins in chloroplast may be regulated by ABA. According to this study the A domain may be a substrate for other kinases and the

regulatory function of the Toc159 in protein import may also be modulated by other mechanisms.

### *Proteolysis of the Toc159A-domain*

Toc159 was first identified as 86kD protein associating with a translocating preprotein in pea (Hirsch et al., 1994; Kessler et al., 1994; Schnell et al., 1994). The sequence of the Arabidopsis genome revealed that Toc159 is in fact a much larger protein and that in pea the entire N-terminal A-domain had been missing (Bölter et al., 1998). It appears likely that in pea the A-domain is entirely proteolytically cleaved. This may partially be the case in Arabidopsis were the A-domain is present both as a domain of intact full length Toc159 as well as a separate protein in the cytosol (Hiltbrunner et al., 2001b). However, the ratio of full length to proteolytically processed Toc159 is not precisely known. The A-domain is dispensable *in vivo* and Toc159 variants lacking the A-domain complemented the lethal, albino *toc159* mutant (*ppi2*) (Bauer et al., 2000). The fact that the A-domain is a) dispensable, b) proteolytically cleavable and c) multiply phosphorylated suggests that it has regulatory functions. However, these regulatory functions are not completely understood. The differences in the function in protein import activity of Toc159 family are probably related with the A domains (Inoue et al., 2010).

### *Regulation by ubiquination*

During plastid developmental transitions (i.e. from non-photosynthetic etioplasts in the dark to active chloroplasts in the light), the composition of the protein import machinery is modified to accommodate different client proteins. This implicates the ubiquitin proteasome system (UPS) and the chloroplast outer membrane RING-type E3-ligase SP1 (suppressor of *ppi1* locus 1; *ppi1* is a mutant lacking Toc33) (Ling et al., 2012). SP1 was identified using forward genetics in Arabidopsis. It is anchored in the outer membrane of the chloroplast by two transmembrane helices separated by an intermembrane-space domain. SP1 exposes a C3HC4-type RING-finger domain at the C-terminal to the cytosol (Ling et al., 2012). SP1 is an E3-ligase and together with E2 and E1 enzymes ubiquitinates the TOC complex components and targets them for degradation by the proteasome. The *sp1 ppi1* double mutant is larger, greener and contains more extensively developed chloroplasts than *ppi1*; this indicates that

loss of ubiquitination activity rescues the pale phenotype in the *ppi1* mutant (Ling et al., 2012; Kessler, 2012). It was demonstrated that in the absence of SP1 in the *ppi1 sp1* double mutant, the level of other Toc components (for example Toc75) increases (degradation is reduced) and compensates the import deficiency of the *ppi1* mutant.

# Aim of the thesis

The import of preproteins is essential in chloroplast biogenesis, the main components of the TOC-TIC import machinery were isolated, identified and their roles well characterized. Recently, research was extended to the study of import regulation; the post-translational modifications of the A-domain of Toc159 was suggested to be a regulatory mechanism for the import activity. Although the A domain of Toc159 family members is dispensable for viability and import activity, it has been demonstrated to be essential for protein client specificity. The A-domain is a target of CKII, a cytosolic kinase that phosphorylates the A-domains of Toc159, Toc120 and Toc132. Moreover, additional kinases (for instance an envelope-associated kinase activity and SnRK2) are known to phosphorylate Toc159.

The aim of the present thesis is the identification and characterization of new regulatory kinases using a variety of approaches. Any kinases interacting with Toc159 will be co-isolated with an N-terminally TAP-tagged version of Toc159 (NTAP-TOC159). IgG purification followed by mass spectrometry will be used to co-isolate and identify candidate interacting proteins including kinases. Amino acid sequences will be analysed and data bases searched to confirm a strong status of any candidate (e.g kinase motifs, known chloroplast outer membrane association) before proceeding with the experimentation. Any confirmed candidate kinase will be TAP-tagged and used to demonstrate the interaction with Toc159 in reverse by IgG affinity chromatography followed by mass spectrometry. In the case of a positive outcome the candidate kinase will be further characterized:

1) I*n vivo* function by isolation and analysis of the knock out mutants in comparison with wild type (import efficiency of isolated chloroplasts, survival rate of mutants upon de-etiolation and senescence progression); Mutants will be complemented with the wild type gene and later with kinase mutants (e.g. kinase dead ).

2) *In vivo* localization of the protein using fluorescence tagging and confocal microscopy.

3) Analysis of membrane topology and association using sucrose gradient floatation for membrane isolation followed by protease resistance, alkaline carbonate extraction and Triton X-100 solubilization assays.

The majority of the results presented in this thesis were recently published (Zufferey et al, J. Biol. Chem, 2017, doi: 10.1074/jbc.M117.776468) (Zufferey et al., 2017).

# Material and methods

## DNA constructs

Recombinant KOC1 proteins were expressed from constructs obtained by PCR amplification from AT4G32250 cDNA using primers (Table 1): KOC1_NheI_F and KOC1_NotI_R for pET21d-KOC1$_{(FL)}$; KOC1_NcoI_F and KOC1_NcoI_R for pET21d-KOC1$_{(1-343)}$; KOC1_NheI_F and KOC1_NcoI_R2 for pET21d-KOC1$_{(1-547)}$, and cloned in fusion with a C-terminal hexahistidinyl tag into the pET21d plasmid (Constructs provided by Bastien Christ, Master thesis 2009).

The pCHF8-NTAP-KOC1 vector was obtained by inserting KOC1 (amplified using primers KOC1_FL_NcoI_F and KOC1_FL_XbaI_R from plasmid pET21d-KOC1$_{(FL)}$) (Table 1), digested with NcoI and ligated into the BspHI-XbaI-digested vector pCHF8-NTAPi (Agne et al. 2010). To obtain the pCHF7-NTAP-TOC159-cmyc construct, a DNA fragment was amplified using primers Toc159-StuI-F and Toc159-cmyc-GibR (Table 1) from plasmid pCHF7-NTAPi-Toc159 (Agne et al., 2009), and from this fragment a second DNA fragment was amplified using primers Toc159-StuI-F and cmyc-uniGibR and assembled into the S*tu*I-X*ba*I-digested vector pCHF7-NTAPi-Toc159 by Gibson technology, following the manufacturer instructions (Gibson Assembly Kit, NEB). For pEG104-N-YFP-KOC1 construct the sequence coding for KOC1 was amplified using primers attB-KOC1-FW and attB-KOC1-REV (Table 1) and recombined in pEG104 using the Gateway system (Invitrogen™ Gateway® Technology with Clonase™ II) (Construct provided by Axel Meyrat, Master thesis 2012). For pEG101-C-YFP-EMB2004 construct the sequence coding for EMB2004 (AT1G10510) was amplified using primers attB-EMB2004-FW and attB-EMB2004-REV (Table 1) and recombined in pEG101 vector using the Gateway system (Construct provided by Cyrille Montandon, PhD thesis 2015).

## Plant material and growth conditions

Plants were grown *in vitro* under long-day conditions (16 hours light, 8 hours dark), or short day conditions (8 hours light- 16 hours dark) at 100-120 µmol.m$^{-2}$ s$^{-1}$ and 21°C. Agar plates contained 0.8% (w/v) Phytoagar (Duchefa), 0.5X Murashige and Skoog (MS) medium (Duchefa), and 0, 0.5, 0.8 or 1% (w/v) sucrose (for survival, import, sub-plastidial fractionation or protoplast isolation, or IgG purification experiments, respectively).

The following Arabidopsis (*Arabidopsis thaliana*) mutants TAP-TOC159:*ppi2* (NTAP-TOC159) and TAP:WT were described previously (Agne et al., 2009). The homozygous T-DNA insertion lines SALK_083378 (*koc1-1)*, SALK_051823 (*koc1-2*) were selected on 0.5X MS medium containing kanamycin (50mg/L) and screened by PCR amplification using the primers koc1-2_LP with koc1-2_RP, koc1-1_LP with koc1-1_RP, and koc1-2LP, koc1-2_RP or koc1-1_LP with LbB1.3. Wild type Arabidopsis plants Columbia-0 (Col-0) were used. The transgenic plants NTAP-KOC1:*koc1-1* (NTAP-KOC1) and NTAP-TOC159-cmyc: *ppi2* (NTAP-Toc159-cmyc), were obtained by transformation of homozygous *koc1-1* or heterozygous *ppi2* plants (Bauer et al., 2000), using the vectors pCHF8-NTAP-KOC1 or pCHF7-NTAP-TOC159-cmyc, respectively. Plants were selected as described (Agne et al., 2009).

## Purification of recombinant KOC1 proteins

KOC1 recombinant proteins were purified on $Ni^{2+}$-nitrilotriacetic acid ($Ni^{2+}$-NTA) agarose beads under denaturing conditions. Bacterial pellets were resuspended in buffer L1 (100 mM $NaH_2PO_4$, 10 mM Tris-HCL pH 8, 10 mM imidazole, 8 M urea) (5ml/gr pellet) and incubated during 3 hours at room temperature. The lysate was centrifuged for 20 min at 40,000g to obtain a clear supernatant. The supernatant was incubated during 1 hour at room temperature with $Ni^{2+}$-NTA agarose (1ml of beads/10 ml supernatant). The resin was washed three times with 10 ml of buffer L1 pH 6.3. $His_6$-tagged recombinant proteins were eluted in a buffer L1 containing 100 mM imidazole pH 4.5 (Qiaexpressionist$^{TM}$, QIAGEN).

Recombinant $KOC1_{(FL)}$ was purified for phosphorylation assays. Native recombinant $KOC1_{(FL)}$ was purified from bacterial pellets resuspended in buffer L2 (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 0.1% (v/v) Triton X100 (TX100), 1mM PMSF, 0.2% (v/v) protease inhibitor cocktail PIC (Sigma), pH 8.0). Bacteria cells were disrupted by high pressure using a French press, incubated during 30 min with DNAse (Roche) (0.2µl/ml) and centrifuged for 30 min at 40,000g. The supernatant was filtered (0.2µm) and incubated during 1 hour with $Ni^{2+}$-NTA beads in microtubes. The resin was washed two times with buffer L2 containing 20 mM Imidazole and one time with the same buffer containing 0.5% n-Dodecyl-β-D-maltoside (DDM) instead of TX100. Recombinant $KOC1_{(FL)}$ proteins were eluted with buffer L2 containing 250 mM Imidazole and 0.5% DDM instead of TX100. Eluates were dialyzed against

30 mM Tris-HCl pH 7.5, 75 mM NaCl, 75 mM KCl, 1mM PMSF and 0.5% DDM. All procedures were done at 4°C.

## Purification of recombinant Toc159A protein

The TOC159 A-domain ($TOC159_{(1-740)}$-His-6x) (TOC159A) was overexpressed in transformed *E. coli* strain BL21(DE3) cells. The cells were lysed in buffer L3 (50 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF) by high pressure using a French press. The lysate was centrifuged 30 min at 40,000g. The proteins were purified from the supernatant fraction by $Ni^{2+}$-NTA agarose on a ÄKTA Prime® system. The column was washed three times with buffer L3. The proteins were eluted in a buffer L3 containing 250 mM imidazole. The eluate was dialyzed against 20 mM piperazine pH 5.5, 50 mM NaCl. The dialyzed eluate was applied to the DEAE ion exchange column using ÄKTA Prime® (Richardson et al., 2009). All procedures were done at 4°C.

## Purification of recombinant Toc132A and Toc120A proteins

$TOC120_{(1-343)}$-His-6x (TOC120A), $TOC132_{(1-431)}$-His-6x (TOC132A) (Ivanova et al., 2004) were overexpressed in transformed *E. coli* BL21(DE3) cells. The cells were lysed in buffer L4 (50 mM $NaH_2PO_4$, 10 mM Tris-HCl pH 8.0, 10 mM imidazole, 0.1% (v/v) TX100, 1mM PMSF) by high pressure using a French press. The lysate was centrifuged for 30 minutes at 40,000g. The supernatant was incubated during 2 hours with $Ni^{2+}$-NTA agarose in microtubes. The resin was washed two times with buffer L4. The proteins were eluted in a buffer L4 containing 250 mM imidazole. Eluates were dialyzed against 10 mM Tris-HCl pH 8, 50 mM NaCl. All procedures were done at 4°C.

## Antibodies

Antibodies for TOC and TIC components were described previously (Hiltbrunner et al., 2001b; Ma et al., 1996; Köhler et al., 2015; Bauer et al., 2000). Antibodies against PGL35 were described previously (Vidi et al., 2006). Antibodies against CBP were purchased in Genscript, LHCB2 were from Agrisera and c-myc and IgG were from Cell signaling.

For α-KOC1 antibody production, two forms of recombinant KOC1, ($KOC1_{(1-343)}$ and $KOC1_{(1-547)}$) purified by $Ni^{2+}$-NTA affinity chromatography (described before) were pooled and

injected into rabbits for polyclonal antibody production (Eurogentec, Seraing, Belgium). To affinity purify antibodies from the serum, purified recombinant $KOC1_{FL}$ was crosslinked to Affi-Gel 10 (Bio-Rad) according to the commercial protocol. The serum was incubated with $KOC1_{(FL)}$ Affi-Gel column. The column was washed two times with 10 ml of PBS buffer. The α-KOC1 antibodies were eluted with 0.2 M Glycine pH 2.2 buffer and immediately neutralized with 1M Tris pH 8.0.

## IgG Affinity purification

The procedure has already been described (Agne et al., 2010) and was applied with few modifications. All steps were performed at 4°C. Plants grown *in vitro* (10g FW) were ground in a mortar in a total volume of 18 ml of buffer G (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 1mM PMSF, 5 mM NaF, 0.2% (v/v) PIC). The 100,000g pellet fraction of the NTAP-KOC1 and NTAP-TOC159 was resuspended in buffer G and centrifuged for an additional 1h at 100,000g. Pellet-associated proteins were solubilized in buffer G containing 1.65% (v/v) TX100 and 10% glycerol (buffer $G_{KOC}$) for NTAP-KOC1, or 0.375% (v/v) TX100 and 5% glycerol (buffer $G_{TOC}$) for NTAP-TOC159. Proteins of TAP:WT plants were extracted directly in buffer $G_{KOC}$ or $G_{TOC}$. Solubilized proteins were incubated overnight with 100µl of IgG sepharose resin. The beads were washed once with 35 ml and 6 times with 5 ml in buffer $G_{KOC}$ for NTAP-KOC1 or $G_{TOC}$ for NTAP-TOC159. The last wash was done with the same buffer without proteases inhibitors. The proteins were eluted in a buffer 50 mM Tris-HCl pH 8, 0.5 mM EDTA, 100 mM NaCl, 1 mM dithiothreitol (DTT), TX100 (1.65 % or 0.375%) glycerol ( 5% or 10%) for NTAP-KOC1 or NTAP-TOC159 for 2 hours at 16°C with 50 units of AcTEV protease (Tobacco Etch Virus protease, Invitrogen). 50 µg of proteins of the "total" fractions (T: Total, L: load; Ft: flow-through, W1: first wash) or 10% of the fraction (W5: last wash and TEV: eluate ) were loaded on SDS-PAGE, and transfer by Western blot in Dunn buffer on nitrocellulose membrane.

## Transient expression in Arabidopsis protoplasts

For protoplast isolation and transformation we used a polyethylene glycol based method adapted from (Jin et al., 2001) and (Yoo et al., 2007). Wild-type (Col-0) Arabidopsis plants (4-week-old) grown under short day conditions on agarose plates containing 0.5X MS medium with 0.8% (w/v) sucrose were harvested in a buffer (400 mM sorbitol, 5 mM MES, 8 mM

$CaCl_2$, pH 5.6). After mincing the plants with a scalpel the buffer was replaced by the same buffer containing 1.5 % (w/v) Cellulase "Onozuka" and 0,375 % (w/v) Macerozyme, R-10 (SERVA). Digestion was allowed to proceed for four hours at 25°C.

The protoplasts were released by gentle shaking, the solution was filtered (100 µm nylon mesh) and centrifuged at 100g for 5 min. Protoplasts were washed once in 10-15 ml W5 solution (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 5 mM glucose, 1.5 mM MES, pH 5.6), resuspended in cold W5 (final concentration of $2x10^6$ protoplasts/ml) and incubated on ice for 30 min. The protoplasts were centrifuged and resuspended in 400 mM sorbitol, 15 mM $MgCl_2$, 5 mM MES, pH 5.6. For transformation, 100 µl of protoplast suspension was mixed with 20 µg of plasmid and carefully mixed with 110 µl PEG-CMS (1 g PEG4000, 375 µl water, 1 ml 500 mM sorbitol, 250 µl 1 M calcium nitrate). After incubation for 20 min at 23°C, the protoplasts were washed with W5 and centrifuged for 2 min (100g). The protoplasts were washed in 1 ml of protoplast-culture medium (4.4 g/l MS medium (Duchefa), 350 mM sorbitol, 50 mM glucose, 3 mM $CaCl_2$, pH 5.8) and finally resuspended in 0.5 ml of protoplast culture medium. After incubation in the dark for approximately 16 h the samples were analysed by confocal laser scanning microscopy (LEICA TCS 4D microscope, LEICA Microsystems). Protoplasts were transformed with plasmids, pEG104-N-YFP-KOC1 for the localization of YFP-KOC1, pEG101-C-YFP-EMB2004 as envelope marker and pCL60GFP (Stratagene) as a control.

## Isolation of intact chloroplasts

For the isolation of intact chloroplasts we used the protocols from (Smith et al., 2003) and (Agne et al., 2009) with the following modifications. Chloroplasts were obtained from Arabidopsis plants (Col-0 or *koc1-1*) (2-week-old) grown *in vitro* on agarose plates containing 0.5X MS medium with 0.5% (w/v) sucrose under long-day conditions. The tissue (5-9g) was enzymatically digested using 1.5% (w/v) cellulase and 0.375 % (w/v) macerozyme. The digestion was extended to 12 hours at 19°C.

## Chloroplast protease treatment and extraction

Intact chloroplasts were subjected to thermolysin treatment according to (Smith et al., 2003). Chloroplast pellet corresponding to 20µg of chlorophyll was resuspended in 100µl of

HEPES-sorbitol buffer (HS) and incubated for 1 hour on ice with 20µl of thermolysin (2mg/ml). For alkaline extraction we used chloroplast corresponding to 20 µg of chlorophyll incubated for 10 min on ice with 600 µl of 0.1 M $Na_2CO_3$ pH: 11. The fractions were separated by centrifugation for 1 hour at 100,000g.

## Chloroplast fractionation

Fractionation of intact chloroplasts was carried out according to (Vidi et al., 2006) and (Hiltbrunner et al., 2001b) with some modifications. NTAP-KOC1:*koc1-1* plants (140g FW) grown on soil during 8 weeks under short-day conditions were ground in a blender in 400 ml of HB Buffer (450 mM Sorbitol, 20 mM Tricine-KOH pH: 8.4, 10 mM EDTA pH: 8.4, 10 mM $NaHCO_3$, 1 mM $MnCl_2$, 5 mM Na-Ascorbate, 1 mM PMSF). The lysate was filtered through two layers of Miracloth and centrifuged at 4°C for 5 min at 600g. The pellet was resuspended in 10 ml of RB Buffer (300 mM Sorbitol, 20 mM Tricine-KOH pH: 8.4, 2.5 mM EDTA pH: 8.4 and 5 mM $MgCl_2$). Intact chloroplasts were purified on a Percoll step gradient (40% (v/v) and 85% (v/v) in RB Buffer). Intact chloroplast were washed with 50 ml of RB Buffer and centrifuged for 5 min at 700 g. After centrifugation, the chloroplasts were hypertonically lysed in 0.6 M sucrose TED Buffer (500 mM Tricine pH: 7.5, 20 mM EDTA, 20 mM DTT) at -80°C overnight. The thawed suspension was resuspended using a Potter homogenizer and centrifuged at 100,000g for 1 hour at 4°C. The membrane pellet was resuspended in 45% sucrose in TED buffer (3.5 mg/ml) using a Potter. The total membrane fraction (corresponding to 12 mg of chlorophyll) was separated on a linear sucrose gradient (5-45%) in TED buffer and centrifugation for 16 hours at 100,000g. After centrifugation 37 fractions (1 ml) were collected. Proteins from uneven fractions were precipitated, separated by SDS-PAGE and transferred to nitrocellulose, stained with amido black and probed with antibodies.

## Chloroplast protein import assay

Chloroplast import experiments were performed according to (Agne et al., 2009) with some modifications. For each import reaction we used chloroplasts corresponding to 20 µg of chlorophyll and 4µl of *in vitro* translated [$^{35}$S] methionine-labeled preproteins, prepared according to the commercial protocol (TNT® T7 Quick-coupled Transcription/ Translation

System, Promega). The *in vitro* translocated, [$^{35}$S]-labeled preproteins of the small subunit of Rubisco (preSSu) and the alpha subunit of pyruvate dehydrogenase E1-alpha (preE1α) (pET21d-preE1-alpha-DHFR-(6x- His) (Inoue et al., 2010) were used as substrates. The pre-proteins were incubated with chloroplasts and import was allowed to proceed for 0, 5 and 15 min. Total proteins of the samples were precipitated, separated by SDS-PAGE and dried gels were analyzed using a PhosphorImager (Molecular imager® FX (BIO-RAD) and Quantity One 4.6 software for quantification.

## De-etiolation survival test

For the de-etiolation survival test, we used seeds that were collected from the parental plants grown simultaneously in the same growth chamber with the same light conditions. *koc1-1*, *koc1-2*, WT (Col-0) and NTAP-KOC1:*koc1-1* plants were grown on 0.5X MS medium, seeds were cold treated for 3 days at 4°C to synchronize germination. The seeds were exposed 4 hours to standard light (100-120 µmol.m$^{-2}$ s$^{-1}$ and 21°C) and then grown in the dark. After 6 days, the plants were exposed to standard light conditions for two weeks. The germination and survival rates were calculated.

## Dark-induced senescence test in Arabidopsis rosette leaves

Arabidopsis wild type plants (Col-0) and *koc1* mutants (*koc1-1* and *koc1-2)* were grown on soil during 7 weeks under short-day conditions. The rosettes were cut and placed in the dark for 11 days to induce senescence. The rosettes were placed in Petri dishes on Whatman® paper moistened with water to prevent drying and covered with aluminium foil. Each day, a set of three rosettes were removed from the dark and plant tissues were ground to a fine powder under liquid nitrogen, that was used to extract protein and RNA. Proteins were precipitated and separated by SDS-PAGE, transferred to nitrocellulose membrane and immunodetected with antibodies against KOC1, TOC75, TOC159A and Actin as a loading control.

## RNA extraction, cDNA synthesis and qPCR

RNA, extracted from wild type senescent plants, was isolated using the NucleoSpin RNA Plant Kit (Macherey Nagel) which included a DNase treatment. An additional DNase

treatment step was performed to avoid genomic DNA contamination. RNA was then purified with NucleoSpin RNA clean-up kit (Macherey Nagel). 1 µg of RNA was used for reverse transcription with the GoScript™ Reverse Transcriptase (Promega) according to the supplier's recommendations. cDNA were diluted 5x for qPCR reactions. The following primers pairs were used: for KOC1:QPCR-KOC1-F and QPCR-KOC1-R, for SGR1: SGR1-S and SGR1-AS (Table 1) and for S16 as control were obtained from Qiagen (S16 QT00833819). FastStart Essential DNA Green Master was used to performed qPCR using on the LightCycler (Roche).

## Phosphorylation assays

KOC1 purified from NTAP-KOC1:*koc1-1* plants by the IgG Affinity purification was incubated for 30 min at 25°C with 3-5µg of either purified recombinant TOC159A, TOC120A or TOC132A in phosphorylation buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 1 mM DTT, 50 µM ATP) in presence of 10 µCi of [γ-$^{33}$P]ATP. In addition, KOC1 denatured (10 min at 65°C) was also incubated with TOC159A in presence of [γ-$^{33}$P]ATP. As a negative control, the recombinant proteins were incubated separately with [γ-$^{33}$P]ATP. Reactions were stopped by diluting in ice-cold phosphorylation buffer followed by $CHCl_3$-methanol precipitation (Wessel, 1984). The samples were separated by SDS-PAGE and dried gels were analyzed using a PhosphorImager (Molecular imager® FX (BIO-RAD) and Quantity One 4.6 software for quantification.

Native recombinant $KOC1_{(FL)}$ (20µg) purified on $Ni^{2+}$-NTA agarose beads was incubated for 30 min at 25°C with 10 µg of purified TOC159A in phosphorylation buffer containing 0.2% of DDM and [γ-$^{33}$P] ATP. The reactions were treated as described in the paragraph before.

## Mass spectrometric identification of KOC-interacting proteins

KOC-interacting proteins were isolated by a TAP-tagged version of KOC compared to a control. Proteins were identified and quantified after Nano-LC separation using the data-independent HD-MSE data acquisition method as previously described (Helm et al., 2014). In brief, LC separation and HD-MSE data acquisition were performed using 1 µl from each of the in solution digested samples on a nanoACQUITY UPLC System coupled to a Synapt G2-S mass spectrometer (Waters, Eschborn, Germany). MS acquisition range was set to 50–5000

Da. Data analysis was carried out by ProteinLynx Global Server (PLGS 3.0.1, Apex3D algorithm v. 2.128.5.0, 64 bit, Waters, Eschborn, Germany) with automated determination of chromatographic peak width as well as MS TOF resolution. Lock mass value for charge state 2 was defined as 785.8426 Da/e and the lock mass window was set to 0.25 Da. Databank search query (PLGS workflow) was carried out as follows: Peptide and fragment tolerances were set to automatic (resulting in a maximum mass tolerance of 30 ppm), two fragment ion matches per peptide, five fragment ions for protein identification, and two peptides per protein. The false discovery rate (FDR) was set to 4% at the protein level. MSE data were searched against the modified A. thaliana database (TAIR10, ftp://ftp.arabidopsis.org) containing common contaminants such as keratin (ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) and glycogen phosphorylase B from rabbit (Uniprot ID: P00489). Quantification was performed based on the intensity of the three most abundant proteotypic peptides (Hi3-method, (Silva, 2005)).The glycogen phosphorylase B was used with 10 fmol/µL as internal quantification standard (The method was provided by Prof. Sacha Baginsky).

# Results

## KOC1 co-purifies with Toc159

Arabidopsis plants (NTAP-TOC159:*ppi2*) expressing N-terminally TAP-tagged TOC159 were used to isolate potential TOC159 interactors by IgG-affinity chromatography (Köhler et al., 2015). Plants expressing the TAP-tag alone (TAP:WT) were used as a negative control. The TAP-tag contains an IgG-binding domain separated from a calmodulin-binding peptide (CBP) sequence by a tobacco etch mosaic virus (TEV) protease site. TX-100 detergent extracts were prepared and subjected to IgG-affinity chromatography. TEV protease elution was applied to release either TOC159 still carrying the CBP (together with any interacting proteins) or the negative control CBP. Proteins in the TEV eluates were identified by mass spectrometry (Köhler et al., 2015). The mass spectrometric data revealed a member of the protein kinase superfamily protein (AT4G32250) that we named KOC1 (*K*inase at the *O*uter membrane of the *C*hloroplast *1*). KOC1 co-purified with NTAP-TOC159 but not with TAP control (Fig. 9). Specific antibodies against recombinant KOC1 were raised in rabbit and affinity purified.

To confirm the association of KOC1 with the TOC159 complex, aliquots of the sequential steps of the IgG affinity purification experiment were separated by SDS-PAGE and analyzed by Western blot (Fig. 9). A 66 kD band corresponding to the expected size of KOC1 was detected in the total detergent extract loads (L) and in the flow-throughs (Ft) of the NTAP-TOC159 and TAP:WT samples (Fig. 9). The KOC1 band was present only in the TEV eluate of the NTAP-TOC159 purification but not that of the TAP-tagged negative control (TAP:WT). In addition, TOC75 and -33 as well as TIC110 co-purified with NTAP-TOC159 (Fig. 9). The thylakoid and plastoglobule marker proteins, LHCB2 and FBN1a (PGL35) respectively, were detected by Western blotting in the load (L) and flow through (Ft) fractions but not in the TEV eluates.

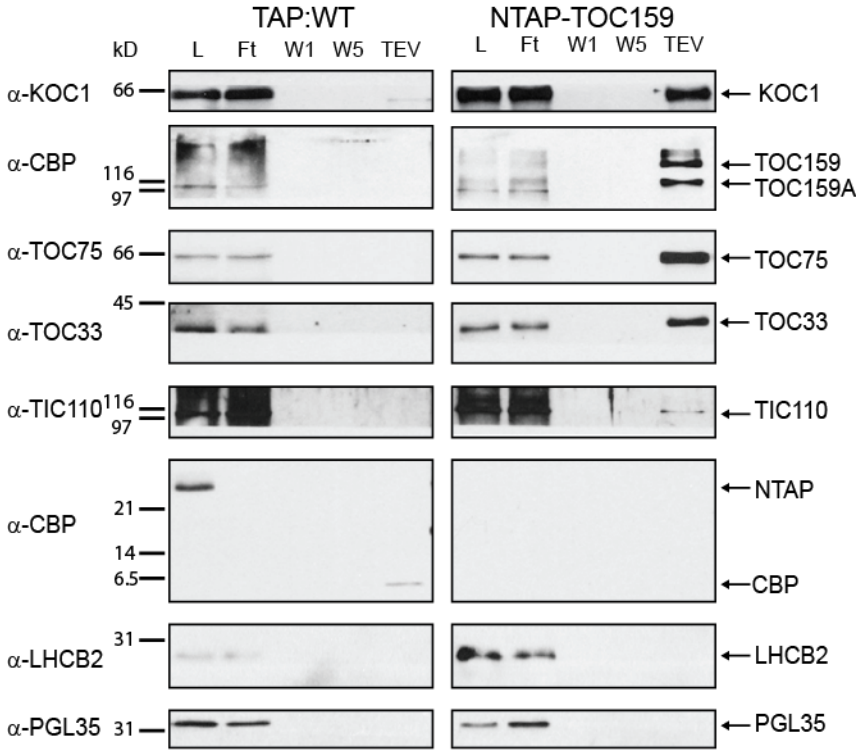


**Figure 9. KOC1 co-purified with TOC159 and TOC complex components.** Sequential fractions of NTAP-TOC159 IgG affinity purification were analyzed by Western blotting. The membrane was probed with antibodies against KOC1, CBP, TOC75, TOC33, TIC110, LHCB2 and PGL35. (L: load; Ft: flow-through, W1: first wash; W5: last wash, TEV: eluate. 50µg of proteins loaded (L, Ft, W1) or 10% of the fractions (W5, TEV). Several identical blots were used for immunoblotting. TAP:WT was used as a negative control.

## TOC complex components co-purified with KOC1

To confirm the interaction of KOC1 with the TOC complex proteins, NTAP-KOC1: *koc1-1* plants expressing NTAP-tagged KOC1 were used to isolate KOC1 interactors by IgG-affinity chromatography. TAP:WT plants were used as a negative control. Specific antibodies against recombinant KOC1 detected the protein in the total (T), load (L), flow-through and the TEV eluate (TEV) extracts of the NTAP-KOC1 purification (Fig. 10). Due to TEV cleavage it had a noticeably smaller size than NTAP-KOC1 in the total extract (T) and the load (L). TOC159, TOC75, TOC33 and TIC56 were also detected in the eluate of NTAP-KOC1 but not in the eluate of the negative control TAP:WT (Fig. 10). The thylakoid marker protein LHCB2 was not detectable in the eluate. Altogether these results demonstrate that KOC1 associates with TOC complex *in vivo*.

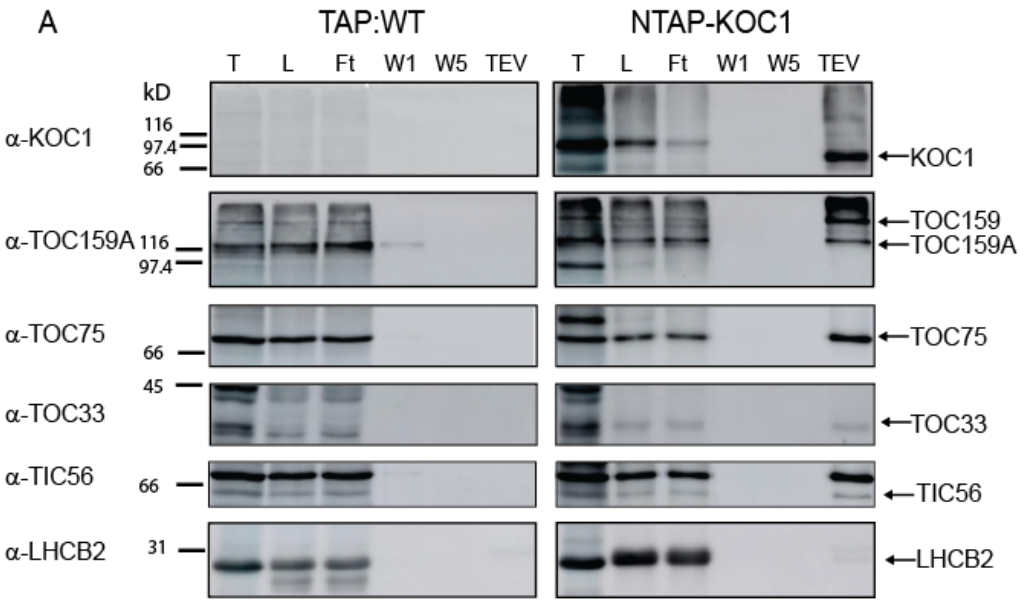


**Figure 10**. **TOC complex components co-purified with KOC1.** Sequential fractions of the IgG affinity purification of NTAP-KOC1 were analyzed by Western blotting. The membrane was probed with antibodies against KOC1, TOC159A, TOC75, TOC33, TIC56 and LHCB2. (T: Total, L: load; Ft: flow-through, W1: first wash; W5: last wash, TEV: eluate). 50µg of proteins loaded (T, L, Ft, W1) or 10% of the total fractions (W5, TEV). Several identical blots were used for immunoblotting. TAP:WT was used as a negative control.

## KOC1 proteomics analysis

The TEV eluate of the NTAP-KOC1 purification experiment was subjected to quantitative mass spectrometry. The experiment was carried out in two biological replicates. A list of common proteins in the two experiments was created and contained 191 proteins (Table 2). Among the 15 most abundant interaction candidates were 7 TOC and TIC components. In order of decreasing abundance in femtomoles these were TOC75, TOC159, YCF1.2, TIC110, TOC33, TOC34 and ClpC (Table 2). To identify interesting potential candidates, we removed certain proteins from the list: photosynthesis-associated proteins, proteins localized in other organelles than chloroplasts and proteins of known function other than import, ribosome, protease or transport, and a reduced list with 88 proteins was obtained (Table 3). In this list, in summary, 18% of proteins were components of TOC-TIC import machinery, 32% constituents of ribosomes, 12% known or predicted proteases, 14% implicated in various transport processes, 12% unknown or known as chloroplast protein and finally 11% potential KOC1 interacting proteins (Table 3). The majority of proteins considered potential interacting proteins are unknown proteins and may cooperate in KOC1 functions.

When the protein list established for NTAP-KOC1 (Table 2) was compared to a list for NTAP-TOC159 consisting of 43 proteins (not shown) weighted for enrichment a high overlap of 83% was detected. Notably, 11 known components of the TOC and TIC complexes as well as components of the 1 MD complex were represented in this list and for which the overlap between TOC159 and KOC1 protein list was complete.

We filtered the list of KOC1-interacting proteins by protein abundance and enrichment factor to identify specific interactions at high stringency (Table 4). We first selected proteins that were identified in both biological replicates but not in the negative controls. We then calculated an enrichment factor from the ratio between protein abundance in isolated chloroplasts and in the TAP-purified fraction of KOC interacting proteins and retained those proteins with an enrichment factor >1 in the list. And lastly, we requested interacting proteins to have a minimum abundance of 2% of the bait protein (i.e. KOC1) to select for specific KOC/protein interactions. This filtering resulted in 51 proteins that fulfill requirements (Table 4). These proteins were plotted into an interaction network using the STRING database (STRING Version 10.0 software (http://string-db.org)). This analysis identified three complexes interacting with KOC1, the TOC-complex, an FtsH/FtsHi protease complex and the cytoplasmic ribosome (Fig. 11) (Analysis provided by Prof. Sacha Baginsky).

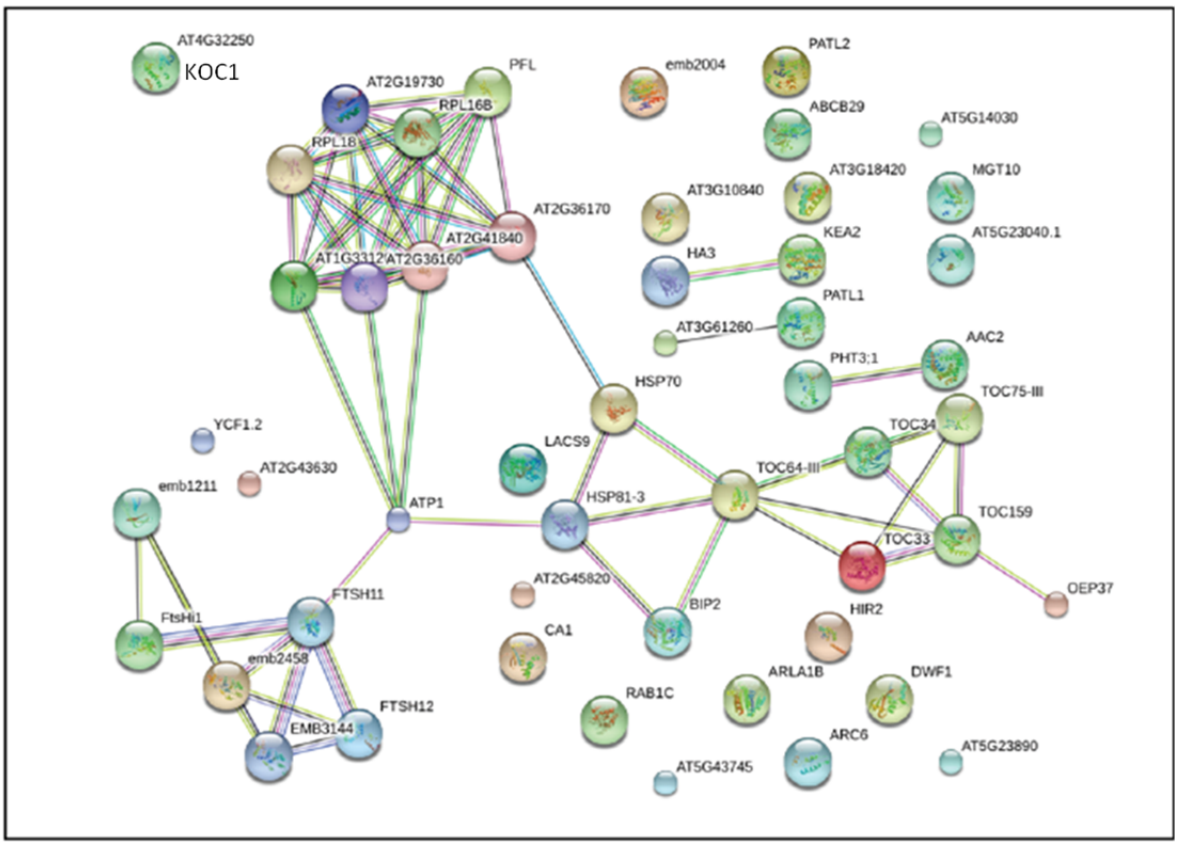


**Figure 11**. **TOC complex components co-purified with KOC1.** Interaction networks of KOC1 using the list of co-immunopurified proteins and STRING 10.0 program. Three main groups of proteins interacting with KOC1 were identified: 1) The proteins implicated in the import activity and TOC-complex family members, 2) Proteins in the FtsH/FtsHi protease complex and 3) Proteins interacting with cytoplasmic ribosome (Analysis provided by Prof. Sacha Baginsky).

## KOC1 is a predicted membrane-anchored kinase

KOC1 has 611 amino acids residues. It contains a kinase domain (amino acids 39-306) predicted by the Prosite software (http://prosite.expasy.org). The C-terminal half of KOC1 contains a HERC2-related region (HECT (**H**omologous to the **E**6-AP **C**arboxyl **T**erminus) and RLD (**R**CC1-**l**ike **d**omain) containing E3 Ubiquitin Protein Ligase 2, amino acids 376-531) (Fig.12A-B). The HERC2-related region in KOC1 is followed by a 24 amino acid stretch (549-572) enriched in hydrophobic amino acids predicted to form a transmembrane helix (TM) by the TMpred software (http://embnet.vital-it.ch/cgi-bin/TMPRED_form_parser) (Fig.12A-B). The ChloroP software (http://www.cbs.dtu.dk/services/ChloroP/) does not predict a transit peptide (Fig.12B). Still, KOC1 was identified in chloroplast proteome studies (Ferro et al., 2010; Zybailov et al., 2008).

KOC1 most closely resembles to the KEG (KEEP ON GOING) protein (AT5G13530), a RING E3 ubiquitin ligase. In addition to the RING sequence, KEG contains ankyrin and HERC2-like repeats and a kinase domain (Stone et al., 2006). The RING sequence and the ankyrin repeats are absent from KOC1. But, both the kinase domain and the HERC2-related region in KOC1 are homologous to those in KEG. The sequence analysis suggests that KOC1 functions as a kinase and an implication in ubiquitination also appears remotely possible.

The protein sequence of KOC1 was used to search putative orthologs in different species. We performed the analysis using the program BLAST (Basic Local Alignment Search Tool) to scan the protein sequence database of the NCBI (National Center for Biotechnology Information) https://www.ncbi.nlm.nih.gov/. The BLAST alignment compares the query sequence to sequence databases to find regions of similarity between sequences and calculate the statistical significance of the matches. The alignment was done searching similarity with protein sequences in plants, *Physcomitrella*, Cyanobacteria and human databases. The most similar protein sequences (Identity 91-81%) identified were from species in the Brassicales order, these species are close to Arabidopsis. The majority of proteins are named as "Predicted E3-ubiquitin protein ligase". The alignment revealed also proteins from *Prunus persica* (peach), *Malus domestica* (apple) and *Fragaria vesca* (wild strawberry) in the Rosales order that share around 70% of identity with KOC1. Uncharacterized proteins from plants as *Cephalotus follicularis* (carnivorous pitcher plant), *Theobroma cacao* (cacao tree), *Manihot esculenta* (manioc), *Vitis vinifera* (grape), *Nicotiana tabacum* (tobacco) and *Glycine max* (soybean) share homology with KOC1 with the identity between 61-68%. The result indicates that KOC1 is well conserved in plants. Interestingly in *Physcomitrella patens*, we found an 537 amino-acid long unknown protein (XP_001784625.1) that shares homology with KOC1 (Identity 42%). This protein is predicted as a "Serine-Threonine kinase". *In Physcomitrella patents*, there are also some "predicted kinase" proteins that have similarity only with the kinase domain of KOC1 (aa39-306) with an identity of around 28%. The alignment with the Cyanobacterium database revealed one protein (WP_015220094) that shows similarity (25%) with KOC1 at the kinase domain. Finally, some proteins predicted as "serine-threonine protein kinase" from human share homology only with the kinase domain of KOC1 around 29%.

## Isolation of *koc1* mutants and NTAP-KOC1:*koc1-1* plants

To obtain information on the function of KOC1 *in vivo*, T-DNA mutant collections were searched for insertions in the KOC1 gene (AT4G32250). Two independent mutant lines, SALK_083378 termed: *koc1-1* and SALK_051823 termed: *koc1-2*, were identified and homozygous lines were isolated (Fig. 13, Fig. 12 C-D). The *koc1-1* line contained a single T-DNA insertion at 841 base pairs after the start codon. The *koc1-2* line contained a double T-DNA insertion at 2269 base pairs after the start codon (Fig. 13).

Immunoblotting using KOC1 antibodies demonstrated the absence of KOC1 protein (Fig. 12D) and confirmed the knockout nature of the mutants. NTAP-KOC1:*koc1-1* plants were obtained by introducing a T-DNA construct encoding a N-terminally TAP tagged KOC1 (NTAP-KOC1) in *koc1-1* mutant background. The *koc1-1 and koc1-2* mutants as well as the homozygous NTAP-KOC1:*koc1-1* plants displayed a wild type phenotype under standard growth conditions (long-day: 16 hours light: 8 hours dark) (Fig. 12C).

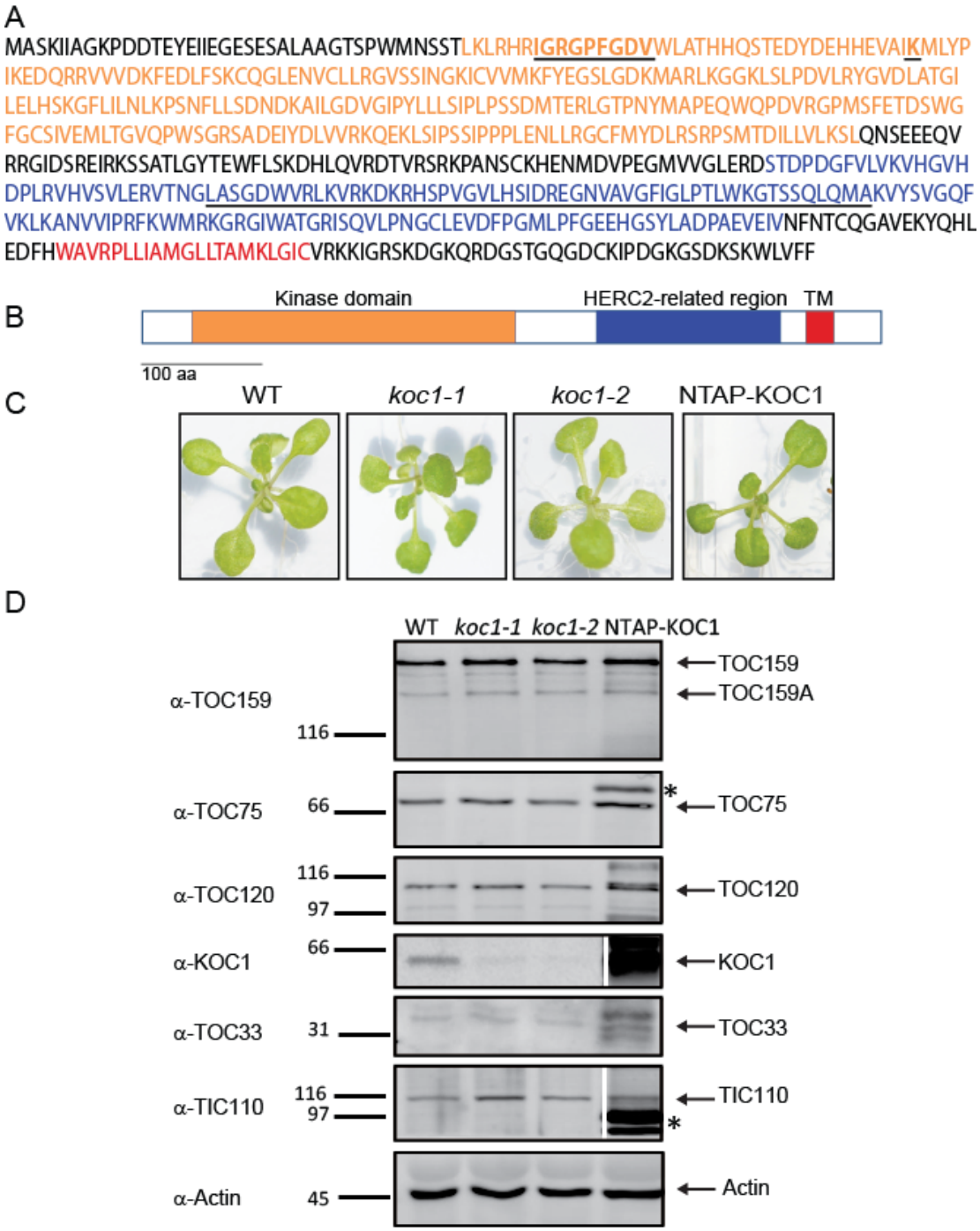


**Figure 12**. **KOC1 sequence analysis and mutant characterization. A-B.** Amino acid sequence of KOC1 contains a kinase domain (orange, aa39-306). Underlined aa45-53 are conserved in the catalytic domain, K74 in bold print is predicted to bind ATP. In the HERC2-related region (blue, aa376-531) underlined amino acids share homology with the KEG protein. A predicted transmembrane stretch (TM, aa549-572) is highlighted in red. **C.** Images of 2-week-old plants WT, *koc1-1*, *koc1-2* and NTAP-KOC1 :*koc1-1* grown *in vitro*. **D.** Total protein extracts of 2-week-old plants (WT, *koc1-1, koc1-2* and NTAP-KOC1:*koc1-1*) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Antibodies against TOC159A, TOC75, TOC120, KOC1, TOC33 and TIC110 were used. Actin was used as a loading control. (*)Bands corresponding to overexpressed NTAP-KOC1.

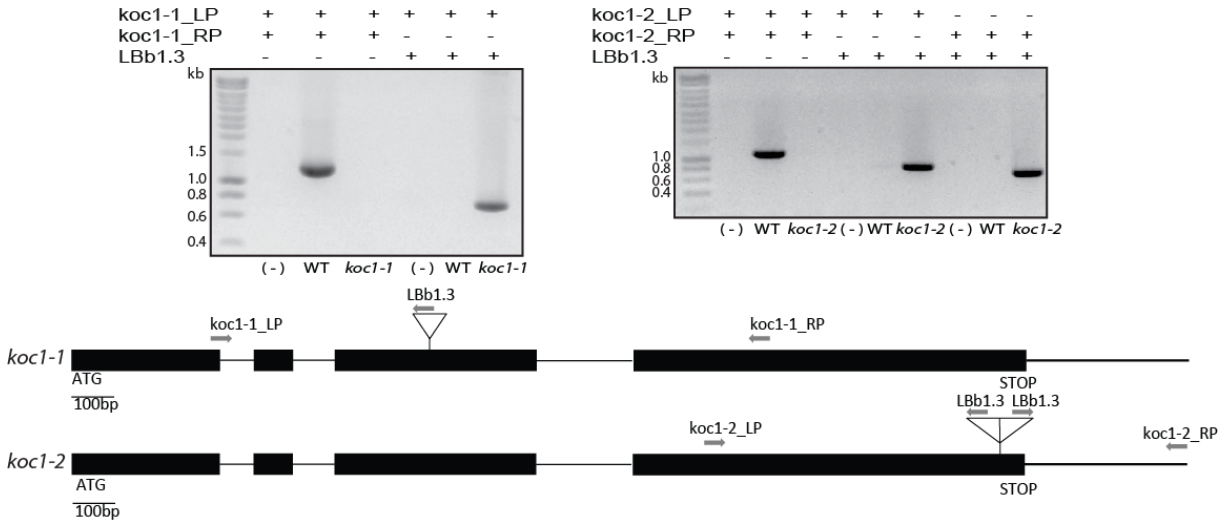


**Figure 13. Genotyping of *koc1* lines.** Identification of the *koc1* homozygous T-DNA insertion mutants (*koc1-1 and koc1-2)* by PCR. Col-0 (WT) was used as a control, (-) negative control without DNA. Schematic representation of KOC1 gene indicating the T-DNA insertions (triangles), exons (black boxes) introns (thin lines) and primers (koc1-1 or koc1-2 _LP or _RP see Table 1 for sequence).

## TOC components accumulate normally in *koc1* mutants

Sequence analysis of KOC1 revealed two potential biochemical functions: that of a kinase and potentially that of a factor in ubiquitination and subsequent proteasome-mediated degradation. Both potentially affect assembly and stability of TOC and TIC components. Therefore, the two *koc1* mutants were compared with the wild type and NTAP-KOC1 :*koc1-1* overexpressing lines by Western blotting. The levels of the components of the TOC core complex (TOC159, -75 and -33) as well as TOC120 and TIC110 appeared unchanged in the *koc1* mutants. This was more difficult to judge in the overexpressing line due to the proximity of NTAP-containing bands that give strong signals with any kind of IgG and which precluded quantification. Visibly, however, no major changes appeared to occur (Fig. 12D). The results suggest that the TOC and TIC components are normally assembled and stable in the *koc1* and overexpressing backgrounds.

## KOC1 localizes at the outer chloroplast membrane

For *in vivo* localization, isolated Arabidopsis wild type protoplasts were transformed with vectors: pEG104-N-YFP-KOC1 (coding for KOC1 with a N-terminal YFP tag: YFP-KOC1), pEG101-C-YFP-EMB2004 (coding for an envelope protein at the inner membrane fused to YFP: EMB2004-YFP) and pCL60-GFP. Transient expression of YFP-KOC1, EMB2004-YFP and

GFP was analyzed by confocal microscopy. Chloroplasts were identified by red chlorophyll autofluorescence (Fig. 14, Chlorophyll). YFP-KOC1 gave a ring-like fluorescence pattern (Fig. 14, YFP-KOC1). The merge between the YFP-KOC1 and chlorophyll signal shows that YFP-KOC1 was localized at the chloroplast periphery (Fig. 14, Merge). EMB2004-YFP gives a fluorescence pattern strongly resembling that of YFP-KOC1 (Fig. 14, EMB2004-YFP), whereas GFP results in a pattern typical for the cytosol and is distinct from that of YFP-KOC1 (Fig. 14, GFP).

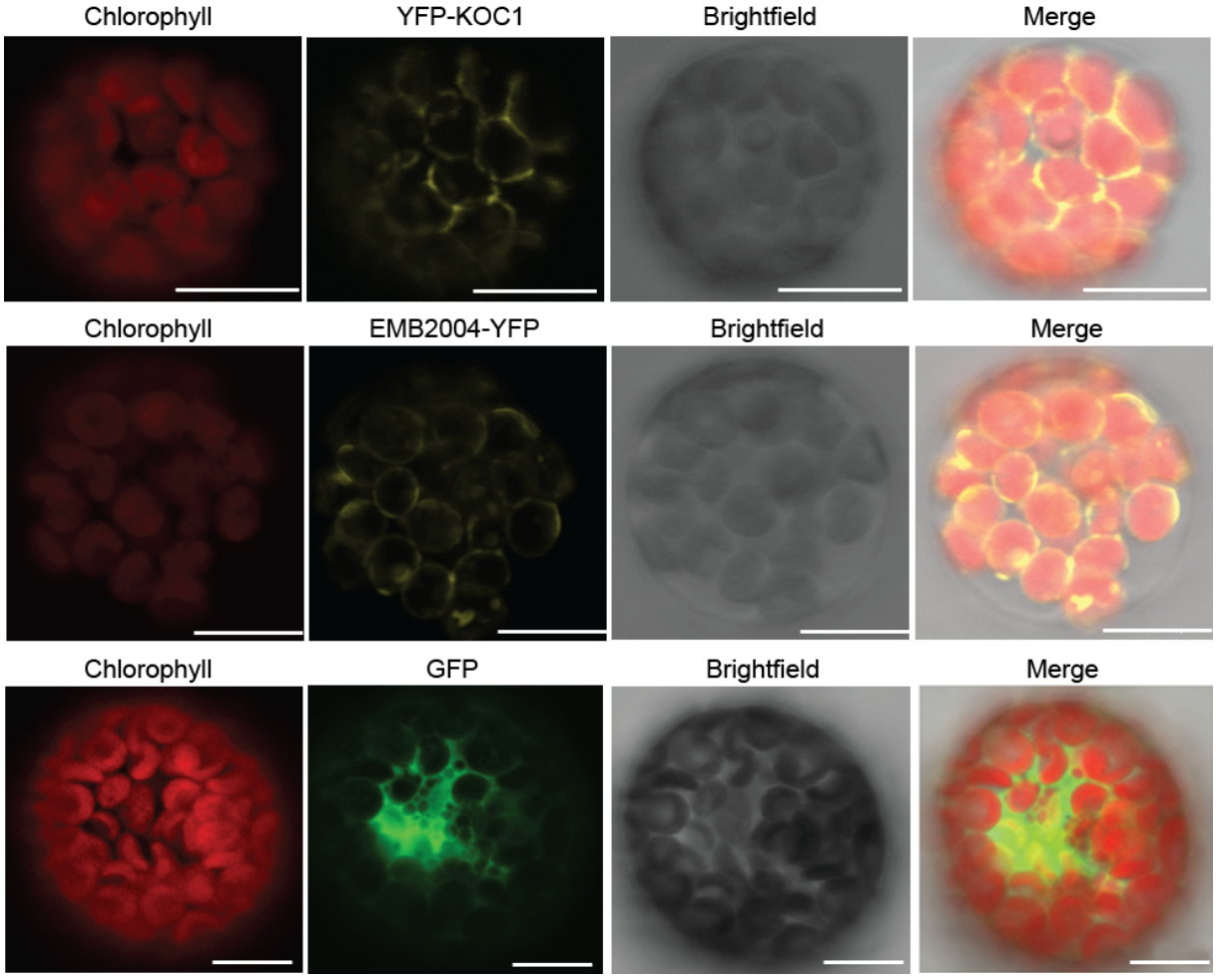


**Figure 14. KOC1 is localized at the outer membrane of chloroplasts.** Confocal microscopy images of isolated protoplasts expressing YFP-KOC1, EMB2004-YFP and GFP. Chlorophyll fluorescence in red identifies chloroplasts, the signal of YFP-KOC1 and EMB2004-YFP appears yellow and GFP in green. Intact protoplasts were visualized by bright field. Merge shows the overlay of chlorophyll, fluorescence of proteins and bright field images. Scale bars: 10µm.

## KOC1 is present in the chloroplast envelope fraction

To localize NTAP-KOC1 in chloroplast membrane compartments, chloroplasts were isolated from NTAP-KOC1:*koc1-1* plants. A total chloroplast membrane fraction was prepared and separated into thylakoids, envelope membranes and plastoglobules by floatation on a linear 5-45% sucrose gradient. Fractions were analyzed by Western blotting (Fig. 15A). LHCB2 was present mostly in fractions 27-37 indicating they contain thylakoids. FIB1a/PGL35 was found in fractions 1-13 indicating the presence of plastoglobules. FIB1a/PGL35 was also detected in denser fractions (23-31) likely due to plastoglobule association with thylakoids. NTAP-KOC1 was detected mostly in fractions 17-31 and was well separated from the fractions enriched in thylakoid and plastoglobule markers. Moreover, TOC159 and TOC75 co-fractionated with NTAP-KOC1 (Fig. 15A) supporting KOC1 localization at the chloroplast envelope.

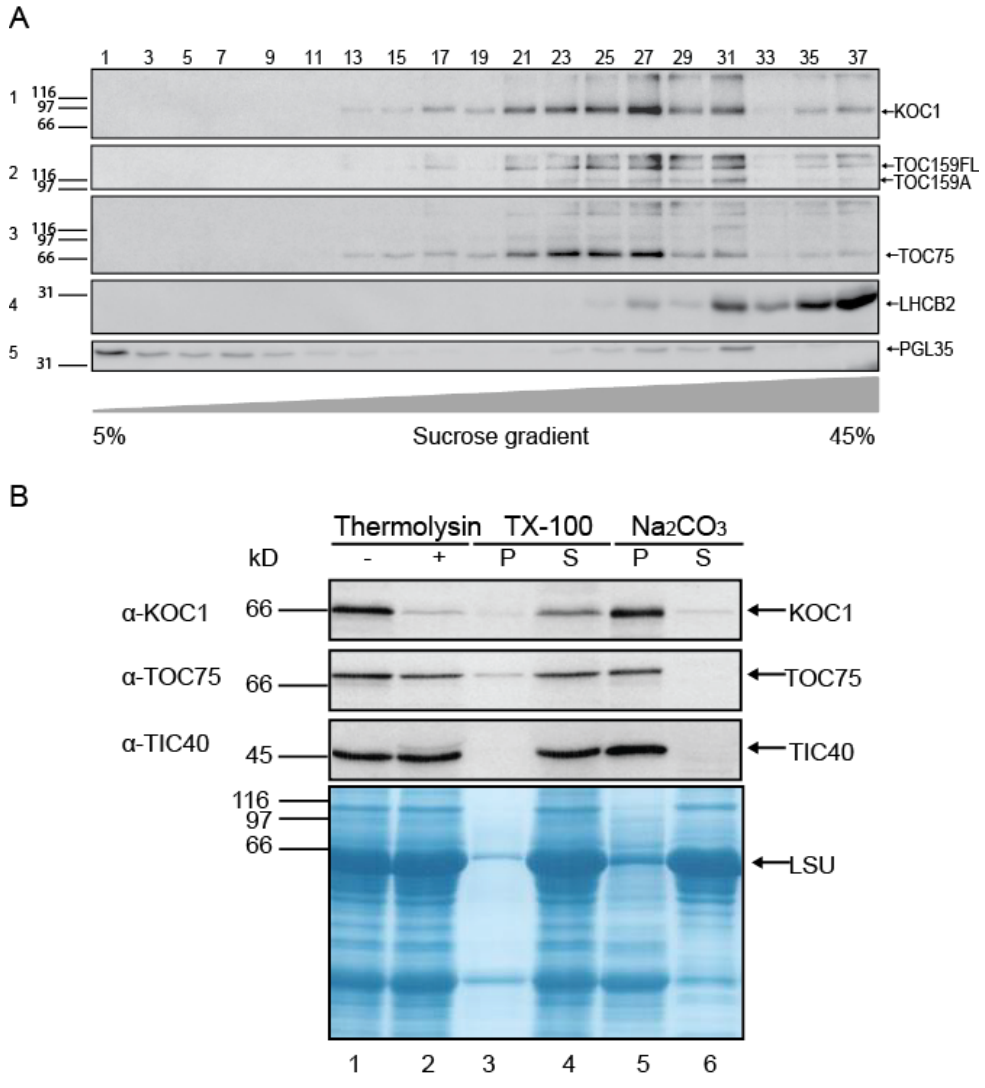

**Figure 15**. **KOC1 is localized at the outer membrane of the chloroplasts**. **A.** Total membranes from NTAP-KOC1 chloroplasts were separated on a continuous sucrose gradient (5-45%) and 37 fractions were collected. Proteins from uneven fractions were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies (1) CBP, (2) TOC159A, (3) TOC75, (4) LHCB2 and (5) PGL35. **B.** Col-0 chloroplasts were subjected (+) to thermolysin treatment or not (-), Triton X-100 solubilization (TX-100) and alkaline extraction (Na2CO3) (P: pellet/ S: supernatant). Samples were separated by SDS-PAGE, transferred to nitrocellulose, stained with amido black (lower part) and probed with antibodies against KOC1, TOC75 and TIC40.

## KOC1 is exposed at the chloroplast surface

To investigate KOC1 localization at the chloroplast envelope, we treated isolated chloroplasts (Col-0) with thermolysin protease (Fig. 15B). Thermolysin degrades surface-exposed proteins but does not access the intermembrane space. Thermolysin treated chloroplasts were separated by SDS-PAGE and probed by immunoblotting. Specific antibodies revealed that the KOC1 band was strongly diminished by thermolysin (Thermolysin +) whereas the known thermolysin-resistant outer membrane protein TOC75 and the inner membrane protein TIC40 were largely unaffected (Fig. 15B), indicating that KOC1 was accessible at the outer surface of the outer envelope membrane.

## KOC1 is an integral membrane protein

To analyze the membrane association of KOC1, extraction experiments were carried out. Isolated chloroplasts (Col-0) were extracted with alkaline carbonate buffer ($Na_2CO_3$) or solubilized with Triton X-100 (Fig. 15B). Upon centrifugation of the alkaline carbonate extraction, KOC1 remained in the pellet fraction (P) but was found in the supernatant (S) upon Triton X-100 solubilization. The known integral membrane proteins TOC75 and TIC40 behaved in the same way. We therefore conclude that KOC1 is an integral membrane protein.

## Purification of recombinant full length KOC1 for phosphorylation assays

Full length KOC1 was overexpressed in *E. coli SoluBL21* and purified on $Ni^{2+}$-NTA agarose under native conditions. During the purification, aliquots of the sequential steps of the affinity purification experiment were collected; the proteins were precipitated, separated by SDS-PAGE and analyzed by Western blotting (Fig. 16). Antibodies against recombinant KOC1 were used to verify that the protein was well purified, and amido black staining of proteins to verify the quality of the purification. The bands corresponding to KOC1 were detected at around 66 kD in the load fraction (L), flow-through (Ft) and the elution samples (E1-E9) (Fig.

16). The membrane stained with amido black shows some residual bands probably corresponding to bacterial proteins contaminating the eluate fractions, however the majority of protein purified corresponds to KOC1.

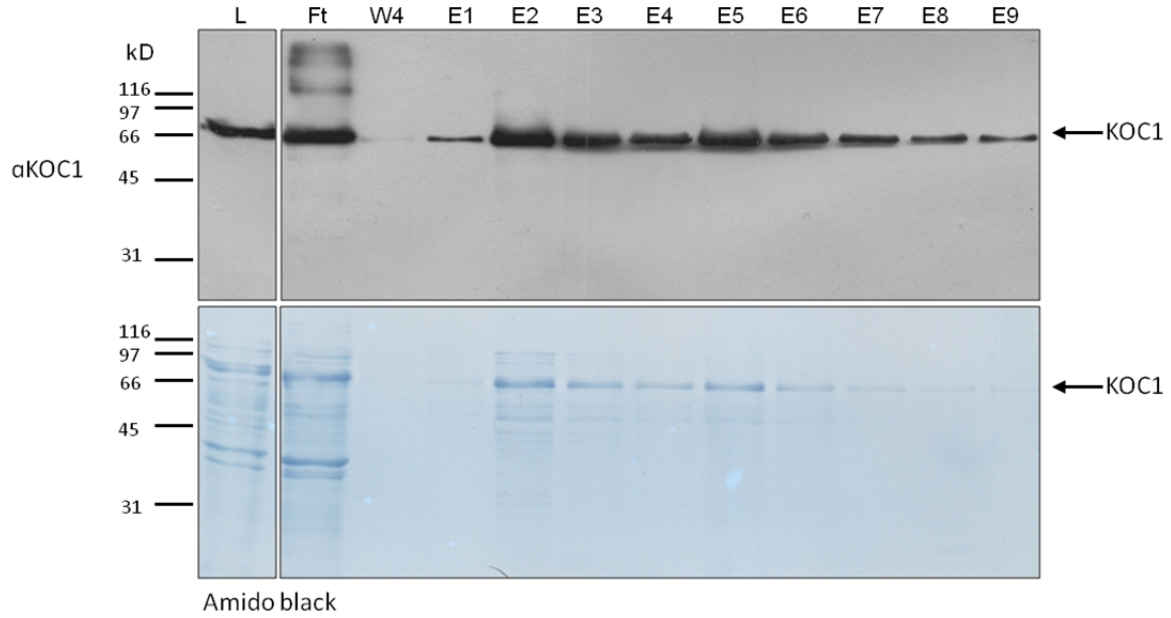


**Figure 16**. **Purification of recombinant KOC1$_{(FL)}$ by Ni$^{2+}$-NTA chromatography.** Sequential fractions of the KOC1$_{(FL)}$ purification were precipitated and separated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane. The membrane was stained with amido black (lower part) and subsequently probed with antibodies against KOC1. L: load; Ft: flow-through, W4: last wash, E1-E9: eluates.

## Recombinant KOC1 expressed and purified from *E. coli* phosphorylates the A domain of Toc159 *in vitro*

The A-domain of TOC159 is hyperphosphorylated *in vivo*. To examine whether KOC1 phosphorylates TOC159A, we purified recombinant KOC1$_{(FL)}$ (Fig. 16) and TOC159A from bacteria. For the *in vitro* phosphorylation assays (Fig. 17) we incubated 20µg of purified KOC1$_{(FL)}$ with 10µg of TOC159A in the presence of detergent (DDM), and [γ$^{33}$P]ATP (Fig.17, line 3). As a negative control, the recombinant proteins KOC1$_{(FL)}$ and TOC159A were incubated separately with [γ-$^{33}$P]ATP (Fig.17, line 1-2) to detect phosphorylation of endogenous protein in the samples. The samples were analyzed by SDS-PAGE followed by autoradiography. Only the band corresponding to TOC159A was detected and phosphorylated by KOC1$_{(FL)}$ showing that the A domain is an *in vitro* target of KOC1. In the control sample (TOC159A alone) (Fig.17, line 2) we did not detect phosphorylated bands, therefore the phosphorylation of TOC159A was clearly dependent on recombinant KOC1.

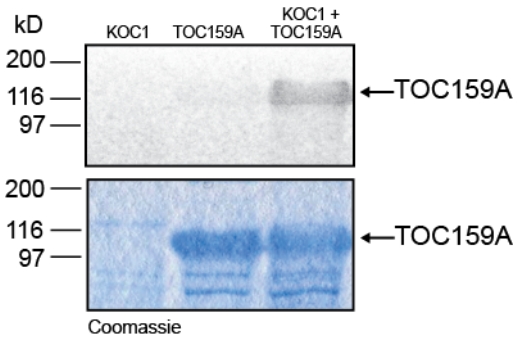


**Figure 17. TOC159A is phosphorylated by recombinant KOC1$_{(FL)}$ expressed in *E. coli*.** Purified TOC159A (10 µg) was incubated with [γ-$^{33}$P]ATP and with recombinant KOC1 (20 µg) purified from bacteria (lane 3). KOC1 and TOC159A were incubated alone with [γ-$^{33}$P]ATP as negative controls. Samples were separated by SDS-PAGE and analyzed using a PhosphorImager (Molecular imager® FX BIO-RAD) and Quantity One 4.6 software. The data shown are representative experiments out of several replicates.

## KOC1 purified from transgenic plants, phosphorylates the A domain of TOC159 *in vitro*

To obtain additional evidence for *in vitro* phosphorylation we tested whether KOC1 purified from transgenic plants phosphorylates TOC159A. We isolated KOC1 from NTAP-KOC1:*koc1-1* plants (see above Fig. 10) and performed an *in vitro* phosphorylation experiment on recombinant purified TOC159A (3 µg) in the presence of [γ$^{33}$P]ATP. The experiment resulted in strong phosphorylation of TOC159A (Fig. 18A, lane 1). The control experiments carried out with KOC1 and TOC159A alone did not result in detectable phosphorylation (Fig. 18A, lanes 2 and 3) whereas denatured KOC1 (De-KOC1) showed slight residual activity (Fig. 18A, lane 4).

## KOC1 purified from transgenic plants, phosphorylates the A domain of TOC120 and TOC132

The A-domains of the TOC159 homologs, TOC120 and TOC132 are also known as phosphoproteins. We incubated recombinant purified TOC120A and TOC132A (5 µg) with KOC1 purified from NTAP-KOC1:*koc1-1* plants and [γ-$^{33}$P]ATP. The result shows that KOC1 is able to phosphorylate the A-domais *in vitro* (Fig. 18B, lanes 2-3). Altogether the *in vitro* phosphorylation experiments demonstrate that TOC159 and its homologs are substrates of KOC1 *in vitro*, providing additional evidence for the kinase function of KOC1. The phosphorylation of TOC159A appears to be less efficient when incubated with recombinant KOC1, the conformation and folding of the protein produced in bacteria is probably less stable and less active. The experiments confirm that KOC1 is a kinase protein.

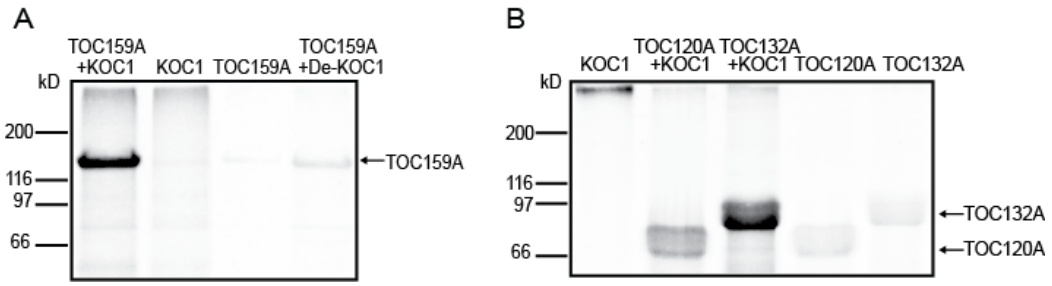


**Figure 18**. **A-domains of TOC159 and family members are phosphorylated by KOC1. A.** TOC159A was incubated with KOC1 isolated from NTAP-KOC1 :*koc1-1* plants (lane 1) or incubated with heat-denatured KOC1 (De-KOC1) (lane 4) in presence of [γ-$^{33}$P]ATP. **B** Purified TOC120A (lane 2) and TOC132A (lane 3) were incubated with [γ-$^{33}$P]ATP and KOC1 isolated from NTAP-KOC1:*koc1-1* plants. In A and B KOC1, TOC159A, -120A and -132A were incubated alone with [γ-$^{33}$P] ATP as negative controls. The proteins were separated by SDS-PAGE and analyzed using a PhosphorImager (Molecular imager®FX BIO-RAD) and Quantity One 4.6 software. The data shown are representative experiments out of several replicates.

## A large percentage of Toc159 exists as the full-Length protein

TOC159 occurs both with and without its A-domain but the ratio of the two forms is unknown. Regulation of protein import at the A-domain is only plausible if the A-domain is present. To determine to what extent this is the case we engineered a TOC159 construct encoding a N-terminal NTAP tag and a C-terminal myc-tag (Fig.19).

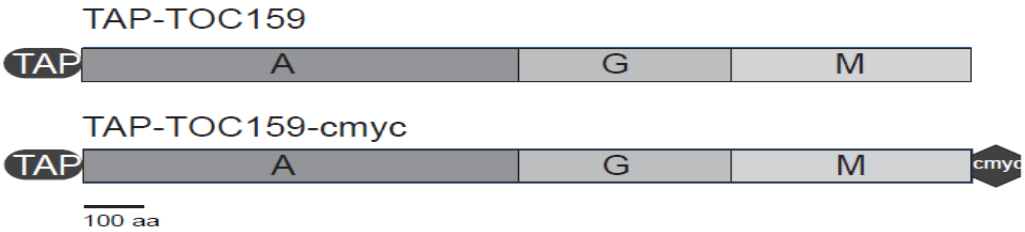


**Figure 19. NTAP-TOC159 constructs**. Schematic representation of TAP-TOC159 and TAP-TOC159-cmyc constructs. The domains of TOC159 are illustrated: the N-terminal A domain, G domain and M domain.

The C-terminal myc-tag allows to detect full length TOC159 (TOC159-FL) as well as TOC159 lacking the A-domain (TOC159GM). By Western blotting, this should result in two bands corresponding to TOC159-FL and TOC159GM. A Western blotting experiment was carried out on equal amounts of total protein of *ppi2*, WT, NTAP-TOC159:*ppi2* and NTAP-TOC159-cmyc:*ppi2* plants (Fig. 20). To detect the myc-tag with minimal interference from the TAP tag (which binds IgG), the NTAP tag was first saturated with non-specific rabbit IgG. In a second incubation, the blot was incubated with mouse anti-myc antibodies and developed

with goat-anti-mouse IgG coupled to HRP (Fig. 20, anti-cmyc). The anti-myc antibodies detected two specific bands in the NTAP-TOC159-cmyc extract that were not present in NTAP-TOC159, one at above 200 kD and the other at around 100 kD corresponding to TOC159-FL and TOC159GM, respectively (Fig. 20, lane 4).

The ratio between TOC159-FL and TOC159GM was around 2:1 indicating that around two thirds of TOC159 exist in the full-length form whereas around one third lacks the A-domain. A third band in between the two was observed in the NTAP-TOC159 and NTAP-TOC159-cmyc extracts and most likely corresponded to the free A-domain (TOC159A) that had not been completely saturated by the non-specific IgG. Finally the blot was incubated with goat-anti-rabbit antibodies coupled to HRP to detect the NTAP-tag (Fig. 20, rabbit IgG). The patterns and intensity of bands observed for NTAP-TOC159 and NTAP-TOC159-cmyc were highly similar suggesting that the two proteins were expressed at similar levels in their respective genetic backgrounds. The anti-rabbit antibody detected levels of free TOC159A that appeared greater than those of TOC159-FL suggesting that the TOC159A is stable in the cytosol whereas lower levels of TOC159-FL are maintained at the chloroplast outer membrane.

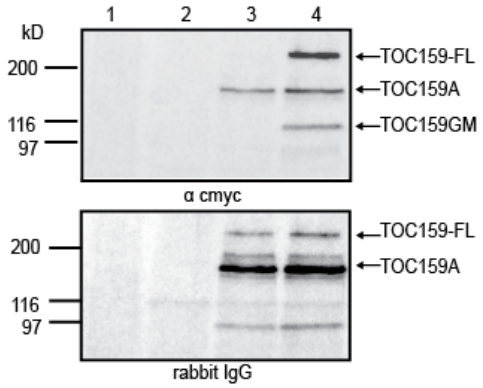


**Figure 20**. **Ratio of full length TOC159 to TOC159 without A-domain.** Total proteins extracts of seedlings 1 (*ppi2*), 2 (WT), 3 (NTAP-TOC159 :*ppi2*) and 4 (NTAP-TOC159-cmyc :*ppi2*) were analyzed by Western blotting. The membrane was consecutively probed with antibodies against cmyc and rabbit IgG. The data shown are representative experiments out of several replicates.

## KOC1 supports full import activity

Since KOC1 can interact and phosphorylate TOC components, we tested whether KOC1 plays a role in preprotein import. Chloroplasts were isolated from 2-week-old wild-type and *koc1-1* mutant seedlings. The isolated chloroplasts were incubated with either [$^{35}$S]-pSSu (a client

preprotein of the TOC159 receptor) (Fig. 21A) or [$^{35}$S]-pE1α (a client preprotein of the TOC120 and -132 receptors) (Fig. 21B) for 0', 5' and 15' and one sample was treated with thermolysin after 15' (15'T). The proteins were precipitated and separated by SDS-PAGE, followed by Phosphorimager analysis. Import was defined as the accumulation of mature [$^{35}$S]SSu and [$^{35}$S]E1α. In *koc1-1* chloroplasts the accumulation of both mature SSu (47%) and E1α (60.3%) after 15 min was reduced in comparison with wild type chloroplasts (100%) (Fig. 21A-B).

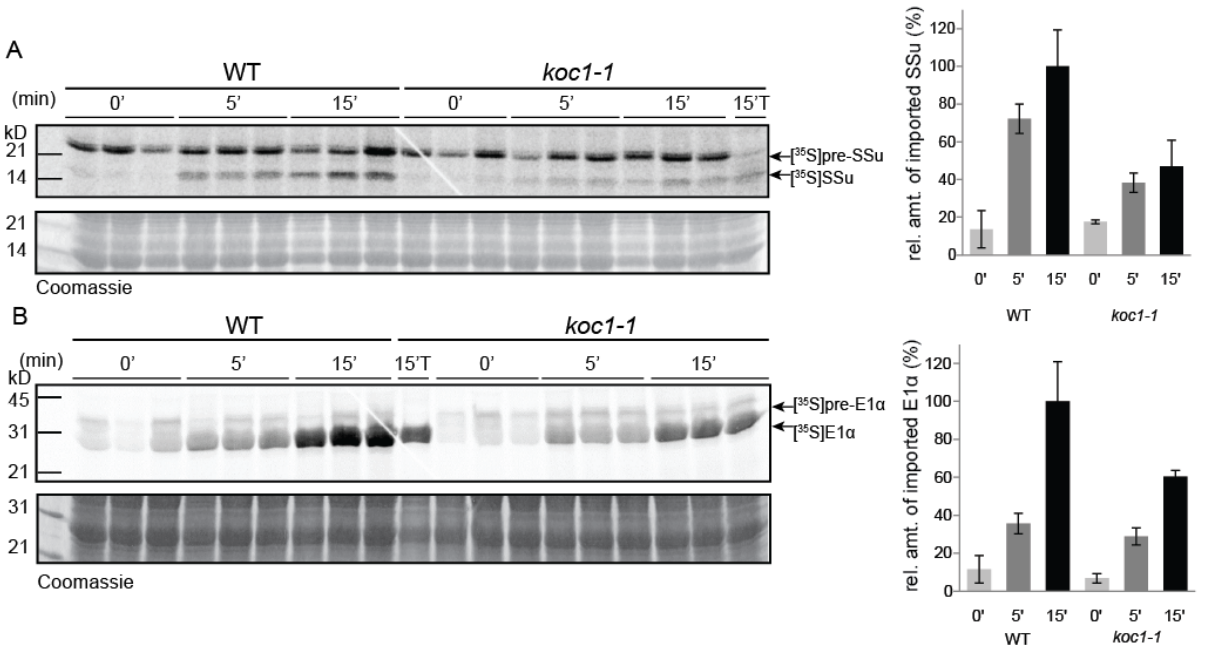


**Figure 21. KOC1 supports full import activity**. Isolated chloroplasts from *koc1-1* and Col-0 (WT) plants were incubated with [35S]Met-labeled preproteins of pSSu in **A** and E1-α in **B**. The pre-proteins were incubated with chloroplasts and import was allowed to proceed for 0, 5 and 15 min (0', 5' and 15'), one sample was treated with thermolysin after 15' (15'T). Proteins from chloroplasts were separated by SDS-PAGE followed by Phosphorimager analysis. The graphs show the quantification of the bands corresponding to imported, mature SSu and E1α at 0, 5 and 15 min averaged over three technical replicates. Error bars represent S.D. The amount of mature protein imported into WT chloroplasts at 15 min was arbitrarily set to 100%. The qualitatively similar results were obtained in five independent experiments.

## KOC1 is required for survival during de-etiolation

During de-etiolation, rapid import of proteins occurs to accomplish the transition to photoautotrophic growth. To analyze a potential role for KOC1 in the process, wild type, *koc1-1*, *koc1-2* and NTAP-KOC1:*koc1-1* plants were grown in the dark for 6 days and then exposed to standart light conditions. To guarantee identical seed quality, the parental plants

had been grown simultaneously and allowed to set seed in the same growth chamber. For all four genotypes the germination rate was close to 100%. However the survival rate differed for the different genotypes. Approximately 34% of the wild type plants survived. In comparison only around 9% of the *koc1-2* and 13% *koc1-1* plants survived which differed significantly from wild type survival rates (Fig. 22). The survival rate of NTAP-KOC1:*koc1-1* plants was around 23% and statistically indistinguishable from WT. This provides evidence that NTAP-KOC1 expressed in *koc1-1* plants is functional and complements the *koc1-1* de-etiolation phenotype.

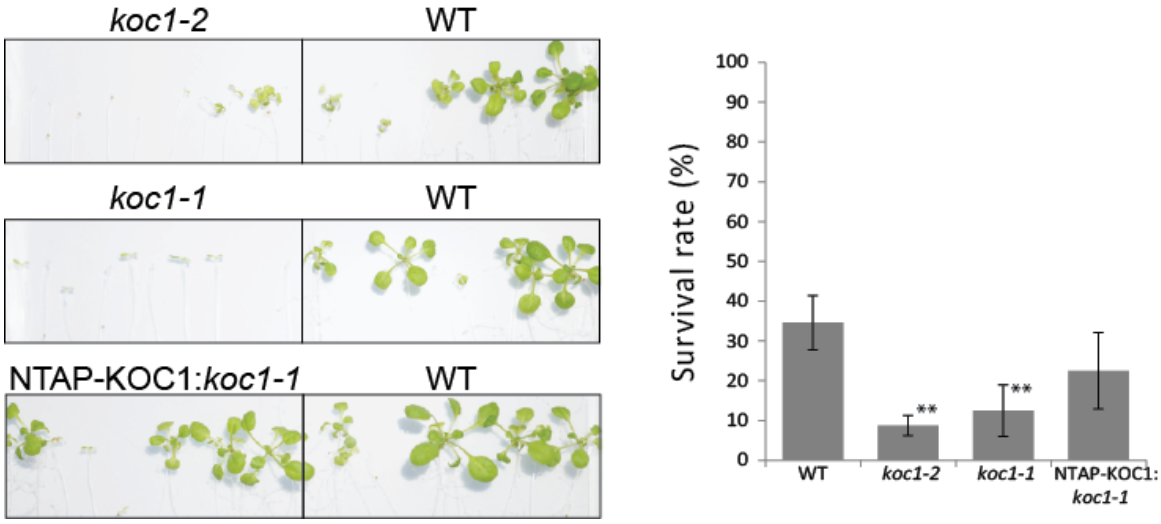


**Figure 22. KOC1 is required for survival during de-etiolation**. Images of surviving *koc1-2, koc1-1,* WT and NTAP-KOC1:*koc1-1* plants upon exposure to long day conditions for two weeks after etiolation for 6 days in dark. The germination and survival rates were calculated. The germination rate was around 100% for all genotypes. The survival rates were: WT: 34.6%, NTAP-KOC1:*koc1-1*: 22.5%, *koc1-2*: 8.8% and *koc1-1*: 12.5 %. Student's t-Test: '**= pValue<0.01; *=pValue<0.05 (*n: 80* for *koc1-1* and *koc1-2*; *n: 240* for WT). This experiment was repeated three times with comparable results.

## KOC1 is highly expressed in senescent leaves

To identify in which conditions KOC1 is expressed, we used the information available on Genevestigator®. According to the data in this public repository, the expression level of KOC1 remained unchanged at a medium level at different stages of development in Arabidopsis. Increased expression was observed in rosette leaves at the silique and senescence stages (Fig. 23).

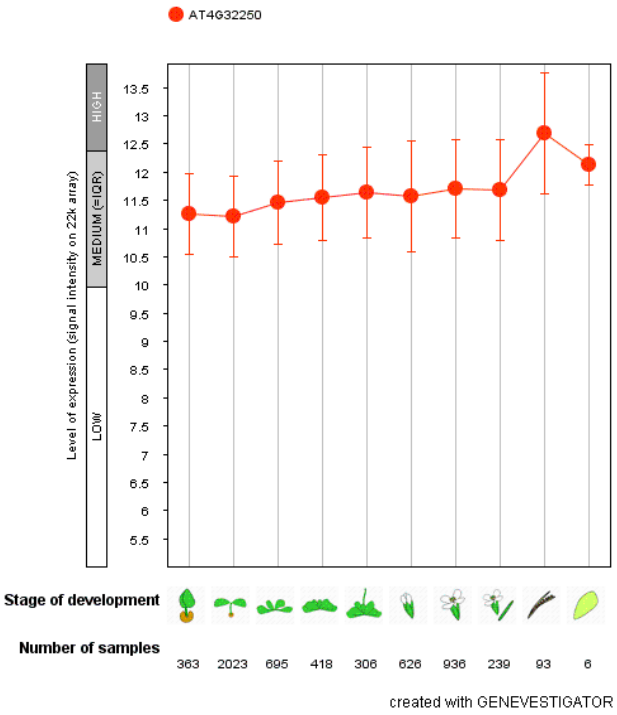


**Figure 23**. **Level of expression of KOC1** (AT4G32250) at different stages of development in Arabidopsis obtained from Genevestigator®.

## KOC1 is strongly expressed in senescent Arabidopsis rosette leaves

To independently confirm that KOC1 expression is increased in senescent leaves, the rosettes of Arabidopsis wild type plants (WT) and *koc1* mutants (*koc1-1* and *koc1-2)* grown on soil, were cut and placed in darkness for 11 days to induce senescence. A set of rosettes were removed from the dark, photographed daily and grounded to extract total proteins and RNA. No visible yellowing occurred from 0 to 4 days in the dark (Fig. 24A), however WT plants appeared to be paler green when compared to the *koc1* mutant after 7 days. The proteins were extracted, separated and analysed by Western blotting and immunodetection (Fig. 24B). KOC1 was detected with antibodies (αKOC1) on samples after day 2 in darkness and a significant increase was detected in the day 11 sample (Fig. 24B). To determine whether the accumulation of KOC1 is correlated with the abundance and stability of the other TOC members, antibodies against TOC159A and TOC75 were used. The abundance of TOC75 and TOC159 appears to slightly decrease during the senescence, however the result was not entirely clear, there are some samples where TOC159 was not well detected. Based on these results we cannot conclude whether the stability of TOC components correlate with KOC1 abundance. RNA extracted from wild type senescent plants was used to reverse

transcription to obtain cDNAs. qPCR analysis were carried out for three genes, KOC1, TOC159 and SRG1 (senescence-inducible chloroplast stay-green protein 1) as a control. The results revealed that the relative expression of KOC1 was increased. At day 5, WT plants had more than 15-fold greater expression level of KOC1 than at day 0. Moreover, at day 11 the expression level increased more than 40-fold (Fig. 24 C). The relative expression of TOC159 was very low compare to KOC1 and the variation was undetectable. The senescence test is a preliminary result, the experiment was done once and need to be confirmed.

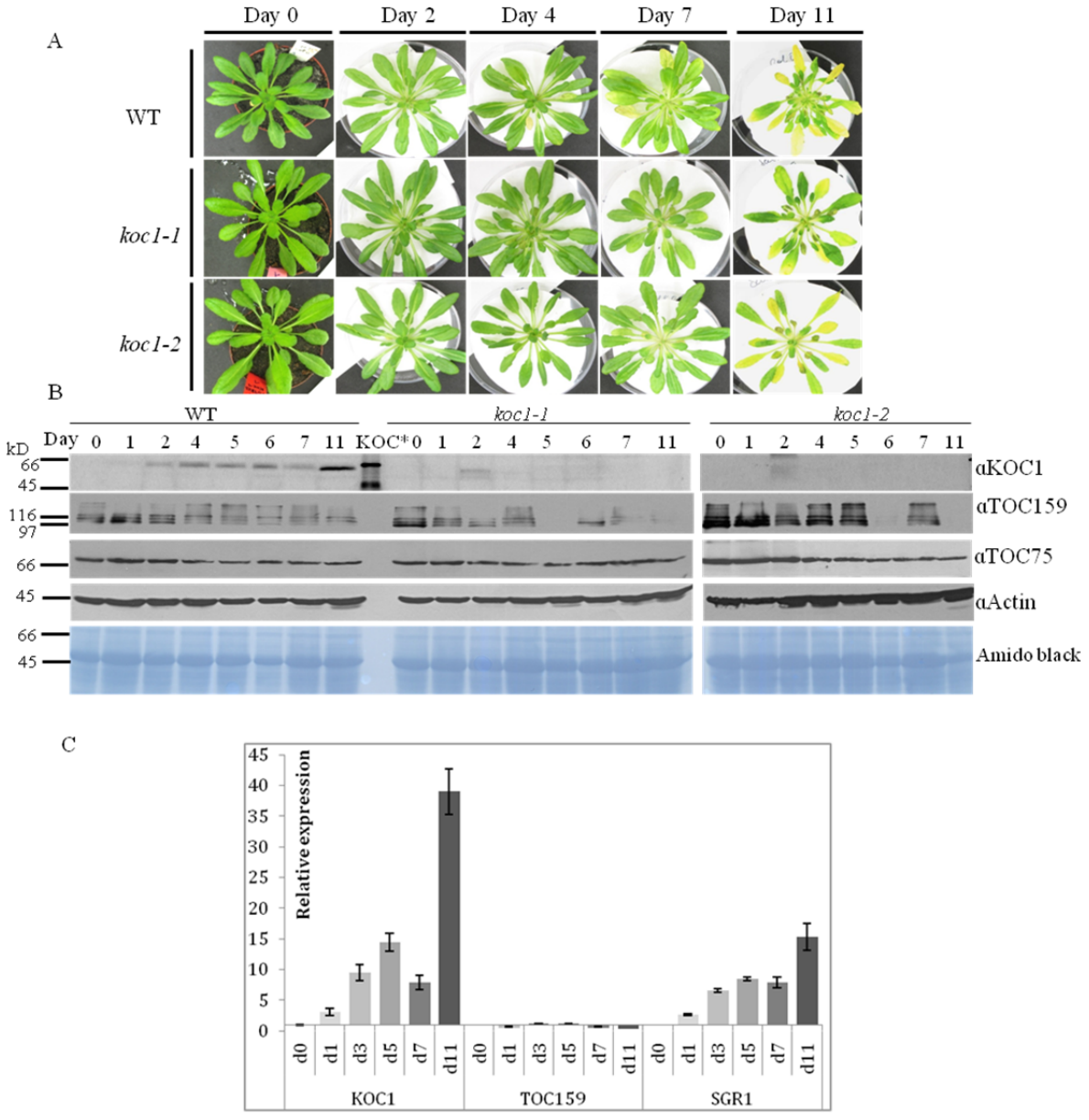


**Figure 24. Upregulating of KOC1 in dark-induced senescence in Arabidopsis rosette leaves**. **A**. Rosettes from Col-0 (WT), *koc1-1* and *koc1-2* plants were placed in the darkness to induce senescence. During the treatment, some rosettes were photographed (days: 0, 2, 4, 7 and 11). **B**.

Protein extracts from rosettes (days: 0, 1, 2, 4, 5, 6, 7 and 11) were separated by SDS-PAGE and analyzed by Western blotting with antibodies against KOC1, TOC75, TOC159A and actin that was used as a loading control. KOC*: 5ng of purified recombinant $KOC1_{(FL)}$. **C**. Relative expression of KOC1, TOC159 and SRG1 as a control from senescent plants (days: 0, 1, 3, 5, 7 and 11).

# Discussion

Phosphorylation is emerging as an important mechanism in regulating the assembly and activity of protein import complexes in protein translocation systems. CKII and SnRK2, two cytosolic kinases are known to phosphorylate the TOC159 A-domain. Here, we identify KOC1, an integral chloroplast envelope kinase, that phosphorylates the A-domain. We demonstrate that KOC1 affects import activity but does not interfere with composition and abundance of import components.

N-terminally TAP-tagged TOC159 (NTAP-TOC159) was used to isolate the protein import machinery and to identify new potential interaction partners of TOC159 *in vivo* (Fig. 9) (Köhler et al., 2015). Among the identified proteins was the predicted kinase AT4G32250 that we named KOC1. In the reverse experiment NTAP-KOC1 was used as the bait (Fig. 10). NTAP-KOC1 was most likely fully functional as it 1) complemented the *koc1-1* phenotype in the de-etiolation survival assay (Fig. 22) and 2) phosphorylated recombinant A-domains after affinity purification (Fig. 18). TOC159 as well as other recognized components of the import machinery co-isolated together with NTAP-KOC1. Overall, the lists of proteins identified by mass spectrometry associating with NTAP-TOC159 and NTAP-KOC1, respectively, were highly overlapping. The combination of the two co-isolation experiments provided strong evidence for the association of KOC1 with the chloroplast protein import machinery.

By mass spectrometric analysis, not TOC159 but TOC75 gave the highest score in femtomoles of any component of the import machinery associating with KOC1. This indicates that TOC159 may not be the primary interaction partner of KOC1. KOC1 may also associate with multiple TOC complexes containing the different TOC159 homologs, as suggested by the ability of KOC1 to phosphorylate the A-domains of TOC132 and -120 (Fig. 18). The NTAP-KOC1-associated proteins belonged to three distinct networks: the TOC-complex, the intra-chloroplastic FtsH proteases and the cytosolic ribosome (Fig. 11). The interaction with the FtsH network hints at an interaction between the import and protein quality control systems within the chloroplast. The interaction between KOC1 and FtsH proteins is most likely indirect and may involve interaction with TIC components that also co-isolated with KOC1. Interaction with the cytosolic ribosome suggests coordination of

preprotein synthesis with the import process. These hypotheses should be experimentally explored in future studies.

KOC1 lacks a predicted, cleavable transit peptide at the N-terminus and is therefore not a predicted chloroplast protein (Fig. 12A). The absence of a cleavable transit peptide, however, is typical of chloroplast outer envelope proteins. KOC1 has a single predicted transmembrane region near its C-terminus. It is therefore probable that KOC1 belongs to the category of tail-anchored outer membrane proteins (Kim and Hwang, 2013; Dhanoa et al., 2010). Outer envelope localization was supported by three lines of evidence (fluorescence, membrane fractionation, protease sensitivity) (Fig. 14 and 15). Based on resistance to alkaline extraction, KOC1 behaves as an integral membrane protein (Fig. 15B). The predicted transmembrane sequence at the C-terminus in combination with protease sensitivity indicated that the bulk of the KOC1 protein faces the cytosol. The only other known organellar kinase facing the cytosol apart from KOC1 is mitochondrial CKI (casein kinase I) which phosphorylates TOM20 and stimulates assembly of the TOM complex (Gerbeth et al., 2013).

The KOC1 protein contains two striking elements in addition to the transmembrane region: a predicted N-terminal kinase domain and a C-terminal HERC2-related region. The sequence of KOC1 is most closely related to that of KEG (Keep On Going, AT5G13530). KEG contains a kinase domain and 12 HERC2 repeats with similarity to KOC1. In addition to those domains KEG also a RING domain responsible for its function as an E3 ligase (Stone et al., 2006). The RING domain is absent from the KOC1 sequence suggesting that it does not function as an E3 ligase. It cannot be excluded however that KOC1 functions in conjunction with other E3 ligases under specific conditions. One such candidate is the outer membrane E3-ligase SP1 that is involved in ubiquitination and turnover of TOC159 and -75 (Ling et al., 2012). However, the components of the TOC complex as well as TIC110 were present in both *koc1* lines at similar concentrations as in the wild type (Fig. 12D). This indicated that loss of KOC1 does not affect composition and abundance of the components of the import machinery under standard growth conditions.

A question of central interest was whether KOC1 kinase phosphorylates the A-domains of TOC159 and its homologs. KOC1 purified from transgenic plants phosphorylated the recombinant TOC159, -132 and -120 A-domains (Fig. 18). The A-domains of TOC159 and TOC132/-120 determine preprotein specificity (TOC159 specializing in photosynthesis-associated proteins and TOC132/-120 in housekeeping proteins) (Inoue et al., 2010). The ability of KOC1 to phosphorylate all three A-domains suggests a function in import of both photosynthesis-associated and housekeeping proteins. It is important to note that the majority of TOC159 is full length (Fig. 20) and therefore such a scenario is plausible. However, we were not able to reliably identify KOC1-dependent phosphorylation sites in the recombinant TOC159 A-domain using mass spectrometry. Their identification will allow site-specific mutagenesis to test the role of KOC1-dependent phosphorylation in protein import. While TOC159 family members are likely *in vivo* targets of KOC1 others at the chloroplast outer membrane or in the cytosol could also exist. Such targets could be identified in the future using phosphoproteomics approaches.

An outer membrane kinase of 70 kD (OEK70) phosphorylating the C-terminal G- and M-domains of TOC159 in pea has been characterized earlier but was never identified at the molecular level (Fulgosi and Soll, 2002). Based on the similar mass of Arabidopsis KOC1, it is tempting to speculate that OEK70 may be a KOC1 homolog in pea.

The proposition that KOC1 affects import of TOC159 as well as TOC132/-120 dependent import substrates (pSSU for TOC159 and pE1α for TOC132/-120 (Inoue et al., 2010)) was tested in *in vitro* protein import assays (Fig. 21). In both cases, import into *koc1-1* mutant chloroplasts was reduced by about 40%. This suggests that KOC1 is required for efficient protein import in both pathways. As the concentrations of the TOC components in *koc1* chloroplasts were similar to wild type (Fig. 12D) it appears probable that KOC1 directly regulates the activity of the import receptors. This is likely to implicate phosphorylation but we cannot exclude other possibilities. Based on the diminished import activity in the mutant chloroplasts the effect of KOC1 is predicted to be positive. In contrast PKA-dependent phosphorylation of Tom70 inhibited receptor activity and import of metabolite carriers in mitochondria (Schmidt et al., 2011).

Despite the diminished import efficiency the *koc1* mutant had a wild type phenotype under standard growth conditions. Similarly, TOC159 and TOC33 GTPase mutants had diminished

import efficiency *in vitro* but were phenotypically indistinguishable from the wild type (Aronsson et al., 2010; Agne et al., 2009). Possibly the mutant plants are able to compensate for the chloroplast protein import deficit over time. A phenotype in such mutants might only occur at specific points in development where high import capacity is required. Such a point in development is the switch from etioplasts (chloroplast precursor organelle in the dark grown plants) to chloroplasts that develop when plants are moved into the light, a process known as de-etiolation.

In the de-etiolation assay (Fig. 22) (Ling et al., 2012) dark grown seedlings near seed depletion were moved to the light. Under these conditions chloroplast biogenesis must be completed quickly in order to prevent starvation and initiate photoautotrophic growth. In the de-etiolation assay, the *koc1* seedlings had a lower survival rate than the wild type and NTAP-KOC1:*koc1-1* seedlings, corroborating the proposition that KOC1 activity is required when protein import demand is high.

SnRK2 kinase phosphorylates the A-domain of TOC159 in an ABA dependent fashion. This suggests hormonal control of TOC159 phosphorylation and import activity. It appears likely that various regulatory pathways converge at TOC159 and its homologs via pathway-associated kinases.

Genevestigator® microarray data demonstrate that the mRNA expression level of KOC1 increases in senescent leaves compared to rosette leaves. This was confirmed at the protein level by Western blotting and qPCR analysis of senescent leaf extracts. An increase of KOC1 was detected in wild type plants upon dark-induced senescence (Fig. 24). Interestingly, KOC1 was undetectable in rosettes before the treatment indicating a correlation of KOC1 expression with the senescence process. No visible yellowing was observed in rosettes from 0 to 4 days in the dark. Nevertheless, after 7 days, the oldest leaves at the bottom of the WT plant turned to yellow, in contrast to *koc1* mutants that appeared green. Suggesting that senescence may be delayed in *koc1* mutants, probably due to diminished chlorophyll degradation and thylakoid disassembly. During senescence import capacity is still required by the chloroplast, as senescence related enzymes and proteins have to be imported. In this case, we hypothesize that KOC1 has a role in the regulation of the import of senescence-related enzymes.

# Conclusion

Phosphorylation is considered one of the most important regulatory mechanisms in development and maintenance of cells. In plant cells, phosphorylation is known to modulate vital metabolic functions, for instance, gene expression and photosynthesis. In chloroplast and mitochondria, the import of proteins is essential and appears to be regulated by phosphorylation. Recent progress provided new insights into phosphorylation targets, however, the kinases proteins implicated remained unknown. In the import of proteins in mitochondria, only one organelle-associated kinase (CKI) is known. In chloroplasts, KOC1 is the first chloroplast outer membrane-associated kinase identified as a part of the import machinery. The amino acid sequence of KOC1 reveals a unique domain organization and it cannot be included in an existing inventory. KOC1 has a kinase domain as well as a HERC2-related region. In this thesis, we demonstrated that KOC1 regulates the import activity of the TOC complex by phosphorylation. However, the role of the HERC2-related region should also be studied, and for this we provide a useful list of KOC1-interacting proteins. Among the candidates, unknown proteins constitute a platform to study other unexpected roles of KOC1 and a novel biological function of phosphorylation in chloroplasts. SnRK2 kinase phosphorylates TOC159 in an abscisic acid-dependent way, suggesting that the import activity could be hormonally regulated. In future experiments, the hormonal control of the import process, may be explored using *koc1* knock out mutants. Preliminary results show that KOC1 is upregulated in dark-induced senescent plants. The function of KOC1 during senescence could be another exciting challenge to understand new mechanisms of regulation of the TOC complex.

# Outlook

KOC1 was identified as a kinase phosphorylating TOC159 family members. However, KOC1 is probably able to phosphorylate other proteins. In this study we provided a list of proteins identified associated with KOC1, some unknown proteins could be eventually substrates of KOC1. To verify the strong interaction of KOC1 with the candidates, “reverse” IgG affinity purification is an excellent tool. In this group of KOC1-interacting proteins identified by mass spectrometry analysis, there is a cluster annotated as “Cytoplasmic ribosome complex”. The interaction of KOC1 with the ribosome would be an interesting subject of study.

Signal transduction pathways involved in developmental programs often consist of a cascade of processes acting via protein phosphorylation and dephosphorylation. KOC1 kinase appears to be good candidate to participate together with other senescence-associated proteins in the phosphorylation cascade. The expression of KOC1 is known to be strongly increased during the senescence process. To obtain more information about how KOC1 is implicated in the senescence process, the dark-induced senescence test using NTAP-KOC1:*koc1-1* plants is an interesting possibility. On the other hand, the purification by IgG affinity chromatography of KOC1 interacting proteins in senescent NTAP-KOC1:*koc1-1* plants could help us to better understand its interaction partners and thereby the function of KOC1.

A role of KOC1 in the ubiquitination process during senescence as well as other developmental stages is a possibility. There is evidence for involvement of ubiquitination in transition from chloroplast to gerontoplast, as well as from the etioplast to chloroplast. Phosphorylation of proteins by KOC1 may initiate bulk protein degradation by the ubiquitin pathway via a functional interaction with chloroplast outer membrane E3-ligase SP1 is imaginable.

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# Annex

## Table 1. List of primers used in this project.

| Primer name | Primer sequence |
|---|---|
| KOC1_NheI_F | 5’-GAGAGAGCTAGCATGGCTTCAAAGATTATT-3’ |
| KOC1_NotI_R | 5’-TCTCTCGCGGCCGCAAAGAACACAAGCCA-3’ |
| KOC1_NcoI_F | 5’-GAGACCATGGCTTCAAAGATTATTGC-3’ |
| KOC1_NcoI_R | 5’- TCTCCCATGGCTTGCAGATGATCCTTTGAA-3’ |
| KOC1_NcoI_R2 | 5’- TCTCTCGCGGCCGCATCCTCCAGATGTTGA-3’ |
| KOC1_FL_NcoI_F | 5’-TACCATGGCTAGCATGGCTTCAAAG-3’ |
| KOC1_FL_XbaI_R | 5’-TCTCTAGACTAAAAGAACACAAGCCATTTAG-3’ |
| LBb1.3Salk Institute | 5’-ATTTTGCCGATTTCGGAAC-3’ |
| koc1-1_LP | 5’- CAGGGGAGTCTCTAGTATCA-3’ |
| koc1-1_RP | 5’-TAG CAC AAA TCC ATC TGG GTC-3’ |
| koc1-2_LP | 5’-AGAACATGGATGTGCCAGAAG-3’ |
| koc1-2_RP | 5’-CGCTGCATATACCATGTGATG-3’ |
| Toc159-StuI-F | 5’-CAAATTCTCTATTGAGGCCTCAAGAACCATTG-3’ |
| Toc159-cmyc-Gib-R | 5’-CTTCTTCAGAGATCAGTTTCTGTTCTACGTACATGCTGTACTTGTC-3’ |
| cmyc-uniGib-R | 5’-CTGCAGGTCGACTCTAGATTACAGATCTTCTTCAGAGATC-3’ |
| attB-KOC1-FW | 5’- ACAAGTTTGTACAAAAAAGCAGGCTCCGCTTCAAAGATTATTGCTGG-3’ |
| attB-KOC1-REV | 5’- ACCACTTTGTACAAGAAAGCTGGGTCTAAAAGAACACAAGCCATT-3’ |
| attB-EMB2004-FW | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTTCTTCTTCCACCAG-3’ |
| attB-EMB2004-REV | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGGAAGAAAATTTCTACTTCC-3’ |
| QPCR-KOC1-F | 5’-GTTTTGAGATACGGGGTTG-3’ |
| QPCR-KOC1-R | 5’-TCAGAACTTGGTAAAGGGATAC-3’ |
| SGR1-S | 5’-TGGGCAAATAGGCTATACCG-3’ |
| SGR1-AS | 5’-AAGTTCCCATCTCCATGCAC-3’ |

## Table 2. List of proteins identified in the NTAP-KOC1 TEV eluate (Fig. 10) by mass-spectrometry.

The list contains the common proteins identified in two biological replicates. The list was filtered by abundance in femtomoles (fmol). Among the 15 most abundant interaction candidates there are 7 TOC and TIC components in bold.

| | | KOC1 | |
|---|---|---|---|
| | **Accession** | **Protein (fmol)** | **Gene Description** |
| 1 | AT4G32250 | 34.9472 | KOC1 Protein kinase superfamily protein, kinase activity, ATP binding; in chloroplast |
| **2** | **AT3G46740** | **8.042533333** | **TOC75, Forms the outer envelope translocation channel (beta-barrel), in chloroplast.** |
| 3 | AT4G00120 | 7.145166667 | ATPase alpha subunit of ATP synthase, In the thylakoid membrane. |
| 4 | AT3G63410 | 6.8946 | MPBQ/MSBQ methyltransferase in chloroplast inner membrane, involved in tocopherol biosynthesis. |
| **5** | **AT4G02510** | **6.4308** | **TOC159, integral membrane GTPase, transit-sequence receptor for the import of proteins** |
| 6 | AT5G23060 | 6.126233333 | CAS, a chloroplast-localized protein that modulates cytoplasmic Ca2+ concentration. |
| 7 | AT4G03280 | 3.821266667 | Rieske FeS center of cytochrome b6f complex, in shoot but not in root. |
| **8** | **ATCG01130** | **3.657866667** | **YCF1.2, protein binding, in chloroplast membrane** |
| 9 | AT5G02940 | 3.473433333 | Protein of unknown function, in chloroplast envelope. |
| **10** | **AT1G06950** | **3.453233333** | **TIC110, involved in protein import into the chloroplast and chloroplast biogenesis.** |
| **11** | **AT1G02280** | **3.2534** | **TOC33, GTP-binding GTP-ase, Component of the chloroplast protein import machinery.** |
| 12 | AT1G07890 | 3.080633333 | Cytosolic ascorbate peroxidase APX1, scavenge hydrogen peroxide in plant cells. |
| **13** | **AT5G05000** | **3.002133333** | **TOC34, GTPase protein in import of proteins into the chloroplast.** |
| 14 | AT3G08940 | 2.899366667 | Lhcb4.2 involved in the light harvesting complex of photosystem II The mRNA is cell-to-cell mobile. |
| **15** | **AT5G50920** | **2.775733333** | **CLPC, involved in protein import in chloroplast, provide ATP that drives TIC machinery.** |
| 16 | AT2G41840 | 2.6055 | Ribosomal protein S5 family protein, structural constituent of ribosome |
| 17 | AT2G43950 | 2.5375 | OEP37, constitutes a peptide sensitive ion channel in chloroplast outer membranes. |
| 18 | AT3G61260 | 2.452433333 | Remorin, process unknown, plasma membrane, vacuole |
| 19 | AT3G47520 | 2.444 | Protein with NAD-dependent malate dehydrogenase activity, located in chloroplasts. |
| 20 | AT3G01500 | 2.4029 | Putative beta-carbonic anhydrase CA1, regulates CO2-controlled stomatal movements in guard cells. |
| 21 | AT5G13490 | 2.2424 | Encodes mitochondrial ADP/ATP carrier |
| 22 | AT4G17530 | 2.147866667 | AtRabD2c encodes a Rab GTPase, roles in pollen development, germination and tube elongation. |

| | | | |
|---|---|---|---|
| 23 | AT5G57350 | 2.0477 | Member of Plasma membrane H+-ATPase family |
| 24 | AT1G01790 | 2.007533333 | K efflux antiporter KEA1 |
| 25 | AT4G04640 | 1.9817 | One of two genes (with ATPC2) encoding the gamma subunit of Arabidopsis chloroplast ATP synthase. |
| 26 | AT5G42020 | 1.8882 | Luminal binding protein (BiP2) involved in polar nuclei fusion during proliferation of endosperm nuclei. |
| 27 | AT3G17970 | 1.868833333 | TOC64 Integral chloroplast outer membrane protein, Tetratricopeptide repeat (TPR) proteins |
| 28 | AT3G19820 | 1.804 | Involved in the conversion of the brassinosteroid precursor 24-methylenecholesterol to campesterol. |
| 29 | AT2G36170 | 1.767566667 | Ribosomal protein L40e, structural constituent of ribosome; Ubiquitin extension protein 1 |
| 30 | ATCG00470 | 1.762933333 | ATPase epsilon subunit |
| 31 | AT5G46110 | 1.752733333 | Chloroplast triose phosphate / 3-phosphoglycerate translocator |
| 32 | AT5G22640 | 1.748233333 | EMB1211 is a MORN involved in embryo development and chloroplast biogenesis. |
| 33 | AT1G10510 | 1.735966667 | EMB2004, embryo development ending in seed dormancy, match RAN GTPase activating protein 1 |
| 34 | AT5G17090 | 1.696933333 | Cystatin/monellin superfamily protein |
| 35 | AT5G01590 | 1.653166667 | Unknown protein, function unknown, in chloroplast envelope |
| 36 | AT1G22530 | 1.619933333 | PATELLIN 2 (PATL2),transporter activity, alpha-tocopherol transport |
| 37 | AT1G22780 | 1.604033333 | S18 ribosomal protein involved in the binding of f-Met tRNA during initiation of mRNA translation. |
| 38 | AT5G54270 | 1.5768 | Lhcb3 protein is a component of light harvesting chlorophyll a/b-protein complex of Photosystem II. |
| 39 | ATCG00130 | 1.523733333 | ATPase F subunit. |
| 40 | AT1G33120 | 1.517566667 | Ribosomal protein L6 family, structural constituent of ribosome, rRNA binding. |
| 41 | ATMG01190 | 1.4399 | ATPase subunit 1 |
| 42 | AT2G43630 | 1.4308 | Function unknown, chloroplast thylakoid membrane. |
| 43 | AT1G30380 | 1.415166667 | Encodes subunit K of photosystem I reaction center. The mRNA is cell-to-cell mobile. |
| 44 | AT3G06510 | 1.396666667 | Encodes a protein with beta-glucosidase and galactosyltransferase activity. |
| 45 | AT2G45820 | 1.3562 | Remorin family protein, DNA binding. |
| 46 | AT5G23890 | 1.309933333 | Unknown protein, in Mitochondrion, thylakoid membrane, chloroplast envelope |
| 47 | AT1G72150 | 1.2719 | Novel cell-plate-associated protein, related in sequence to proteins involved in membrane trafficking |
| 48 | AT3G16290 | 1.217 | EMB2083, chloroplast, chloroplast envelope, InterPro: ATPase, AAA+ type, core, Peptidase M41 |
| 49 | AT4G09650 | 1.215633333 | Encodes the chloroplast ATPase delta-subunit. The mRNA is cell-to-cell mobile. |
| 50 | AT1G77590 | 1.2093 | Encodes major plastidic long chain acyl-CoA synthetase |
| 51 | AT5G19620 | 1.1585 | AtOEP80 is paralog to the chloroplastic protein translocation channel Toc75. |
| 52 | AT3G04340 | 1.141966667 | Embryo defective 2458, nucleoside-triphosphatase, ATPase, metalloendopeptidase activity |

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| 53 | AT1G79560 | 1.095133333 | Encodes an FtsH protease localized to the chloroplast. Mutations result in embryo lethality. |
| 54 | AT5G24690 | 1.0896 | Process unknown, mitochondrion, chloroplast inner membrane, chloroplast envelope |
| 55 | AT5G14740 | 1.069333333 | Encodes a beta carbonic anhydrase likely to be localized in the cytoplasm. |
| 56 | ATCG00340 | 1.060133333 | Encodes the D1 subunit of photosystem I reaction center. |
| 57 | AT5G53170 | 1.059533333 | encodes an FtsH protease that is localized to the chloroplast and the mitochondrion |
| 58 | AT4G00630 | 1.0542 | Encodes a K(+)/H(+) antiporter that modulates monovalent cation, pH homeostasis in chloroplasts |
| 59 | AT5G56030 | 1.0272 | A member of heat shock protein 90 (HSP90) gene family. |
| 60 | AT3G20320 | 1.0002 | Encodes a permease-like component of an ABC transporter involved in lipid transfer ER to chloroplast. |
| 61 | AT5G64580 | 0.999133333 | AAA-type ATPase protein, metalloendopeptidase activity, nucleotide binding, ATP binding |
| 62 | AT5G23040 | 0.993633333 | Encodes a protein that enables protochlorophillide's binding to pPORA's transit sequence |
| 63 | AT5G22830 | 0.975 | Transmembrane magnesium transporter. One of nine family members. |
| 64 | AT5G42480 | 0.9325 | Shows homology to the cyanobacterial cell division protein Ftn2. |
| 65 | AT4G32260 | 0.928833333 | ATPase chloroplast, hydrogen ion transmembrane transporter |
| 66 | AT2G30950 | 0.908833333 | Metalloprotease that functions in thylakoid biogenesis. Involved in the repair of PSII |
| 67 | AT1G55670 | 0.905733333 | Subunit G of photosystem I, 11-kDa membrane protein, role in electron transport |
| 68 | AT5G14040 | 0.896133333 | Encodes a mitochondrial phosphate transporter. Modulates plant responses to salt stress. |
| 69 | AT1G74470 | 0.8872 | Encodes for a multifunctional protein with geranylgeranyl reductase activity |
| 70 | AT5G03910 | 0.8718 | Member of ATH subfamily |
| 71 | AT5G43745 | 0.8634 | Protein of unknown function, chloroplast envelope, |
| 72 | AT2G36160 | 0.846 | Ribosomal protein S11 family protein; structural constituent of ribosome; |
| 73 | AT5G14030 | 0.8145 | translocon-associated protein beta (TRAPB) family protein |
| 74 | AT3G10840 | 0.814033333 | alpha/beta-Hydrolases protein, hydrolase and catalytic activity,chloroplast envelope |
| 75 | AT2G16640 | 0.789666667 | TOC132, transmembrane receptor activity, protein targeting to chloroplast |
| 76 | AT2G01250 | 0.784033333 | Ribosomal protein L30/L7 family protein; structural constituent of ribosome, |
| 77 | AT5G08540 | 0.7602 | Unknown protein; molecular_function unknown, chloroplast envelope |
| 78 | AT2G18960 | 0.754 | Encodes a plasma membrane proton ATPase. Mutants have a reduced ability to close their stomata |
| 79 | AT1G11860 | 0.738733333 | Glycine cleavage T-protein, aminomethyltransferase activity, response to cadmium ion |
| 80 | AT5G45775 | 0.738066667 | Ribosomal L5P family protein, structural constituent of ribosome |
| 81 | AT2G28900 | 0.737966667 | AtOEP16, 16-KDa plastid outer membrane protein, import of protochlorophyllide oxidoreductase A. |
| 82 | AT4G23940 | 0.723966667 | Encodes FtsHi1, chloroplast envelope membrane, Functions in chloroplast |

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| | | | biogenesis and division. |
| 83 | AT2G19730 | 0.721033333 | Ribosomal L28e protein family |
| 84 | AT2G24820 | 0.707066667 | TIC55-II, translocon at the inner envelope membrane of chloroplasts 55-II |
| 85 | AT4G14070 | 0.702333333 | Plastidic acyl activating enzyme, elongation of exogenous fatty acids to 16- and 18-carbon fatty acids. |
| 86 | AT4G21520 | 0.6969 | Transducin/WD40 repeat-like protein, 86 |
| 87 | AT1G30360 | 0.688266667 | Early-responsive to dehydration 4 (ERD4, response to water deprivation |
| 88 | AT1G06430 | 0.6816 | encodes a FtsH protease that is localized to the chloroplast |
| 89 | AT2G33040 | 0.6776 | Gamma subunit of Mt ATP synthase (ATP3), zinc ion binding, proton transport |
| 90 | AT5G08670 | 0.672366667 | Mitochondrial ATP synthase beta-subunit |
| 91 | AT1G62960 | 0.658033333 | Aminotransferase with specificity for aspartate and aromatic amino acids tyrosine and phenylalanine. |
| 92 | AT5G19760 | 0.6395 | Mitochondrial carrier transporting both dicarboxylates and tricarboxylates |
| 93 | AT1G65260 | 0.629266667 | Encodes a protein required for thylakoid membrane formation. |
| 94 | AT2G38670 | 0.620733333 | Mitochondrial ethanolamine-phosphate cytidylyltransferase, phosphatidylethanolamine biosynthesis. |
| 95 | AT1G20010 | 0.6043 | Beta tubulin |
| 96 | AT1G44575 | 0.5957 | PSII-S (CP22), a ubiquitous pigment-binding protein associated with photosystem II (PSII) of plants. |
| 97 | AT5G66570 | 0.574933333 | Extrinsic subunit of photosystem II, play a central role in stabilization of the catalytic manganese cluster |
| 98 | AT3G25520 | 0.573266667 | Ribosomal protein L5, binds to 5S ribosomal RNA and export from the nucleus to the cytoplasm. |
| 99 | AT2G45740 | 0.570966667 | Peroxin11 (PEX11) gene family, integral to peroxisome membrane, controls peroxisome proliferation |
| 100 | AT5G64816 | 0.5676 | Unknown protein |
| 101 | AT4G21280 | 0.564333333 | Encodes the PsbQ subunit of the oxygen evolving complex of photosystem II. |
| 102 | AT3G05560 | 0.562833333 | Ribosomal L22e protein, structural constituent of ribosome, cytosolic ribosome, ribosome |
| 103 | AT5G42270 | 0.5454 | VAR1 contains a conserved motif for ATPase and a metalloprotease characteristic to FtsH proteins |
| 104 | AT5G51070 | 0.542133333 | ATP-dependent Clp protease regulatory subunit The mRNA is cell-to-cell mobile. |
| 105 | AT1G06680 | 0.528866667 | 23 kD extrinsic protein,part of photosystem II and participates in the regulation of oxygen evolution. |
| 106 | AT1G78900 | 0.526033333 | Catalytic subunit A of the vacuolar ATP synthase. |
| 107 | AT1G76030 | 0.522533333 | Vacuolar ATP synthase subunit B1. Interact with the gene product of hexokinase1 (ATHXK1). |
| 108 | AT3G56940 | 0.516533333 | ZIP protein with varying mRNA accumulation in leaves, stems and roots. |
| 109 | AT3G01290 | 0.513866667 | SPFH/Band 7/PHB domain-containing membrane-associated protein family; |
| 110 | AT3G49860 | 0.510233333 | ARF-like GTPase family, ARF and ARF-like (ARL) GTPases. |
| 111 | AT4G27680 | 0.498333333 | P-loop containing nucleoside triphosphate hydrolases, ATPase activity |
| 112 | AT2G44640 | 0.491233333 | Protein of unknown function |

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| 113 | AT2G44120 | 0.491033333 | Ribosomal protein L30/L7 family protein, structural constituent of ribosome, transcription regulator |
| 114 | AT3G53560 | 0.490466667 | Tetratricopeptide repeat (TPR)-like superfamily protein, binding, biological_process unknown |
| 115 | AT5G27540 | 0.484166667 | Encodes a protein with similarity to GTPases that is localized to the mitochondrion. |
| 116 | AT5G16620 | 0.483466667 | TIC40 chloroplast protein import |
| 117 | AT4G35000 | 0.481766667 | Microsomal ascorbate peroxidase APX3. Ascorbate peroxidases |
| 118 | AT3G46780 | 0.481166667 | Plastid transcriptionally active 16 (PTAC16), binding, catalytic activity |
| 119 | AT1G67730 | 0.4802 | YBR159w beta-ketoacyl reductase (KCR), which catalyzes the first reduction during VLCFA |
| 120 | AT2G30490 | 0.4802 | Cinnamate-4-hydroxylase, Mutations impact phenylpropanoid metabolism, growth and development. |
| 121 | AT1G67430 | 0.480166667 | Ribosomal protein L22p/L17e family protein; FUNCTIONS IN: structural constituent of ribosome |
| 122 | AT1G50250 | 0.4795 | FTSH protease, in chloroplast. Involved in the D1 repair cycle of Photosystem II. |
| 123 | AT1G52230 | 0.479266667 | Phosphorylation of this protein is dependent on calcium. The mRNA is cell-to-cell mobile. |
| 124 | AT5G20290 | 0.478066667 | Ribosomal protein S8e family protein, structural constituent of ribosome |
| 125 | AT4G35790 | 0.476066667 | Protein with phospholipase D activity, Involved in phospolipase metabolism. |
| 126 | AT5G42960 | 0.471333333 | Unknown protein, molecular_function unknown |
| 127 | AT3G05590 | 0.470133333 | Encodes cytoplasmic ribosomal protein L18. |
| 128 | AT5G46800 | 0.467166667 | Seedling lethal mutation; Mitochondrial Carnitine Acyl Carrier-Like Protein |
| 129 | AT3G47370 | 0.466733333 | Ribosomal protein S10p/S20e family protein, structural constituent of ribosome |
| 130 | AT5G12470 | 0.462366667 | Protein of unknown function |
| 131 | AT1G43170 | 0.455533333 | Encodes a cytoplasmic ribosomal protein. The mRNA is cell-to-cell mobile. |
| 132 | AT3G18420 | 0.450733333 | Prenylyltransferase superfamily protein, biological_process unknown, in chloroplast envelope |
| 133 | AT2G17360 | 0.4417 | Ribosomal protein S4 (RPS4A) family protein; structural constituent of ribosome |
| 134 | AT4G30190 | 0.439266667 | belongs to the P-type ATPase superfamily of cation-transporting ATPases |
| 135 | AT4G35250 | 0.435566667 | NAD(P)-binding Rossmann-fold superfamily protein, binding, catalytic activity |
| 136 | AT3G52930 | 0.435133333 | Aldolase protein, copper ion binding in response to cadmium ion, to karrikin, to salt stress |
| 137 | AT5G62690 | 0.434 | Tubulin beta-2/beta-3 chain The mRNA is cell-to-cell mobile. |
| 138 | AT5G64040 | 0.419566667 | Encodes the only subunit of photosystem I located entirely in the thylakoid lumen. |
| 139 | AT3G13070 | 0.4151 | CBS domain-containing protein / transporter associated, molecular_function unknown |
| 140 | AT5G53540 | 0.413866667 | P-loop containing nucleoside triphosphate hydrolases superfamily protein |
| 141 | AT1G80300 | 0.407033333 | Encodes an ATP/ADP transporter. The mRNA is cell-to-cell mobile. |
| 142 | ATCG00800 | 0.403 | Chloroplast ribosomal protein S3, a constituent of the small subunit of the ribosomal complex |
| 143 | AT5G17170 | 0.3981 | Enhancer of sos3-1 (ENH1), electron carrier activity, metal ion binding, |

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|---|---|---|---|
| | | | chloroplast |
| 144 | AT5G17920 | 0.390933333 | Cytosolic cobalamin-independent methionine synthase, methionine regeneration via SAM cycle |
| 145 | AT5G59840 | 0.3821 | Ras-related small GTP-binding family protein, GTP binding, protein transport, |
| 146 | AT5G18380 | 0.3709 | Ribosomal protein S5 domain 2-like, structural constituent of ribosome, translation |
| 147 | AT2G38280 | 0.370633333 | Encodes a protein with in vitro AMP deaminase activity that is involved in embryogenesis. |
| 148 | AT2G18020 | 0.3697 | Embryo defective 2296 (EMB2296), structural constituent of ribosome |
| 149 | AT3G44880 | 0.368266667 | Pheide a oxygenase (PAO). Accelerated cell death (acd1) mutants show rapid |
| 150 | AT1G19800 | 0.366666667 | Permease-like protein involved in lipid transfer from the ER to the chloroplast |
| 151 | AT4G17170 | 0.366033333 | Member of RAB gene family |
| 152 | AT4G17390 | 0.3656 | Ribosomal protein L23/L15e, structural constituent of ribosome |
| 153 | AT2G38750 | 0.3644 | Annexins, calcium dependent membrane binding proteins, involved in Golgi mediated secretion. |
| 154 | AT4G31780 | 0.362833333 | Encodes an A-type monogalactosyldiacylglycerol (MGDG) synthase. |
| 155 | AT3G46830 | 0.361233333 | RAB GTPase homolog A2C (RABA2c), protein transport, small GTPase mediated signal transduction. |
| 156 | AT3G08510 | 0.354433333 | Phosphoinositide-specific phospholipase C, hydrolysis of phosphatidylinositol 4,5-bisphosphate |
| 157 | AT5G14220 | 0.3511 | PPO2, a putative protoporphyrinogen oxidase, also known as MEE61 (maternal effect embryo arrest 61) |
| 158 | AT1G71500 | 0.3462 | PSB33, plastid lineage, associated with the chloroplast provides stability to Photosystem II. |
| 159 | AT4G12800 | 0.3449 | Encodes subunit L of photosystem I reaction center. |
| 160 | AT1G56070 | 0.3444 | Translation elongation factor 2-like protein that is involved in cold-induced translation. |
| 161 | AT5G58870 | 0.340833333 | FtsH protease that is localized to the chloroplast |
| 162 | AT4G14960 | 0.3356 | Alpha-tubulin isoform required for right handed helical growth. |
| 163 | AT3G17390 | 0.3351 | S-adenosylmethionine synthetase |
| 164 | AT2G35800 | 0.334833333 | Encodes a predicted calcium-dependent S-adenosyl methionine carrier. |
| 165 | AT3G14110 | 0.331533333 | TPR domain containing protein,chloroplast membrane and is involved in chlorophyll biosynthesis. |
| 166 | AT2G26250 | 0.330833333 | Epidermis-specific, encodes KCS10, a putative 3-ketoacyl-CoA synthase. |
| 167 | AT3G05970 | 0.3306 | Encode peroxisomal long-chain acyl-CoA synthetase (LACS) isozymes |
| 168 | AT3G20370 | 0.325766667 | TRAF-like family protein, molecular_function unknown |
| 169 | AT1G32220 | 0.320666667 | NAD(P)-binding Rossmann-fold superfamily protein, coenzyme binding, binding, catalytic activity |
| 170 | AT5G64940 | 0.304733333 | ABC1K8, protein kinase family that is induced by heavy metals. |
| 171 | AT3G57090 | 0.304033333 | Similarity to yeast FIS proteins, membrane anchored proteins bind DRP prot. during organelle division. |
| 172 | AT5G10360 | 0.2939 | RPS6A and RPS6B are fully redundant and essential during gametogenesis. |
| 173 | AT5G61170 | 0.290433333 | Ribosomal protein S19e family protein, structural constituent of ribosome |

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| 174 | AT4G37270 | 0.287133333 | P1B-type ATPases, chloroplast envelope and is involved in the transport of Cu into chloroplasts. |
| 175 | AT4G29130 | 0.2762 | Hexokinase (HXK1) in the plant glucose-signaling network, glucose sensor to interrelate nutrient, light |
| 176 | AT1G70410 | 0.2743 | Putative beta-carbonic anhydrase betaCA4, regulates CO2-controlled stomatal movements in guard cells. |
| 177 | AT5G67560 | 0.271266667 | ARF-like GTPase family |
| 178 | AT4G28750 | 0.268766667 | Mutant has decreased effective quantum yield of photosystem II, Pale green plants, Reduced growth |
| 179 | AT5G02870 | 0.259266667 | Ribosomal protein L4/L1, structural constituent of ribosome |
| 180 | AT3G04840 | 0.250233333 | Ribosomal protein S3Ae, structural constituent of ribosome |
| 181 | AT4G00100 | 0.246266667 | Cytoplasmic ribosomal protein S13 homologue involved in early leaf development |
| 182 | AT3G09630 | 0.245766667 | Ribosomal protein L4/L1, constituent of ribosome |
| 183 | AT1G23310 | 0.244633333 | Protein having glyoxylate aminotransferase activity, peroxisome, involved in photorespiration |
| 184 | AT5G27770 | 0.243366667 | Ribosomal L22e protein family,Structural constituent of ribosome |
| 185 | AT3G12580 | 0.2278 | Heat shock protein 70 (HSP70), ATP binding |
| 186 | AT2G21870 | 0.222366667 | Encodes the FAd subunit of mitochondrial F1F0-ATP synthase. Essential for pollen formation. |
| 187 | AT1G18210 | 0.210566667 | Calcium-binding EF-hand, calcium ion binding |
| 188 | AT1G75780 | 0.178966667 | Beta tubulin gene downregulated by phyA-mediated far-red light high-irradiance and the phyB |
| 189 | AT5G23860 | 0.163066667 | Beta-tubulin, preferentially expressed in endodermal and phloem cells of primary roots |
| 190 | AT2G24520 | 0.1385 | H(+)-ATPase 5 (HA5), ATPase activity, cation transport, metabolic process, ATP biosynthetic process |
| 191 | AT1G58380 | 0.097266667 | XW6, structural constituent of ribosome, small ribosomal subunit, plasma membrane, chloroplast |

## Table 3. Reduced list of KOC-interacting proteins identified by mass-spectrometry in the NTAP-KOC1 TEV eluate.

The list of proteins obtained in Table 2 was reduced to identify putative KOC-interacting candidates. Some proteins were removed from the list: photosynthesis-associated proteins, proteins localized in other organelles than chloroplasts, proteins of known function other than import, ribosome, protease or transport.

| | Accession | (fmol) | Protein name |
|---|---|---|---|
| 1 | AT4G32250 | 34.9472 | KOC1, Protein kinase superfamily protein, kinase activity, ATP binding; in chloroplast |
| 2 | AT3G46740 | 8.04253333 | TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 75-III (TOC75-III) |
| 3 | AT4G02510 | 6.4308 | TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 159 (TOC159) |
| 4 | AT5G23060 | 6.12623333 | CALCIUM SENSING RECEPTOR (CaS) |
| 5 | ATCG01130 | 3.65786667 | (YCF1.2) protein binding, in chloroplast membrane |
| 6 | AT5G02940 | 3.47343333 | Protein of unknown function, in chloroplast envelope |
| 7 | AT1G06950 | 3.45323333 | TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 110 (TIC110) |
| 8 | AT1G02280 | 3.2534 | TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 33 (TOC33) |
| 9 | AT5G05000 | 3.00213333 | TRANSLOCON AT THE OUTER ENVELOPE MEMBRANE OF CHLOROPLASTS 34 (TOC34) |
| 10 | AT5G50920 | 2.77573333 | CLPC HOMOLOGUE 1 (CLPC1) |
| 11 | AT2G41840 | 2.6055 | Ribosomal protein S5 family protein, structural constituent of ribosome |
| 12 | AT2G43950 | 2.5375 | CHLOROPLAST OUTER ENVELOPE PROTEIN 37 (OEP37) |
| 13 | AT4G17530 | 2.14786667 | RAB GTPASE HOMOLOG 1C (RAB1C) |
| 14 | AT1G01790 | 2.00753333 | K+ EFFLUX ANTIPORTER 1 (KEA1) |
| 15 | AT3G17970 | 1.86883333 | TRANSLOCON AT THE OUTER MEMBRANE OF CHLOROPLASTS 64-III (TOC64-III) |
| 16 | AT2G36170 | 1.76756667 | UBIQUITIN EXTENSION PROTEIN 2 (UBQ2) |
| 17 | AT5G22640 | 1.74823333 | EMBRYO DEFECTIVE 1211 (emb1211) |
| 18 | AT1G10510 | 1.73596667 | EMBRYO DEFECTIVE 2004 (emb2004) |
| 19 | AT5G17090 | 1.69693333 | Cystatin/monellin superfamily protein |
| 20 | AT5G01590 | 1.65316667 | TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 56 (TIC56) |
| 21 | AT1G22780 | 1.60403333 | POINTED FIRST LEAVES (PFL) S18 ribosomal protein |
| 22 | AT1G33120 | 1.51756667 | Ribosomal protein L6 family, structural constituent of ribosome, rRNA binding. |
| 23 | AT2G43630 | 1.4308 | Unknown protein |
| 24 | AT5G23890 | 1.30993333 | Unknown protein |
| 25 | AT3G16290 | 1.217 | EMBRYO DEFECTIVE 2083 (EMB2083) ATPase, AAA+ type, core, Peptidase M41 |
| 26 | AT5G19620 | 1.1585 | OUTER ENVELOPE PROTEIN OF 80 KDA (OEP80) |
| 27 | AT3G04340 | 1.14196667 | EMBRYO DEFECTIVE 2458 (emb2458) ATPase, metalloendopeptidase activity |
| 28 | AT1G79560 | 1.09513333 | FTSH PROTEASE 12 (FTSH12) |
| 29 | AT5G24690 | 1.0896 | Unknown protein |
| 30 | AT5G53170 | 1.05953333 | FTSH PROTEASE 11 (FTSH11) |
| 31 | AT5G56030 | 1.0272 | HEAT SHOCK PROTEIN 81-2 (HSP81-2) |
| 32 | AT5G64580 | 0.99913333 | EMBRYO DEFECTIVE 3144 (EMB3144) ATPase protein, metalloendopeptidase activity |
| 33 | AT5G23040 | 0.99363333 | CELL GROWTH DEFECT FACTOR 1 (CDF1) |

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|---|---|---|---|
| 34 | AT2G30950 | 0.90883333 | VARIEGATED 2 (VAR2) Metalloprotease that functions in thylakoid biogenesis. |
| 35 | AT5G43745 | 0.8634 | Unknown protein |
| 36 | AT2G36160 | 0.846 | Ribosomal protein S11 family protein; structural constituent of ribosome |
| 37 | AT2G16640 | 0.78966667 | MULTIMERIC TRANSLOCON COMPLEX IN THE OUTER ENVELOPE MEMBRANE 132 (TOC132) |
| 38 | AT2G01250 | 0.78403333 | Ribosomal protein L30/L7 family protein; structural constituent of ribosome, |
| 39 | AT5G08540 | 0.7602 | Unknown protein |
| 40 | AT5G45775 | 0.73806667 | Ribosomal L5P family protein, structural constituent of ribosome |
| 41 | AT2G28900 | 0.73796667 | OUTER PLASTID ENVELOPE PROTEIN 16-1 (OEP16-1) |
| 42 | AT4G23940 | 0.72396667 | FTSH INACTIVE PROTEASE 1 (FTSHI1) |
| 43 | AT2G19730 | 0.72103333 | Ribosomal L28e protein family |
| 44 | AT2G24820 | 0.70706667 | TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 55-II (TIC55-II) |
| 45 | AT1G06430 | 0.6816 | FTSH PROTEASE 8 (ftsh8) |
| 46 | AT1G65260 | 0.62926667 | PLASTID TRANSCRIPTIONALLY ACTIVE 4 (PTAC4) |
| 47 | AT1G20010 | 0.6043 | TUBULIN BETA-5 CHAIN (TUB5) |
| 48 | AT3G25520 | 0.57326667 | RIBOSOMAL PROTEIN L5 (ATL5) |
| 49 | AT5G64816 | 0.5676 | Unknown protein |
| 50 | AT3G05560 | 0.56283333 | Ribosomal L22e protein, structural constituent of ribosome, cytosolic ribosome, ribosome |
| 51 | AT5G42270 | 0.5454 | VARIEGATED 1 (VAR1) conserved motif for ATPase and a metalloprotease characteristic |
| 52 | AT2G44640 | 0.49123333 | Unknown protein |
| 53 | AT2G44120 | 0.49103333 | Ribosomal protein L30/L7 family protein, structural constituent of ribosome |
| 54 | AT3G53560 | 0.49046667 | Tetratricopeptide repeat (TPR)-like family, binding, biological_process unknown |
| 55 | AT5G27540 | 0.48416667 | MIRO-RELATED GTP-ASE 1 (MIRO1) |
| 56 | AT5G16620 | 0.48346667 | TRANSLOCON AT THE INNER ENVELOPE MEMBRANE OF CHLOROPLASTS 40 (TIC40) |
| 57 | AT3G46780 | 0.48116667 | PLASTID TRANSCRIPTIONALLY ACTIVE 16 (PTAC16) |
| 58 | AT1G67430 | 0.48016667 | Ribosomal protein L22p/L17e family protein, structural constituent of ribosome |
| 59 | AT1G50250 | 0.4795 | FTSH PROTEASE 1 (FTSH1) |
| 60 | AT5G20290 | 0.47806667 | Ribosomal protein S8e family protein, structural constituent of ribosome |
| 61 | AT5G42960 | 0.47133333 | Unknown protein |
| 62 | AT3G05590 | 0.47013333 | RIBOSOMAL PROTEIN L18 (RPL18) |
| 63 | AT3G47370 | 0.46673333 | Ribosomal protein S10p/S20e family protein, structural constituent of ribosome |
| 64 | AT5G12470 | 0.46236667 | RETICULATA-RELATED 4 (RER4) |
| 65 | AT1G43170 | 0.45553333 | RIBOSOMAL PROTEIN 1 (RP1) |
| 66 | AT3G18420 | 0.45073333 | SLOW GREEN 1 (SG1) |
| 67 | AT2G17360 | 0.4417 | Ribosomal protein S4 (RPS4A) family protein; structural constituent of ribosome |
| 68 | AT5G59840 | 0.3821 | Ras-related small GTP-binding family protein, GTP binding, protein transport, |
| 69 | AT5G18380 | 0.3709 | Ribosomal protein S5 domain 2-like, structural constituent of ribosome, translation |
| 70 | AT2G18020 | 0.3697 | EMBRYO DEFECTIVE 2296 (EMB2296),structural constituent of ribosome |
| 71 | AT1G19800 | 0.36666667 | ATP-BINDING CASSETTE I14 (ABCI14) |
| 72 | AT4G17170 | 0.36603333 | RAB GTPASE HOMOLOG B1C (RABB1C) |
| 73 | AT4G17390 | 0.3656 | Ribosomal protein L23/L15e, structural constituent of ribosome |
| 74 | AT3G46830 | 0.36123333 | RAB GTPASE HOMOLOG A2C (RABA2c) |
| 75 | AT5G58870 | 0.34083333 | FTSH PROTEASE 9 (ftsh9) |

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|---|---|---|---|
| 76 | AT3G57090 | 0.30403333 | (BIGYIN) membrane anchored proteins bind DRP proteins during organelle division |
| 77 | AT5G10360 | 0.2939 | EMBRYO DEFECTIVE 3010 (EMB3010) |
| 78 | AT5G61170 | 0.29043333 | Ribosomal protein S19e family protein, structural constituent of ribosome |
| 79 | AT5G67560 | 0.27126667 | ADP-RIBOSYLATION FACTOR-LIKE A1D (ARLA1D) |
| 80 | AT5G02870 | 0.25926667 | RIBOSOMAL LARGE SUBUNIT 4 (RPL4) |
| 81 | AT3G04840 | 0.25023333 | Ribosomal protein S3Ae, structural constituent of ribosome |
| 82 | AT4G00100 | 0.24626667 | RIBOSOMAL PROTEIN S13A (RPS13A) |
| 83 | AT3G09630 | 0.24576667 | SUPPRESSOR OF ACAULIS 56 (SAC56) Ribosomal protein L4/L1, constituent of ribosome |
| 84 | AT5G27770 | 0.24336667 | Ribosomal L22e protein family,Structural constituent of ribosome |
| 85 | AT3G12580 | 0.2278 | HEAT SHOCK PROTEIN 70 (HSP70) |
| 86 | AT1G75780 | 0.17896667 | TUBULIN BETA-1 CHAIN (TUB1) |
| 87 | AT5G23860 | 0.16306667 | TUBULIN BETA 8 (TUB8) |
| 88 | AT1G58380 | 0.09726667 | (XW6) structural constituent of ribosome, small ribosomal subunit, |
| Protein implicates in the importation in chloroplasts | | | |
| Ribosomal proteins | | | |
| Protease | | | |
| Protein implicates in transport | | | |
| Protein candidate | | | |
| Other proteins | | | |

## Table 4: Filtered list of KOC-interacting proteins and quantification by MSE.

Proteins interacting with KOC were quantified by MSE and their quantities related to their abundance in a chloroplast reference proteome. The enrichment index is arbitrary and calculated from the abundance of the protein in the eluates divided by their abundance in complete chloroplasts without prior enrichment. Only those proteins identified in two biological replicates, no detection in the negative controls, an enrichment index above 1 and an absolute abundance of at least 2% of the bait were retained in the list and are displayed here.

| Identifier | Annotation | fmol on column (negative control, replicate 1) | fmol on column (eluate, replicate 1) | fmol on column (negative control, replicate 2) | fmol on column (eluate, replicate 2) | Complete chloroplast reference | Complete chloroplast reference (fmol) | Enrichment index 1st replicate | Enrichment index 2nd replicate | avrg enrichment index | avrg fmol in eluate | % of bait |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT4G32250 | Protein kinase superfamily protein; FUNCTIONS IN: protein kinase activity, kinase activity, ATP binding; INVOLVED IN: protein amino acid phosphorylation; LOCATED IN: chloroplast; | 0.62613333333 | 34.9472 | 0.33516666667 | 10.9877 | not detected | n.a. | n.a. | n.a. | infinite | 22.96745 | 100 |
| AT4G02510 | An integral membrane GTPase that functions as a transit-sequence receptor required for the import of proteins necessary for chloroplast biogenesis. Located in the outer chloroplast membrane. | N.D. | 6.4308 | N.D. | 1.45093333 | not detected | n.a. | n.a. | n.a. | infinite | 3.94086667 | 17.1584859 |
| AT5G53170 | encodes an FtsH protease that is localized to the chloroplast and the mitochondrion | N.D. | 1.05953333 | N.D. | 2.45403333 | not detected | n.a. | n.a. | n.a. | infinite | 1.75678333 | 7.64901342 |
| AT1G22530 | PATELLIN 2 (PATL2); FUNCTIONS IN: transporter activity; INVOLVED IN: transport; LOCATED IN: plasma membrane, chloroplast | N.D. | 1.61993333 | N.D. | 1.7089 | not detected | n.a. | n.a. | n.a. | infinite | 1.66441667 | 7.24685007 |
| AT5G13490 | Encodes mitochondrial ADP/ATP carrier | N.D. | 2.2424 | N.D. | 0.9345 | not detected | n.a. | n.a. | n.a. | infinite | 1.58845 | 6.91609212 |
| AT4G17530 | AtRabD2c encodes a Rab GTPase, which plays important roles in pollen development, germination and tube elongation. | N.D. | 2.14786667 | N.D. | 0.4689 | not detected | n.a. | n.a. | n.a. | infinite | 1.30838333 | 5.69668524 |
| AT2G41840 | Ribosomal protein S5 family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic small ribosomal subunit, cytosolic ribosome, nucleolus, membrane | N.D. | 2.6055 | N.D. | | not detected | n.a. | n.a. | n.a. | infinite | 1.30275 | 5.67215777 |
| AT5G22640 | EMB1211 is a MORN (multiple membrane occupation and recognition nexus) motif containing protein involved in embryo development and chloroplast biogenesis. The mRNA is cell-to-cell mobile. | N.D. | 1.74823333 | N.D. | 0.74466667 | not detected | n.a. | n.a. | n.a. | infinite | 1.24645 | 5.42702825 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT2G36170 | Ubiquitin supergroup;Ribosomal protein L40e; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic large ribosomal subunit, nucleolus; | N.D. | 1.76756667 | N.D. | 0.56953333 | not detected | n.a. | n.a. | n.a. | infinite | 1.16855 | 5.08785259 |
| AT1G72150 | novel cell-plate-associated protein that is related in sequence to proteins involved in membrane trafficking in other | N.D. | 1.2719 | N.D. | 1.01236667 | not detected | n.a. | n.a. | n.a. | infinite | 1.14213333 | 4.97283474 |
| AT3G05590 | Encodes cytoplasmic ribosomal protein L18. | N.D. | 0.47013333 | N.D. | 1.681 | not detected | n.a. | n.a. | n.a. | infinite | 1.07556667 | 4.68300428 |
| AT5G57350 | member of Plasma membrane H+-ATPase family | N.D. | 2.0477 | N.D. | | not detected | n.a. | n.a. | n.a. | infinite | 1.02385 | 4.45783054 |
| AT3G19820 | Involved in the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol. Brassinosteroids affect cellular elongation. Mutants have dwarf phenotype. DWF1 is a Ca2+ dependent calmodulin-binding protein. | N.D. | 1.804 | N.D. | 0.23593333 | not detected | n.a. | n.a. | n.a. | infinite | 1.01996667 | 4.44092255 |
| AT1G22780 | S18 ribosomal protein involved in the binding of f-Met tRNA during initiation of mRNA translation. | N.D. | 1.60403333 | N.D. | 0.34843333 | not detected | n.a. | n.a. | n.a. | infinite | 0.97623333 | 4.25050815 |
| AT2G45820 | Remorin family protein; FUNCTIONS IN: DNA binding; INVOLVED IN: biological_process unknown;LOCATED IN: plasma membrane; | N.D. | 1.3562 | N.D. | 0.58653333 | not detected | n.a. | n.a. | n.a. | infinite | 0.97136667 | 4.22931874 |
| ATMG01190 | ATPase subunit 1 | N.D. | 1.4399 | N.D. | 0.37563333 | not detected | n.a. | n.a. | n.a. | infinite | 0.90776667 | 3.95240511 |
| AT1G33120 | Ribosomal protein L6 family; FUNCTIONS IN: structural constituent of ribosome, rRNA binding; INVOLVED IN: translation; LOCATED IN: in 7 components | N.D. | 1.51756667 | N.D. | 0.19406667 | not detected | n.a. | n.a. | n.a. | infinite | 0.85581667 | 3.72621543 |
| AT3G04340 | embryo defective 2458 (emb2458); FUNCTIONS IN: nucleoside-triphosphatase activity, ATPase activity, metalloendopeptidase activity, nucleotide binding, ATP binding; INVOLVED IN: embryo development ending in seed dormancy; LOCATED IN: chloroplast | N.D. | 1.14196667 | N.D. | 0.2685 | not detected | n.a. | n.a. | n.a. | infinite | 0.70523333 | 3.07057742 |
| AT1G79560 | Encodes an FtsH protease that is localized to the chloroplast. Mutations in this locus result in embryo lethality. | N.D. | 1.09513333 | N.D. | 0.30153333 | not detected | n.a. | n.a. | n.a. | infinite | 0.69833333 | 3.0405349 |
| AT5G43745 | Protein of unknown function (DUF1012); LOCATED IN: chloroplast, chloroplast envelope; | N.D. | 0.8634 | N.D. | 0.4881 | not detected | n.a. | n.a. | n.a. | infinite | 0.67575 | 2.94220734 |
| AT5G22830 | Transmembrane magnesium transporter. One of nine family members. | N.D. | 0.975 | N.D. | 0.3706 | not detected | n.a. | n.a. | n.a. | infinite | 0.6728 | 2.92936308 |
| AT5G64580 | AAA-type ATPase family protein; FUNCTIONS IN: nucleoside-triphosphatase activity, metalloendopeptidase activity, nucleotide binding, ATP binding; INVOLVED IN: embryo development; LOCATED IN: chloroplast, chloroplast envelope | N.D. | 0.99913333 | N.D. | 0.26546667 | not detected | n.a. | n.a. | n.a. | infinite | 0.6323 | 2.75302657 |
| AT5G42480 | Shows homology to the cyanobacterial cell division protein Ftn2, mutant only has two mesophyll cell chloroplasts. Protein was localized to a ring at the center of the chloroplasts. | N.D. | 0.9325 | N.D. | 0.18863333 | not detected | n.a. | n.a. | n.a. | infinite | 0.56056667 | 2.4407005 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT2G36160 | Ribosomal protein S11 family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic small ribosomal subunit, cytosolic ribosome, plasma membrane, chloroplast, membrane; | N.D. | 0.846 | N.D. | 0.2729 | not detected | n.a. | n.a. | n.a. | infinite | 0.55945 | 2.43583855 |
| AT3G01290 | SPFH/Band 7/PHB domain-containing membrane-associated protein family; INVOLVED IN: N-terminal protein myristoylation; LOCATED IN: mitochondrion, plasma membrane, vacuole, membrane | N.D. | 0.51386667 | N.D. | 0.59723333 | not detected | n.a. | n.a. | n.a. | infinite | 0.55555 | 2.41885799 |
| AT5G03910 | member of ATH subfamily | N.D. | 0.8718 | N.D. | 0.23696667 | not detected | n.a. | n.a. | n.a. | infinite | 0.55438333 | 2.41377834 |
| AT5G56030 | A member of heat shock protein 90 (HSP90) gene family. Expressed in all tissues and abundant in root apical meristem, | N.D. | 1.0272 | N.D. | 0.04696667 | not detected | n.a. | n.a. | n.a. | infinite | 0.53708333 | 2.33845435 |
| AT5G14030 | translocon-associated protein beta (TRAPB) family protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; | N.D. | 0.8145 | N.D. | 0.18113333 | not detected | n.a. | n.a. | n.a. | infinite | 0.49781667 | 2.16748776 |
| AT2G19730 | Ribosomal L28e protein family; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation, ribosome | N.D. | 0.72103333 | N.D. | 0.2547 | not detected | n.a. | n.a. | n.a. | infinite | 0.48786667 | 2.12416558 |
| AT4G23940 | Encodes FtsHi1. Localizes to the chloroplast envelope membrane. Functions in chloroplast biogenesis and division. | N.D. | 0.72396667 | N.D. | 0.25003333 | not detected | n.a. | n.a. | n.a. | infinite | 0.487 | 2.12039212 |
| AT5G45775 | Ribosomal L5P family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic large ribosomal subunit, vacuole | N.D. | 0.73806667 | N.D. | 0.22373333 | not detected | n.a. | n.a. | n.a. | infinite | 0.4809 | 2.09383279 |
| AT3G61260 | Remorin family protein; FUNCTIONS IN: binding; INVOLVED IN: biological_process unknown; LOCATED IN: plasma membrane, vacuole; EXPRESSED IN: 24 plant structures; EXPRESSED | N.D. | 2.45243333 | N.D. | 1.38516667 | not detected | 0.077866667 | 31.4952911 | 17.7889555 | 24.6421233 | 1.9188 | 8.35443203 |
| AT3G17970 | Integral chloroplast outer membrane protein. Belongs to one of the 36 carboxylate clamp (CC)- tetratricopeptide repeat (TPR) proteins (Prasad 2010, Pubmed ID: 20856808) with potential to interact with Hsp90/Hsp70 as co-chaperones. | N.D. | 1.86883333 | N.D. | 0.5175 | AT3G17970 | 0.109733333 | 17.0306804 | 4.71597813 | 10.8733293 | 1.19316667 | 5.19503326 |
| AT5G05000 | Outer membrane GTPase protein that may function in import of nuclear encoded proteins into the chloroplast. Phosphorylation of the G-domains regulate translocon assembly | N.D. | 3.00213333 | N.D. | 1.28286667 | AT5G05000 | 0.216633333 | 13.858132 | 5.92183413 | 9.88998307 | 2.1425 | 9.32841913 |
| AT5G42020 | Luminal binding protein (BiP2) involved in polar nuclei fusion during proliferation of endosperm nuclei. | N.D. | 1.8882 | N.D. | 0.65423333 | AT5G42020 | 0.1303 | 14.4911742 | 5.02097723 | 9.75607572 | 1.27121667 | 5.53486202 |
| ATCG01130 | YCF1.2; FUNCTIONS IN: protein binding; LOCATED IN: chloroplast, membrane; EXPRESSED IN: cultured cell, leaf; CONTAINS InterPro DOMAIN/s: Ycf1 (InterPro:IPR008896); BEST Arabidopsis thaliana protein match is: Ycf1 protein (TAIR:ATCG01000.1). | N.D. | 3.65786667 | N.D. | 0.7971 | ATCG01130 | 0.265366667 | 13.784198 | 3.00376837 | 8.39398317 | 2.22748333 | 9.69843554 |
| AT3G49860 | A member of ARF-like GTPase family. A thaliana has 21 members, in two subfamilies, ARF and ARF-like (ARL) GTPases. | N.D. | 0.51023333 | N.D. | 0.1792 | AT3G49860 | 0.071166667 | 7.16955504 | 2.51803279 | 4.84379391 | 0.34471667 | 1.5008922 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT1G10510 | embryo defective 2004 (emb2004); INVOLVED IN: embryo development ending in seed dormancy; LOCATED IN: mitochondrion, chloroplast, plastid, chloroplast envelope | N.D. | 1.73596667 | N.D. | 0.48246667 | AT1G10510 | 0.336333333 | 5.16144698 | 1.43448959 | 3.29796829 | 1.10921667 | 4.82951597 |
| AT1G02280 | Encodes a GTP-binding GTP-ase. Component of the chloroplast protein import machinery. Required for import of POR B into plastids. Toc33 phosphorylation may not play an important role in vivo. | N.D. | 3.2534 | N.D. | 0.7088 | AT1G02280 | 0.653133333 | 4.98121874 | 1.08523017 | 3.03322446 | 1.9811 | 8.62568548 |
| AT3G18420 | Protein prenylyltransferase superfamily protein; FUNCTIONS IN: binding; INVOLVED IN: biological_process unknown; LOCATED IN: chloroplast, chloroplast envelope | N.D. | 0.45073333 | N.D. | 0.30356667 | AT3G18420 | 0.145233333 | 3.10351159 | 2.09019968 | 2.59685563 | 0.37715 | 1.64210655 |

# Publication

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# The Novel Chloroplast Outer Membrane Kinase KOC1 Is a Required Component of the Plastid Protein Import Machinery

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Running title: *KOC1 kinase of the chloroplast import machinery*

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## ABSTRACT

The biogenesis and maintenance of cell organelles such as mitochondria and chloroplasts requires the import of many proteins from the cytosol, a process that is controlled by phosphorylation. In the case of chloroplasts, the import of hundreds of different proteins depends on Translocons at the Outer and Inner Chloroplast membrane (TOC and TIC, respectively) complexes. The essential protein TOC159 functions thereby as an import receptor. It has an N-terminal acidic (A) domain that extends into the cytosol, controls receptor specificity, and is highly phosphorylated *in vivo*. However, kinases that phosphorylate the TOC159 A-domain to enable protein import have remained elusive. Here, using co-purification with TOC159 from Arabidopsis, we discovered a novel component of the chloroplast import machinery, the regulatory Kinase of the Outer Chloroplast membrane 1 (KOC1). We found that KOC1 is an integral membrane protein facing the cytosol and stably associates with TOC. Moreover, KOC1 phosphorylated the A-domain of TOC159 *in vitro*, and in mutant *koc1* chloroplasts, pre-protein import efficiency was diminished. *koc1* Arabidopsis seedlings had reduced survival rates after transfer from the dark to the light in which protein import into plastids is required to rapidly complete chloroplast biogenesis. In summary, our data indicate that KOC1 is a functional component of the TOC machinery that phosphorylates import receptors, supports pre-protein import, and contributes to efficient chloroplast biogenesis.

Biogenesis and maintenance of the chloroplast requires the import of proteins from the cytosol. During evolution, the majority of chloroplast genes were lost or transferred to the nucleus (1). Chloroplast genes that were successfully transferred to the nucleus acquired additional sequences that encode cleavable N-terminal targeting peptides, known as transit peptides (2). Cytoplasmic ribosomes synthesize the preproteins (chloroplast protein with a transit peptide). The transit sequence is recognized by the chloroplast protein import machinery, which initiates import. It consists of translocon complexes at the outer (TOC) and inner membrane of the chloroplast (TIC) (3–5).

The TOC core in Arabidopsis contains three components, TOC159, TOC33 and TOC75. TOC159 and TOC33 are exposed to the cytoplasm and function as preprotein co-receptors (6–8). TOC75 embedded in the outer membrane forms a protein-conducting channel (9). The central component of the TIC complex is TIC20 that contributes to the protein conducting channel across the inner membrane (10). During preprotein translocation, TIC20 associates with other TIC components including TIC110 and TIC40 that recruits chaperones to the exit of the TIC complex (11, 12). The ClpC and cpHsp70 chaperones have both been reported to provide the driving force for preprotein import into the chloroplast (13–15). Recently, a 1MD complex has been described: in addition to

TIC20 it includes TIC56, TIC100 as well as the chloroplast encoded TIC214 (YCF1) (16, 17). The 1MD complex associates with the translocating preprotein and forms a preprotein sensitive channel when reconstituted into planar lipid bilayers. TIC components (both absent and present in the 1 MD complex) co-purified with TOC159 suggesting that they cooperate in preprotein import (17).

TOC159 and TOC33 belong to a small family of GTP-binding proteins sharing homology in their GTP-binding domains (18, 19). In Arabidopsis TOC33 has one homolog (TOC34) and TOC159 has three, TOC90, -120 and -132 (20, 21). In addition, to the GTP-binding (G-) domain the TOC159 homologs have a C-terminal membrane anchoring (M-) domain and a N-terminal acidic (A-) domain (7, 22).

The functions of the A-domain are not completely understood. A domain swapping study (23) analyzing the roles of the A-domains of TOC159 and TOC132 indicated that they mediate pre-protein specificity (TOC159 specializing in photosynthesis-associated proteins and TOC132 in house-keeping plastid proteins). Removal of the A-domain reduced pre-protein specificity resulting in receptors with overlapping specificity (23). In contrast to the G- and M-domains, the A-domain is dispensable *in vivo* (24–26). TOC159 is present with and without the A-domain in isolated chloroplasts and may be cleaved by an unknown protease. The ratio between the cleaved and uncleaved forms is unknown.

Regulation of protein import has been studied in the past. TOC159 and TOC33 are GTP-binding proteins, suggesting regulation of import by a GTPase cycle (19, 27). Specific point mutations in the GTP-binding motifs (reducing binding or hydrolysis of GTP) altered protein import kinetics but did not result in visible phenotypes (26, 28, 29).

In addition to GTP, ubiquitination and phosphorylation affect the TOC159 homologs (22). Ubiquitination and degradation by the ubiquitin proteasome system (UPS) play an important role during plastid developmental transitions (for example from non-photosynthetic etioplasts in the dark to active chloroplasts in the light) (30). To achieve this, the composition of the protein import machinery is modified to accommodate massive import of photosynthesis-associated proteins. This implicates degradation by the UPS of one kind of TOC159 homolog and replacement by another (31). The chloroplast outer membrane RING-type E3-ligase SP1 mediates the ubiquitination reaction (30). SP1 also mediates the depletion of the TOC components by the UPS under conditions inducing oxidative stress. This diminishes import of photosystem components and thereby limits production of harmful reactive oxygen species (32).

Phosphorylation has been reported to regulate the activity of the pea homologs of both TOC33 (psTOC34) and TOC159 (psTOC159) (33, 34). psTOC34 is the target of a protein kinase at the outer membrane. The phosphorylation of psTOC34 decreases the affinity of psTOC34 for GTP and regulates the GTP-dependent interaction with the preprotein. The interaction between TOC34 and the preprotein is strongly enhanced by phosphorylation of the transit peptide (35). An outer envelope kinase of 98 kD (OEK98) has been implicated in the phosphorylation of psTOC34 but has not been identified (33). In Arabidopsis, TOC33 can be phosphorylated at S181 (36). However, mutation of this residue to either non-phosphorylable alanine or to phospho-mimick aspartate or glutamate had no detectable effect *in vivo* (36). The G-domain of psTOC159 is the target of an outer envelope kinase of 70 kD (OEK70). Phosphorylation by OEK70 inhibits association of TOC159 with the TOC complex (33).

Recent large-scale phosphoproteomics projects in Arabidopsis revealed hyperphosphorylation of the A-domain (PhosphAT 4.0) but provided no evidence for the phosphorylation of either the G- and M-domains of TOC159. The A-domain has many predicted and experimentally identified casein kinase II (CKII) sites. CKII efficiently phosphorylates recombinant A-domain *in vitro* (37). However, some of the phosphorylation sites within the A-domain do not resemble CKII sites suggesting that additional kinases may be involved. SnRK2 (Sucrose nonfermenting 1 (SNF1)-related protein kinase 2) phosphorylates T692 in response to ABA (abscisic acid) activation (38). Interestingly, ABA-dependent phosphorylation of TOC159 homologs, TOC132 and -120, was also reported but only in the SnRK2 deficient mutant background. Wang and colleagues proposed that chloroplast protein import activity may be regulated by ABA via SnRK2 dependent phosphorylation of the A-domain (38). Additional A-domain kinases other than CKII and SnRK2 have been predicted but not identified (37).

Here, a TAP (tandem affinity purification)-tagged version of TOC159 (NTAP-TOC159) was used to co-isolate new interaction partners by IgG-affinity chromatography. This approach has already proven useful in the identification of TIC56 in a TOC159-containing super complex (17). Here, we identify KOC1 as a new component in this complex

and show that it is required for full import activity and successful de-etiolation.

## RESULTS

*KOC1 Co-purifies with TOC159*–Arabidopsis plants (NTAP-TOC159:*ppi2*) expressing N-terminally TAP-tagged TOC159 were used to isolate potential TOC159 interactors by IgG-affinity chromatography (17). Plants expressing the TAP-tag alone (TAP:WT) were used as a negative control. The TAP-tag contains an IgG-binding domain separated from a calmodulin-binding peptide (CBP) sequence by a tobacco etch mosaic virus (TEV) protease site. TX-100 detergent extracts were prepared and subjected to IgG-affinity chromatography. TEV protease elution was applied to release either TOC159 still carrying the CBP (together with any interacting proteins) or the negative control CBP. Proteins in the TEV eluates were identified by mass spectrometry (17). The mass spectrometric data revealed a member of the protein kinase superfamily protein (AT4G32250) that we named KOC1 (*K*inase at the *O*uter membrane of the *C*hloroplast *1*). KOC1 co-purified with NTAP-TOC159 but not with TAP control (Fig. 1). Specific antibodies against recombinant KOC1 were raised in rabbit and affinity purified.

To confirm the association of KOC1 with the TOC159 complex, aliquots of the sequential steps of the IgG affinity purification experiment were separated by SDS-PAGE and analyzed by Western blot (Fig. 1). A 66 kD band corresponding to the expected size of KOC1 was detected in the total detergent extract loads (L) and in the flow-throughs (Ft) of the NTAP-TOC159 and TAP:WT samples (Fig. 1). The KOC1 band was present only in the TEV eluate of the NTAP-TOC159 purification but not that of the TAP-tag negative control (TAP:WT). In addition, TOC75 and -33 as well as TIC110 co-purified with NTAP-TOC159 (Fig. 1). The thylakoid and plastoglobule marker proteins, LHCB2 and FBN1a (PGL35), were detected by Western blotting in the load (L) and flow through (Ft) fractions but not in the TEV eluates.

To confirm the interaction of KOC1 with the TOC complex proteins, NTAP-KOC1:*koc1-1* plants (see below) were used for IgG-affinity purification. KOC1 was present in the TEV eluate (Fig. 2A). Due to TEV cleavage it had a noticeably smaller size than NTAP-KOC1 in the total extract (T) and the load (L). TOC159, TOC75, TOC33 and TIC56 were also detected in the eluate of NTAP-KOC1 but not in the eluate of the negative control TAP:WT. The thylakoid marker protein LHCB2 was not detectable in the eluate. Altogether these results demonstrate that KOC1 associates with TOC complex *in vivo*.

The TEV eluate of the NTAP-KOC1 purification experiment was subjected to quantitative mass spectrometry. The experiment was carried out in two biological replicates. A list of common proteins in the two experiments was created and contained 191 proteins (not shown). Among the 15 most abundant interaction candidates were 7 TOC and TIC components. In order of decreasing abundance in femtomoles these were TOC75, TOC159, YCF1.2, TIC110, TOC33, TOC34 and ClpC. When the protein list established for NTAP-KOC1 was compared to a list for NTAP-TOC159 consisting of 43 proteins weighted for enrichment a high overlap of 83% was detected. Notably, 11 known components of the TOC and TIC complexes as well as components of the 1 MDa complex were represented in this list and for which the overlap between TOC159 and KOC1 protein list was complete.

We filtered the list of KOC1-interacting proteins by protein abundance and enrichment factor to identify specific interactions at high stringency (Table S1). We first selected proteins that were identified in both biological replicates but not in the negative controls. We then calculated an enrichment factor from the ratio between protein abundance in isolated chloroplasts and in the TAP-purified fraction of KOC interacting proteins and retained those proteins with an enrichment factor >1 in the list. And lastly, we requested interacting proteins to have a minimum abundance of 2% of the bait protein (i.e. KOC1) to select for specific KOC/protein interactions. This filtering resulted in 51 proteins that fulfill requirements (Table S1). These proteins were plotted into an interaction network using the STRING database (STRING Version 10.0 software (http://string-db.org)). This analysis identified three complexes interacting with KOC1, the TOC-complex, an FtsH/FtsHi protease complex and the cytoplasmic ribosome (Fig. 2B).

*KOC1 is a Predicted Transmembrane Kinase*–KOC1 has 611 amino acids residues. It contains a kinase domain (amino acids 39-306) predicted by the Prosite software (http://prosite.expasy.org). The C-terminal half of KOC1 contains a HERC2-related region (HECT (**H**omologous to the **E**6-AP **C**arboxyl **T**erminus) and RLD (**R**CC1-**l**ike **d**omain) containing E3 Ubiquitin Protein Ligase 2, amino acids 376-531). The HERC2-related region in KOC1 is followed by a 24 amino acid stretch (549-572) enriched in hydrophobic amino acids predicted to form a transmembrane helix (TM) (Fig. 3A) by the TMpred

software (http://embnet.vital-it.ch/software/TMPRED_form.html). The ChloroP software (http://www.cbs.dtu.dk/services/ChloroP/) does not predict a transit peptide (Fig. 3B). Still, KOC1 was identified in chloroplast proteome studies (39, 40).

KOC1 most closely resembles the KEG (KEEP ON GOING) protein (AT5G13530), a RING E3 ubiquitin ligase. In addition to the RING sequence, KEG contains ankyrin and HERC2-related repeats and a kinase domain (41). The RING sequence and the ankyrin repeats are absent from KOC1. But, both the kinase domain and the HERC2-related region in KOC1 are homologous to those in KEG. The sequence analysis suggests that KOC1 functions as a kinase and an implication in ubiquitination also appears remotely possible.

*Isolation of koc1 Mutants and NTAP-KOC1:koc1-1 Plants*–To obtain information on the function of KOC1 *in vivo*, T-DNA mutant collections were searched for insertions in the KOC1 gene (AT4G32250). Two independent mutant lines, SALK_083378 termed: *koc1-1* and SALK_051823 termed: *koc1-2*, were identified and homozygous lines were isolated (Fig. S1, Fig. 3C-D). The *koc1-1* line contained a single T-DNA insertion at 841 base pairs after the start codon. The *koc1-2* line contained a double T-DNA insertion at 2269 base pairs after the start codon (Fig. S1).

Immunoblotting using KOC1 antibodies demonstrated the absence of KOC1 protein (Fig. 3D) and confirmed the knockout nature of the mutants. NTAP-KOC1:*koc1-1* plants were obtained by introducing a T-DNA construct encoding a N-terminally TAP tagged KOC1 (NTAP-KOC1) in *koc1-1* mutant background. The *koc1-1* and *koc1-2* mutants as well as the homozygous NTAP-KOC1:*koc1-1* plants displayed a wild type phenotype under standard growth conditions (long-day: 16 hours light: 8 hours dark) (Fig. 3C).

*TOC Components Accumulate Normally in koc1 Mutants*–Sequence analysis of KOC1 revealed two potential biochemical functions: that of a kinase and potentially that of a factor in ubiquitination and subsequent proteasome-mediated degradation. Both potentially affect assembly and stability of TOC and TIC components. Therefore, the two *koc* mutants were compared with the wild type and NTAP-KOC1 overexpressing lines by Western blotting. The levels of the components of the TOC core complex (TOC159, -75 and -33) as well as TOC120 and TIC110 appeared unchanged in the *koc1* mutants. This was more difficult to judge in the overexpressing line due to the proximity of NTAP-containing bands that give strong signals with any kind of IgG and which precluded quantification. Visibly, however, no major changes appeared to occur (Fig. 3D). The results suggest that the TOC and TIC components are normally assembled and stable in the *koc1* and overexpressing backgrounds.

*KOC1 Localizes at the Outer Chloroplast Membrane*–For *in vivo* localization, isolated Arabidopsis wild type protoplasts were transformed with a vector (pEG104-N-YFP-KOC1) coding for KOC1 with a N-terminal YFP tag (YFP-KOC1), with vectors pEG101-C-YFP-EMB2004 and pCL60-GFP and analyzed by confocal microscopy. Transient expression of EMB2004 (an envelope protein at the inner membrane that also co-isolated with KOC1) fused to YFP (EMB2004-YFP) and GFP alone. Chloroplasts were identified by red chlorophyll autofluorescence (Fig. 4A, Chlorophyll). YFP-KOC1 gave a ring-like fluorescence pattern (Fig. 4A, YFP-KOC1). The merge between the YFP-KOC1 and chlorophyll signal shows that YFP-KOC1 was localized at the chloroplast periphery (Fig. 4A, Merge) EMB2004-YFP gives a fluorescence pattern strongly resembling that of YFP-KOC1 (Fig. S3), whereas GFP results in a pattern typical for the cytosol and is distinct from that of YFP-KOC1 (Fig. S3).

*KOC1 is Present in the Chloroplast Envelope Fraction*–To localize NTAP-KOC1 in chloroplast membrane compartments, chloroplasts were isolated from NTAP-KOC1:*koc1-1* plants. A total chloroplast membrane fraction was prepared and separated into thylakoids, envelope membranes and plastoglobules by floatation on a linear 5-45% sucrose gradient. Fractions were analyzed by Western blotting (Fig. 4B). LHCB2 was present mostly in fractions 27-37 indicating thylakoids. FIB1a/PGL35 was found in fractions 1-13 indicating the presence of plastoglobules. FIB1a/PGL35 was also detected in denser fractions (23-31) likely due to plastoglobule association with thylakoids. NTAP-KOC1 was detected mostly in fractions 17-31 and was well separated from the fractions enriched in thylakoid and plastoglobule markers. Moreover, TOC159 and TOC75 co-fractionated with NTAP-KOC1 (Fig. 4B) supporting KOC1 localization at the chloroplast envelope.

*KOC1 is Exposed at the Chloroplast Surface*–To investigate KOC1 localization at the chloroplast envelope, we treated isolated chloroplasts with thermolysin protease (Fig. 4C). Thermolysin degrades surface-exposed proteins but does not access the intermembrane space. Thermolysin treated chloroplasts were separated by SDS-PAGE and probed by immunoblotting. The KOC1 band was strongly diminished by thermolysin

(Thermolysin +) whereas the known thermolysin-resistant outer membrane protein TOC75 and the inner membrane protein TIC40 were largely unaffected (Fig. 4C), indicating that KOC1 was accessible at the outer surface of the outer envelope membrane.

*KOC1 is an Integral Membrane Protein*–To analyze the membrane association of KOC1, extraction experiments were carried out. Isolated chloroplasts were extracted with alkaline carbonate buffer ($Na_2CO_3$) or solubilized with Triton X-100 (Fig. 4C). Upon centrifugation of the alkaline carbonate extraction, KOC1 remained in the pellet fraction (P) but was found in the supernatant (S) after Triton X-100 solubilization. The known integral membrane proteins TOC75 and TIC40 behaved in the same way. We therefore conclude that KOC1 is an integral membrane protein.

*KOC1 Phosphorylates the A domain of TOC159 in vitro*–The A-domain of TOC159 is hyperphosphorylated *in vivo*. To test whether KOC1 kinase phosphorylates TOC159A, we isolated KOC1 from NTAP-KOC1:*koc1-1* plants (see above) and performed an *in vitro* phosphorylation experiment on recombinant TOC159-A in the presence of radioactive ATP. The experiment resulted in strong phosphorylation of TOC159A (Fig. 5A, lane 1). The control experiments carried out with KOC1 and TOC159A alone did not result in detectable phosphorylation (Fig. 5A, lanes 2-3) whereas denatured KOC1 showed slight residual activity (Fig. 5A, lane 4). Recombinant KOC1 purified from *E. coli* bacteria also phosphorylated TOC159A *in vitro* (Fig. 5B) providing additional evidence for the kinase function. The A-domains of the TOC159 homologs, TOC120 and TOC132 are also known phosphoproteins and recombinant TOC120A and -132A were phosphorylated by KOC1 kinase *in vitro* (Fig. 5C, lanes 2-3). Altogether the *in vitro* phosphorylation experiments demonstrate that TOC159 and its homologs are substrates of KOC1 kinase *in vitro*.

*A Large Percentage of TOC159 Exists as the Full-Length Protein*–TOC159 occurs both with and without its A-domain but the ratio of the two forms is unknown. Regulation of protein import at the A-domain is only plausible if the A-domain is present. To determine to what extent this is the case we engineered a TOC159 construct encoding a N-terminal NTAP tag and a C-terminal myc-tag (Fig. 5D, Fig. S2). The C-terminal myc-tag allows to detect full length TOC159 (TOC159-FL) as well as TOC159 lacking the A-domain (TOC159GM). By Western blotting, this should result in two bands corresponding to TOC159-FL and TOC159GM. A Western blotting experiment was carried out on equal amounts of total protein of *ppi2*, WT, NTAP-TOC159:*ppi2* and NTAP-TOC159-cmyc:*ppi2* plants (Fig. 5D). To detect the myc-tag with minimal interference from the TAP tag (which binds IgG), the NTAP tag was first saturated with non-specific rabbit IgG. In a second incubation, the blot was incubated with mouse anti-myc antibodies and developed with goat-anti-mouse IgG coupled to HRP (Fig. 5D, anti-cmyc). The anti-myc antibodies detected two specific bands in the NTAP-TOC159-cmyc extract that were not present in NTAP-TOC159, one at above 200 kD and the other at around 100 kD corresponding to TOC159-FL and TOC159GM, respectively (Fig. 5D, lane 4).

The ratio between TOC159-FL and TOC159GM was around 2:1 indicating that around two thirds of TOC159 exist in the full-length form whereas around one third lacks the A-domain. A third band in between the two was observed in the NTAP-TOC159 and NTAP-TOC159-cmyc extracts and most likely corresponded to the free A-domain (TOC159A) that had not been completely saturated by the non-specific IgG. Finally the blot was incubated with goat-anti-rabbit antibodies coupled to HRP to detect the NTAP-tag (Fig. 5D, rabbit IgG). The patterns and intensity of bands observed for NTAP-TOC159 and NTAP-TOC159-cmyc were highly similar suggesting that the two proteins were expressed at similar levels in their respective genetic backgrounds. The anti-rabbit antibody detected levels of free TOC159A that appeared greater than those of TOC159-FL suggesting that the TOC159A is stable in the cytosol whereas lower levels of TOC159-FL are maintained at the chloroplast outer membrane.

*KOC1 Supports Import Activity*–Since KOC1 can interact and phosphorylate TOC components, we tested whether KOC1 plays a role in preprotein import. Chloroplasts were isolated from 2-week-old wild type and *koc1-1* mutant seedlings. The isolated chloroplasts were incubated with either [$^{35}$S]-pSSu (a client preprotein of the TOC159 receptor) (Fig. 6A) or [$^{35}$S]-pE1α (a client preprotein of the TOC120 and -132 receptors) (Fig. 6B) for 0', 5' and 15', one sample was treated with thermolysin after 15' (15'T). The samples analyzed by SDS-PAGE, followed by Phosphorimager analysis. Import was defined as the accumulation of mature [$^{35}$S]SSu and [$^{35}$S]E1α. In *koc1-1* chloroplasts the accumulation of both mature SSu (47%) and E1α (60.3%) after 15 min was reduced in comparison with wild type chloroplasts (100%) (Fig. 6 A-B).

*KOC1 is Required for Survival During De-etiolation*–During de-etiolation, rapid import of

proteins occurs to accomplish the transition to photoautotrophic growth. To analyze a potential role for KOC1 in the process, wild type, *koc1-1, koc1-2* and NTAP-KOC1:*koc1-1* plants were grown in the dark for 6 days and then exposed to standard light conditions. To guarantee identical seed quality, the parental plants had been grown simultaneously and allowed to set seed in the same growth chamber. For all four genotypes the germination rate was close to 100%. However the survival rate differed for the different genotypes. Approximately 34% of the wild type plants survived. In comparison only around 9% of the *koc1-2* and 13% *koc1-1* plants survived which differed significantly from wild type survival rates (Fig. 6 C). The survival rate of NTAP-KOC1:*koc1-1* plants was around 23% and statistically indistinguishable from WT. This provides evidence that NTAP-KOC1 expressed in *koc1-1* plants is functional and complements the *koc1-1* de-etiolation phenotype.

**DISCUSSION**

Phosphorylation is emerging as an important mechanism in regulating the assembly and activity of protein import complexes in protein translocation systems. CKII and SnRK2, two cytosolic kinases are known to phosphorylate the TOC159 A-domain. Here, we identify KOC1, an integral chloroplast envelope kinase that phosphorylates the A-domain. We demonstrate that KOC1 affects import activity but does not interfere with composition and abundance of import components.

N-terminally TAP-tagged TOC159 (NTAP-TOC159) was used to isolate the protein import machinery and to identify new potential interaction partners of TOC159 *in vivo* (Fig. 1) (17). Among the identified proteins was the predicted kinase AT4G32250 that we named KOC1. In the reverse experiment NTAP-KOC1 was used as the bait (Fig. 2A). NTAP-KOC1 was most likely fully functional as it 1) complemented the *koc1-1* phenotype in the de-etiolation survival assay (Fig. 6C) and 2) phosphorylated recombinant A-domains after affinity purification (Fig.5A and B). TOC159 as well as other recognized components of the import machinery co-isolated together with NTAP-KOC1. Overall, the lists of proteins identified by mass spectrometry associating with NTAP-TOC159 and NTAP-KOC1, respectively, were highly overlapping. The combination of the two co-isolation experiments provided strong evidence for the association of KOC1 with the chloroplast protein import machinery.

By mass spectrometric analysis, not TOC159 but TOC75 gave the highest score in femtomoles of any component of the import machinery associating with KOC1. This indicates that TOC159 may not be the primary interaction partner of KOC1. KOC1 may also associate with multiple TOC complexes containing the different TOC159 homologs, as suggested by the ability of KOC1 to phosphorylate the A-domains of TOC132 and -120. The NTAP-KOC1-associated proteins belonged to three distinct networks: the TOC-complex, the intra-chloroplastic FtsH proteases and the cytosolic ribosome (Fig. 2B). The interaction with the FtsH network hints at an interaction between the import and protein quality control systems within the chloroplast. The interaction between KOC1 and FtsH proteins is most likely indirect and may involve interaction with TIC components that also co-isolated with KOC1. Interaction with the cytosolic ribosome suggests coordination of preprotein synthesis with the import process. These hypotheses should be experimentally explored in future studies.

KOC1 lacks a predicted, cleavable transit peptide at the N-terminus and is therefore not a predicted chloroplast protein (Fig. 3A). The absence of a cleavable transit peptide, however, is typical of chloroplast outer envelope proteins. KOC1 has a single predicted transmembrane region near its C-terminus. It is therefore probable that KOC1 belongs to the category of tail-anchored outer membrane proteins (42, 43). Outer envelope localization was supported by three lines of evidence (fluorescence, membrane fractionation, protease sensitivity) (Fig. 4). Based on resistance to alkaline extraction, KOC1 behaves as an integral membrane protein (Fig. 4C). The predicted transmembrane sequence at the C-terminus in combination with protease sensitivity indicated that the bulk of the KOC1 protein faces the cytosol. The only other known organelle-associated kinase facing the cytosol apart from KOC1 is mitochondrial CK1 (casein kinase 1) which phosphorylates TOM20 and stimulates assembly of the TOM complex (44).

The KOC1 protein contains two striking elements in addition to the transmembrane region: a predicted N-terminal kinase domain and a C-terminal HERC2-like region. The sequence of KOC1 is most closely related to that of KEG (Keep On Going, AT5G13530). KEG contains a kinase domain and 12 HERC2 repeats with similarity to KOC1. In addition to those domains KEG also a RING domain responsible for its function as an E3 ligase (41). The RING domain is absent from the KOC1 sequence suggesting that it does not function as an E3 ligase. It cannot be excluded however that KOC1 functions in conjunction with other E3 ligases under specific conditions. One such candidate is the outer

membrane E3-ligase SP1 that is involved in ubiquitination and turnover of TOC159 and -75 (30). However, the components of the TOC complex as well as TIC110 were present in both *koc1* lines at similar concentrations as in the wild type (Fig. 3D). This indicated that loss of KOC1 does not affect composition and abundance of the components of the import machinery under standard growth conditions.

A question of central interest was whether KOC1 kinase phosphorylates the A-domains of TOC159 and its homologs. KOC1 purified from transgenic plants phosphorylated the recombinant TOC159, -132 and -120 A-domains (Fig. 5A and C). The A-domains of TOC159 and TOC132/-120 determine preprotein specificity (TOC159 specializing in photosynthesis-associated proteins and TOC132/-120 in housekeeping proteins) (23). The ability of KOC1 to phosphorylate all three A-domains suggests a function in import of both photosynthesis-associated and housekeeping proteins. It is important to note that the majority of TOC159 is full length (Fig. 5D) and therefore such a scenario is plausible. However, we were not able to reliably identify KOC1-dependent phosphorylation sites in the recombinant TOC159 A-domain using mass spectrometry. Their identification will allow site-specific mutagenesis to test the role of KOC1-dependent phosphorylation in protein import. While TOC159 family members are likely *in vivo* targets of KOC1 others at the chloroplast outer membrane or in the cytosol could also exist. Such targets could be identified in the future using phosphoproteomics approaches.

An outer membrane kinase of 70 kD (OEK70) phosphorylating the C-terminal G- and M-domains of TOC159 in pea has been characterized earlier but was never identified at the molecular level (33). Based on the similar mass of Arabidopsis KOC1, it is tempting to speculate that OEK70 may be a KOC1 homolog in pea.

The proposition that KOC1 affects import of TOC159 as well as TOC132/-120 dependent import substrates (pSSu for TOC159 and pE1α for TOC132/-120 (23)) was tested in *in vitro* protein import assays (Fig. 6A and B). In both cases, import into *koc1-1* mutant chloroplasts was reduced by about 40%. This suggests that KOC1 is required for efficient protein import in both pathways. As the concentrations of the TOC components in *koc1* chloroplasts were similar to wild type (Fig.3D) it appears probable that KOC1 directly regulates the activity of the import receptors. This is likely to implicate phosphorylation but we cannot exclude other possibilities. Based on the diminished import activity in the mutant chloroplasts the effect of KOC1 is predicted to be positive. In contrast PKA-dependent phosphorylation of Tom70 inhibited receptor activity and import of metabolite carriers in mitochondria (45).

Despite the diminished import efficiency the *koc1* mutant had a wild type phenotype under standard growth conditions. Similarly, TOC159 and TOC33 GTPase mutants had diminished import efficiency *in vitro* but were phenotypically indistinguishable from the wild type (26, 29). Possibly the mutant plants are able to compensate for the chloroplast protein import deficit over time. A phenotype in such mutants might only occur at specific points in development where high import capacity is required. Such a point in development is the switch from etioplasts (chloroplast precursor organelle in the dark grown plants) to chloroplasts that develop when plants are moved into the light, a process known as de-etiolation.

In the de-etiolation assay (Fig. 6C) (30) dark grown seedlings near seed depletion were moved to the light. Under these conditions chloroplast biogenesis must be completed quickly in order to prevent starvation and initiate photoautotrophic growth. In the de-etiolation assay, the *koc1* seedlings had a lower survival rate than the wild type and NTAP-KOC1:*koc1-1* seedlings, corroborating the proposition that KOC1 activity is required when protein import demand is high.

SnRK2 kinase phosphorylates the A-domain of TOC159 in an ABA dependent fashion. This suggests hormonal control of TOC159 phosphorylation and import activity. It appears likely that various regulatory pathways converge at TOC159 and its homologs via pathway-associated kinases.

## EXPERIMENTAL PROCEDURES

*DNA constructs*–Recombinant KOC1 proteins were expressed from constructs obtained by PCR amplification from AT4G32250 cDNA using primers: KOC1_NheI_F and KOC1_NotI_R for $KOC1_{(FL)}$; KOC1_NcoI_F and KOC1_NcoI_R for $KOC1_{(1-343)}$; KOC1_NheI_F and KOC1_NcoI_R2 for $KOC1_{(1-547)}$ (see Table S2), and cloned in fusion with a C-terminal hexahistidinyl tag into the pET21d plasmid. The pCHF8-NTAP-KOC1 vector was obtained by inserting KOC1 (amplified using primers KOC1_FL_NcoI_F and KOC1_FL_XbaI_R from plasmid pET21d-KOC1-FL) (Table S2), digested with NcoI and ligated into the BspHI- XbaI-digested vector pCHF8-NTAPi (37). To obtain the pCHF7-NTAP-TOC159-cmyc construct, a DNA fragment was amplified using primers Toc159-StuI-

F and Toc159-cmyc-Gib-R (Table S2) from plasmid pCHF7-NTAPi-Toc159 (26), and from this fragment a second DNA fragment was amplified using primers Toc159-StuI-F and cmyc-uniGib-R and assembled into the S*tu*I- X*ba*I- digested vector pCHF7-NTAPi-Toc159 by Gibson technology, following the manufacturer instructions (Gibson Assembly Kit, NEB). For pEG104-N-YFP-KOC1 construct the sequence coding for KOC1 was amplified using primers attB-KOC1-FW and attB-KOC1-REV (Table S2), and recombined in pEG104 using the Gateway system (Invitrogen™ Gateway® Technology with Clonase™ II).
For pEG101-C-YFP-EMB2004 construct the sequence coding for EMB2004 (AT1G10510) was amplified using primers attB-EMB2004-FW and attB-EMB2004-REV and recombined in pEG101 vector using the Gateway system.

*Plant Material*–Plants were grown *in vitro* under long-day conditions (16 hours light, 8 hours dark), or short day conditions (8 hours light- 16 hours dark) at 100-120 µmol.$m^{-2}$ $s^{-1}$ and 21°C. Agar plates contained 0.8% (w/v) Phytoagar (Duchefa), 0.5X Murashige and Skoog (MS) medium (Duchefa), and 0, 0.5, 0.8 or 1% (w/v) sucrose (for survival, import, sub-plastidial fractionation or protoplast isolation, or IgG purification experiments, respectively). The following Arabidopsis (*Arabidopsis thaliana*) mutants NTAP-TOC159:*ppi2* (NTAP-TOC159) and TAP:WT were described previously (26). The homozygous T-DNA insertion lines SALK_083378 (*koc1-1)*, SALK_051823 (*koc1-2*) (46) were selected on 0.5 X MS medium containing kanamycin (50mg/L) and screened by PCR amplification using the primers koc1-2_LP with koc1-2_RP, koc1-1_LP with koc1-1_RP, and koc1-2_LP, koc1-2_RP or koc1-1_LP with LbB1.3. Wild type plants Columbia-0 (Col-0) were used. The transgenic plants NTAP-KOC1:*koc1-1* (NTAP-KOC1) and NTAP-TOC159-cmyc: *ppi2* (NTAP-TOC159-cmyc), were obtained by transformation of homozygous *koc1-1* or heterozygous *ppi2* plants (25), using the vectors pCHF8-NTAP-KOC1 or pCHF7-NTAP-TOC159-cmyc, respectively. Plants were selected as described (26) and displayed a wild type phenotype under standard growth conditions.

*Protein Expression and Purification*–$KOC1_{FL}$, $KOC1_{(1-343)}$ and $KOC1_{(1-547)}$ were overexpressed in *Escherichia coli* SoluBL21 (Genlantis) transformed with the corresponding expression vector. For antibody (α-KOC1) production and purification, recombinant proteins were purified on $Ni^{2+}$-nitrilotriacetic acid ($Ni^{2+}$-NTA) agarose beads under denaturing conditions according to manufacturer instructions (Qiaexpressionist™, QIAGEN).

Native recombinant $KOC1_{FL}$ was purified from bacterial pellets resuspended in buffer L (50 mM $NaH_2PO_4$, 300 mM NaCl, 20 mM imidazole, 0.1% (v/v) Triton X100 (TX100), 1mM PMSF, 0.2% (v/v) protease inhibitor cocktail PIC (Sigma), pH 8.0). Bacterial cells were disrupted by high pressure using a French press, incubated during 30 min with DNAse (Roche) (0.2µl/ml) and centrifuged for 30 min at 40,000g. The supernatant was filtered (0.2µm) and incubated during 1 hour with $Ni^{2+}$-NTA beads. The resin was washed two times with buffer L containing 20 mM Imidazole and one time with the same buffer containing 0.5% n-Dodecyl-β-D-maltoside (DDM) instead of Triton. Recombinant $KOC1_{FL}$ was eluted with buffer L containing 250 mM Imidazole and 0.5% DDM. Eluates were dialyzed against 30 mM Tris-HCl pH 7.5, 75 mM NaCl, 75 mM KCl, 1mM PMSF and 0.5% DDM. All procedures were done at 4°C.

TOC120A ($TOC120_{(1-343)}$-His-6x), TOC132A ($TOC132_{(1-431)}$-His-6x) (21) and TOC159A ($TOC159_{(1-740)}$-His-6x), were overexpressed in transformed *E. coli* strain BL21 (DE3) cells. The cells were lysed in buffer L' (50 mM Tris-HCl pH 8, 300 mM NaCl, 5 mM imidazole, 1 mM PMSF) by high pressure using a French press. After centrifugation for 30 min at 40,000 g the supernatant fraction was passed on $Ni^{2+}$-NTA column using ÄKTA Prime® system (for TOC159A) or incubated during 2 hours with $Ni^{2+}$-NTA beads in microtubes (for TOC132A and TOC120A). The column was washed three times with Buffer L'. The proteins were eluted in a buffer L' containing 250 mM Imidazole. TOC120A and TOC132A were dialyzed against 10 mM Tris-HCl pH 8, 50 mM NaCl. TOC159A was dialyzed against 20 mM piperazine pH 5.5, 50 mM NaCl and further purified on DEAE ion exchange column using ÄKTA Prime® as described in (47). The proteins were precipitated by the $CHCl_3$-methanol extraction methods (48).

*Antibodies*–Antibodies for TOC and TIC components were described previously (17, 25, 49, 50). Antibodies against PGL35 were described previously (51). Antibodies against CBP were purchased in Genscript, LHCB2 were from Agrisera and c-myc and IgG were from Cell signaling. For anti-KOC1 antibody production, two forms of recombinant KOC1, ($KOC1_{(1-343)}$ and $KOC1_{(1-547)}$) purified by $Ni^{2+}$-NTA affinity chromatography were pooled and injected into rabbits for polyclonal antibody production (Eurogentec, Seraing, Belgium). To affinity purify antibodies from the serum,

purified recombinant $KOC1_{FL}$ was crosslinked to Affi-Gel 10 (Bio-Rad) according to the commercial protocol. The serum was incubated with $KOC1_{FL}$ Affi-Gel column. The column was washed twice with 10 ml of PBS buffer. The anti-KOC1 antibodies were eluted with 0.2 M Glycine pH 2.2 buffer and immediately neutralized with 1M Tris pH 8.

*IgG Affinity Purification*–The procedure has already been described (37) and was applied with few modifications. All steps were performed at 4°C. Plants grown *in vitro* (10g FW) were ground in a mortar in a total volume of 18 ml of buffer G (50 mM Tris-HCL, pH 7.5, 100 mM NaCl, 1mM PMSF, 5 mM NaF, 0.2% (v/v) PIC). The 100,000g pellet fraction of the NTAP-KOC1 and NTAP-TOC159 was resuspended in buffer G and centrifuged for an additional 1h at 100,000g. Pellet-associated proteins were solubilized in buffer G containing 1.65% (v/v) TX100 and 10% glycerol (buffer $G_{KOC}$) for NTAP-KOC1, or 0.375% (v/v) TX100 and 5% glycerol (buffer $G_{TOC}$) for NTAP-TOC159. Proteins of TAP:WT plants were extracted directly in buffer $G_{KOC}$ or $G_{TOC}$. Solubilized proteins were incubated overnight with 100µl of IgG sepharose resin. The beads were washed once with 35 ml and 6 times with 5 ml in buffer $G_{KOC}$ for NTAP-KOC1 or $G_{TOC}$ for NTAP-TOC159. The last wash was done with the same buffer without proteases inhibitors. The proteins were eluted in 50 mM Tris-HCl pH 8, 0.5 mM EDTA, 100 mM NaCl, 1 mM DTT, TX100 (1.65 % or 0.375%) glycerol ( 5% or 10%) for NTAP-KOC1 or NTAP-TOC159 for 2 hours at 16°C with 50 units of AcTEV protease (Tobacco Etch Virus protease, Invitrogen). 50 µg of proteins of the "total" fraction (Total, load, Ft and W1 fractions) or 10% of the fraction (W5 and TEV fraction) were loaded on SDS-PAGE, and transfer by Western blot in Dunn buffer on nitrocellulose membrane.

*Transient Expression in Arabidopsis Protoplasts*–Protoplasts were isolated from Col-0 plants (4-week-old) grown in short day conditions, and transformed using a polyethylene glycol based method adapted from (52, 53) as described in Köhler *et al.*(17). Protoplasts were transformed with plasmids pEG104-N-YFP-KOC1 for the localization of YFP-KOC1, pEG101-C-YFP-EMB2004 as envelope marker and pCL60 (Stratagene) as a control.

*Chloroplast Isolation*–For the isolation of intact chloroplasts we used the protocols by Smith, *et al.*(54) and Agne, *et al.*(26) with the following modifications. Chloroplasts were obtained from Arabidopsis plants (2-week-old) grown *in vitro* under long-day conditions. The tissue (5-9 g) was enzymatically digested using 1.5% (w/v) cellulase "Onozuka" and 0.375 % (w/v) macerozyme, R-10 (SERVA). The digestion was extended to 12 hours at 19°C.

*Chloroplast Protease Treatment and Extraction*–Intact chloroplasts were subjected to thermolysin treatment according to Smith, *et al* (54). Chloroplast pellet corresponding to 20µg of chlorophyll was resuspended in 100µl of HEPES-sorbitol buffer (HS) and incubated for 1 hour on ice with 20µl of thermolysin (2mg/ml). For alkaline extraction we used chloroplast corresponding to 20 µg of chlorophyll incubated for 10 min on ice with 600 µl of 0.1 M $Na_2CO_3$ pH 11. The fractions were separated by centrifugation for 1 hour at 100,000g.

*Chloroplast fractionation*–Fractionation of intact chloroplasts was carried out according to Vidi, *et al.*(51) and Hiltbrunner, *et al.*(49) with some modifications. NTAP-KOC1:*koc1-1* plants (140g FW) grown on soil during 8 weeks under short-day conditions were ground in a blender in 400 ml of HB Buffer. The lysate was filtered through two layers of Miracloth and centrifuged at 4°C for 5 min at 600g. The pellet was resuspended in 10 ml of RB Buffer. Intact chloroplasts were purified on a Percoll step gradient (40% v/v and 85% v/v in RB Buffer). Intact chloroplast were washed with 50 ml of RB Buffer and centrifuged for 5 min at 700 g. After centrifugation, the chloroplasts were hypertonically lysed in 0.6 M sucrose TED Buffer at -80°C overnight. The thawed suspension was resuspended using a Potter homogenizer and centrifuged at 100,000g for 1 hour at 4°C. The membrane pellet was resuspended in 45% sucrose in TED buffer (3.5 mg/ml) using a Potter. The total membrane fraction (corresponding to 12 mg of chlorophyll) was separated on a linear sucrose gradient (5-45%) in TED buffer and centrifugation for 16 hours at 100,000g.

*Chloroplast protein import assay*–Chloroplast import experiments were performed according to Agne, *et al.*(26) with some modifications. For each reaction we used chloroplasts corresponding to 20 µg of chlorophyll and 4µl of *in vitro* translated [$^{35}$S] methionine-labeled preproteins (TNT® T7 Quick-coupled Transcription/ Translation System, Promega). The *in vitro* translocated, [$^{35}$S]-labeled preproteins of the small subunit of Rubisco (preSSu) and the alpha subunit of pyruvate dehydrogenase E1-alpha (preE1α) (pET21d-preE1-alpha-DHFR-(6x-His) (23) were used as substrates. The proteins of the import experiments were separated by SDS-PAGE and dried gels were analyzed using a Phosphorimager (Molecular imager® FX (BIO-RAD) and Quantity One 4.6 software for quantification.

*De-etiolation Survival Test–koc1-1*, *koc1-2*, WT and NTAP-KOC1:*koc1-1* plants were grown on 0.5X MS medium, seeds were cold treated for 3 days at 4°C to synchronize germination. The seeds were exposed 4 hours to standard light and then grown in the dark. After 6 days, the plants were exposed to standard light conditions for two weeks. The germination and survival rates were calculated.

*Phosphorylation Assays*–KOC1 purified from NTAP-KOC1:*koc1-1* plants by the IgG Affinity purification was incubated for 30 min at 25°C with 3-5µg of either purified TOC159A, TOC120A or TOC132A in phosphorylation buffer (50 mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 5 mM $CaCl_2$, 1 mM dithiothreitol (DTT), 50 µM ATP) in the presence of 10 µCi of [$\gamma$-$^{33}$P] ATP. In addition, KOC1 denatured (10 min at 65°C) was also incubated with TOC159A in presence of [$\gamma$-$^{33}$P] ATP. Reactions were stopped by diluting in ice-cold phosphorylation buffer followed by $CHCl_3$-methanol precipitation (48). The samples were separated by SDS-PAGE and examined by autoradiography.
Native recombinant $KOC1_{FL}$ (20µg) purified on ($Ni^{2+}$-NTA) agarose beads was incubated for 30 min at 25°C with 10 µg of purified TOC159A in phosphorylation buffer containing 0.2% of DDM and [$\gamma$-$^{33}$P] ATP. The reactions were treated as described in the paragraph before.

*Mass Spectrometric Identification of KOC-interacting Proteins*–KOC-interacting proteins were isolated by a TAP-tagged version of KOC compared to a control. Proteins were identified and quantified after Nano-LC separation using the data-independent HD-MSE data acquisition method as previously described (55). In brief, LC separation and HD-MSE data acquisition were performed using 1 µl from each of the in solution digested samples on a nanoACQUITY UPLC System coupled to a Synapt G2-S mass spectrometer (Waters, Eschborn, Germany). MS acquisition range was set to 50–5000 Da. Data analysis was carried out by ProteinLynx Global Server (PLGS 3.0.1, Apex3D algorithm v. 2.128.5.0, 64 bit, Waters, Eschborn, Germany) with automated determination of chromatographic peak width as well as MS TOF resolution. Lock mass value for charge state 2 was defined as 785.8426 Da/e and the lock mass window was set to 0.25 Da. Databank search query (PLGS workflow) was carried out as follows: Peptide and fragment tolerances were set to automatic (resulting in a maximum mass tolerance of 30 ppm), two fragment ion matches per peptide, five fragment ions for protein identification, and two peptides per protein. The false discovery rate (FDR) was set to 4% at the protein level. MSE data were searched against the modified A. thaliana database (TAIR10, ftp://ftp.arabidopsis.org) containing common contaminants such as keratin (ftp://ftp.thegpm.org/fasta/cRAP/crap.fasta) and glycogen phosphorylase B from rabbit (Uniprot ID: P00489). Quantification was performed based on the intensity of the three most abundant proteotypic peptides (Hi3-method (56)).The glycogen phosphorylase B was used with 10 fmol/µL as internal quantification standard.

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**Author contributions**: MZ, CM, VD and ED performed and analyzed experiments. MZ, BA, SB and FK designed the study, analyzed the results and prepared the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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## FIGURE LEGENDS

**FIGURE 1.** Co-purification of KOC1 with TOC159 and TOC components. Sequential fractions of NTAP-TOC159 IgG affinity purification were analyzed by Western blotting. The membrane was probed with antibodies against KOC1, CBP, TOC75, TOC33, TIC110, LHCB2 and PGL35. (L: load; Ft: flow-through, W1: first wash; W5: last wash, TEV: eluate). 50µg of proteins loaded (L, Ft and W1) or 10% of the fractions (W5 and TEV). Several identical blots were used for immunoblotting. TAP:WT was used as a negative control.

**FIGURE 2.** Co-purification of TOC components with KOC1. *A.* Sequential fractions of NTAP-KOC1 IgG affinity purification were analyzed by Western blotting. The membrane was probed with antibodies against KOC1, TOC159, TOC75, TOC33, TIC56 and LHCB2. (T: Total, L: load; Ft: flow-through, W1: first wash; W5: last wash, TEV: eluate). 50µg of proteins loaded (T, L, Ft and W1) or 10% of the fractions (W5 and TEV). Several identical blots were used for immunoblotting. TAP:WT was used as a negative control. *B.* Interaction networks of KOC1 using the list of co-immunopurified proteins and STRING 10.0 program.

**FIGURE 3.** KOC1 sequence analysis and mutant characterization. *A-B.* Amino acid sequence of KOC1 contains a kinase domain (orange, aa39-306). Underlined aa45-53 are conserved in the catalytic domain, aa74 is predicted to bind ATP. In the HERC2-related region (blue, aa376-531) underlined amino acids share homology with the KEG protein. A predicted transmembrane stretch (TM, aa549-572) is highlighted in red. *C.* Images of 2-week-old plants WT, *koc1-1*, *koc1-2* and NTAP-KOC1:*koc1-1* grown *in vitro*. *D.* Total protein extracts of 2-week-old plants (WT, *koc1-1*, *koc1-2* and NTAP-KOC1) were separated by SDS-PAGE and transferred to nitrocellulose membrane. Antibodies against TOC159A, TOC75, TOC120, KOC1, TOC33 and TIC110 were used. Actin was used as a loading control. (*) Bands corresponding to overexpressed NTAP-KOC1.

**FIGURE 4.** Localization of KOC1 at the outer membrane of the chloroplast. *A.* Confocal microscopy images of an isolated representative protoplast expressing YFP-KOC1. Chlorophyll fluorescence in red identifies chloroplasts, the signal of YFP-KOC1 appears yellow, intact protoplasts were visualized by bright field. Merge shows the overlay of chlorophyll, YFP-KOC1 and bright field images. Scale bars: 10μm. *B.* Total membranes from NTAP-KOC1 chloroplasts were separated on a continuous sucrose gradient (5-45%) and 37 fractions were collected. Proteins from uneven fractions were separated by SDS-PAGE, transferred to nitrocellulose and probed with antibodies (1) CBP, (2) TOC159A, (3) TOC75, (4) LHCB2 and (5) PGL35. *C.* Col-0 chloroplasts were subjected (+) to thermolysin treatment or not (-), Triton X-100 solubilization (TX- 100) and alkaline extraction ($Na_2CO_3$) (P: pellet/ S: supernatant). Samples were separated by SDS-PAGE, transferred to nitrocellulose, stained with amido-black (lower part) and probed with antibodies against KOC1, TOC75 and TIC40.

**FIGURE 5.** Phosphorylation of the A-domain by KOC1 and its presence in TOC159 *A.* TOC159A was incubated with KOC1 isolated from NTAP-KOC1:*koc1-1* plants (lane 1) or same, but heat-denatured KOC1 (De-KOC1) (lane 4) in the presence of [γ-$^{33}$P]ATP. *B.* Purified TOC159A was incubated with [γ-$^{33}$P]ATP and with recombinant full length KOC1 purified from bacteria (lane 3). *C.* Purified TOC120A (lane 2) and TOC132A (lane 3) were incubated with [γ-$^{33}$P]ATP and KOC1 isolated from NTAP-KOC1:*koc1-1* plants. In *A* - C the samples were separated by SDS-PAGE and analyzed using a Phosphorimager (Molecular imager® FX BIO-RAD) and Quantity One 4.6 software. KOC1, TOC159A, TOC120A and TOC132A were incubated alone with [γ-$^{33}$P]ATP as negative controls. *D.* Total protein extracts of seedlings 1 (*ppi2*), 2 (WT), 3 (NTAP-TOC159:*ppi2*) and 4 (NTAP-TOC159-cmyc:*ppi2*) were analyzed by Western blotting. The membrane was consecutively probed with antibodies against cmyc and rabbit IgG. The data shown are representative experiments out of several replicates.

**FIGURE 6.** Requirement of KOC1 for efficient chloroplast protein import and de-etiolation. *A-B* Isolated chloroplasts from *koc1-1* and Col-0 (WT) plants were incubated with [$^{35}$S]Met-labeled preproteins of pSSu in *A* and E1-α in *B*. The pre-proteins were incubated with chloroplasts and import was allowed to proceed for 0, 5 and 15 min (0',5',15'), one sample was treated with thermolysin after 15' (15'T). Proteins form chloroplasts were separated by SDS-PAGE followed by Phosphorimager analysis. The graphs show the quantification of the bands corresponding to imported, mature SSu and E1α at 0, 5 and 15 min averaged over three technical replicates. The amount of mature protein imported into WT chloroplasts at 15 min was arbitrarily set to 100%. The qualitatively similar results were obtained in five independent experiments. *C.* Images of surviving *koc1- 2*, *koc1-1* WT and NTAP-KOC1:*koc1-1* plants upon exposure to long day conditions for two weeks after etiolation for 6 days in dark. The germination and survival rates were calculated. The germination rate was around 100% for all genotypes. The survival rates were: WT: 34.6%, NTAP-KOC1:*koc1-1*: 22.5%, *koc1-2*: 8.8% and *koc1-1*: 12.5 %. Student's t-Test: '**= pValue<0.01; *=pValue<0.05 (*n: 80* for *koc1-1* and *koc1-2*; *n: 240* for WT). This experiment was repeated three times with comparable results.

**FIGURES**

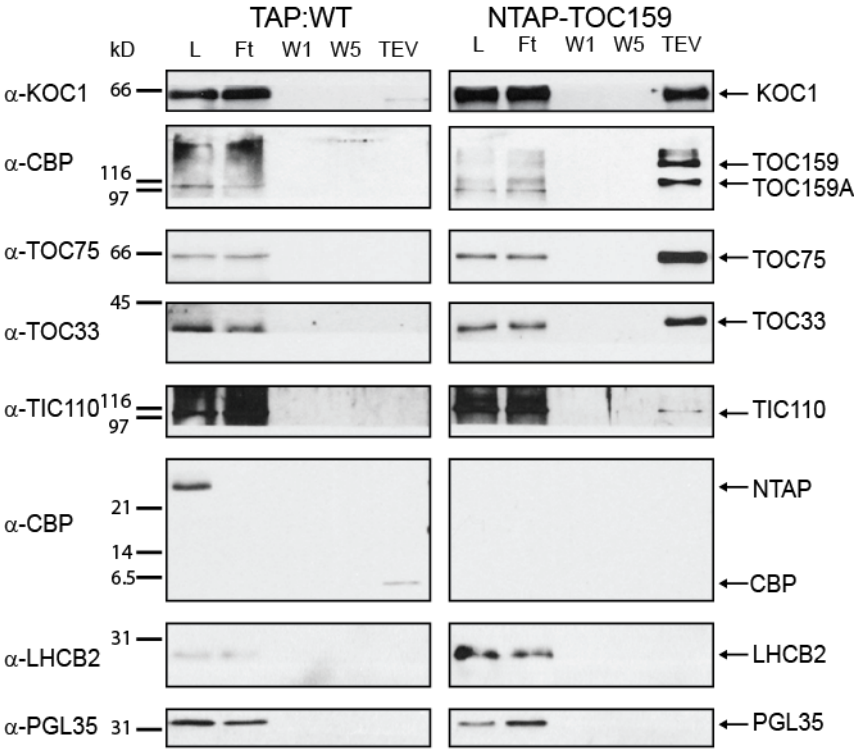


Figure 1.

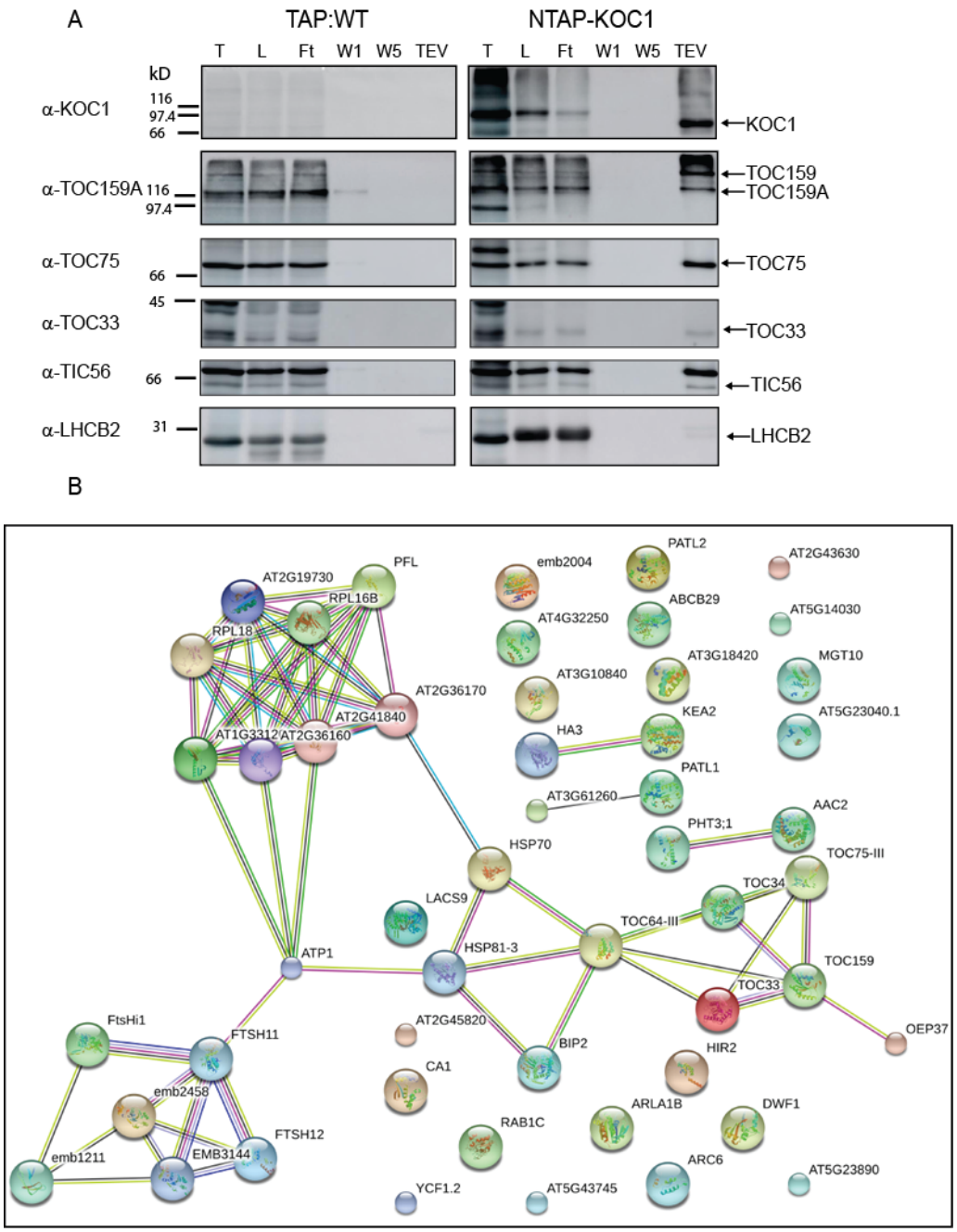
A
TAP:WT
NTAP-KOC1
T L Ft W1 W5 TEV
T L Ft W1 W5 TEV
kD
α-KOC1
116
97.4
66
KOC1
α-TOC159A
116
97.4
TOC159
TOC159A
α-TOC75
66
TOC75
45
α-TOC33
TOC33
α-TIC56
66
TIC56
α-LHCB2
31
LHCB2
B
AT2G19730
PFL
RPL16B
RPL18
AT2G36170
AT2G41840
AT1G3312
AT2G36160
ATP1
FtsHi1
FTSH11
emb2458
FTSH12
emb1211
EMB3144
emb2004
PATL2
AT2G43630
AT4G32250
ABCB29
AT5G14030
AT3G10840
AT3G18420
MGT10
KEA2
AT5G23040.1
HA3
PATL1
AT3G61260
AAC2
PHT3;1
HSP70
TOC75-III
TOC34
LACS9
TOC64-III
HSP81-3
TOC159
TOC33
AT2G45820
OEP37
BIP2
HIR2
CA1
ARLA1B
DWF1
RAB1C
ARC6
AT5G23890
YCF1.2
AT5G43745

Figure 2.

A

MASKIIAGKPDDTEYEIIEGESESALAAGTSPWMNSSTLKLRHRIGRGPFGDVWLATHHQSTEDYDEHHEVAIKMLYP
IKEDQRRVVVDKFEDLFSKCQGLENVCLLRGVSSINGKICVVMKFYEGSLGDKMARLKGGKLSLPDVLRYGVDLATGI
LELHSKGFLILNLKPSNFLLSDNDKAILGDVGIPYLLLSIPLPSSDMTERLGTPNYMAPEQWQPDVRGPMSFETDSWG
FGCSIVEMLTGVQPWSGRSADEIYDLVVRKQEKLSIPSSIPPPLENLLRGCFMYDLRSRPSMTDILLVLKSLQNSEEEQV
RRGIDSREIRKSSATLGYTEWFLSKDHLQVRDTVRSRKPANSCKHENMDVPEGMVVGLERDSTDPDGFVLVKVHGVH
DPLRVHVSVLERVTNGLASGDWVRLKVRKDKRHSPVGVLHSIDREGNVAVGFIGLPTLWKGTSSQLQMAKVYSVGQF
VKLKANVVIPRFKWMRKGRGIWATGRISQVLPNGCLEVDFPGMLPFGEEHGSYLADPAEVEIVNFNTCQGAVEKYQHL
EDFHWAVRPLLIAMGLLTAMKLGICVRKKIGRSKDGKQRDGSTGQGDCKIPDGKGSDKSKWLVFF

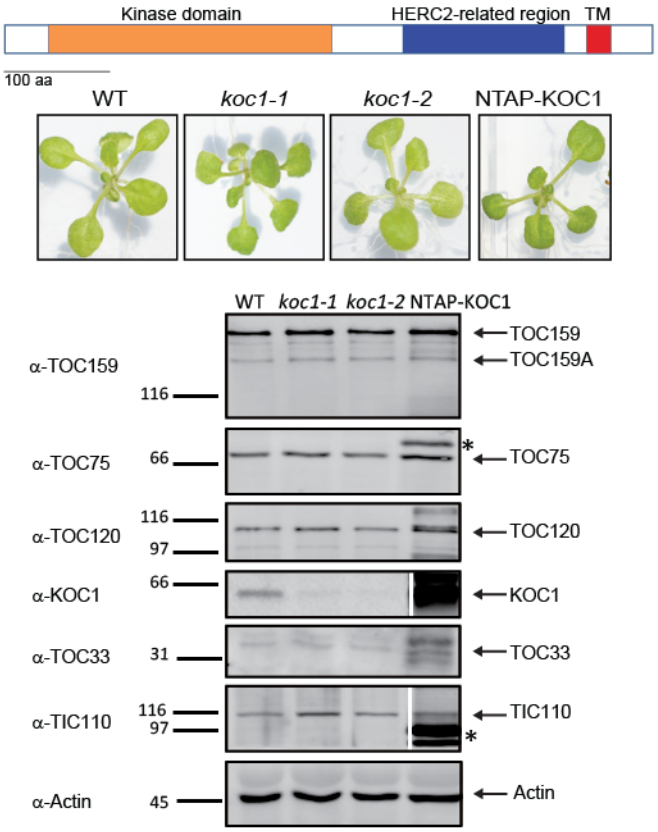


Figure 3.

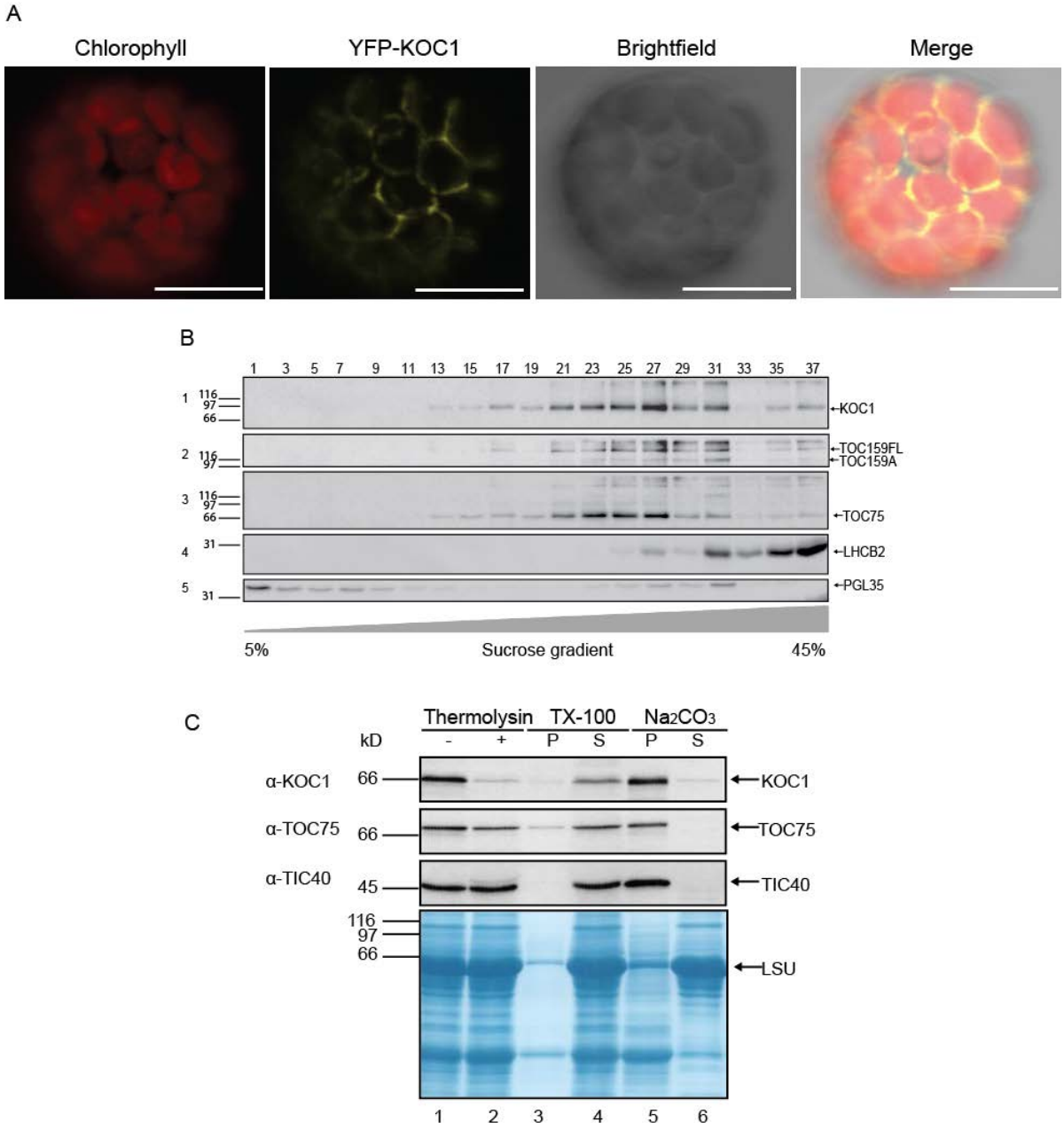
A
Chlorophyll
YFP-KOC1
Brightfield
Merge
B
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 35 37
116
97
66
KOC1
TOC159FL
TOC159A
TOC75
LHCB2
PGL35
5%
Sucrose gradient
45%
C
Thermolysin
TX-100
Na2CO3
kD
-
+
P
S
P
S
α-KOC1
α-TOC75
α-TIC40
66
66
45
116
97
66
KOC1
TOC75
TIC40
LSU
1 2 3 4 5 6

Figure 4.

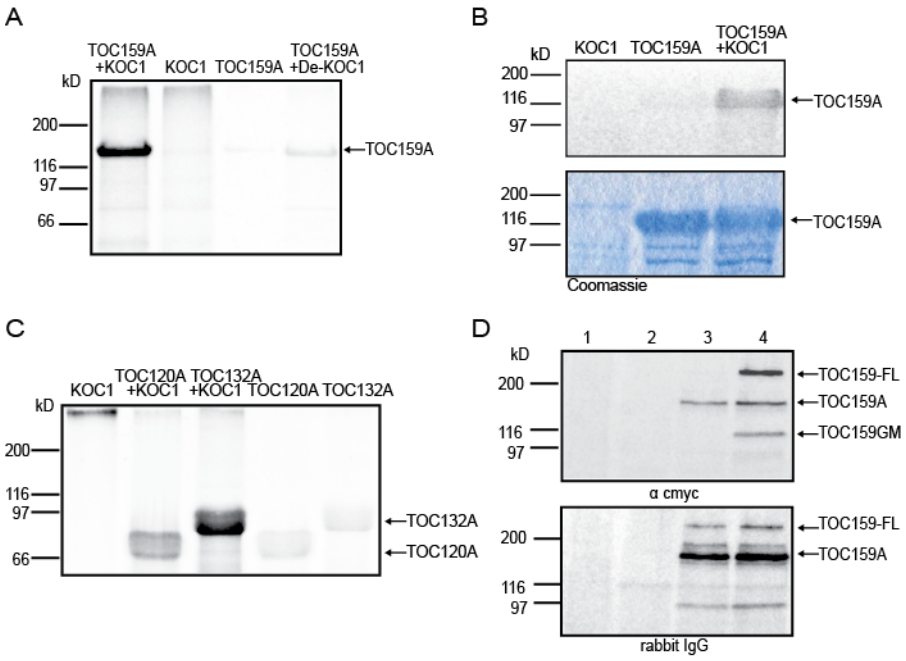
A
TOC159A +KOC1
KOC1
TOC159A
TOC159A +De-KOC1
kD
200
116
97
66
TOC159A
B
kD
KOC1
TOC159A
TOC159A +KOC1
200
116
97
TOC159A
200
116
97
TOC159A
Coomassie
C
KOC1
TOC120A +KOC1
TOC132A +KOC1
TOC120A
TOC132A
kD
200
116
97
66
TOC132A
TOC120A
D
1
2
3
4
kD
200
116
97
TOC159-FL
TOC159A
TOC159GM
α cmyc
200
116
97
TOC159-FL
TOC159A
rabbit IgG

Figure 5.

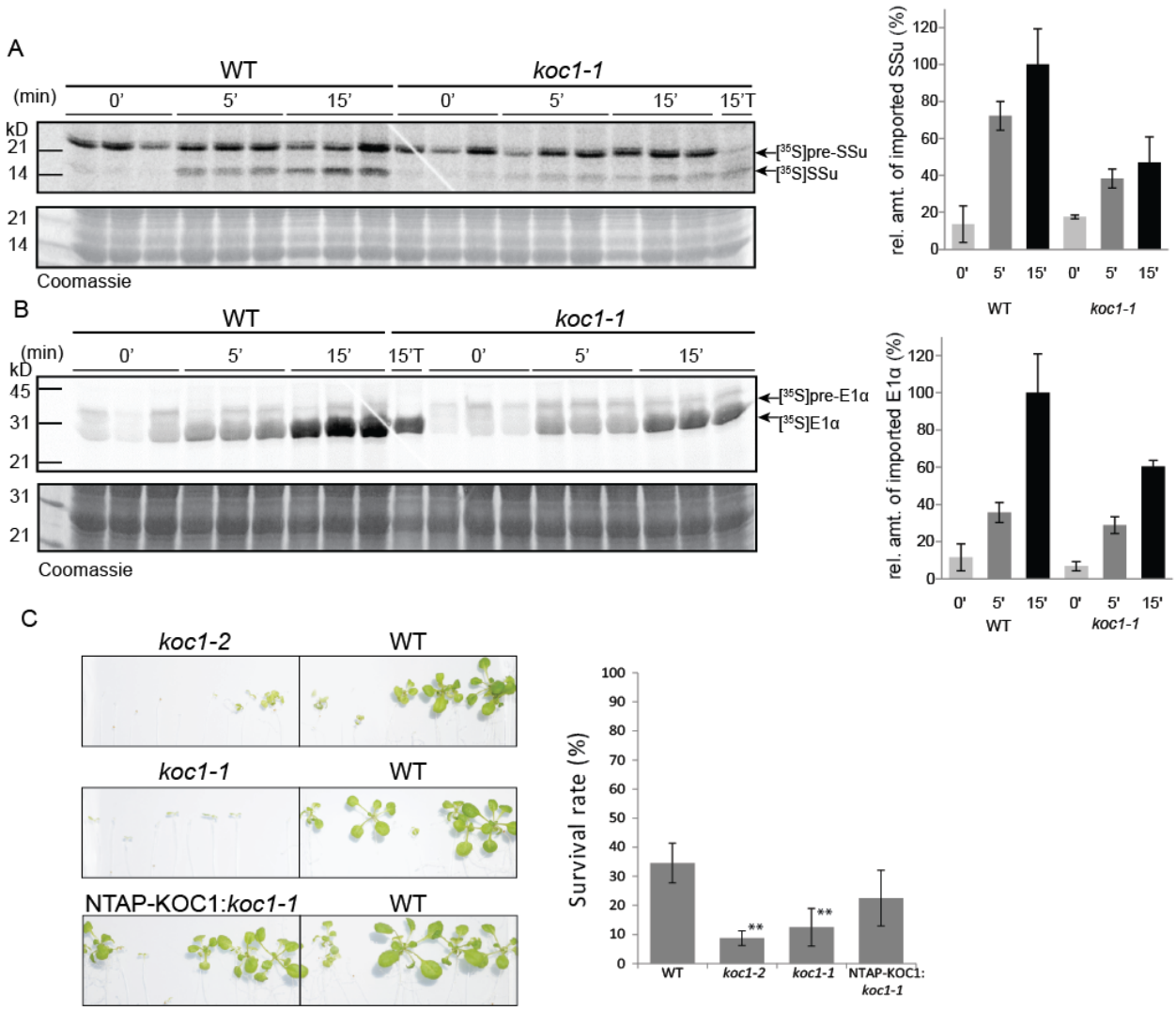
A
WT
koc1-1
(min)
0'
5'
15'
0'
5'
15'
15'T
kD
21
14
[35S]pre-SSu
[35S]SSu
21
14
Coomassie
rel. amt. of imported SSu (%)
120
100
80
60
40
20
0
0'
5'
15'
0'
5'
15'
WT
koc1-1
B
WT
koc1-1
(min)
0'
5'
15'
15'T
0'
5'
15'
kD
45
31
21
[35S]pre-E1α
[35S]E1α
31
21
Coomassie
rel. amt. of imported E1α (%)
120
100
80
60
40
20
0
0'
5'
15'
0'
5'
15'
WT
koc1-1
C
koc1-2
WT
koc1-1
WT
NTAP-KOC1:koc1-1
WT
Survival rate (%)
100
90
80
70
60
50
40
30
20
10
0
**
**
WT
koc1-2
koc1-1
NTAP-KOC1:
koc1-1

Figure 6.

**The Novel Chloroplast Outer Membrane Kinase KOC1 Is a Required Component of the Plastid Protein Import Machinery**

Mónica Zufferey, Cyrille Montandon, Véronique Douet, Emilie Demarsy, Birgit Agne, Sacha Baginsky and Felix Kessler

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The Novel Chloroplast Outer Membrane Kinase KOC1 Is a Required Component of the Plastid Protein Import Machinery

**Mónica Zufferey[1], Cyrille Montandon[2], Véronique Douet[1], Emilie Demarsy[3], Birgit Agne[4], Sacha Baginsky[4] and Felix Kessler[1]**

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**Supplemental figures:**

1. Figure S1: Genotyping of *koc1* lines
2. Figure S2: NTAP-TOC159 constructs
3. Figure S3: Localization of EMB2004-YFP and GFP by confocal microscopy

**Supplemental tables:**

1. Table S1: Identification of KOC-interacting proteins and quantification by MSE
2. Table S2: List of primers used for cloning and genotyping

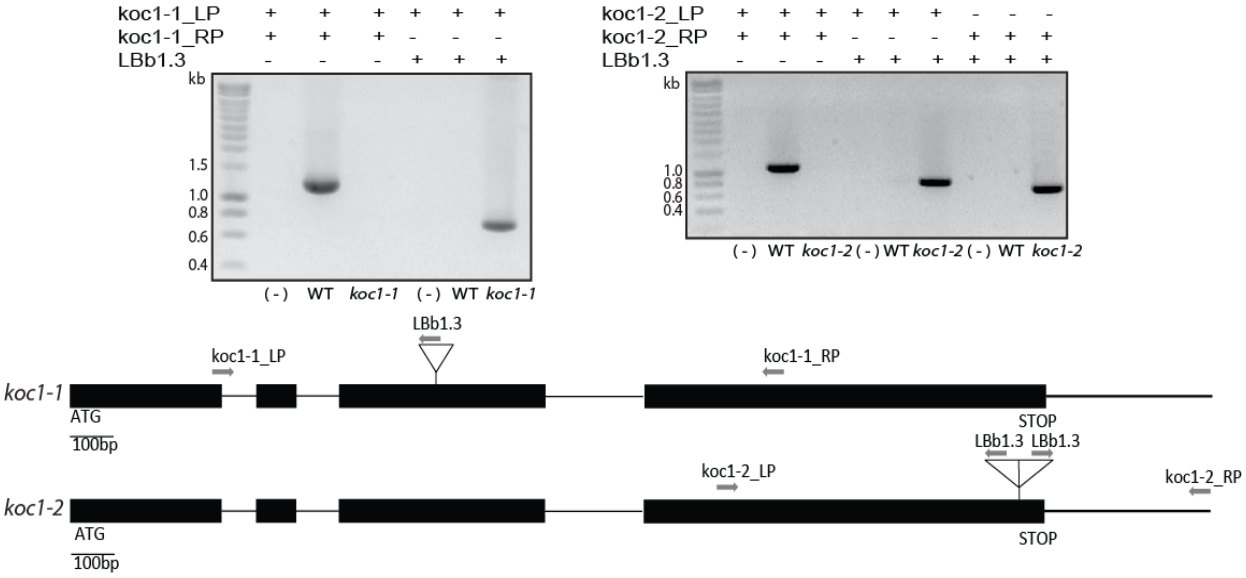


Figure S1.

**FIGURE S1.** Genotyping of *koc1* lines. Identification of the *koc1* homozygous T-DNA insertion mutants (*koc1-1* and *koc1-2*) by PCR. WT (Col-0) was used as a control, (-) negative control without DNA. Schematic representation of KOC1 gene indicating the T-DNA insertions (triangles), exons (black boxes) introns (thin lines) and primers koc1-1 or koc1-2 _LP or _RP and LBb1.3 (arrows).

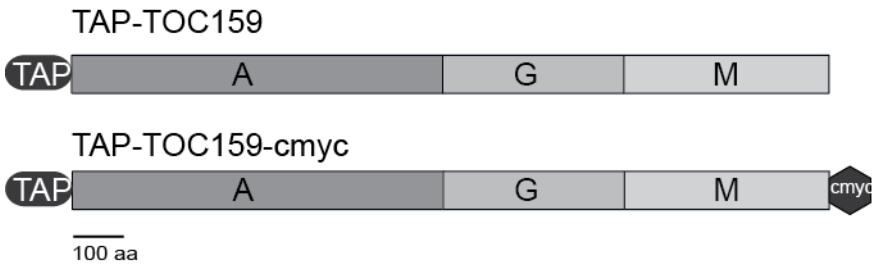


Figure S2.

**FIGURE S2.** NTAP-TOC159 constructs. Schematic representation of TAP-TOC159 and TAP-TOC159-cmyc constructs. The domains of TOC159 are illustrated: the N-terminal A domain, G domain and M domain.

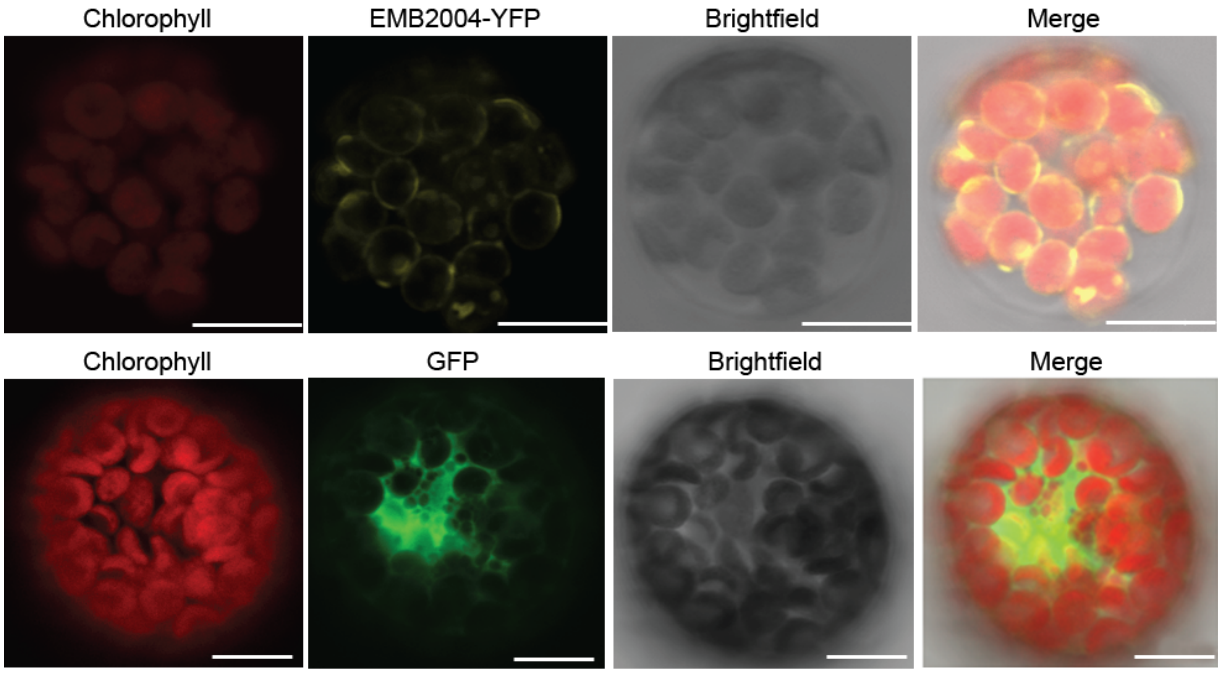


Figure S3.

**FIGURE S3.** Confocal microscopy images of an isolated representative protoplast expressing EMB2004-YFP and GFP. Chlorophyll fluorescence visualized in red identifying chloroplasts, EMB2004-YFP appears yellow, GFP in green. Intact protoplasts were visualized by bright field. Merge shows the overlay of chlorophyll, fluorescence of proteins and bright field images. Scale bars: 10µm.

| Identifier | Annotation | fmol on column (negative control, replicate 1) | fmol on column (eluate, replicate 1) | fmol on column (negative control, replicate 2) | fmol on column (eluate, replicate 2) | Complete chloroplast reference | Complete chloroplast reference (fmol) | Enrichment index 1st replicate | Enrichment index 2nd replicate | avrg enrichment index | avrg fmol in eluate | % of bait |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT4G32250 | Protein kinase superfamily protein; FUNCTIONS IN: protein kinase activity, kinase activity, ATP binding; INVOLVED IN: protein amino acid phosphorylation; LOCATED IN: chloroplast; | 0.62613333333 | 34.9472 | 0.33516666667 | 10.9877 | not detected | n.a. | n.a. | n.a. | infinite | 22.96745 | 100 |
| AT4G02510 | An integral membrane GTPase that functions as a transit-sequence receptor required for the import of proteins necessary for chloroplast biogenesis. Located in the outer chloroplast membrane. | N.D. | 6.4308 | N.D. | 1.45093333 | not detected | n.a. | n.a. | n.a. | infinite | 3.94086667 | 17.1584859 |
| AT5G53170 | encodes an FtsH protease that is localized to the chloroplast and the mitochondrion | N.D. | 1.05953333 | N.D. | 2.45403333 | not detected | n.a. | n.a. | n.a. | infinite | 1.75678333 | 7.64901342 |
| AT1G22530 | PATELLIN 2 (PATL2); FUNCTIONS IN: transporter activity; INVOLVED IN: transport; LOCATED IN: plasma membrane, chloroplast | N.D. | 1.61993333 | N.D. | 1.7089 | not detected | n.a. | n.a. | n.a. | infinite | 1.66441667 | 7.24685007 |
| AT5G13490 | Encodes mitochondrial ADP/ATP carrier | N.D. | 2.2424 | N.D. | 0.9345 | not detected | n.a. | n.a. | n.a. | infinite | 1.58845 | 6.91609212 |
| AT4G17530 | AtRabD2c encodes a Rab GTPase, which plays important roles in pollen development, germination and tube elongation. | N.D. | 2.14786667 | N.D. | 0.4689 | not detected | n.a. | n.a. | n.a. | infinite | 1.30838333 | 5.69668524 |
| AT2G41840 | Ribosomal protein S5 family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic small ribosomal subunit, cytosolic ribosome, nucleolus, membrane | N.D. | 2.6055 | N.D. |  | not detected | n.a. | n.a. | n.a. | infinite | 1.30275 | 5.67215777 |
| AT5G22640 | EMB1211 is a MORN (multiple membrane occupation and recognition nexus) motif containing protein involved in embryo development and chloroplast biogenesis. The mRNA is cell-to-cell mobile. | N.D. | 1.74823333 | N.D. | 0.74466667 | not detected | n.a. | n.a. | n.a. | infinite | 1.24645 | 5.42702825 |
| AT2G36170 | Ubiquitin supergroup;Ribosomal protein L40e; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic large ribosomal subunit, nucleolus; | N.D. | 1.76756667 | N.D. | 0.56953333 | not detected | n.a. | n.a. | n.a. | infinite | 1.16855 | 5.08785259 |
| AT1G72150 | novel cell-plate-associated protein that is related in sequence to proteins involved in membrane trafficking in other eukaryotes The mRNA is cell-to-cell mobile. | N.D. | 1.2719 | N.D. | 1.01236667 | not detected | n.a. | n.a. | n.a. | infinite | 1.14213333 | 4.97283474 |
| AT3G05590 | Encodes cytoplasmic ribosomal protein L18. | N.D. | 0.47013333 | N.D. | 1.681 | not detected | n.a. | n.a. | n.a. | infinite | 1.07556667 | 4.68300428 |
| AT5G57350 | member of Plasma membrane H+-ATPase family | N.D. | 2.0477 | N.D. |  | not detected | n.a. | n.a. | n.a. | infinite | 1.02385 | 4.45783054 |
| AT3G19820 | Involved in the conversion of the early brassinosteroid precursor 24-methylenecholesterol to campesterol. Brassinosteroids affect cellular elongation. Mutants have dwarf phenotype. DWF1 is a Ca2+ dependent calmodulin-binding protein. | N.D. | 1.804 | N.D. | 0.23593333 | not detected | n.a. | n.a. | n.a. | infinite | 1.01996667 | 4.44092255 |
| AT1G22780 | S18 ribosomal protein involved in the binding of f-Met tRNA during initiation of mRNA translation. Expression restricted to meristems. Mutant phenotype-pointed first leaves,reduced fresh weight, growth retardation. | N.D. | 1.60403333 | N.D. | 0.34843333 | not detected | n.a. | n.a. | n.a. | infinite | 0.97623333 | 4.25050815 |
| AT2G45820 | Remorin family protein; FUNCTIONS IN: DNA binding; INVOLVED IN: biological_process unknown; LOCATED IN: plasma membrane; | N.D. | 1.3562 | N.D. | 0.58653333 | not detected | n.a. | n.a. | n.a. | infinite | 0.97136667 | 4.22931874 |
| ATMG01190 | ATPase subunit 1 | N.D. | 1.4399 | N.D. | 0.37563333 | not detected | n.a. | n.a. | n.a. | infinite | 0.90776667 | 3.95240511 |
| AT1G33120 | Ribosomal protein L6 family; FUNCTIONS IN: structural constituent of ribosome, rRNA binding; INVOLVED IN: translation; LOCATED IN: in 7 components | N.D. | 1.51756667 | N.D. | 0.19406667 | not detected | n.a. | n.a. | n.a. | infinite | 0.85581667 | 3.72621543 |
| AT3G04340 | embryo defective 2458 (emb2458); FUNCTIONS IN: nucleoside-triphosphatase activity, ATPase activity, metalloendopeptidase activity, nucleotide binding, ATP binding; INVOLVED IN: embryo development ending in seed dormancy; LOCATED IN: chloroplast | N.D. | 1.14196667 | N.D. | 0.2685 | not detected | n.a. | n.a. | n.a. | infinite | 0.70523333 | 3.07057742 |
| AT1G79560 | Encodes an FtsH protease that is localized to the chloroplast. Mutations in this locus result in embryo lethality. | N.D. | 1.09513333 | N.D. | 0.30153333 | not detected | n.a. | n.a. | n.a. | infinite | 0.69833333 | 3.0405349 |
| AT5G43745 | Protein of unknown function (DUF1012); LOCATED IN: chloroplast, chloroplast envelope; | N.D. | 0.8634 | N.D. | 0.4881 | not detected | n.a. | n.a. | n.a. | infinite | 0.67575 | 2.94220734 |
| AT5G22830 | Transmembrane magnesium transporter. One of nine family members. | N.D. | 0.975 | N.D. | 0.3706 | not detected | n.a. | n.a. | n.a. | infinite | 0.6728 | 2.92936308 |
| AT5G64580 | AAA-type ATPase family protein; FUNCTIONS IN: nucleoside-triphosphatase activity, metalloendopeptidase activity, nucleotide binding, ATP binding; INVOLVED IN: embryo development; LOCATED IN: chloroplast, chloroplast envelope | N.D. | 0.99913333 | N.D. | 0.26546667 | not detected | n.a. | n.a. | n.a. | infinite | 0.6323 | 2.75302657 |
| AT5G42480 | Shows homology to the cyanobacterial cell division protein Ftn2, mutant only has two mesophyll cell chloroplasts. Protein was localized to a ring at the center of the chloroplasts. | N.D. | 0.9325 | N.D. | 0.18863333 | not detected | n.a. | n.a. | n.a. | infinite | 0.56056667 | 2.4407005 |
| AT2G36160 | Ribosomal protein S11 family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic small ribosomal subunit, cytosolic ribosome, plasma membrane, chloroplast, membrane; | N.D. | 0.846 | N.D. | 0.2729 | not detected | n.a. | n.a. | n.a. | infinite | 0.55945 | 2.43583855 |
| AT3G01290 | SPFH/Band 7/PHB domain-containing membrane-associated protein family; INVOLVED IN: N-terminal protein myristoylation; LOCATED IN: mitochondrion, plasma membrane, vacuole, membrane | N.D. | 0.51386667 | N.D. | 0.59723333 | not detected | n.a. | n.a. | n.a. | infinite | 0.55555 | 2.41885799 |
| AT5G03910 | member of ATH subfamily | N.D. | 0.8718 | N.D. | 0.23696667 | not detected | n.a. | n.a. | n.a. | infinite | 0.55438333 | 2.41377834 |
| AT5G56030 | A member of heat shock protein 90 (HSP90) gene family. Expressed in all tissues and abundant in root apical meristem, pollen and tapetum. Expression is NOT heat-induced but induced by IAA and NaCl. Interacts with HsfA1d in the cytosol and the nucleus and | N.D. | 1.0272 | N.D. | 0.04696667 | not detected | n.a. | n.a. | n.a. | infinite | 0.53708333 | 2.33845435 |
| AT5G14030 | translocon-associated protein beta (TRAPB) family protein; FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; | N.D. | 0.8145 | N.D. | 0.18113333 | not detected | n.a. | n.a. | n.a. | infinite | 0.49781667 | 2.16748776 |
| AT2G19730 | Ribosomal L28e protein family; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation, ribosome biogenesis; LOCATED IN: in 6 components | N.D. | 0.72103333 | N.D. | 0.2547 | not detected | n.a. | n.a. | n.a. | infinite | 0.48786667 | 2.12416558 |
| AT4G23940 | Encodes FtsHi1. Localizes to the chloroplast envelope membrane. Functions in chloroplast biogenesis and division. | N.D. | 0.72396667 | N.D. | 0.25003333 | not detected | n.a. | n.a. | n.a. | infinite | 0.487 | 2.12039212 |
| AT5G45775 | Ribosomal L5P family protein; FUNCTIONS IN: structural constituent of ribosome; INVOLVED IN: translation; LOCATED IN: cytosolic large ribosomal subunit, vacuole | N.D. | 0.73806667 | N.D. | 0.22373333 | not detected | n.a. | n.a. | n.a. | infinite | 0.4809 | 2.09383279 |
| AT3G61260 | Remorin family protein; FUNCTIONS IN: binding; INVOLVED IN: biological_process unknown; LOCATED IN: plasma membrane, vacuole; EXPRESSED IN: 24 plant structures; EXPRESSED DURING: 16 growth stages; CONTAINS InterPro DOMAIN/s: Remorin, C-terminal (InterPro: | N.D. | 2.45243333 | N.D. | 1.38516667 | not detected | 0.077866667 | 31.4952911 | 17.7889555 | 24.6421233 | 1.9188 | 8.35443203 |
| AT3G17970 | Integral chloroplast outer membrane protein. Belongs to one of the 36 carboxylate clamp (CC)-tetratricopeptide repeat (TPR) proteins (Prasad 2010, Pubmed ID: 20856808) with potential to interact with Hsp90/Hsp70 as co-chaperones. | N.D. | 1.86883333 | N.D. | 0.5175 | AT3G17970 | 0.109733333 | 17.0306804 | 4.71597813 | 10.8733293 | 1.19316667 | 5.19503326 |
| AT5G05000 | Outer membrane GTPase protein that may function in import of nuclear encoded proteins into the chloroplast. Phosphorylation of the G-domains regulate translocon assembly. | N.D. | 3.00213333 | N.D. | 1.28286667 | AT5G05000 | 0.216633333 | 13.858132 | 5.92183413 | 9.88998307 | 2.1425 | 9.32841913 |
| AT5G42020 | Luminal binding protein (BiP2) involved in polar nuclei fusion during proliferation of endosperm nuclei. | N.D. | 1.8882 | N.D. | 0.65423333 | AT5G42020 | 0.1303 | 14.4911742 | 5.02097723 | 9.75607572 | 1.27121667 | 5.53486202 |
| ATCG01130 | YCF1.2; FUNCTIONS IN: protein binding; LOCATED IN: chloroplast, membrane; EXPRESSED IN: cultured cell, leaf; CONTAINS InterPro DOMAIN/s: Ycf1 (InterPro:IPR008896); BEST Arabidopsis thaliana protein match is: Ycf1 protein (TAIR:ATCG01000.1). | N.D. | 3.65786667 | N.D. | 0.7971 | ATCG01130 | 0.265366667 | 13.784198 | 3.00376837 | 8.39398317 | 2.22748333 | 9.69843554 |
| AT3G49860 | A member of ARF-like GTPase family. A thaliana has 21 members, in two subfamilies, ARF and ARF-like (ARL) GTPases. | N.D. | 0.51023333 | N.D. | 0.1792 | AT3G49860 | 0.071166667 | 7.16955504 | 2.51803279 | 4.84379391 | 0.34471667 | 1.5008922 |
| AT1G10510 | embryo defective 2004 (emb2004); INVOLVED IN: embryo development ending in seed dormancy; LOCATED IN: mitochondrion, chloroplast, plastid, chloroplast envelope | N.D. | 1.73596667 | N.D. | 0.48246667 | AT1G10510 | 0.336333333 | 5.16144698 | 1.43448959 | 3.29796829 | 1.10921667 | 4.82951597 |
| AT1G02280 | Encodes a GTP-binding GTP-ase. Component of the chloroplast protein import machinery. Required for import of POR B into plastids. Toc33 phosphorylation may not play an important role in vivo. | N.D. | 3.2534 | N.D. | 0.7088 | AT1G02280 | 0.653133333 | 4.98121874 | 1.08523017 | 3.03322446 | 1.9811 | 8.62568548 |
| AT3G18420 | Protein prenylyltransferase superfamily protein; FUNCTIONS IN: binding; INVOLVED IN: biological_process unknown; LOCATED IN: chloroplast, chloroplast envelope | N.D. | 0.45073333 | N.D. | 0.30356667 | AT3G18420 | 0.145233333 | 3.10351159 | 2.09019968 | 2.59685563 | 0.37715 | 1.64210655 |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| AT2G43630 | FUNCTIONS IN: molecular_function unknown; INVOLVED IN: biological_process unknown; LOCATED IN: chloroplast thylakoid membrane, chloroplast, nucleus, chloroplast envelope | N.D. | 1.4308 | N.D. | 0.65796667 | AT2G43630 | 0.484433333 | 2.95355398 | 1.35821923 | 2.1558866 | 1.04438333 | 4.54723242 |
| AT5G14040 | Encodes a mitochondrial phosphate transporter. Modulates plant responses to salt stress. | N.D. | 0.89613333 | N.D. | 0.25456667 | AT5G14040 | 0.3131 | 2.86213137 | 0.81305227 | 1.83759182 | 0.57535 | 2.50506695 |
| AT3G46740 | Component of the translocon outer membrane (TOC) complex. Forms the outer envelope translocation channel (beta-barrel). Plays a role in preprotein conductance. Imported into chloroplast. Expressed in young dividing photosynthetic tissues. | N.D. | 8.04253333 | N.D. | 1.84283333 | AT3G46740 | 2.8509 | 2.82105066 | 0.64640406 | 1.73372736 | 4.94268333 | 859.074187 |
| AT5G23040 | Encodes a protein that enables protochlorophillide's binding to pPORA's transit sequence, regulating pPORA's translocation into the plastid stroma, and blocking movement of the translocating polypeptide chain back into the cytosol. | N.D. | 0.99363333 | N.D. | 0.31663333 | AT5G23040 | 0.4071 | 2.44075985 | 0.77777778 | 1.60926881 | 0.65513333 | 2.85244262 |
| AT3G12580 | heat shock protein 70 (HSP70); FUNCTIONS IN: ATP binding; INVOLVED IN: in 9 processes; LOCATED IN: cytosol, mitochondrion, cell wall, plasma membrane. | - | 0.2278 | N.D. | 0.16866667 | AT3G12580 | 0.1409 | 1.61674947 | 1.19706648 | 1.40690797 | 0.19823333 | 0.86310554 |
| AT4G00630 | Encodes a K(+)/H(+) antiporter that modulates monovalent cation and pH homeostasis in plant chloroplasts or plastids. | N.D. | 1.0542 | N.D. | 0.2698 | AT4G00630 | 0.472666667 | 2.2303244 | 0.57080395 | 1.40056417 | 0.662 | 2.88234001 |
| AT2G43950 | Constitutes a peptide sensitive ion channel in chloroplast outer membranes. Accumulates in germinating seeds and developing embryos. | N.D. | 2.5375 | N.D. | 0.7826 | AT2G43950 | 1.199833333 | 2.11487707 | 0.65225726 | 1.38356716 | 1.66005 | 7.22783766 |
| AT3G10840 | alpha/beta-Hydrolases superfamily protein; FUNCTIONS IN: hydrolase activity, catalytic activity; LOCATED IN: chloroplast envelope | N.D. | 0.81403333 | N.D. | 0.29863333 | AT3G10840 | 0.4654 | 1.74910471 | 0.64167025 | 1.19538748 | 0.55633333 | 2.42226862 |
| AT5G23890 | LOCATED IN: mitochondrion, chloroplast thylakoid membrane, chloroplast, plastid, chloroplast envelope; EXPRESSED IN: 24 plant structures; EXPRESSED DURING: 14 growth stages; CONTAINS InterPro DOMAIN/s: S-layer homology domain (InterPro:IPR001119) | N.D. | 1.30993333 | N.D. | 0.239 | AT5G23890 | 0.653966667 | 2.00305826 | 0.36546205 | 1.18426016 | 0.77446667 | 3.37201852 |
| AT1G77590 | Encodes major plastidic long chain acyl-CoA synthetase with a slight substrate preference of oleic acid over any of the other fatty acids. | N.D. | 1.2093 | N.D. | 0.30783333 | AT1G77590 | 0.704 | 1.71775568 | 0.43726326 | 1.07750947 | 0.75856667 | 3.30279011 |
| AT3G01500 | Encodes a putative beta-carbonic anhydrase betaCA1. Together with betaCA4 (At1g70410) regulates CO2-controlled stomatal movements in guard cells. | N.D. | 2.4029 | 0.59603333333 | 32.4081667 | AT3G01500 | 16.37386667 | 0.14675214 | 1.97926167 | 1.06300691 | 17.4055333 | 75.783482 |

**Table S1**: Identification of KOC-interacting proteins and quantification by MSE. Proteins interacting with KOC were quantified by MSE and their quantities related to their abundance in a chloroplast reference proteome. The enrichment index is arbitrary and calculated from the abundance of the protein in the eluates divided by their abundance in complete chloroplasts without prior enrichment. Only those proteins identified in two biological replicates, no detection in the negative controls, an enrichment index above 1 and an absolute abundance of at least 2% of the bait were retained in the list and are displayed here.

| Primer name | Primer sequence |
|---|---|
| KOC1_NheI_F | 5’-GAGAGAGCTAGCATGGCTTCAAAGATTATT-3’ |
| KOC1_NotI_R | 5’-TCTCTCGCGGCCGCAAAGAACACAAGCCA-3’ |
| KOC1_NcoI_F | 5’-GAGACCATGGCTTCAAAGATTATTGC-3’ |
| KOC1_NcoI_R | 5’- TCTCCCATGGCTTGCAGATGATCCTTTGAA-3’ |
| KOC1_NcoI_R2 | 5’- TCTCTCGCGGCCGCATCCTCCAGATGTTGA-3’ |
| KOC1_FL_NcoI_F | 5’-TACCATGGCTAGCATGGCTTCAAAG-3’ |
| KOC1_FL_XbaI_R | 5’-TCTCTAGACTAAAAGAACACAAGCCATTTAG-3’ |
| LBb1.3 (Salk Institute) | 5’-ATTTTGCCGATTTCGGAAC-3’ |
| koc1-1_LP | 5’- CAGGGGAGTCTCTAGTATCA-3’ |
| koc1-1_RP | 5’-TAG CAC AAA TCC ATC TGG GTC-3’ |
| koc1-2_LP | 5’-AGAACATGGATGTGCCAGAAG-3’ |
| koc1-2_RP | 5’-CGCTGCATATACCATGTGATG-3’ |
| Toc159-StuI-F | 5’-CAAATTCTCTATTGAGGCCTCAAGAACCATTG-3’ |
| Toc159-cmyc-Gib-R | 5’-CTTCTTCAGAGATCAGTTTCTGTTCTACGTACATGCTGTACTTGTC-3’ |
| cmyc-uniGib-R | 5’-CTGCAGGTCGACTCTAGATTACAGATCTTCTTCAGAGATC-3’ |
| attB-KOC1-FW | 5’- ACAAGTTTGTACAAAAAAGCAGGCTCCGCTTCAAAGATTATTGCTGG-3’ |
| attB-KOC1-REV | 5’- ACCACTTTGTACAAGAAAGCTGGGTCTAAAAGAACACAAGCCATT-3’ |
| attB-EMB2004-FW | 5’-GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTTCTTCTTCCACCAG-3’ |
| attB-EMB2004-REV | 5’-GGGGACCACTTTGTACAAGAAAGCTGGGTGGAAGAAAATTTCTACTTCC-3’ |

**TABLE S2**: List of primers used for cloning and genotyping