Chup1 – a chloroplast movement protein and its interactions

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vorgelegt von
Serena Schmidt von Braun

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Erstgutachter: Prof. Dr. Enrico Schleiff
Zweitgutachter: Prof. Dr. Manfred Schliwa

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[...] Nach Mohl's[1]) Angabe sind in der mittleren Schichte des Blattes von *Orontium (Rhodea) japonicum* die Chlorophyllkörner in der Mitte der Zelle zu einem Haufen zusammengeballt. Ich habe diese Pflanze oft untersucht, fand aber immer die Chlorophyllkörner an der Zellwandung anliegen. Bei der grossen Aufmerksamkeit jedoch, mit der ich die Chlorophyllkörner der verschiedenen *Sedum*-Arten untersuchte, zeigte sich mir eine höchst interessante Erscheinung. Ich brachte nämlich mehrere Arten derselben mit cylindrischen Blättern ins warme Haus, dessen Fenster sich gegen Süden öffneten, um vielleicht in den Blättern der unter diesen Umständen sich rasch entwickelnden Triebe über die jugendlichen Zustände der Chlorophyllkörner einigen Aufschluss zu erhalten. Zufälliger Weise untersuchte ich sie längere Zeit hindurch täglich zur Mittagsstunde, und ward nicht wenig überrascht, stets sämmtliche Chlorophyllkörner zu einer Gruppe vereinigt irgend einer Stelle der Zellwandung anliegend zu finden [...]

Figure 1 Excerpt from: Böhm JA (1856) Beiträge zur näheren Kenntnis des Chlorophylls. S.B. Akad. Wiss. Wien, Math.-nat. Kl. 22: 479-498

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1.1 Abstract

The molecular mechanisms of light dependent chloroplast movement could for a long time not be unravelled. But the recent discovery of a mutant deficient in chloroplast movement sparked new impulses in the field. This study investigates the molecular mechanisms of chloroplast movement based on the protein Chup1 and the interactions of Chup1 and cytoskeletal effectors. It is demonstrated that Chup1 is exclusively and directly targeted to the chloroplast surface in an N-terminus dependent manner.

Analyzing a putative role of Chup1 as a linker between chloroplasts and the cytoskeleton, an interaction with actin is demonstrated which is independent on the filament status of actin. In accordance with this, binding of actin to the outer envelope of chloroplasts is demonstrated. Adding to the understanding of chloroplast movement, it is shown that Chup1 interacts with profilin. Furthermore, an enhancing effect of Chup1 on the interaction of profilin to actin could be demonstrated. As profilin is an actin binding protein and a potent modifier of the polymerisation status of actin filaments, a key role of profilin in chloroplast movement is suggested. For Chup1, an important role as a linker molecule in bridging chloroplasts to actin filaments and a regulatory function in actin polymerization is discussed.

The investigation of the global expression profile revealed the effects of light treatment on *chup1* mutant plants and the effects of blue light on wildtype plants. From cluster analysis, gene products participating in blue-light induced signalling are suggested. Furthermore, it is suggested, that gene expression is not involved in the regulation of chloroplast movement. A conclusive model of chloroplast movement can be presented.

1.2 Zusammenfassung

Die molekularen Mechanismen der Chloroplastenbewegung waren für lange Zeit rätselhaft. Erst die kürzliche Entdeckung einer Mutante, die Defekte in der Chloroplastenbewegung aufwies, brachte neue Impulse in die Forschergemeinschaft. In dieser Arbeit werden die molekularen Mechanismen der Chloroplastenbewegung im Zusammenhang mit dem Protein Chup1 und die Interaktionen von Chup1 mit Zytoskelett - Effektoren untersucht. Dabei wird gezeigt, dass Chup1, abhängig von einem N-terminalen Signal, ausschließlich und auf direktem Weg an die Chloroplastenoberfläche geleitet wird. In dieser Arbeit wird die Rolle von Chup1 als putatives Verbindungsglied zwischen dem Chloroplasten und dem Aktin-Zytoskelett untersucht. Dabei kann eine Interaktion von Aktin und Chup1 gezeigt werden, die unabhängig vom Aktin-Filamentstatus ist. In Übereinstimmung hiermit wird gezeigt, dass Chup1 an die äußere Hüllmembran des Chloroplasten binden kann.

Die Tatsache dass eine Interaktion von Chup1 und Profilin gezeigt werden kann bringt einen Erkenntnisgewinn für die Regulation der Chloroplastenbewegung. Zudem kann eine Verstärkung der Interaktion von Aktin und Profilin durch Chup1 nachgewiesen werden. Da Profilin ein Aktin bindendes Protein ist und darüber hinaus eine zentrale Komponente in der Aktin-Zytoskelett Dynamik darstellt, wird eine Schlüsselrolle für Profilin in der Regulation der Chloroplastenbewegung vorgeschlagen. Für Chup1 wird eine wichtige Funktion als Bindeglied zwischen dem Aktin-Zytoskelett und dem Chloroplasten und eine Rolle in der Regulation der Aktin-Polymerisation diskutiert. Durch eine Analyse des globalen Expressionsprofils konnten die Effekte von Starklicht auf die *chup1* Mutante und von Blaulicht auf den Wildtyp untersucht werden. Durch eine Clusteranalyse konnten zudem Einblicke in die Signalkette der Lichtregulation gewonnen werden. Darüber hinaus wird postuliert, dass Genexpression keinen Anteil an der Regulation von blaulichtgesteuerten Signalketten in der Chloroplastenbewegung hat. Abschließend kann ein verbessertes Modell für die Chloroplastenbewegung vorgeschlagen werden.

2 Introduction

2.1 Chloroplast Movement

Chloroplasts are semi-autonomous organelles in plant cells that evolved from an ancient cyanobacterium taken up by a host cell (Sagan 1967, Martin et al 1998, Cavalier-Smith 2000). The major function of chloroplasts is the conversion of light energy to utilizable energy for the cell. The importance of chloroplasts is paramount for life that is dependent on biomass produced with energy from the sun (which is of course most life on earth). Therefore it is astonishing that the principal mechanisms of chloroplast movement remained a mystery for a long time. The apparent movement of chloroplasts in response to light per se however, can be observed with simple instruments. For this reason the research on the phenomenon of the moving chloroplasts has a history of 150 years. One of the first to publish observations on chloroplast movement was Böhm in 1856 (Figure 1). He made the observation that the so called "chlorophyll particles" (chloroplasts) in leaves of *Sedum* plants could have different organizations in the cell under different light conditions. The movements that chloroplasts undergo to take different spatial organizations in variable light are today referred to as "accumulation movement" under low light and "avoidance movement" under high light conditions (Wada et al. 2003) (Figure 2). The distribution of chloroplasts in mesophyll cells in low light conditions is an arrangement at the cell walls perpendicular to the light. Under high fluence rates the chloroplasts arrange at the walls parallel to the light. In darkness, the chloroplasts can adopt a third position that varies among different species (Inoue and Shibata, 1974), depends on the growth conditions (Trojan and Gabrys, 1996) and can be an intermediate form or an accumulation at the bottom of the cell. This positioning is termed dark positioning or dark accumulation (Suetsugu et al. 2005b).

Light is not the only trigger for chloroplast movement, other triggers for movement have been found in fern (*Adiantum capillus-veneris*). The chloroplasts in this species react to mechano-stimulation and wounding (Sato et al. 1999).

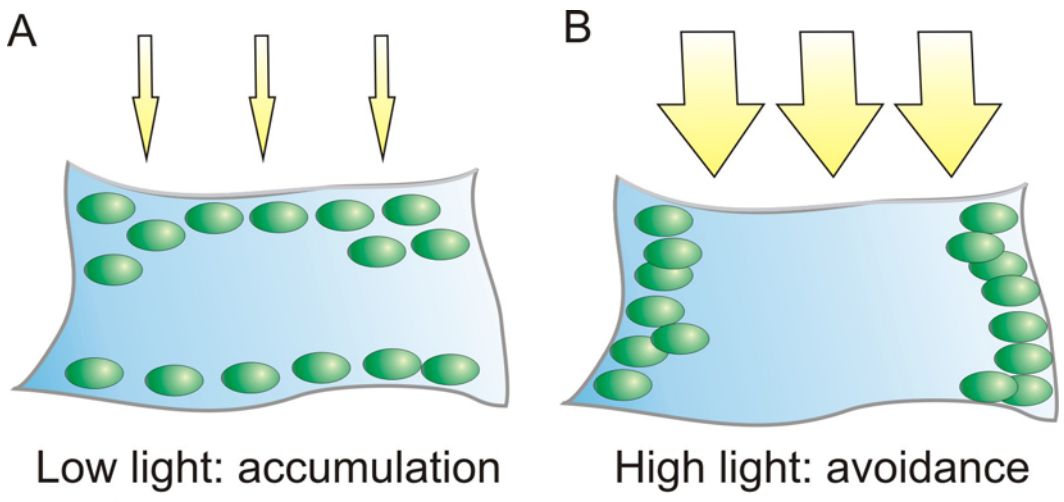

Figure 2 Chloroplast distribution under different light conditions. A Chloroplasts accumulate under low light conditions at the periclinial walls to gather more light. **B** Chloroplasts undergo avoidance movement under high light conditions and distribute at the anticlinial walls to avoid high light. The high light distribution of chloroplasts allows them to avoid photodamage, while the low light distribution enables them to gather light efficiently for photosynthesis.

The physiological reason for chloroplast arrangements under different light conditions was early proposed to be the optimization of photosynthesis (Zurzycki et al. 1955). The light absorption would thus be maximized by the low light arrangement of chloroplasts in light conditions when the photon flux is below the saturation point of the photosystems. Vice versa, the photosystems are protected from photodamage in high light by the arrangement at the anticlinial walls, where mutual shading is optimized (Zurzycki 1957, Park et al 1996). This hypothesis was confirmed 2002 by Kasahara and colleagues, as chloroplasts in mutant plants, that could not perform chloroplast movement in high light were more sensitive to photodamage than wild type plants.

In the past, other reasons for chloroplast movement were discussed as well, like altered CO_2 diffusion, but no evidence for these hypotheses could be presented so far (e.g. Gorton et al. 2003).

Chloroplast movement thus is an adaptation to light in between short term regulation of the rate of photosynthesis - e.g. by phosphorylation/degradation - and long term photoprotection mechanisms like reduction of grana thylakoids or transcription control.

2.2 The Signal

2.2.1 The Light Receptors

Early on it was clear that light itself is the signal. More specifically, blue light induces the relocation reaction of chloroplasts. This is true for most plants (Sato et al. 2000). Exceptions are several cryptogams (the algae, fern and moss *Mougeotia*, *Adiantum*, and *Mesotaenium*) however, where red light together with blue light is used for the regulation of directional chloroplast movement (Wada et al. 1993). The red light receptor phytochrome has been demonstrated to function as a light receptor for chloroplast movement in algae (Haupt et al. 1969), mosses (Sato et al. 2001), ferns (Yatsuhashi 1996) and in the aquatic angiosperm *Vallisneria* (Dong et al. 1995). To some effect, red light can also slightly modulate the blue light induced chloroplast movement in other plants (investigated in *Arabidopsis*, Kagawa and Wada 2000, DeBlasio et al. 2005). The response to red in contrast to blue light might reflect the shifting of light requirements of the photosystems (see Schmidt von Braun and Schleiff 2007).

The search for a flavoprotein began as it became clear that blue light (390–500 nm) and ultraviolet-A (320–390 nm) light was the trigger for chloroplast movement and blue light induced morphological changes in most plants. Flavoproteins were the favoured candidates because the action spectrum of phototropism and chloroplast movement closely resembled the excitation spectrum of flavoproteins (Briggs and Christie 2002).

This led to the discovery of a new family of photoreceptors, the so called phototropins (Liscum and Briggs 1995, Huala et al. 1997, Christie et al. 1999). Phototropins are the blue light sensitive receptors that convey the signal for the light-induced movements of chloroplasts (Jarillo et al. 2001, Kagawa et al. 2001, Sakai et al. 2001). They additionally mediate phototropism (Huala et al. 1997, Liscum and Briggs, 1995), blue-light-induced stomatal opening (Kinoshita et al. 2001) and other blue light dependent reactions like the rapid inhibition of hypocotyl growth (Folta and Spalding, 2001).

Two phototropins have been identified in *Arabidopsis* to date: Phot1 and Phot2 (former names NPH1 and NPL1) and subsequently been found in other plants. They differ in the sensitivities to blue light: Phot1 being susceptible to lower fluence rates than Phot2 (Kagawa et al. 2001, Jarillo et al. 2001, Sakai et al. 2001). Phot1 mediates accumulation over a broad fluence range of light (from 0.4 to 100 μmol), whereas Phot2 mediates accumulation at low fluence at a higher rate than Phot1 (2 to 16 μmol) and avoidance at high fluence rates (32 and

100 µmol) in mesophyll cells (all figures for blue light (390-500 nm), Harada and Shimazaki 2007). This explains the requirement of Phot1 in the accumulation response and Phot2 in both accumulation and avoidance response.

The structure of the phototropins comprises a serine/threonine kinase in the C-terminal part of the protein and two domains found in signalling proteins: the light, oxygen or voltage (LOV) domain (Huala et al. 1997). The LOV domains function as the binding site for two flavin mononucleotide (FMN) chromophores (Christie et al. 1999, Sakai et al. 2001). The FMN is non-covalently bound in the dark, but forms an adduct with the LOV domain in blue light (Salomon et al. 2000). This is thought to activate the kinase domain by a structural change in the protein through the release of the binding of the LOV2 domain to the kinase domain in light (Matsuoka and Tokutomi, 2005). The Phot2 protein is slightly shorter than Phot1 (Briggs et al. 2001) but what actually is responsible for the difference in light sensitivity is not known yet. The phototropins can undergo autophosphorylation upon light reception. Phosphorylation of other substrates by the kinase domain has not been detected so far, but seems quite likely (see Discussion).

In *Adiantum* as well as in *Mougeotia* - both showing chloroplast movement in response to blue and red light - a chimeric photoreceptor resulting from gene fusion between the N-terminus of the red light receptor phytochrome and a phototropin was found which was termed neochrome, and is responsible for chloroplast movement in these organisms (Nozue et al. 1998, Suetsugu et al. 2005a).

Both phototropins are localized at the plasma membrane (Christie et al. 2002, Harada et al. 2003). Phot1 was also seen to be localized in part in the cytoplasm during blue light illumination (Sakamoto and Briggs, 2002), the consequence of the relocalization was not detected. In 2006 it was reported by Kong et al. that Phot2 relocalizes from the plasma membrane to the Golgi apparatus upon blue light illumination. The kinase domain was found to be essential for the relocalization. An implication for signal transduction was concluded from this observation (see Discussion) and even a chloroplast localization is suggested (Harada and Shimazaki 2007; Weber, Düsseldorf, personal communication). However, the consequence of the delocalization of Phot1 and Phot2 from the plasma membrane remains to be studied.

2.2.2 The modulator calcium

Upon blue light illumination, a phototropin-mediated increase in calcium levels in the cytoplasm has been reported (Baum et al. 1999, Babourina et al. 2002, Stoelzle et al. 2003). In line, it was shown that calcium participates as modulator downstream of phototropin signal transduction in chloroplast movement with different intensities in high- and low light (Harada et al. 2003). In the case of chloroplast movement, unlike the situation in phototropism (Baum et al. 1999), calcium was reported to be released from internal stores and not by influx from the apoplast (Tlalka and Gabrys 1993, Tlalka and Fricker 1999, Sato et al. 2001, Stoelzle et al. 2003). A Phot2 dependent calcium release from internal stores (like for instance the ER, the vacuole or the Golgi) has been concluded from calcium channel inhibitor studies and inhibition of phospholipase C (which can induce calcium release (see Discussion)) in phototropin mutants (Harada et al. 2003). For Phot1 however, which mediates calcium influx from the apoplast, as found by mutant and inhibitor studies (Baum et al. 1999), to date no influence in intracellular calcium release has been found. The controversy is discussed by Harada et al. (2003) who proposed a Phot1 mediated modulation of Phot2 induced calcium increase from internal stores. The differentiation between accumulation in low light and avoidance in high light is accomplished by the different increases in calcium concentration on one hand, by the possible amplification of Phot2-induced signalling by Phot1 on the other hand and also very likely by the relocation of Phot2 to the Golgi in strong light. In the latter case Phot2 is possibly inducing intracellular Ca^{2+} release that could provide a different Ca^{2+} signature in strong light. Furthermore a direct involvement of the chloroplast in signalling is conceivable (see Discussion).

The differentiation for the signalling downstream of phototropins to result in either chloroplast movement, phototropism or other blue light induced changes, could equally be the result of the release of Ca^{2+} from different sources that produce different patterns or "Ca^{2+} signatures" in the cytosol (Allen and Schroeder 2001, Sanders et al. 2002, Harada and Shimazaki 2007) and interact with calcium effectors such as calmodulin or other calcium binding proteins.

Downstream interactors with phototropins have been identified yet only in connection with phototropism and stomata opening. In *Vicia faba*, the protein VfPip with homology to a dynein light chain was identified to interact with Phot1, and was found to interact with microtubules in guard cells. It has been concluded that VfPip is involved in blue-light induced stomata opening (Emi et al 2005). The two proteins identified so far in *Arabidopsis* are Rpt2 and Nph3 and belong to a family of novel plant specific proteins (Sakai et al. 2000,

Motchoulski and Liscum 1999). Rpt2 forms a complex with Phot1 in vivo and both have putative phosphorylation sites, a nuclear localization signal, a BTB/POZ domain, and a coiled-coil domain. Another protein family interacting with Phot1 in phototropin signalling are the Pks proteins (Pks1-4). They could function together with Phot1 and Nph3 to mediate phototropism (Lariguet et al. 2006), as Pks1 is forming a complex with Phot1 and Nhp3. Rpt2 and Nph3 proteins are not involved in signal transduction leading to chloroplast movement, as demonstrated by mutant studies (Inada et al. 2004). The Pks mutants were not tested yet but seem to be predominantly involved in hypocotyl curvature.
From the signal to the actual movement a further component has to be considered - the mechanism of movement which is relying on tracks.

2.3 The Tracks

Plant cells contain two types of cytoskeletal elements, microtubuli and microfilaments, which besides maintaining a solid support also perform numerous other functions including signalling, transport and cell division. Microtubuli are built from tubulin subunits (α- and β-tubulin, with nine and six isoforms in *Arabidopsis*) and microfilaments from actin monomers (eight functional isoforms in *Arabidopsis*, Meagher et al. 1999). The search for intermediate filaments as found in animal cells is still ongoing. Intermediate filaments comprise a family of structurally related alpha-helical proteins with globular tails that form non-polar filament structures. Putative candidates for intermediate filaments in plants are for instance the Filament-like plant proteins (FPP), that seem to be structurally related to animal nuclear lamins, and other large coiled-coil containing proteins (Gindullis et al. 2002).
Plant actins were identified more easily, as plant actin isoforms are typically showing 83 to 88% identity to actins of a wide range of species including animals. This high degree of conservation is interpreted to be a result of the fact that almost the whole surface of actin is involved in protein–protein interactions (Meagher et al. 1999). Actin is involved in many different cellular processes like establishing cell polarity, division plane determination, preprogramming of development and cell wall deposition, cell elongation, tip growth, transmembrane transport and positioning of receptors, mRNA transport within the cell, RNA polymerase I transcription and organelle movement (e.g. Staiger and Lloyd 1991, Meagher et al. 1999, Philimonenko et al. 2004).

It was hypothesized early that chloroplasts use the cytoskeleton to move in the cell. Boresch postulated a model of "pulling fibres" in 1914, which could be interpreted as the first explanation of the involvement of cytoskeletal elements in chloroplast movement.

Proof for the conception of cytoskeletal elements taking part in chloroplast movement was gained by inhibitor studies. In most investigated plants, the mechanism of chloroplast movement is relying on actin filaments, as actin antagonists like cytochalasin-D, m-maleimidobenzoic acid or N-hydroxysuccinimide ester inhibit chloroplast movement (in the green algae *Mougeotia* (Wagner et al. 1972), the fern *A. capillus-veneris* (Kadota and Wada 1992), mosses (Sato et al. 2001), *Lemna triscula*, (Malec et al. 1996), *Alocasia macrorrhiza* Gorton et al. 1999) and angiosperms (Witztum and Parthasarathy 1985, Izutani et al. 1990, Tlalka and Gabrys 1993, Kandasamy and Meagher 1999, *A.thaliana*).

No inhibitory effect on chloroplast movement was found however with microtubule drugs. This is true for most plant species, except for the situation in the mosses *F. hygrometrica* and *P. patens,* where microtubules are also participating in chloroplast movement (Wacker et al. 1988, Quader and Schnepf 1989). Interestingly, in *P. patens* - making the movement mechanism different from that of dicotelydons - red light induced chloroplast movement and rapid longitudinal movement in the dark is relying on microtubules only, whereas blue light induced movement occurs on both, microtubules and microfilaments (Sato et al. 2001).

Microscopic observations with fluorescently stained actin revealed that chloroplasts are surrounded by circular actin structures which appear after the end of accumulation movement and before the start of the avoidance movement in fern (*Adiantum capillus-veneris*) (Kadota and Wada 1992). These actin structures were also observed by Dong et al. (1998) in *Vallisneria gigantea*, who described a honeycomb array surrounding the chloroplasts, which was resistant to centrifugal force. This is evidence for an anchoring of chloroplasts in position at times when no light-induced movement occurs. A more detailed observation of fine basket-like actin structures closely surrounding chloroplasts was made by Kandasamy and Meagher (1999) and Kwok and Hanson (2004). They even observed connections between the fine actin filaments on the chloroplast surface and thicker actin filaments extending to strong microfilament bundles. The latter became more prominent on illumination and extended throughout the cell, presumably to form the tracks on which chloroplast movement could take place.

Actin rearrangement after illumination was also observed by Sakurai et al. (2005) in the aquatic angiosperm *Vallisneria gigantea*. Here, short bundles of actin were observed in the

vicinity of chloroplasts in dark adapted cells that disappeared under illumination, while long straight bundles appeared at the same time in the cell. The reorganization was completed after 10 min which coincided with the time of the onset of movement (Sakurai et al. 2005).

The dynamic nature of the actin cytoskeleton can be made understandable when realizing that most actin filaments have half-lives of approximately 1 min (Theriot and Mitchison 1991) and most cross-links between filaments last less than 1 sec (Wachsstock et al. 1994). These dynamics are possible due to the architecture of the filament which is built from actin monomers. A large pool of monomeric G-actin is present in the cell at the same time as the filamentous F-actin. This is made possible by a large number of actin binding proteins that can modify the polymerisation and depolymerisation speed. Actin filaments have a polarised structure. This means that monomer addition to the filament preferentially takes place at the plus (or barbed end) while monomer loss is happening at the minus (or pointed) end. A rapid restructuring of the filament is done with the help of actin binding proteins, for instance by capping the minus end to avoid monomer loss, by accelerating monomer addition at the plus end, or by maintaining a pool of monomeric actin (e.g. dos Remedios et al. 2003).

A dynamic cytoskeleton thus is a precondition for chloroplast movement. The speed of moving chloroplasts was found to be in a range of 1-1.5 μm/min. Interestingly, the velocity of movement is fluence rate dependent (Kagawa and Wada 2004) and directly linked to the activity of the Phot2 receptor, as the velocity of avoidance movement in heterozygous Phot2 mutants was half of that in wild type (Suetsugu and Wada 2007).

2.4 The motor for movement?

When thinking of an actin-based chloroplast movement, the relation to the actin-associated motor protein myosin is not far from crossing the mind. In *Arabidopsis* the myosin family is made up of the classes VIII, XI and X and contains 17 members. Interestingly, the myosins of class VIII and XI are unique for the plant kingdom (Reddy 2001). As different myosins have different specificities for their cargoes (e.g. organelles) (e.g. Karcher et al. 2002), this fact might be relevant for chloroplast movement.
Indeed, an interaction of putative myosins with chloroplasts as detected by immunolocalization was suggested in a number of plant species (La Claire 1991, La Claire et

al. 1995, Liebe and Menzel, 1995). More specifically, a myosin of the class XI was found to be associated with the surface of maize chloroplasts (Wang and Pesacreta 2004). In a recent analysis of fusion proteins of six *A. thaliana* class XI myosin tails to YFP, a number of organellar localisations of the myosin fusions was detected, but yet no chloroplast localisation (Reisen and Hanson, 2007). Additionally, an influence of myosin XI-K of *Nicotiana benthamiana* on Golgi stack trafficking, mitochondria- and peroxisome movement was observed, but no influence of six other tested myosins on light induced chloroplast movement (Avisar et al. 2008). As there are still seven experimentally untested myosin candidates, the search has to go on.

Different modes of movement have to be considered for chloroplasts. The force for movement could be generated directly between the chloroplast and actin or with the help of linker proteins that connect myosin to the chloroplast. In another scenario, the force of movement could be generated between actin filaments (similar to the situation in muscles) connected to the chloroplast and actin filament tracks (Figure 3).

Recently, it was found that myosins are involved only in accumulation movement but not in avoidance movement in Arabidopsis. This was shown by the inhibition of myosins with three different drugs (Paves and Truve 2007). The implication of this could be that the avoidance movement could possibly rely only on the force generated through actin assembly itself. This could imply a third type of movement conceivable for the chloroplast. An example for this kind of movement can be found for the intracellular pathogen Listeria monocytogenes, which moves by the pushing force resulting from polymerizing actin, forming the so called “comet tails” in its wake (Geese et al. 2000). Comet tails however are not observed in the vicinity of chloroplasts, but the principle could be the same. For vesicle movement, recently a polymerization dependent movement has been identified (Merrifield et al. 1999, May et al. 2000, Rozelle et al. 2000). To exert a pushing force, polymerization has to take place on short actin filaments (30-150 nm) longer filaments tend to bend, unless bundles of 10-30 filaments are formed that build a stiffer structure (van der Honing et al. 2007).

The presence of short actin bundles has been observed on illuminated chloroplasts (see 2.3) which could thus either function in anchoring (as discussed) or in movement itself.

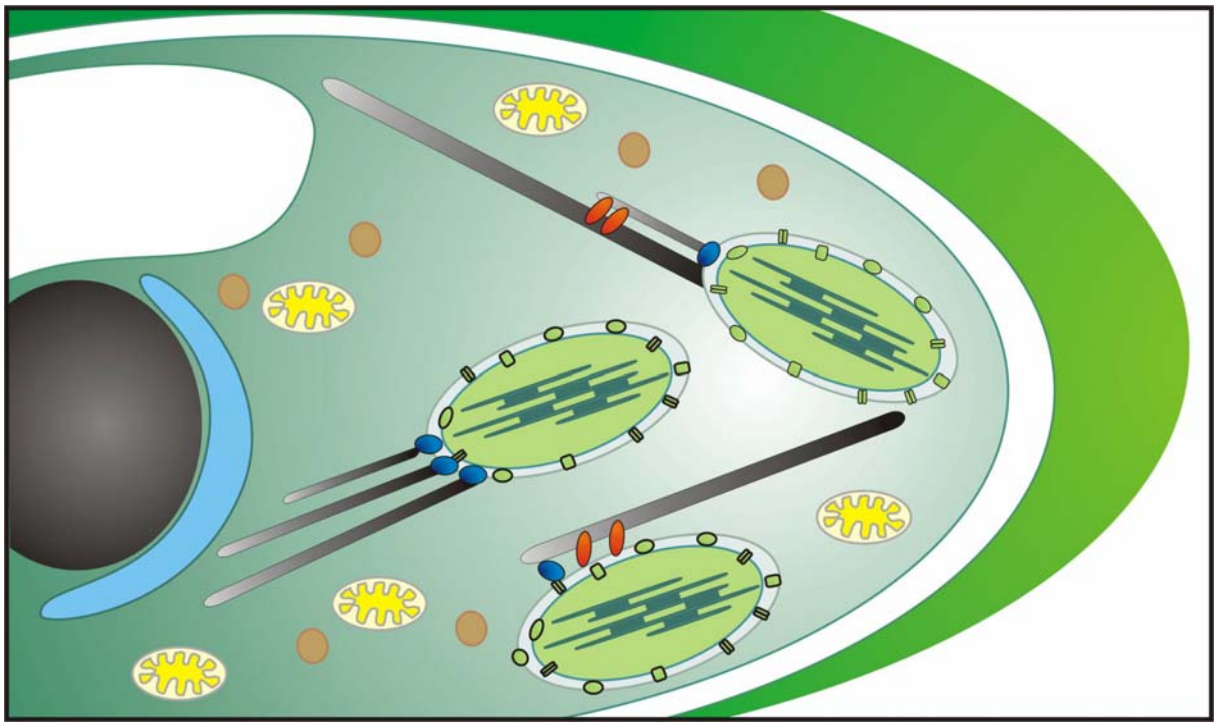

Figure 3 Modes of Movement. Several types of movement mechanistics for chloroplasts are conceivable. Three chloroplasts are depicted with different movement mechanistics. On the upper chloroplast, propulsion force is generated by motor molecules (red) which act between actin filaments, the tethering of the chloroplast to actin is accomplished through linker molecules (blue). For the middle chloroplast, a direct propulsion force is displayed, which is generated directly by actin polymerisation at the chloroplast surface. The lower chloroplast moves by a direct interaction of motors with the chloroplast envelope.

2.5 Components-involved in chloroplast movement

2.5.1 Chup1

In 2002, a mutant was discovered – the first of its kind (apart from photoreceptor mutants) - that was defective in chloroplast movement (Kasahara et al. 2002). The mutant was termed *chup1* for chloroplast unusual positioning 1. In the mutant, chloroplast movement as detectable in wild type was not observed. However, a distribution of chloroplasts on the bottom of the cells was prevailing in all light conditions. This distribution was not due to gravity sedimentation, as the position of the chloroplasts was not altered by an upside-down incubation of the leaves. Peroxisomes were observed to be positioned in the same way as chloroplasts in *chup1* mutants. This is, however, most likely due to the typical close association of chloroplasts and peroxisomes (Mano et al. 2002) and not due to the lack of the Chup1 protein.

By labelling actin filaments with mouse talin-GPF in *Δchup1* plants, according to Oikawa et al. (2003), no apparent change in the cytoskeleton compared to wild type cells was detected.

A study from Sheahan et al. (2004) was pointing out however, that the data presented was flawed by artefacts probably resulting from labelling with talin-GFP, as this is now considered to alter the binding capabilities of actin and cause artificial aggregation of actin. A more detailed observation is needed in future.

Chup1 is a 112 kD protein with several predicted domains. As will be examined in more detail below, Chup1 comprises a hydrophobic N-terminus, a large coiled-coil domain, two putative leucine zippers, a putative actin binding motif and a proline-rich region.

2.5.2 Jac1

Jac1 (J domain protein required for chloroplast accumulation response) is a cytosolic protein and *jac1* mutant plants were found to have defects in the chloroplast accumulation response and in dark-positioning, but were functional in the avoidance response, even though the avoidance movement already set in under a lower fluence rate. Jac1 is therefore indispensable for the accumulation response in low light and in darkness, but not for the avoidance response in high light. (Suetsugu et al. 2005b)

The Jac1 J-domain resembles that of auxilin (e.g. Gall et al. 2000), a clathrin uncoating factor functioning in vesicle transport, but such function could yet not be assigned for Jac1. A function in vesicular traffic could be conceivable since Phot2, a member of the signal transduction machinery relocalizes to the Golgi-apparatus upon illumination with blue light (Kong et al. 2006). Developmental defects as would be expected for an auxilin mutant could not be observed in the *jac1* mutant (e.g. Gall et al. 2000). The authors suggest a possible role for Jac1 in chloroplast movement as a cytosolic signal transducer between phototropins and chloroplasts or so far unidentified proteins (Suetsugu et al. 2005b). Interestingly, Jac1 also functions in Al-uptake in roots. An inhibition of endocytosis was suggested from microscopic observations and a putative function in clathrin-uncoating in the endocytosis process was discussed (Ezaki et al. 2007). A relevance of this function for chloroplast movement has to be investigated.

2.5.3 The Pmi family

Three other mutants with chloroplast movement defects have been published so far by DeBlasio and colleagues (2005) and Luesse et al. (2006). All have been termed plastid movement impaired (Pmi) and display aberrant chloroplast positioning but do not all fall into the same phenotypic groups. *Pmi1* mutants are affected in chloroplast movement under all fluence rates, but do not show the sedimented chloroplast phenotype of *Δchup1*. Pmi1

contains a coiled-coil region at the C-terminus and a rice ortholog was shown to interact with a C2 calcium binding protein in a Yeast Two Hybrid Screen (Cooper et al 2003). A function of Pmi1 in blue-light induced calcium-signalling awaits its investigation.

The *pmi2* mutant displayed attenuated chloroplast movements under medium and high light intensities. The sensitivity to light was observed to be shifted to higher fluence rates in the mutant. Pmi2 is composed of a long coiled-coil region and a putative P-loop (ATP -binding motif A) and is localized in the cytoplasm. Strangely, both, Pmi1 and Pmi2, are expressed in roots as well as in leaves (Luesse et al. 2006).

Pmi15 is similar to Pmi2 but lacks the P loop. The mutant of *pmi15* displayed attenuated chloroplast movement in high-light conditions. *Pmi2* and *pmi15* double mutants show a change in chloroplast movement under all light intensities suggesting a parallel action of the two gene products. Based on the observation of similar phenotypic behaviour of *pim2*, *pmi15* and *phot2*, the authors suggest that Pim2 and Pim15 participate in the Phot2 mediated signal cascade (Luesse et al. 2006).

2.6 Aim

The molecular mechanisms of chloroplast movement and its regulation are poorly understood. The discovery of the *chup1* mutant and the implications for chloroplast movement led the way to a better understanding. So far most investigations focussed on the phenotypic characterization of chloroplast movement. The aim of this work was to biochemically characterize the protein Chup1. Chup1 was known to be involved in chloroplast movement as shown by phenotypic analysis of the mutant. A further characterization on the whole was lacking. As regulation by light is crucial for chloroplast movement, an investigation of global gene expression and a link to signalling pathways was explored. Furthermore, the profile of Chup1 in the context of global light expression was targeted. One goal was to identify the exact nature of the translocation signal for Chup1 localization and to identify the translocation pathway, and to explore a putative link to the secretory pathway. To identify the molecular mechanism of chloroplast movement, the interaction of Chup1 with the actin cytoskeleton and its modifiers was set to be explored. In chloroplast movement, the way of action of Chup1 was aimed to be unravelled, to place Chup1 in a new model of light regulated chloroplast movement.

3 Materials and Methods

3.1 Materials

3.1.1 Chemicals

The chemicals in this study were of analytical grade or better and purchased from Sigma-Aldrich/Fluka (München, Germany), Merk (Darmstadt, Germany), and Roth (Karlsruhe, Germany). Poly-L-proline (MW 10000-30000) was purchased from Sigma. Other materials include MitoTracker Orange CMTMRos from Molecular Probes (Leiden, The Netherlands), [^{35}S] Methionine (10μCi/μl) from Amersham Biosciences (Freiburg, Germany) and dansyl chloride [5 (dimethylamino) naphtalene-1-sulfonyl chloride] from Fluka.

3.1.2 Enzymes and Kits

Restriction enzymes, T4-Ligase, calf intestine alkaline phosphatase (CIAP) were purchased from Fermentas (St.Leon-Rot, Germany), DNaseI and Complete Protease Inhibitor Cocktail Tablets from Roche (Mannheim, Germany), Trypsin and Cellulase from Sigma-Aldrich, Macerozyme Onozuka-RS (Yakult, Japan), PlantRNeasy Kit from Qiagen (Hilden, Germany), Gateway LR and BP Clonase and SuperScriptIII First Strand Synthesis from Invitrogen (Karlsruhe, Germany), Triple Master PCR System and FastPlasmid Mini Kit from Eppendorf (Hamburg, Germany), Nucleobond AX Nucleospin and Nucleospin Extract II kit from Machery-Nagel (Düren, Germany), SMART™ RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA), Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, München, Germany)

3.1.3 *E.coli* strains

DH5α (DE3) from Invitrogen (Karlsruhe, Germany) and TOP10 were used for transformation and amplification of vector DNA.

BL21 (DE3) pLysS from Invitrogen (Karlsruhe, Germany) was used for recombinant expression. pLysS constitutively expresses low levels of T7 lysozyme, and thus inhibits basal levels of T7 RNA polymerase, which results in a reduction of basal expression of recombinant genes.

BL21 (DE3) Rosetta (Novagen, Madison, WI, USA) encoding rare tRNAs.

BL21 (DE3) pMICO encoding 3 rare tRNAs and T7 lysozyme (kindly provided by Dr Ian Menz, Cinquin et al., 2001).

3.1.4 Plant material

Pisum sativum (variety "Arvika") seeds were obtained from Bayerische Futtersaatbau GmbH (Ismaning, Germany). *Arabidopsis thaliana* ecotype Col-0 seeds were obtained from Lehle Seeds (Round Rock, USA). The T-DNA insertion line SALK_129128 resulting in a knock-out of *CHUP1* (At3g25690, ecotype Columbia) was obtained from NASC Stock centre (University of Nottingham, UK, Alonso et al. 2003).

3.1.5 Oligonucleotides

Oligonucleotides used in PCR were synthesized by Operon (Köln, Germany):

Table 1 Oligonucleotides used in this study

Chup1 LP	tggtacccctgaaacaccgaa	SALK line Chup1
Chup1 RP	ccttgtgtctccacatccgct	SALK line Chup1
Lba1	tggttcacgtagtgggccatcg	T-DNA left border primer
Chup1_NheI_fw	ggttaagctagctcatgtttgtccggatagggtttg	Chup1 GFP fusion
Chup1_SalI_rev	aattccgtcgacagtttacagattcttcttcattg	Chup1 GFP fusion
Chup1_dN_Nhe fw	ttaaccgctagcgcatgtccaaaccaagcaaaccatcagat	ΔN-Chup1 GFP fusion
Fim_NheI_fw	ggttaagctagcatgcctcttgaaagagctgaattggttc	fABD2 atFIM1 RFP fusion
Fim_SalI_rev	aattccgtcgactttcgatggatgcttgctctgagac	fABD2 atFIM1 RFP fusion
Pro_NheI_fw	ggttaagctagcatgtcttggcaatcatacgtcgat	atPRF2 GFP fusion
Pro_SalI_rev	aattccgtcgactgagttcagactcgataaggtaatc	atPRF2 GFP fusion
Chup1 consens1 fw	cactttgattggcctga	psChup1 RACE
Chup1 consens2 fw	tacgggaagcatcttttga	psChup1 RACE
Chup1 Erbse 1010 rev	tttgaagtccttccacttgctttg	psChup1 RACE
Chup1 Erbse 1360 rev	ctcaaagatgattctagtgctctttca	psChup1 RACE
psChup1 2230 rev	gtatccttctttgcctcccgtttcatc	psChup1 RACE
psChup1 1470 fw	agctgatgataaggaatgccagtgatagtg	psChup1 RACE
Chup1 Erbse 2430 fw	ctggctagatgaagaactttccttc	psChup1 RACE
RACE UPM	ctaatacgactcactatagggc	RACE Primer Clontech

3.1.6 Vectors

The vectors used in this study were the expression vector pDEST17 (Invitrogen) containing a His-tag C-terminal to the cloning site, and a GFP vector for expression of GFP-fusion proteins under a S35 promoter in plants, the pOL GFP-S65C vector (Peeters et al. 2000). The same vector was also available as RFP construct (pOL RFP). The Golgi marker ST-GFP (rat sialyl transferase fused to GFP, Boevink et al. 1998) was kindly provided by Prof. Chris Hawes from Oxford Brookes University. The constructs cloned from these vectors are described in 3.2.2.2.

3.1.7 Antibodies

Antibodies against Toc34 were raised by Pineda Antibody Service (Berlin, Germany). Anti-profilin (from mouse) and anti-actin (from rabbit) were obtained from Sigma. The secondary antibodies goat anti-rabbit or anti-mouse alkaline phosphatase conjugated were obtained from Sigma.

3.1.8 Other material

Ni-NTA Superflow sepharose was purchased from Qiagen (Hilden, Germany), Nitrocellulose Protran BA-S83 membranes from Schleicher & Schüll (Dassel, Germany), CNBr-activated Sepharose 4B and nProteinA-Sepharose CL-4B and HiTrap Desalting column from Amersham Biosciences (Freiburg, Germany), Wheat Germ Extract from Promega (Mannheim, Germany), Fuji film imaging plates from Fuji (Düsseldorf, Germany), Affymetrix ATH1 arabidopsis genome chip from Affymetrix (High Wycombe, United Kingdom), vermiculite was obtained from Dämmstoff-Fabrik Klein GmbH (Zellertal, Germany)

3.1.9 Services

DNA Sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit from Applied Biosystems (Darmstadt, Germany) on an ABI 3730 by the Sequencing Service Department Biology (München, Germany).

3.1.10 Further Instruments and Equipment

Instruments or equipment used in this study include: BioPhotometer (Eppendorf), Phosphoimager FLA-3000 (Fuji), Ultrafiltration Cell 8050 (Amicon, Beverly, MA, USA), Leica TCS SP5 laser scanning confocal microscope (Leica, Heidelberg, Germany), LS55 Luminescence Spectrometer (Perkin Elmer, Waltham, MA, USA). Light for the White Band Assay and microarray experiments was provided by a cool metal halide lamp Olympus ILH-2A (Olympus, Hamburg, Germany) equipped with a liquid light conductor (5-1800, Olympus) and light intensity was measured with an Almemo FLA603PS5 light sensor (Ahlborn, Holzkirchen, Germany).

3.1.11 Bioinformatic tools

Analysis of Affymetrix expression data was performed with the Affymetrix software package MAS for white light dependent expression delivering the signal intensity and the detection P-

value and data was processed with Sigma Plot (SPSS). Multiple sequence alignment was performed with the program MAFFT (Katoh and Toh 2008) and presented with Jalview (www.jalview.org, Clamp et al. 2004). Pattern and motif search was performed with Prosite (De Castro et al. 2006, http://www.expasy.org/prosite/). Protein parameters (e.g. the pI of proteins) were calculated with ProtParam (http://au.expasy.org/cgi-bin/protparam). Coiled-coil analysis was performed with the PCOILS program (http://toolkit. tuebingen.mpg.de/pcoils, Lupas et al. 1991). The search for sequence similarities in the databases was performed with the Basic Local Alignment Search Tool, BLAST (Altschul et al. 1990, http://www.ncbi.nlm.nih.gov/blast/ Blast.cgi). Sequence annotation was performed with the TAIR 6.0 database (http://www.arabidopsis.org/) in collaboration with Georg Haberer, MIPS, GSF, Neuherberg).

3.2 Methods

3.2.1 General Methods

Gel electrophoresis of DNA on agarose gels, restriction of DNA, ligation and transformation of vector DNA into bacterial strains were performed according to standard protocols (Sambrook et al., 1989). The purification of DNA restriction fragments from agarose gels was done with the Nucleospin Extract II kit. DNA concentration was measured photometrically at 260 nm (Sambrook et al., 1989). Proteins were separated by SDS-PAGE according to Laemmli (1970). Protein concentrations were determined by using the Bio-Rad Protein Assay Kit according to the manufacturer's recommendations and by absorption measurements at 280 nm as described (Sambrook et al., 1989). SDS-PAGE Gels were stained either by Coomassie Brilliant Blue R250 or silver-stained as described (Sambrook et al., 1989). Western blotting (transfer of proteins) was done by semi-dry blotting (Towbin et al., 1979) on nitrocellulose membranes. The immunodecoration with secondary antibody conjugated with alkaline phosphatase and detection with NBT/BCIP were performed as described (Sambrook et al., 1989).

3.2.2 Molecular Methods

3.2.2.1 RNA isolation and cDNA generation

To obtain RNA from plant material, leaf tissue of pea or *A. thaliana* was processed with the Plant RNeasy Kit. Subsequently cDNA was synthesized using the SuperScript III Kit with gene specific reverse primers. To amplify cDNA, a standard PCR (Mullis and Falloona, 1987) was performed using TripleMaster Polymerase according to the manufacturer's recommendations; where necessary restriction enzyme sites were joined to cDNA ends with the use of the respective oligonucleotides (Table 1).

3.2.2.2 Cloning

The following vectors were constructed for use in this study. The delineated GFP/RFP fusions are all C-terminal to the gene of interest. To produce GFP fusion constructs, full-length *CHUP1* and ΔN-*CHUP1* lacking the N-terminal 75 base pairs (hydrophobic domain), were fused to GFP via NheI/SalI using the pOL GFP-S65C vector (Peeters et al. 2000). The second actin binding domain (fABD2) of fimbrin (atFIM1; Sheahan et al. 2004) was fused to RFP via NheI/SalI into pOL RFP. AtPRF2 (AT4G29350, profilin2) was fused to RFP via NheI/SalI

into pOL RFP. Integration of *CHUP1* and *CHUP1-CT* (C-terminus of *CHUP1* from bp 1814-3015) into the expression vector pDEST17 (Invitrogen) was performed using the Gateway system (Invitrogen). All constructs were controlled by sequencing.

3.2.2.3 Protein production

Chup1-CT in the pDEST17 vector was expressed in freshly transformed *E. coli* BL21 (DE3) pLysS cells. The cultures were induced at an OD_{600} of 0.6 to 0.8 with 0.5 mM IPTG and incubated over night at 22 °C. Cells were resuspended in 20 mM Tris/HCl pH 7.0, 150 mM NaCl and lysed with a French Press. After centrifugation for 20 min at 20000 *g* the soluble fraction was purified with NiNTA Superflow matrix according to the manufacturer's instructions. Briefly, the soluble fraction was incubated with NiNTA matrix for 30 min at room temperature, washed with 20 mM Tris/HCl pH 7.0, 5 mM imidazole and eluted with 20 mM Tris/HCl pH 7.0, 500 mM imidazole. To avoid proteolytic degradation, Complete protease inhibitor without EDTA (1 Tbl./40ml) and 0.3 mM PMSF were added to the buffers. Chup1-CT was dialysed with the appropriate buffer before use.

3.2.2.4 In vitro transcription/translation

In vitro translation of *CHUP1* and radioactive labelling was performed in wheat germ extract (Promega). A reaction mixture contained 100µl wheat germ extract, 1-3µg *CHUP1* DNA in pDEST17, 6µl TNT reaction buffer (Promega), 4µl T7 Polymerase (Promega), 4µl amino acid mixture minus methionine (Promega, 1mM), 4-8 µl [^{35}S] methionine (Amersham, 10µCi/µl), 2µl RNase inhibitor (GE Healthcare 40U/µl) and water up to 200µl. The reaction was incubated at 30°C for 2h. Unincorporated [^{35}S] Met was removed by subjecting the reaction to a column containing G25 sephadex medium (Pharmacia). The flowthrough and 50µl of the first elution fraction with 10 mM sodium phosphate buffer pH 8.0 was collected. The translation product was checked by SDS-PAGE analysis and autoradiography by exposing the dried gels on Fuji film imaging plates for 4-16 h. The signal was detected with a Phosphoimager FLA-3000 system.

3.2.3 Cellular Methods

3.2.3.1 Protoplast transformation and fluorescent imaging

Protoplast preparation was performed according to the protocol from Yoo et al. (2007). Mesophyll protoplasts were isolated from 3 to 4 week old *A. thaliana* leaves and transformed with 10-20µg DNA. Fluorescent images were taken with a Leica TCS SP5 laser scanning

confocal microscope. For the visualization of mitochondria, protoplasts were stained with 400 nM MitoTracker Orange CMTMRos in buffer W5 (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7) for 1 h. For studies with brefeldin A (BFA), protoplasts immediately after transformation with DNA (with a final concentration of $1.8*10^5$ cells/ml in 500 µl buffer W5) were mixed with a stock of 20 mM BFA prepared in DMSO to a final concentration of 33 µg/ml (120 nM) and incubated for 24 h in the dark at 16-21°C.

3.2.4 Biochemical Methods

3.2.4.1 Preparation of conjugated CNBr- Sepharose columns

To conjugate proteins or enzymes to sepharose for affinity chromatography, the substances were coupled to CNBr-Sepharose by following the manufacturer's protocol. For the conjugation of DNase I, a ratio of 10mg DNAse I for 2.8 g CNBr-Sepharose was used. For conjugating poly-L-proline to Sepharose, 150 mg poly-L-proline and 1 g CNBr-Sepharose was used. The conjugant was dissolved in coupling buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) and the coupling reaction was performed with shaking at 4°C over night. Remaining active groups were blocked after coupling for 2 hours at room temperature with 0.1 M Tris/HCl pH 8.0. Excess of uncoupled ligand was washed away with five alternating washes of coupling buffer and sodium-acetate buffer (0.1 M Na-acetate pH 4.0, 0.5 M NaCl)**.**

3.2.4.2 Purification of actin from Pisum sativum

Actin was purified from pea leaf by adapting the protocol from Diaz-Camino and Villanueva (1999) for the isolation of actin from *Phaseolus vulgaris.* Leaves from 10-12 day old pea plants were frozen in liquid nitrogen and ground in a mortar. Buffer A (2mM Tris/HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 30 mM sodium phosphate buffer pH 8.0, 1 tablet Complete Protease Inhibitor/100 ml, 0.25mM DTT and 0.005% NaN_3) was added and leaves were ground for 15 min. The leaf extract was filtered through a paper filter and centrifuged at 20000 *g*. The supernatant was applied to a DNase I-Sepharose affinity column (see 3.2.4.1), equilibrated with buffer G (2 mM Tris/HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP, 0.25mM DTT and 0.005% NaN_3). The column was washed with 20 volumes of buffer G plus 0.01% Triton X-100, 20 volumes of buffer G, 3 volumes of buffer G plus 0.6 M NaCl and 3 volumes of buffer C (2 mM Tris/HCl pH 8.0, 2 mM EGTA, 0.2 mM ATP, 0.25 mM DTT, 0.005% NaN_3) and 1.5 volumes of buffer G plus 0.75 M urea. The column was eluted with 1.5

volumes of buffer G plus 4 M urea. The eluate was diluted immediately to maintain functional actin by letting it drop into an excess of 250 ml of buffer G and concentrated in an Amicon ultrafiltration cell, diluted several times with buffer G and concentrated again.

3.2.4.3 Preparation of monomeric or filamentous actin

For preparing monomeric (G) or filamentous (F) actin, rabbit muscle actin (Sigma) or pea leaf actin (see 3.2.4.2) was incubated for 1 h in buffer G (2 mM Tris/HCl pH 8.0, 0.2 mM $CaCl_2$, 0.2 mM ATP) or buffer F (5 mM Tris/HCl pH 8.0, 5 mM $MgCl_2$, 0.1 M KCl, 0.5 mM ATP). The mixture was clarified by centrifugation at 300000 *g* for 1 h at 4°C. The pellet fraction containing F-actin and the supernatant fraction containing G-actin were further processed.

3.2.4.4 Binding of proteins to chloroplast outer envelope membranes

3.2.4.4.1 Binding of Chup1 and Chup1-NT to chloroplast outer envelope

Outer envelope membranes (OE) were prepared from pea chloroplasts according to (Schleiff et al. 2003). Translation products of Chup1 and Chup1-NT (see 3.2.2.4) in 10 mM sodium phosphate buffer pH 8.0 were pre-cleared by centrifugation for 10 min at 256 000 g. Supernatant fractions were incubated with OE for 10 min at room temperature and centrifuged for 10 min at 256 000 g. Supernatant and pellet fractions were subjected to SDS-PAGE and autoradiography.

3.2.4.4.2 Binding of actin to chloroplast outer envelope membranes

Outer envelope was subjected to short sonication pulses in buffer G. For proteolytic digestion of the OE with trypsin, OE was incubated with 1µg/µl trypsin for 2 minutes. The reaction was stopped with a 10 fold excess of trypsin inhibitor. OE and G-actin were incubated for the indicated times and centrifuged for 10 min at 35000 *g* through a 200 mM sucrose cushion in buffer G. Supernatant and pellet fractions were analyzed by SDS-PAGE and western blotting using anti-actin antibody.

3.2.4.5 Binding of actin to Chup1

For co-immunoprecipitation of Chup1 with G-actin and actin-antibodies, G-actin was incubated with radioactively labelled Chup1 protein in buffer G for 30 min and mixed with anti-actin or control antibody (anti-Toc12) coupled to protein A sepharose (GE Healthcare). Coupling of antibodies to protein A sepharose was performed for 30 min in buffer G plus

0.3% BSA. After incubation, the sepharose beads were washed with buffer G and buffer G plus 0.5 M NaCl. Chup1 was eluted from the beads with 0.1 M glycine pH 2.5. The samples were analyzed by SDS-PAGE and autoradiography. For binding studies of F-actin and Chup1, F-actin was incubated with pre-cleared (300 000 *g*, 1h) radioactively labelled Chup1 or control (Toc34) in buffer F. After centrifugation for 45 min at 300 000 *g*, supernatant and pellet fraction were analyzed by SDS-PAGE and autoradiography.

3.2.4.6 Purification of profilin from *Pisum sativum* and *Arabidopsis thaliana*

Profilin was purified from *P. sativum* or *A. thaliana* leaves by poly-L-proline affinity chromatography following the protocol published in (Vidali et al. 1995). Plant leaf material was homogenized in a blender with 4 volumes of extraction buffer (100mM glycine, 100mM KCl, 10 mM Tris/HCl pH 8.0, 1 mM DTT, 1% Triton X-100 and 1 Tbl Complete/200 ml) per gram and extracted at 4°C for 1h while stirring. The extract was filtered through gauze, centrifuged at 24000 g for 30 min and filtered through a paper filter before incubation with 0.1 volume of PLP-sepharose (see 3.2.4.1) equilibrated with extraction buffer without Triton X-100 over night at 4°C. PLP-sepharose was packed into a column and washed with several volumes of extraction buffer and 3 M urea in TBS (20 mM Tris/HCl pH 7.6, 150 mM NaCl). Profilin was eluted with 8 M urea in TBS and diluted immediately in TBS. The eluate was concentrated in an Amicon ultrafiltration cell and diluted several times with TBS before concentrating again.

For some experiments, profilin subsequently was dephosphorylated by incubation with 10 units of calf intestine alkaline phosphatase (CIAP, Fermentas) for 1h at 37°C. The reaction was stopped with EDTA (50 mM final concentration).

3.2.4.7 Fluorescence measurements

3.2.4.7.1 Tryptophan quenching

The spectrum of tryptophan fluorescence was recorded to assay the interaction of actin and profilin. The method makes use of the phenomenon of tryptophan fluorescence quenching upon binding of interaction partners (that contain tryptophan at the interaction sites). The fluorescence of the tryptophans is quenched upon complex formation.

The fluorescence emission spectra of actin and profilin (0.2 µM each) were measured at an excitation wavelength of 295 nm in buffer G on a LS55 Luminescence Spectrometer. Actin

and profilin were mixed at the same concentration as indicated above to obtain the tryptophan emission spectrum of the actin-profilin complex.

3.2.4.7.2 Profilin dansylation

Profilin was labelled with 1mM dansyl chloride [5 (dimethylamino) naphtalene-1-sulfonyl chloride] for 1 h in PBS buffer and quenched with 0.1 M Tris/HCl pH 8.0. Excess dansyl hydroxide was removed by dialysis in buffer G plus 50 mM NaCl for 48 h followed by chromatography on a size exclusion HiTrap desalting column equilibrated with buffer G plus 50 mM NaCl. Elution was performed with the same buffer. For fluorescence measurements actin or Chup1-CT were titrated to dansyl-profilin in buffer G and fluorescence was measured with a LS55 Luminescence Spectrometer with an excitation wavelength of 337 nm.

3.2.4.8 Interactions of profilin

Profilin (or casein/BSA as control protein) was spotted onto nitrocellulose membrane in defined amounts with a dot-blot apparatus. The membrane was blocked with 0.3 % skimmed milk, 0.03 % BSA (BSA was omitted when used as a control) and incubated with radioactively labelled Chup1 in the same buffer over night. Three extended washing steps with blocking buffer were performed and Chup1 protein bound to the membrane was visualized by autoradiography.

Chup1-CT was spotted onto nitrocellulose membrane and blocked respectively. The membrane was incubated with profilin (or without as control) in blocking buffer and immunostained with anti-profilin (Sigma).

3.2.5 Plant physiology

3.2.5.1 Plant Growth

A.thaliana seeds were surface sterilized by a treatment with 70% ethanol followed by 50 % sodium hypochlorite, 0.05 % Tween 20 and a wash step in sterile water. The seeds were plated on MS medium (Murashige and Skoog 1967) supplemented with 1% (w/v) sucrose, stratified for 2 days at 4°C in the dark and grown in climate chambers with the following growth conditions: 14 hour 75 µmol light at 21°C and 10 hour dark at 16°C. Seedlings were transferred to soil after two weeks.

Pisum sativum seeds were imbibed over night in running water, sown on vermiculite or on sand and grown at a 12 h day/12 h night cycle in a climate chamber at 21°C.

3.2.5.2 Mutant analysis

T-DNA containing SALK Lines were analyzed for the harboring of the insertion in the gene of interest by PCR. In a standard PCR reaction, primers for left- and right region (LP and RP) of the gene of interest and the Lba1 primer (Table 1) for the left border of the T-DNA were used to amplify in one reaction the region spanning the left and right primer in case of a wild type genome, amplifying the said region plus the construct from the right primer and the Lba1 primer in case of a heterozygous genome; and amplifying only the latter construct when a homozygous genome was given.

3.2.5.3 White band assay

The phenotypic characterization of chloroplast movement deficient knock-out plants was assayed with the white band assay as described in Kagawa et al. (2001). Leaves of the respective plants were placed on agar to avoid drying and treated with high intensity white light (>400 µmol) applied by a cool metal halide lamp (Olympus) through a narrow slit in a black plate for 1 h.

3.2.5.4 Analysis of Affymetrix Data in high white light dependent expression in WT and *Δchup1*

RNA was extracted from leafs of three sets of biological independent wild type or *Δchup1 A. thaliana* plants (ecotype Columbia) grown for 4 weeks to stage 5.10 (Boyes et al. 2001) at a 14 h 75 µmol light at 21°C and 10 h dark (>0.1 µmol light) 16°C regime before or after 1 h illumination with 400 µmol light at 21°C and immediately frozen in liquid nitrogen. RNA extraction and hybridization was done in collaboration with Prof. Jürgen Soll and Dr. Katrin Phillipar (LMU Munich). Gene chip analysis was performed according to the manufacturer's recommendation in collaboration with Enrico Schleiff. For data analysis, signal intensity and the detection P-value (value for the reliability of the measurement) were analyzed, the standard error for the three independent experiments was calculated and the highest individual detection P-value was considered. Data were first filtered for signals which had in at least one experiment a maximal detection P=0.005. For a comparison of the change in expression, the slope ratio value was determined. The slope ratio is a measurement for the steepness or

"gradient" of expression change calculated from the ratio of the vertical and horizontal distance between two values.

3.2.5.5 Analysis of Affymetrix Data in Blue Light Dependent Expression

For the analysis of gene expression after blue light treatment, RNA was extracted from leaves of at least four biological independent plants of WT ecotype Columbia (Lehle). Plants were grown for 4 weeks to stage 5.10 (Boyes et al. 2001) in a cycle of 16 hours 75 µmol light at 21°C and 10 hours dark (>0.1µmol light) at 16°C. The plants were illuminated with blue light provided by a cool metal halide lamp (Olympus) through blue plexiglass (Degussa, Essen, Germany) in the dark at intensities and time scales indicated in Table 2 and immediately frozen in liquid nitrogen. Hybridization was performed as stated above (3.2.5.4). The analysis of the data and the programs necessary for the analysis were worked out in cooperation together with Oliver Mirus, Georg Haberer und Enrico Schleiff and programmed by Oliver Mirus. For this reason the process of analysis but not the program source code is given in the following.

3.2.5.5.1 Structure of the Primary Data from the Affymetrix Analysis

Introduction to Affymetrix chip architecture/Background

For background information a brief outline of the architecture of a DNA microarray of the Affymetrix type is given here, to help understand the process of analysis in the following paragraphs. The gene chip from Affymetrix was designed initially to comprise a probe set of 11 probe pairs for each sequence. These probes are 25 nucleotides in length, and each probe set consists of 11 match (perfect match to target sequence) and 11 mismatch (a single mismatch in the sequence) oligonucleotide pairs (22 spots in total). The annotation used for the *A. thaliana* ATH1 chip set was from the TIGR ATH1 database as of December 15, 2001. On the chip, 22500 probe sets are spotted, representing approximately 24000 gene sequences on one gene chip array. With new sequence information gained in the last years however, new annotations are required for a precise allocation of the 25mers to specific gene sequences. The new annotation information was drawn from the TAIR 6.0 database. The assignment of the new sequence information to the Affymetrix chip probe sets is outlined in the following.

Three data sets were available. In the first the coordinates of each of the spots for the so called match position are given, including the corresponding sequence. In the second data set the annotation respective to the TAIR 6.0 nomenclature for each spot is included. The third data

set contains the obtained values from the experiments. As data from 7 independent experiments was obtained, this means, that from the type-three data set, 7 files existed, listing the data from the measurements. Table 2 contains the nomenclature of the used files.

	WT	
untreated	G	
	5 min	30 min
1 µmol	A	B
10 µmol	C	D
100 µmol	E	F

Table 2 Experimental conditions. Time scales and light intensities used in themicroarray experiment are encoded in the letters A-G

3.2.5.5.2 Combination of the data

Preceding the analysis and further processing of the data, 7 new files were generated. First, by combination of the data set one and two, a source file was generated, in which the allocation of the spots to match and mismatch and the allocation of the single spots to genes on the basis of the according AGI numbers were stored. All entries which could not be assigned to a gene according to the TAIR 6.0 annotation were deleted.

In a second step, 7 new files were generated, by combining the values to the annotation. During this procedure, entries which had been stored falsely in the Affymetrix file were deleted. Subsequently it was tested, whether at least 6 values still existed for each annotated gene in the data file. If this was not the case, all values for this gene were deleted. In addition, the value <„match“ minus „mismatch“> and the corresponding error <error „match“ plus error „mismatch“> were calculated and subsequently all values for “mismatch” were deleted. The following data structure was thereby obtained: X-coordinate, Y-coordinate, AGI-code, Affymetrix-code, ATOM, Max, value-1, error-1, value-2, error-2. The coordinates match to the position on the chip, ATOM specifies which spot of a gene corresponds to the value, Max specifies, how many spots exist for the corresponding gene, value-1 and error-1 relate to the “match” values and value-2 and error-2 to the “match” minus ”mismatch” values. These files constitute the source files for further calculation.

3.2.5.5.3 Statistical analysis with values for wild type A. thaliana

For the evaluation of data, the values for wild type were analysed first. For this analysis first all mean values (value-1 and value-2) for each gene were calculated by adding all values. Furthermore the standard deviation of the values was calculated (SDA, STA-2). Thereby the generated files now contained only one value per gene. In the next step the individual files were compared, whereupon file C was used as basis for comparison for files A, E and G and file D as basis for comparison for files B, F and G (nomenclature see Table 2). First the respective files were combined. Afterwards it was controlled for each pair of mean value-1 and value-2, whether the distance of the values exceeded half of the error sum. These value pairs were indexed. The value pairs were then analyzed with a linear fit by the least squares method.

3.2.5.5.4 Cluster Analysis

For the cluster analysis of the data, expression categories were defined (see Figure 7). The classification of categories was set depending on the behaviour of gene expression in 1 or 100 µmol blue light in relation to 10µmol blue light. The independent spot values were sorted according to the categories defined and a gene was selected to be in one category if at least 80% of all spot values representing the gene were in that category and if the spot number in this category was larger than 6, because some of the genes had less than 8 spot values after correcting the Affymetrix annotation by the TAIR 6.0 annotation.

4 Results

4.1.1 Affymetrix analysis of blue light dependent gene expression in A. thaliana

4.1.1.1 Analysis of the statistical significance of the obtained data sets

For a specific analysis of physiological reactions to blue light, which, for instance, is involved in stomata regulation and chloroplast movement (see 2.2), the expression profile in reaction to blue light was determined. Wild type plants were illuminated with blue light of 1, 10 and 100 µmol for 5 or 30 min (see Table 2). To begin with, for the analysis, the statistical significance of the data had to be determined. Because of the high costs for Affymetrix chips each condition was conducted in a single experiment (but with several biological independent samples). This raised two questions: what is the statistical significance of the experiments and which value should be used for evaluation: match or match minus mismatch? In literature the problem of the usefulness of the mismatch probe has been discussed (e.g. Naef et al. 2002, Irizarry et al. 2003). To determine the difference in analysis, the average values for match and mismatch were calculated for each gene from those spots which were assigned according to TAIR 6.0 annotation. Using the new TAIR annotation reduced the calculated errors for the signal intensities significantly (not shown).

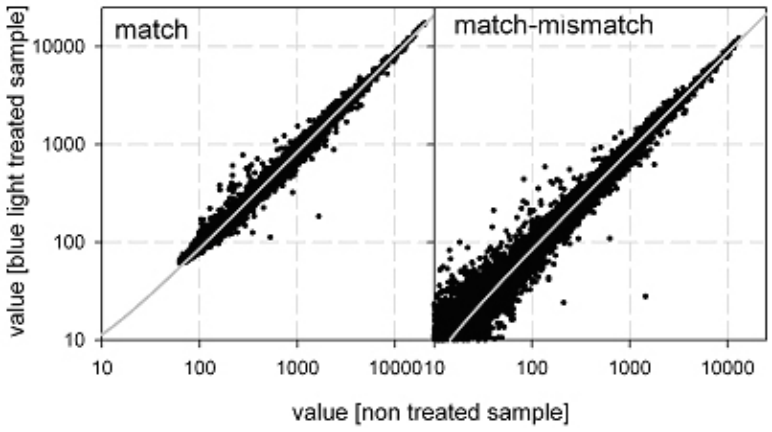

Figure 4 Analysis of raw data and determination of data evaluation. Diagrammed is the comparison between untreated wild type sample (G) and wild type after 5 min treatment with 10µmol blue light. Diagrammed is the comparison between value-1 (“match”, left) and value-2 (“match” minus “mismatch”, right). The grey line indicates the linear regression of all data points.

As is apparent from Figure 4, when calculating with match minus mismatch (value-2) only values with low signal intensity are over-emphasized. Values with high signal intensity are not influenced by the change in calculation. At the same time, the ratio between signal and error of the small signals increases (as subtraction results in addition of errors), so that many

of the signals would have to be removed from the analysis (not shown). Thereby the influence lies only on values with low signal intensities, which can be dispensed with, as low signal intensities are more noise sensitive. Therefore, solely the match values (Value-1) were used for further analysis.

To analyse the results, all genes for which the signal change was smaller than the error of the signals (and therefore, considering the errors, had no signal change) were removed from the analysis (not shown). By this procedure which simplifies the analysis the majority of values can be eliminated. The remaining values were analysed with respect to their expression change values and only genes with a change of more than 1.5 fold in either direction (up- or downregulation) were considered.

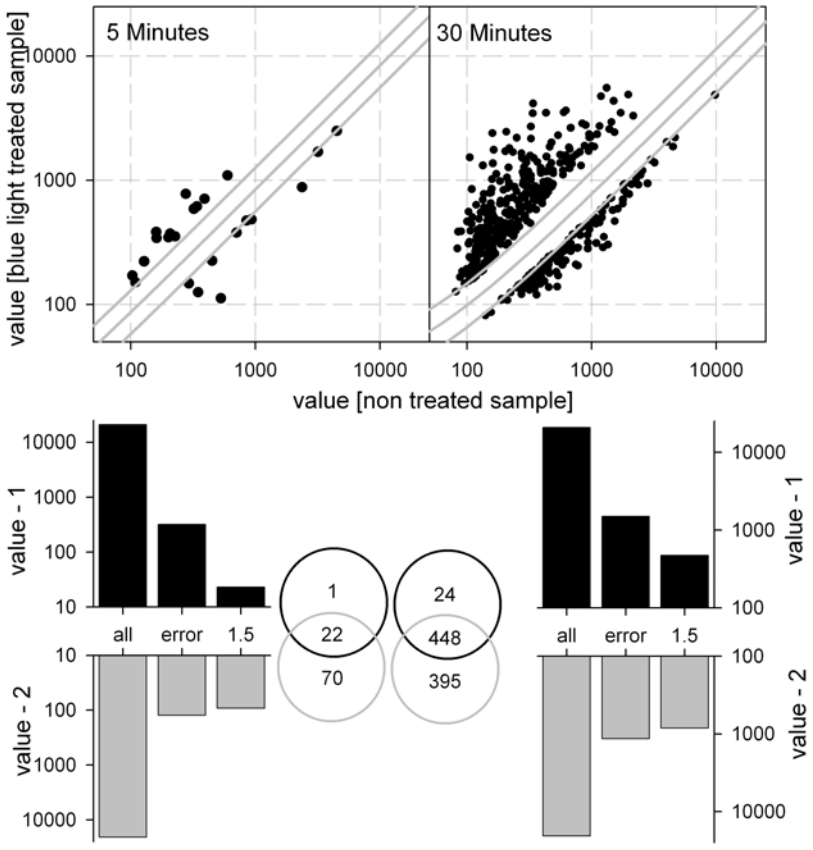

Figure 5 Analysis of the treatment with 10 µmol blue light in comparison to the untreated sample. Upper panel: shown is the comparison of value-1 (match) between untreated wild type sample and the wild type sample after 5 min (left) or 30 min (right) treatment with 10 µmol blue light. Only values remaining after selection by error discrimination and a minimal 1,5 fold change of the signal are shown. Depicted in grey are the borders of 1.5 fold change. Lower panel: The statistics of value-1 (upper part and value-2 selection (lower part) is depicted as bar chart. The circular charts show the overlap between the selected genes by the means of the value-1 (black) and the value-2 (grey).

It is apparent that almost all of the data points found by calculating with value-1 are also found in the pool of data points by taking into account the value-2 (Figure 5), but not *vice*

versa. Furthermore, considering value-2 it becomes obvious that almost all genes passing the error criterion are subsequently selected as up- or downregulated (Figure 5, bottom, value-2, compare error and 1.5). This again shows that errors are introduced while considering value-2, because most of the genes found additionally by value 2 have a low signal intensity (not shown). Additionally, the analysis shows, that only 0.1% of all genes after 5 min and only 2% of genes after 30 min blue light treatment have significantly changed expression signals considering value-1. The conclusion is therefore, that the chosen method can be used for analysis. The statistical variance is low (approximately below 0.1%). To further access this question, the three sets of results for the 5 min blue light treatment were compared. It becomes obvious that the comparison between control and 10µmol blue light treatment for 5 min shows the largest number of regulated genes (Figure 6). Therefore, it can be concluded that the statistical variation is rather low and the consideration of match values only and the concept of program-independent analysis proved to be reliable.

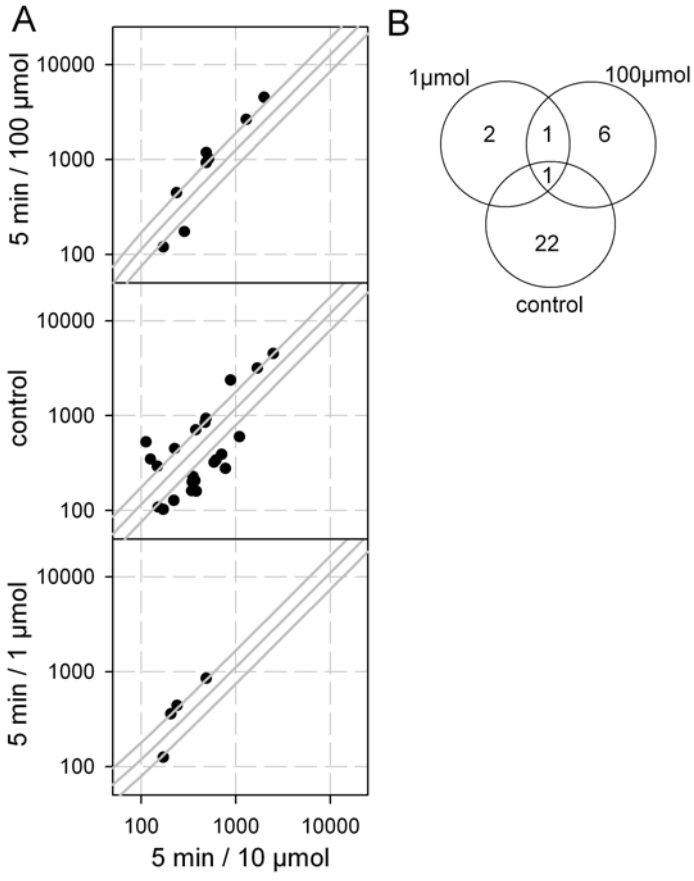

Figure 6 Analysis of the blue light reaction. A Diagrammed is the comparison of the expression values after treatment with 10 µmol blue light for 5 min and the treatment with 100 µmol blue light (above), 1 µmol blue light (below) and the untreated control sample (mid). Depicted in grey are the borders of 1.5 fold regulation. **B** The circular charts show the overlap between the genes selected by value-1.

4.1.1.2 Analysis of the blue light induced gene expression in A. thaliana

After establishing the methodology and error estimation the effects of blue light treatment were analysed but now even based on the individual spot values and no longer on averaged gene values. The program was designed by Enrico Schleiff, programmed by Oliver Mirus and its workflow is outlined in Materials and Methods. This approach was necessary to perform a knowledge based cluster analysis. For the cluster analysis, differential gene expression pattern was classified into different categories. For three different light qualities, nine different categories can be classified depending on up-or downregulation under different fluence rates (Figure 7 A).

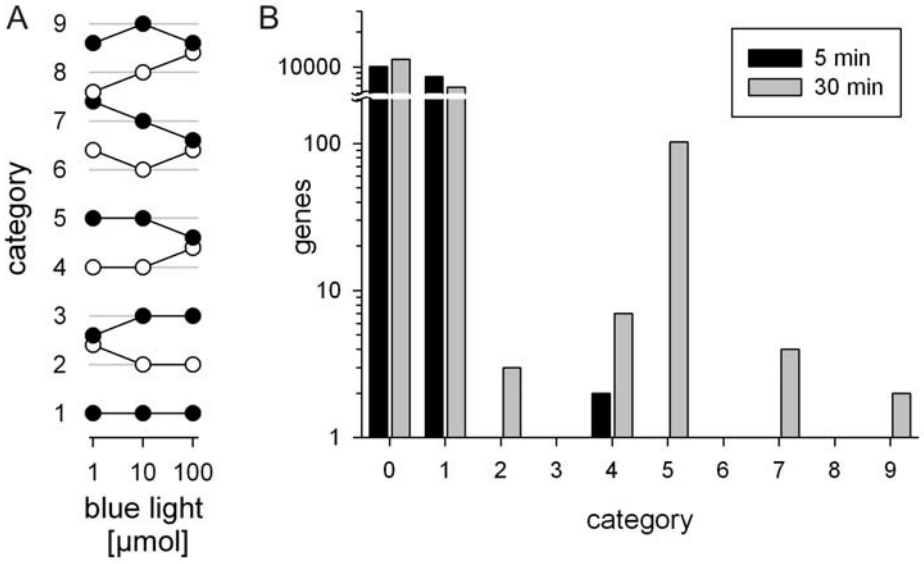

Figure 7 Cluster analysis of blue light dependent gene regulation. Categories assigned to the behaviour of gene expression in 1, 10 or 100 µmol blue light. Dots lower or higher than the central dot indicate a significant lower or higher expression of the gene under the indicated light intensity in comparison to the 10µmol treatment. Class zero (not shown) includes all genes which do not pass the filter described in Materials and Methods. **B** The number of genes according to the clustering obtained by determined spot values for 5 minute (black) or 30 minute treatment (grey) of wild type is shown. The numbers indicate the categories from (A).

Following the described strategy, the values of each spot directly were analyzed. As stated above, the majority of the expression signals are categorized in class 0 and 1, representing genes either not passing the selection filter or being not regulated. Furthermore, as expected from Figure 5, after 30 minutes more genes are found to be differentially regulated, particularly downregulated at 100µmol light treatment (class 5). Hence, the subsequent step was to analyse the genes classified.

4.1.1.2.1 The effect of blue light on the expression profile in wild type plants

After having established a clustering procedure the observed expression profile was analysed. After 5 minutes, only three genes are significantly altered in their expression based on the clustering procedure (Supp. Table 2). One of the genes is upregulated at low light intensities (category 2) and two are upregulated at high light intensities (category 4). All three genes encode proteins with a possible chloroplast localization. The gene of the category 2 encodes a kinase (Supp. Table 2), which is under the control of the Dof transcription factor OBP3 (Kang et al. 2003). The other two genes encode a chloroplast localized copper chaperone for superoxide dismutase (CCS1, Chu et al. 2005) and a chloroplast localized superoxide dismutase (CSD2, Kliebenstein et al. 1998). This could be interesting as reactive oxygen species (ROS) may function in signalling to the actin cytoskeleton in plants (Choi et al. 2008). For the superoxide dismutase (CSD2) a regulation of post-transcriptional mRNA accumulation by the microRNA miR398 was reported (Sunkar et al. 2006). Strikingly, it was observed that miR398 levels are reduced under high light, resulting in enhanced levels of the two Cu/Zn superoxide dismutases, the cytosolic CSD1 and CDS2. In line, CSD2 was found in class 4 (upregulated under high intensities of blue light). To explore whether the observed regulation might be indeed linked to this microRNA, the expression of CSD1 was analyzed by comparison of the expression values determined by averaging the spot values (as in Figure 6). Indeed, an enhanced signal of CSD1 after illumination can be observed (Figure 8). An enhancement of CSD1 and CSD2 transcripts after illumination, which was more pronounced for CSD1, was confirmed by RT-PCR (Petra Lehmann, Frankfurt, personal communication). The relation between blue light regulation and transcript level regulation by miR398 might be interesting to explore in future.

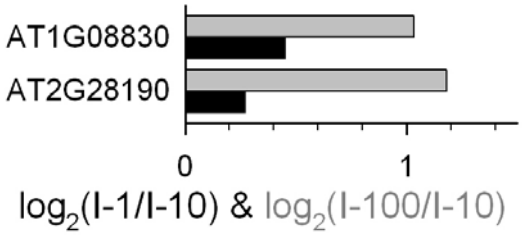

Figure 8 Analysis of treatment with blue light for 5 minutes The expression values determined by spot value averaging were analysed for the two superoxide dismutases CSD2 (encoded by At2g28190) and CSD1 (encoded by At1g08830) and the $\log_2$ of the ratio of the expression after illumination with 1µmol blue light (I-1/I-10) or 100µmol blue light (I-100/I-10) and after illumination with 10µmol blue light is shown.

When analysing the genes found to be regulated after 30 min of blue light, in total 129 genes were identified in the category 2-9. The category 5 (representing no difference between 1 and

10µmol and a down-regulation at 100µmol, Figure 7 A) is dominating. No genes were obtained for category 3 (Figure 7 A).

The genes found to be regulated after 30 min (Supp. Table 2) were analysed by annotation. The majority of genes were transcription factors or of unknown function. For some of the transcription factors, a regulation in response to light has been reported (e.g. RHL41, Iida et al. 2000). Overexpression of RHL41 induced photomorphological changes in the leaves and higher light tolerance (Iida et al. 2000). Interestingly, many kinases (9), calcium binding proteins (3) and calmodulin-binding family proteins (4) or even a kinase from a family of calcium binding kinases (AT5G45820, Gong et al. 2003) were found, which might give a link to signal cascades that involve calcium (see Discussion). The latter kinase (atPKS18, At5g45820) was reported to be sensitive to abscisic acid (Gong et al. 2002). Stomata opening is regulated by blue light and abscisic acid, and a merging of the signal pathways was suggested recently (Warpeha et al. 2007).

A link to blue light induced signal cascades might also be at reach for the receptor like protein kinase that is involved in phosphatidylinositol signalling: (AT5G47070) (see Discussion).

Also, a putative activated protein kinase C receptor (At1g48630) was found. Protein kinase C can be activated by calcium or DAG in animals and the regulation of the cytoskeleton is linked to protein kinase C in animals (e.g. Sohn and Goldschmidt-Clermont 1994). A real homologue has, however, not been reported in plants yet (see Discussion).

The regulation of blue-light induced gene expression on proteins involved in chloroplast movement and proteins reported to be involved in blue-light signalling was analysed separately (Figure 9). However, the expression profiles of the proteins were not drastically influenced by blue light of different fluence rates. In general, most of the genes show a slightly reduced expression after the 30 min treatment with the exception of PMI2, which is not altered in its expression. RPT2 shows a more pronounced downregulation. For the CHUP1 gene a slight upregulation with increasing light at 5 min can be observed, which is not detected at 30 min. Overall, the regulation of the chloroplast movement proteins observed, seems, however, not to be transcriptionally regulated.

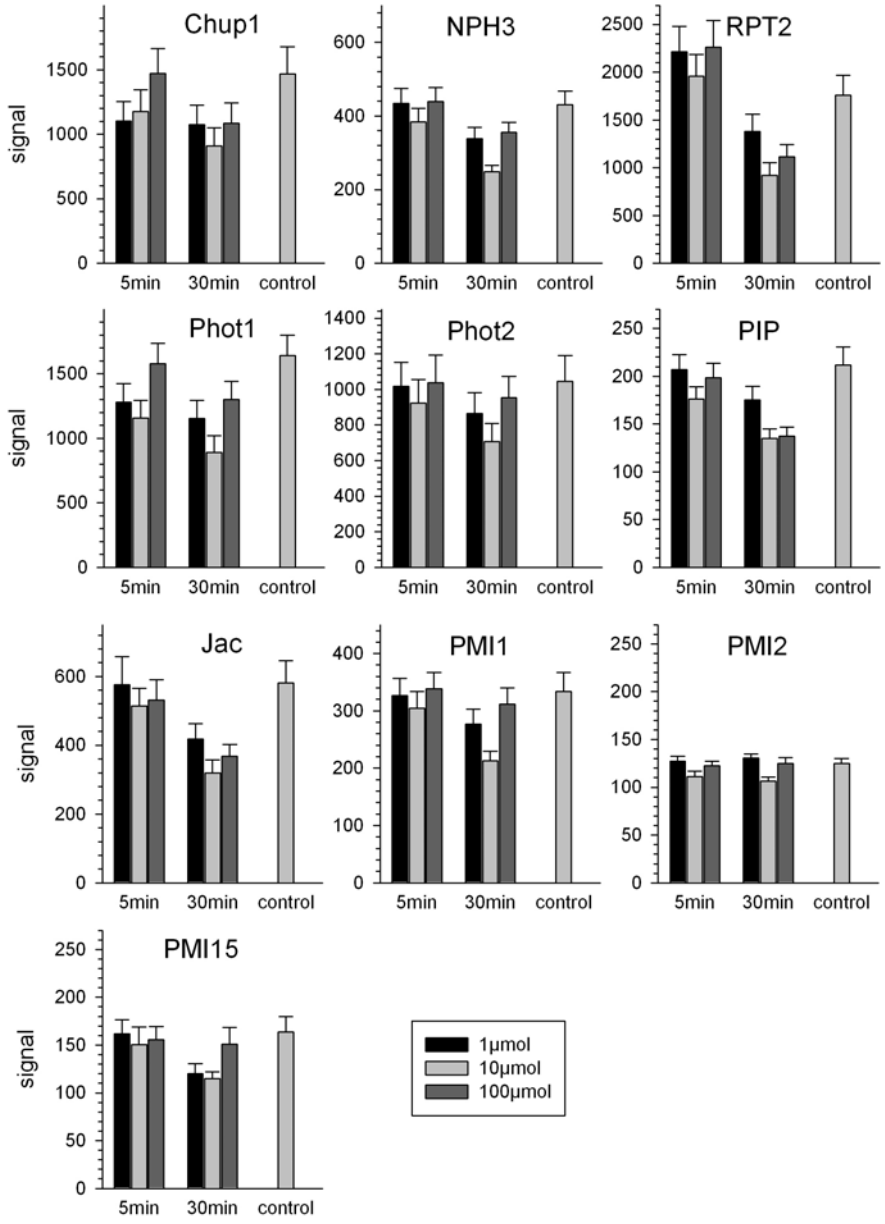

Figure 9 Comparison of the expression profiles of proteins involved in chloroplast movement or blue-light induced phototropic responses under blue light conditions

4.2 The *in vivo* function of Chup1

4.2.1 Confirmation of the T-DNA insertion in *chup1* knockout plants

For a phenotypic characterization of Chup1 function, a mutant line harbouring a T-DNA insertion in the *CHUP1* gene was obtained from the SALK institute and screened by PCR genotyping. To verify the insertion of the T-DNA into the *CHUP1* gene (see Figure 10A), a combination of three different primers (Table 1) was used. In a WT background, the chosen

gene-specific primers CHUP1 LP and CHUP1 RP should yield a WT band of 884 bp. In a heterozygous mutant plant with a T-DNA insertion in the *CHUP1* gene on one chromosome and no insertion on the other, two bands should be visible: the PCR product resulting from the combination of the T-DNA–specific left border primer (Lba1) and the gene-specific primer CHUP1 RP, which give a product of 590 bp. An additional band results from the gene-specific primers CHUP1 LP and CHUP1 RP (WT band). For a homozygous plant, only the PCR product from the Lba1 primer and the CHUP1 RP primer is amplified and results again in a product of 590 bp. The PCR products of the homozygous, heterozygous and wild type plants can be seen in Figure 10B, where the predicted bands of the correct size appear. The T-DNA insertion site in the third exon of the *CHUP1* gene is depicted in Figure 10 A.

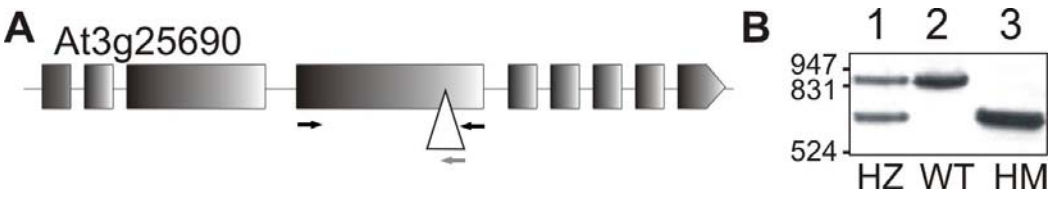

Figure 10 A Intron structure of the *CHUP1* gene (At3g25690), not to scale. The white triangle marks the insertion of the T-DNA. Back arrows mark the position of the gene specific left and right primer (LP, RP); grey arrow the position of Lba1 primer **B** PCR products from HZ (heterozygous, lane 1), WT (wild type, lane 2) and HM (homozygous, lane 3) plants from the progeny of the SALK line 129128 are shown

The knock-out status of the *Δchup1* line was further confirmed by microarray analysis (see chapter 4.1.1). A complete downregulation of the *CHUP1* gene was observed (Figure 12 A).

4.2.2 White Band Assay

A chloroplast movement deficient phenotype was discovered in a screen by Kasahara et al. (2002) resulting from a mutation in the *CHUP1* gene. To verify this phenotype the *Δchup* mutant was tested for loss of chloroplast movement. To assay the chloroplast movement deficient phenotype of *Δchup1*, the white band assay was used (Kagawa et al. 2001). With this screening method, defects in chloroplast movement can be made visible on a macroscopic level. At the illuminated area wild type plants show a paler green colour (Figure 11). This results from an increased transmittance of light through the leaf owing to a lower density of chloroplasts at the periclinial walls (walls perpendicular to the light) and a higher chloroplast density at the anticlinial walls (walls parallel to the light) (see Figure 2, Introduction).

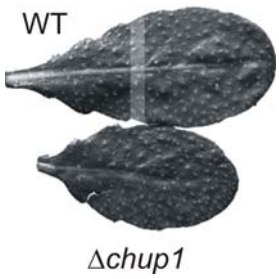

Figure 11 White Band Assay. Leaves from WT (up) or Δ*chup1* (down) were illuminated with strong white light within a narrow area on the leaf. The pale band on the WT leaf denotes the chloroplast movement in this area. On the Δ*chup1* leaf, no pale band is appearing upon illumination with strong light

As can be seen in Figure 11, the leaf of a *Δchup1* plant does not show any sign of chloroplast movement, as no pale band is appearing after illumination. Thus, this is an independent confirmation of the chloroplast movement deficient phenotype of the *chup1* knockout plant which was described by Oikawa et al. (2003).

4.3 Light regulation

To obtain information about the changes in gene expression induced through high light conditions and to explore a mode of function of Chup1 in the network of adaptation to enhanced light intensities, an expression analysis was conducted. A comparison of global gene expression changes in *Δchup1* and wild type plants was obtained through microarray analysis of mRNA from high light treated plants.

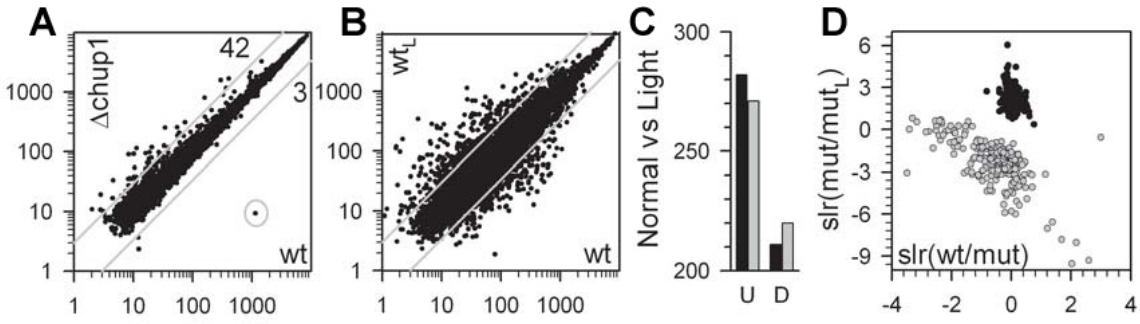

Figure 12 Expression analysis of *Δchup1 plants* **A** The averaged signal intensity of gene expression (three independent experiments) in wild type and *Δchup1* plants at day 30 with a detection P-value equal or smaller than 0.005 in at least one plant type is shown. Lines indicate the border for an at least three fold signal difference and the number of significantly up- or downregulated genes are given (listed in Suppl. Table 1). The signal for CHUP1 is circled. **B** The averaged signal intensity (three independent experiments) at day 30 of the genes in wild type plants before (wt) and after illumination (wtL) with a detection P-value equal to or smaller than 0.005 in at least one plant type is given. Lines indicate the border for an at least threefold signal difference. **C** Comparison of the number of genes significantly up- (U) or down-regulated (D) in response to light in wild type (black) or mutant plants (grey). **D** The slope ratio values (slr) for signals found in wild type versus mutant and mutant versus mutant after light treatment are shown for the genes significantly regulated in wild type in response to light (see panel B). Black dots indicate genes found to be downregulated and grey dots found to be upregulated in wild type in response to light. Positive values correspond to downregulation, negative values to upregulation.

A comparison of the expression signals of wild type and *Δchup1* (Figure 12 A) shows, that only a small number of genes (3) are downregulated. The most drastically downregulated gene is CHUP1, which demonstrates the knock-out status of the mutant. The genes that were found to be upregulated in *Δchup1* are for the most part involved in stress response. This may reflect adaptation to environmental conditions of the mutant and might give a link of the signal cascades of stress induced changes and light stimulus. As the behaviour of gene expression in response to high light in terms of the regulation of chloroplast movement was of major interest, plants treated with strong white light (400µmol) were analysed. The light treatment caused – not surprisingly - a significant change in the expression profile of the wild type compared to non treated wild type (Figure 12 B). With a significance criterion of a three fold enhanced expression change, 282 genes were found to be upregulated and 211 genes downregulated. For high light treated *Δchup1* plants, a similar observation was made (Figure 12 C) while not all of the regulated genes were entirely the same as in wild type.

The most interesting observation was made when the genes found to be regulated in wild type upon light treatment were analysed for their behaviour in the *Δchup1* mutant background (Figure 12 D). Genes that were downregulated in the WT in response to light were not found to be regulated in the non-treated mutant. But this population of genes was slightly downregulated in the mutant in response to light (Figure 12 D black dots). A more drastic differential regulation was observed for genes that were upregulated in high light treated WT: Genes of this population that were found to be upregulated in the mutant compared to WT were found to be not regulated in the mutant after illumination. This is due to the fact that their expression before illumination was already at a comparable high level as reached in WT after illumination. By contrast, genes of that population again (upregulated in high light treated WT), that were downregulated in the mutant in comparison to wild type did strongly enhance their expression in the mutant in response to light to reach a similar expression level after light treatment as obtained in wild type after light treatment (Figure 12 D, grey dots). The exclusive differential regulation of genes in the mutant, which are usually upregulated in response to light in the wild type plants, demonstrates that the mutation causes a shift of the sensing light intensity.

4.4 The Chup1 family in plants

4.4.1 RACE from *Pisum sativum* RNA

To assess whether the Chup1 protein is present in pea and to analyse its domain structure for a comparison of conserved sequence structures, RACE reactions from *Pisum sativum* RNA were performed. The sequence of the *CHUP1* gene from pea (ps*CHUP1*) could be gained from cDNA amplification of 3' and 5' ends (RACE) in consecutive sequencing steps. Highly conserved regions in the *CHUP1* gene, deduced from comparison with sequences from several species (see Table 3) were chosen for the generation of oligonucleotide primers for the first RACE reaction. Primer combinations from the conserved regions were tested on cDNA obtained from *P.sativum* RNA. The primer combination RACE UPM and Chup1 1470 fw (Table 1) resulted in a PCR product.

After sequencing the PCR product, new primer combinations at the 3' and 5' ends of the discovered sequence were generated and another RACE cycle was performed. This strategy was followed until the full-length sequence was obtained. The DNA sequence of *P. sativum CHUP1* was found to be 2958 bp (985 amino acids) long which results in a predicted molecular mass of 110.68 kDa for the protein. The protein is therefore slightly smaller than atChup1, which is 1004 amino acids long and has a mass of 111.91 kDa. The isoelectric point of psChup1 is predicted to be 5.52 and 5.43 for atChup1. No hints for multiple isoforms of the *CHUP1* gene in pea were observed (as is the case in *Physcomitrella patens*) and no splicing variants were encountered in the screen.

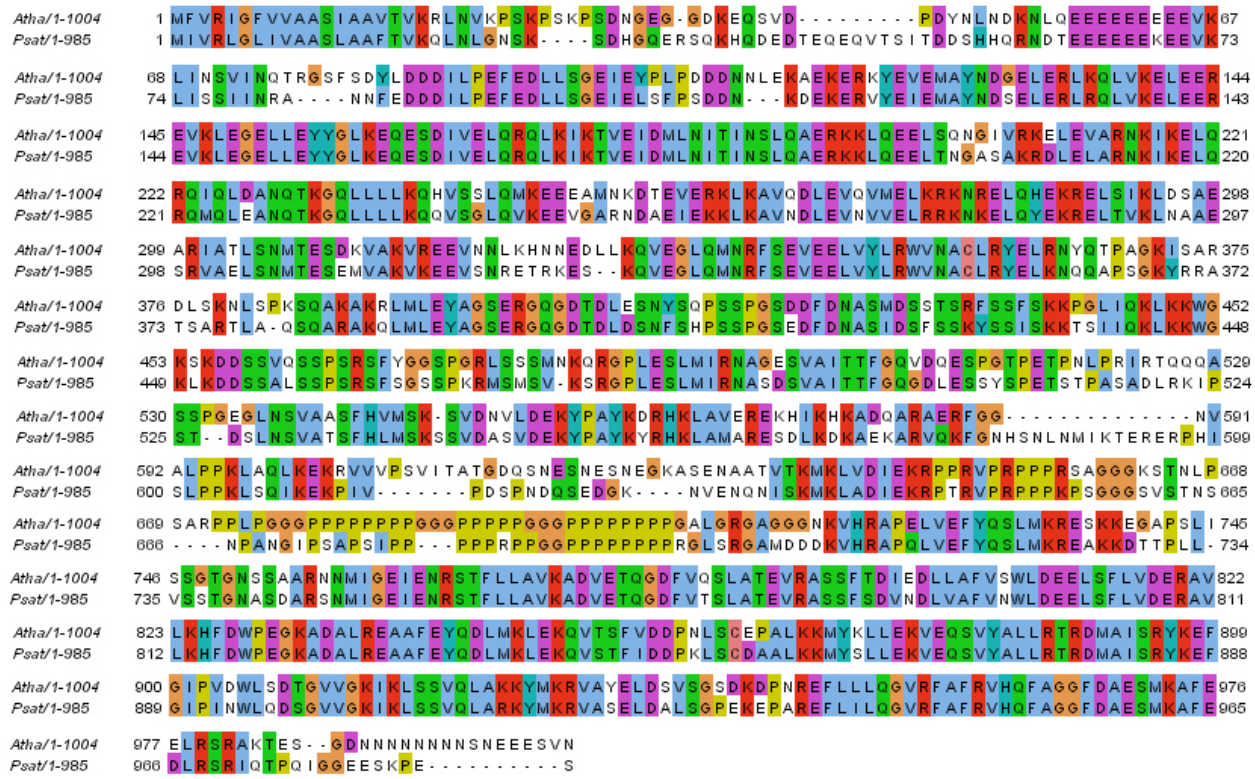

Figure 13 Alignment of Chup1 from Arabidopsis (Atha) and Chup1 from pea (Psat). Alignment of the full-length sequence of atChup1 and the sequence of psChup1 obtained by RACE, performed with MAFFT and depicted with Jalview

4.4.2 The domain structure of Chup1

To further compare psChup1 to atChup1 a sequence analysis based on conserved domains was performed. The analysis of sequence structures that have been conserved in evolution throughout different species is a powerful tool for the assignment of functions to highly conserved parts of proteins. Conserved regions are most likely important for the function of a protein or for the maintenance of the tertiary folding structure. Therefore, the domains postulated by (Oikawa et al. 2003) for the atChup1 protein, were scrutinized with respect to their occurrence and conservedness in orthologs from other species.

The sequences from the following different organisms (Table 3) were found to contain high sequence similarity to the full length Chup1 protein from *Arabidopsis* in a BLAST search (Altschul et al. 1990).

Table 3 Orthologs of atChup1

Abbreviation	Organism	Identifier	Sequence length in aa
Atha	Arabidopsis thaliana	At3g25690	1004
Psat	Pisum sativum	this study	985
Ppat 1A	Physcomitrella patens Chup1A	GI:125659421	1130
Ppat 1B	Physcomitrella patens Chup1B	GI:125659423	1141
Osat	Oryza sativa	GI:115486888	929
Mtru	Medicago truncatula	GI:140058210	986
Vvin	Vitis vinifera	GI:157338727	959

The proteins listed in Table 3 were used for a multiple sequence alignment (MAFFT) including the newly found Chup1 protein from *Pisum sativum*.

4.4.2.1 The N-terminal hydrophobic domain

The selected Chup1 orthologs show a high similarity at the utmost N-terminus (Figure 14), which in the following has only a low conserved structure (see Figure 19). The 18 utmost N-terminal residues are found to be hydrophobic (depicted in blue) with a high occurrence. The N-terminal part of atChup1 was suggested to be acting as a membrane anchor (Oikawa et al. 2003) and is needed for targeting to the chloroplast (chapter 4.5.2).

Figure 14 Multiple sequence alignment of the N-terminal domain of Chup1 orthologs. The sequence from residues 1-28 in atChup1 is shown in a multiple sequence alignment performed with MAFFT. Hydrophobic amino-acids are depicted in blue.

4.4.2.2 The actin binding domain

The atChup1 protein was found to contain an actinin-like (e.g. Gimona et al. 2002) actin binding domain by comparison with prosite patterns [EQ]-x(2)-[ATV]-[FY]-x(2)-W-x-N (Oikawa et al. 2003). This entry was however replaced in April 2006 by the actinin-type actin binding domain signature 1 pattern: ACTININ_1 PS00019 [EQ]-{LNYH}-x-[ATV]-[FY]-{LDAM}-{T}-W-{PG}–N. The amino-acid sequence does, however, not exactly follow the

proposed pattern found in actin-binding proteins from this motif family (Figure 15). This is due to the leucine in position six of the pattern, which is not allowed by the ambiguity code {LDAM}.

Only a low amount of plant actin-binding proteins was used for the calculation of the ambiguity code. The pattern may therefore not depict the reality in plants. When looking at the putative actin-binding domain in atChup1, however, this domain is found to be highly conserved within proteins of the analysed species (Figure 15, actin-binding motif marked in red). Additionally, the domain represents an "island" of conserved amino acids in the structure (Figure 19, marked in red).

Figure 15 Multiple sequence alignment of the actin binding domain in Chup1 orthologs. The sequence from residues 326-369 in atChup1 is shown in a multiple sequence alignment performed with MAFFT. The actinin-like actin binding motif is depicted in red.

4.4.2.3 The profilin binding domain

The atChup1 protein contains a region abundant in prolines. The three repeats of GPPPPP in the sequence can all be classified as proline-rich motif1 (PRM1) which has the consensus motif XPPPPP, where X = G, L, I, S or A (Holt and Koffer, 2001). The PRM1 motif was identified to be a binding motif for profilin.

It is noticeable that among Chup1 proteins the overall arrangement of the prolines is not conserved in a definite structure of the domain (Figure 16), but to a high content in the number of proline repeats itself. Stretches of prolines in proteins by themselves are able to bind profilin, which is reflected in the ability of profilin to bind to poly-L-proline. The following numbers of repeats of the PRM1 motif occur: atChup1 (3), psChup1 (2), oryza (3), physcomitrella 1b (2), physcomitrella 1a (1), medicago (1), vitis (0) (red boxes Figure 16). A conserved part in the proline-rich domain is found at the N-terminal part of this region depicted as a black box in Figure 16, where a triple repeat of prolines is surrounded by the positively charged amino-acids lysine or arginine (in two cases also asparagine or alanine).

This conserved sequence structure was, however, not yet assigned a profilin binding PRM motif.

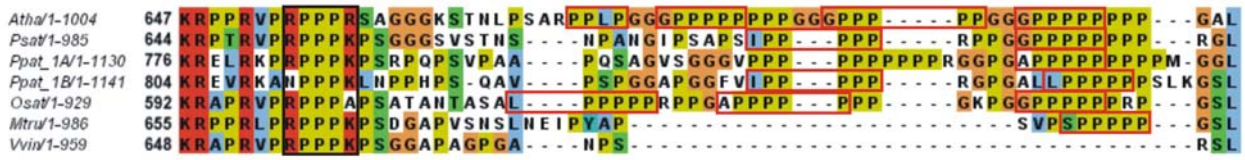

Figure 16 Multiple sequence alignment of the proline-rich region in Chup1 orthologs. The sequence from residues 647-719 in atChup1 is shown in a multiple sequence alignment performed with MAFFT. Red boxes mark PRM motifs, the black box marks a putative conserved PRM motif

4.4.2.4 Dimerization motifs

The atChup1 protein was predicted to contain a coiled-coil motif (Oikawa et al. 2003). Coiled-coils are α-helical structural domains that intertwine to form multimeric supercoils.

The coiled-coil structure is characterized by a heptad repeat pattern. The residues in the first and fourth position are hydrophobic, residues in the fifth and seventh position are mostly charged or polar (Burkhard et al. 2001) A specific class of coiled-coil regions are leucine zippers (see below). Coiled-coil domains are protein oligomerization motifs that function as protein-protein interaction sites in subunit-oligomerization or act as membrane anchors. Interestingly, coiled-coil domains are often a structural feature of cytoskeletal proteins and motor proteins (e.g. myosins) or found in movement processes (e.g. endocytosis or cell adhesion) or in signal transduction (Burkhard et al. 2001)

The existence of coiled-coil domains found in atChup1 (Oikawa et al. 2003) was predicted for the Chup1 orthologs with the PCOILS program. This program was chosen from a range of coiled-coil prediction programs found to outperform competitors based on a comparative analysis by Gruber et al. (2006).

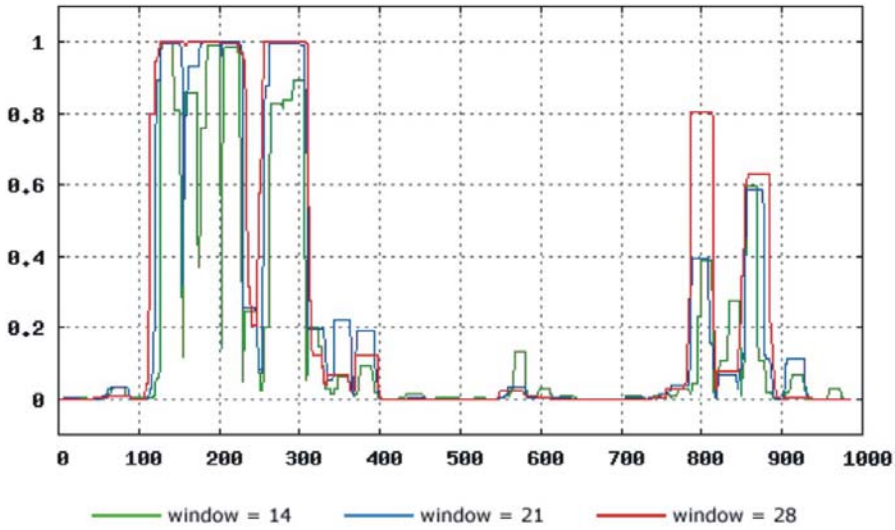

Figure 17 Presence of coiled-coils in psChup1. Exemplary for the Chup1 orthologs, a prediction of coiled-coils by the PCOILS program for psChup1 is shown. X-axis: position in sequence, y-axis: coiled-coil probability.

Figure 17 shows the prediction of coiled-coil domains in psChup1. The predicted coiled-coils are found to be similarly distributed in the orthologs. The coiled-coils have a high prevalence in the N-terminal region of the proteins ranging from a position in sequence of approximately 100-350 aa in atChup1, vitis, psChup1, medicago and oryza and from 200-450 aa in the two *Physcomitrella* orthologs (which is due to an insertion upstream of this region). An additional coiled-coil region can be detected at a position in sequence of approximately 1000 aa in the *Physcomitrella* orthologs and at a position of approximately 800 aa in all other proteins. It is predicted however to have a lower probability. This coiled-coil is formed by the leucine-zipper motif in that region. A short stretch of a possible third coiled-coil conformation is present in the *Physcomitrella* orthologs at around a position of 700 aa.

The coiled-coil prediction gives a picture of the conformational conservation that is not necessarily mirrored in the sequence homology. With the analysis of the coiled-coil structure, the conclusion can be drawn, that although the N-terminal part of the orthologous proteins has overall low sequence conservation, the structural conservation is high. When comparing the coiled-coils predicted in the orthologs, a high similarity in the predicted structure can be detected.

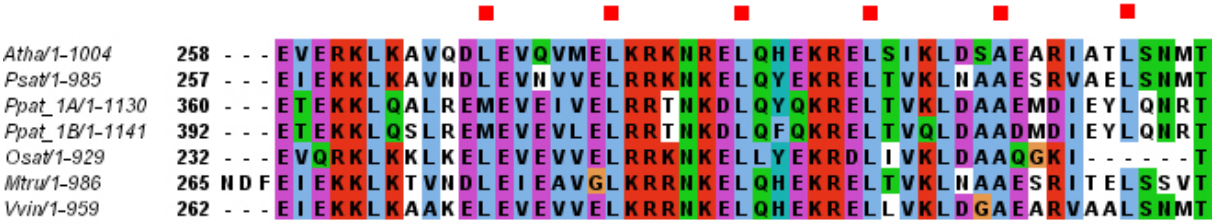

Figure 18 Multiple sequence alignment of the first predicted leucine zipper motif in Chup1 orthologs. Leucine zipper motif from Leu 269 to 304 in atChup1 is shown as a multiple sequence alignment performed with MAFFT. Marked in red are the leucines (in one case the arginines) of the motif

A leucine zipper motif was predicted for Chup1 by (Oikawa et al. 2003). Leucine zippers are sequence structures containing repetitions of leucines (or arginines in basic zipper motifs) at every seventh position. They are often found in transcription factors or function in homodimerization or heterodimerization of proteins. The leucine-zipper motif is a pattern with a high probability of occurrence and as leucine zippers exist in an α-helical conformation, they are also considered to be a subset of the more general coiled-coil structure (O'Shea et al. 1991). This seems to be the case for the Chup1 proteins. The leucine zipper motif (residues from Leu 269 to 304) is indeed only partially conserved (Figure 18). The leucines are replaced by methionines in the Physcomitrella sequences and the medicago sequence is lacking the 6th leucine.

Thus, as the leucine zipper motif is found in the region of the predicted coiled-coil (Figure 17) it could just as well belong to the overlying structure of the predicted coiled-coil. For the second predicted leucine-zipper (four leucines from Leu 802-Leu 823 in atChup1), the sequence conservation is likewise not too pronounced. The first leucine residue is replaced by a valine in oryza, medicago and the two physcomitrella sequences. The remaining three leucine repeats are probably not able to form a functional motif, but may as before belong to the coiled-coil domain (second coiled coil, Figure 17). The overall conserved structure in the alignment of the Chup1 orthologs can be found in Figure 19.

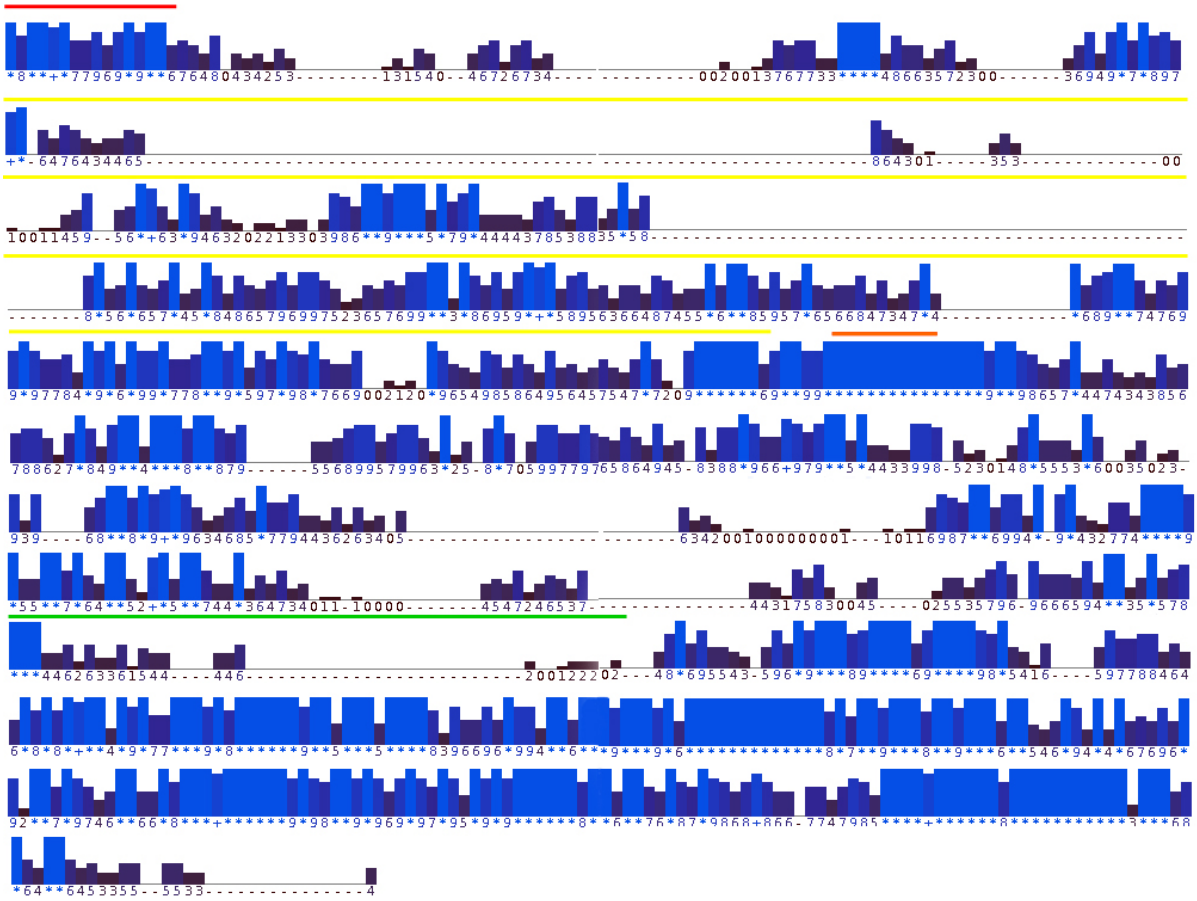

Figure 19 Sequence conservedness of the Chup1 orthologs. Shown is the conservation in sequence for the alignment of the Chup1 orthologs (Table 3). Higher values (high columns, lighter blue) stand for high conservation. Marked in red is the hydrophobic N-terminus, the first coiled-coil in yellow, the actin binding domain in orange and the profilin binding domain in green..

To summarize, from the analysis of the domain structure can be concluded, that psChup1 is a putative functional homologue of atChup1. A model of the domain structure of atChup1, including constructs for further analysis referred to later, is given in (Figure 20).

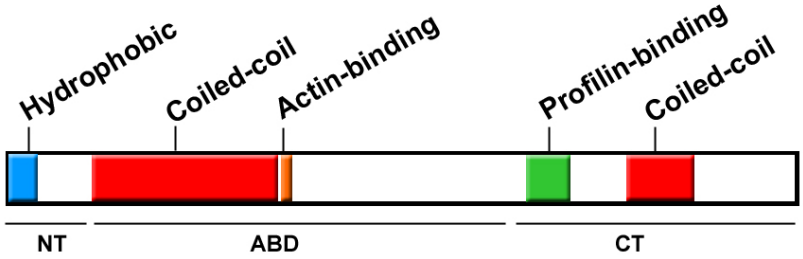

Figure 20 Domains of atChup1 and constructs NT, ABD and CT (not to scale)

4.5 Localization of the Chup1 protein

4.5.1 Chup1 is localized to plastids

To determine the localization of the Chup1 protein in plant cells, GFP fusion constructs were generated. Previously it had been shown by Oikawa et al. (2003) that the N-terminal 25 amino acids of Chup1 are directed to the chloroplast. But a more detailed analysis of the full-length protein and information about the targeting signal were missing, like a confirmation of the exclusive localization of Chup1 to chloroplasts and the dependence of the targeting on the N-terminal domain. This was needed because a component of the signal transduction cascade leading to chloroplast movement was found with at least dual localization. The Phot2 light receptor kinase relocalizes from the plasma membrane to the Golgi apparatus upon illumination with blue light (see 2.2.1, Kong et al. 2006)

To investigate these questions, first GFP fusion constructs of full-length Chup1 and ΔN-Chup1 – the full-length Chup1 without the N-terminal 25 amino acids - were generated.

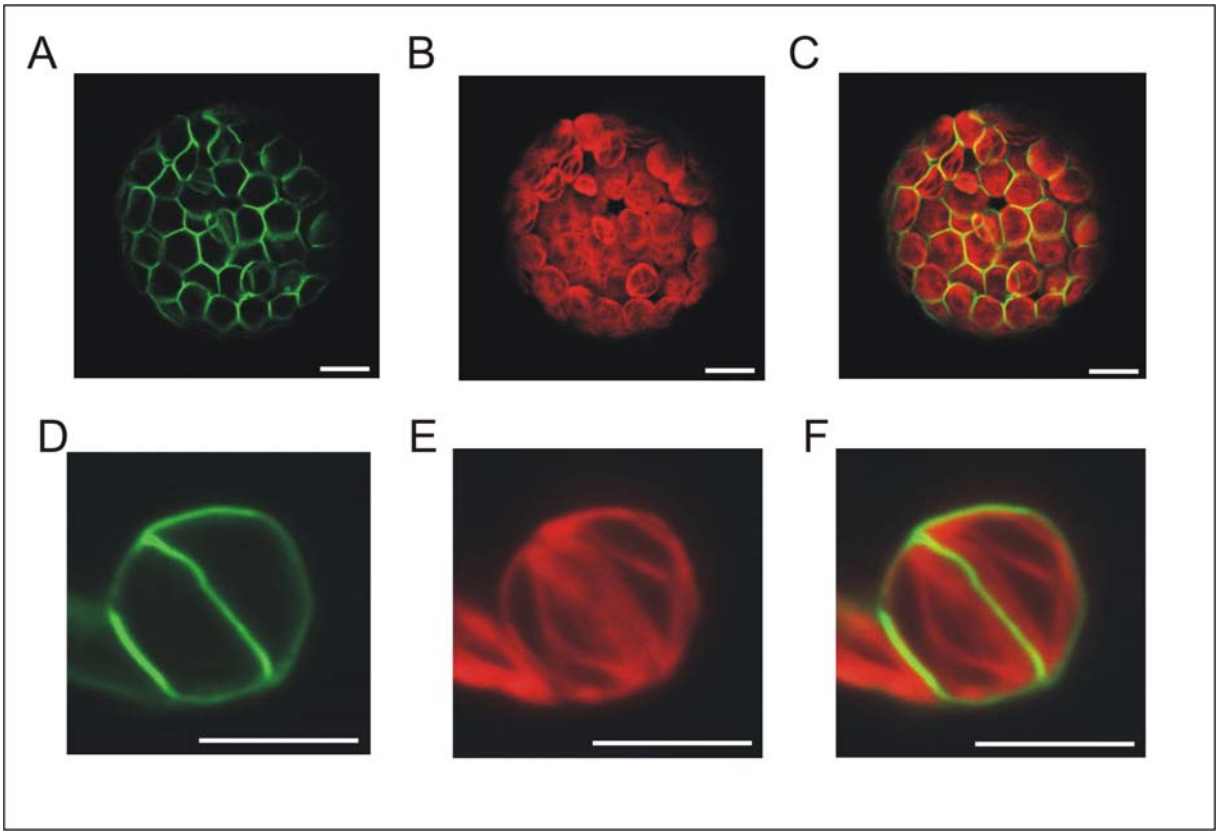

Figure 21 Expression of Chup1-GFP in *A.thaliana* protoplasts. Left panel: GFP fluorescence, middle panel chlorophyll autofluorescence, right panel: overlay picture. A-F Chup1-GFP expression in protoplasts. D-F Close-up of two chloroplasts surrounded by Chup1-GFP fluorescence. Bar = 10 µm.

As can be seen in Figure 21, the full-length Chup1 is directed to the chloroplast. The Chup1-GFP fluorescence signal is evenly surrounding the chloroplast (close-up Figure 21 D). In some experiments, however, the Chup1-GFP signal was found to be distributed in a distinct pattern along the chloroplast surface. Tagged regions occurred, where the signal appeared to be confined to the areas where the chloroplasts are in close contact to each other (Figure 22 A). As the signal intensity was high it is unlikely that the signal is only apparent at the contact sites due to overlap of signals and not visible at the whole chloroplast envelope.

Another observation was that in some cases the signal was more intense in spatially confined areas of the chloroplast surface (Figure 22 D, barbed arrows in G and I). Additionally, in some cases, vesicular structures formed by the Chup1-GFP fluorescence were encountered (Figure 22 G). In rare cases these structures could be observed throughout the cell (Figure 22 H). The physiological relevance of this distribution could, however, not be approached.

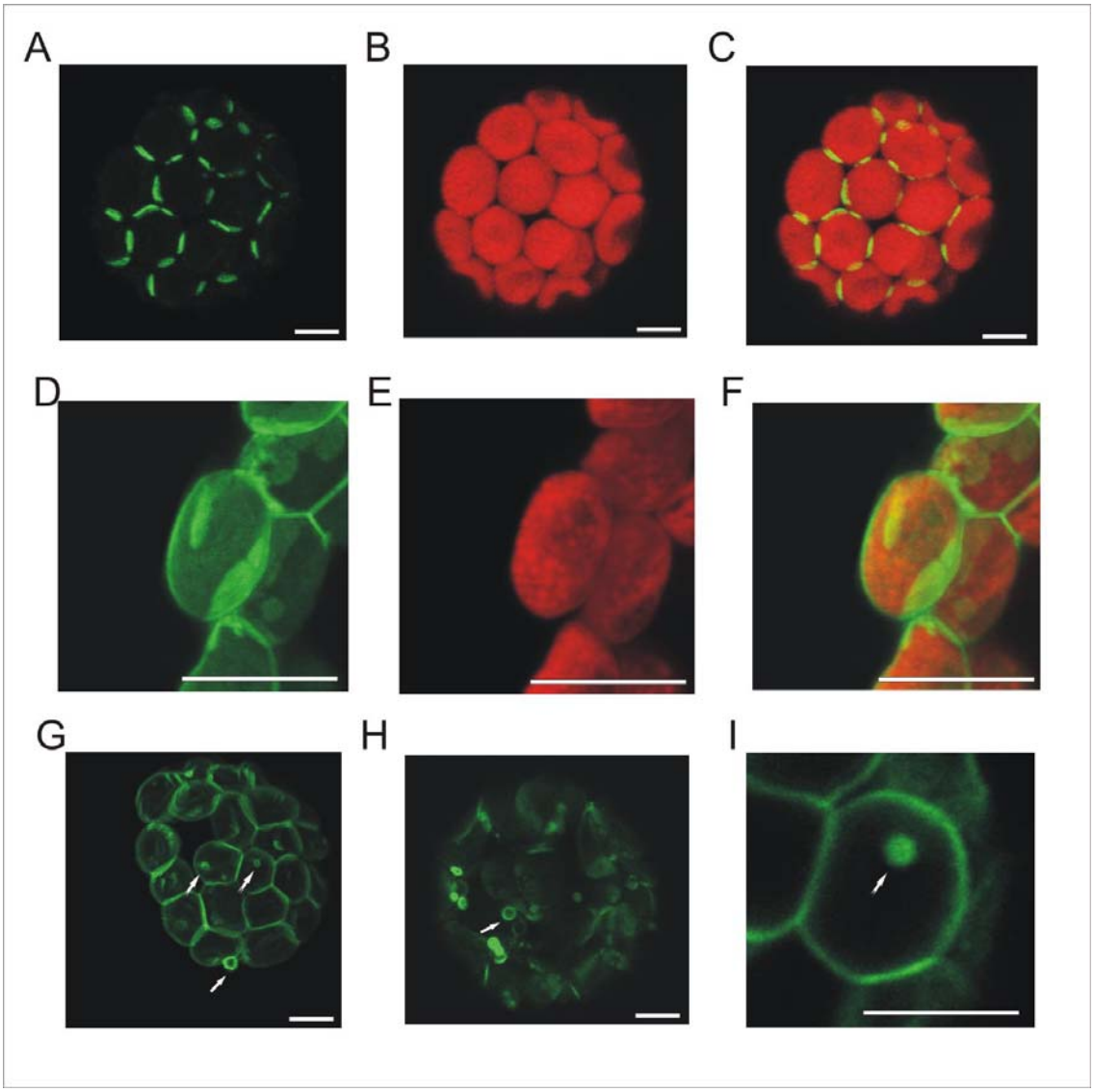

Figure 22 Observed alternative localisation patterns of Chup1-GFP. Chup1-GFP expressed in *A. thaliana* protoplasts (A-I) Left panel: GFP fluorescence, middle panel: chlorophyll autofluorescence, right panel: overlay picture. Arrow points to vesicular structures, barbed arrow to intense regions of GFP fluorescence. Bar = 10 µm.

4.5.2 Chup1 targeting is defined by the conserved N-terminus

When the 25 amino-terminal amino acids of Chup1 were deleted (ΔN-GFP), the distribution of the GFP signal changed (Figure 23 B). The chloroplast localization was abolished. ΔN-GFP was located as distinct spots in the cytoplasm. The spots were not in contact with chloroplasts (Figure 23 E).

Here the question arose, whether information from other Chup1 domains, like the actin binding domain or the PRF1 motif, was able to direct the deletion protein to a specific alternative location. For that reason an association of the speckles with the actin cytoskeleton was checked (Figure 23 F). From known actin markers, the fluorescent protein fusion to the actin binding domain of AtFIM1 (fimbrin) was chosen (Sheahan et al. 2004). Fimbrin is an actin binding protein which has two actin binding domains. The second actin binding domain (fABD2) of fimbrin was fused to RFP to enable co-localization studies (Figure 23). An advantage of fimbrin is, that altered interactions of actin to actin binding proteins upon binding of the tagged protein have not been reported, which is e.g. the case for mouse GFP-talin (Sheahan et al. 2004, see 2.5).

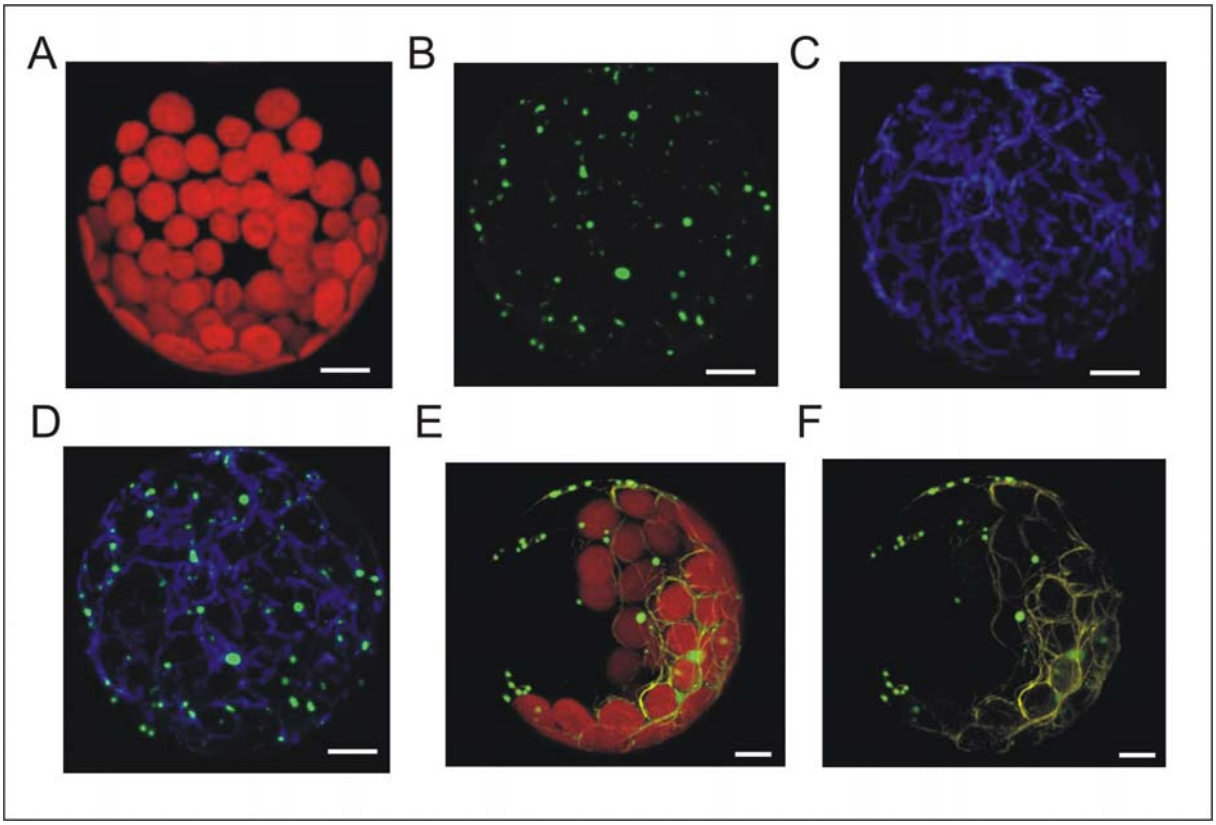

Figure 23 Localization of ΔN-Chup1-GFP. Expression of ΔN-Chup1-GFP in *A.thaliana* protoplasts A Chlorophyll autofluorescence B ΔN-Chup1-GFP C Mitotracker staining D overlay of b and c E overlay of chlorophyll autofluorescence, ΔN-Chup1 GFP and fABD2-RFP F overlay of ΔN-Chup1-GFP and fABD2-RFP. Bar = 10 µm

The actin cytoskeleton was stained effectively by the fimbrin-RFP construct (Figure 23 E,F). Also fine structural elements of actin fibres are visualized by the marker. It should be noted, that the chloroplasts are closely surrounded by the actin cytoskeleton as has previously been observed by other groups (see Introduction, Kandasamy and Meagher 1999, Kwok and Hanson 2004).

The distribution of the ΔN-Chup1-GFP construct was not to be found in association with the actin cytoskeleton, as no co-localization could be observed. In Figure 23 (C, D), protoplasts were stained with Mitotracker to exclude that ΔN Chup1 co-localizes with mitochondria, as for mitochondria a similar pattern can be observed, and indeed no such co-localization was observed. To further analyze the localization of the ΔN-Chup1 construct, the characteristic localization pattern deduced from numerous experiments was compared with the distribution of the Golgi marker ST-GFP. By this comparison it was found very unlikely that the ΔN-Chup1-GFP signal resides in the Golgi (data not shown)

4.5.3 The mode of targeting is independent of vesicle transport

The possibility that ΔN-Chup1 could be targeted to the Golgi apparatus was examined for several reasons. First, Golgi localization was often described to form a punctuate pattern (e.g. Chatre et al. 2005) somewhat similar to that observed for ΔN-Chup1 (Figure 23). Second, the light receptor Phot2, which is the origin of the chloroplast movement signal cascade, was found to have a dual localization (Kong et al. 2006, see 4.5) at the plasma membrane and at the Golgi apparatus. The third reason was that recent findings suggested that some chloroplast proteins are targeted through the secretory pathway to the chloroplast (Villarejo et al. 2005). Moreover, from initial analyses of the biochemical properties of Chup1, a possibility for Chup1 taking the secretory route to the chloroplast could not be excluded. Prediction programs (e.g. TargetP, Emanuelsson et al. 2000) predicted the destination of Chup1 to the secretory pathway. Moreover, a glycosylation site was predicted for Chup1 (e.g. Prosite). By taking the secretory pathway, Chup1 would be able to target different cellular membranes. Analysis of the targeting of Chup1 in respect to a possible route to the chloroplast via the Golgi apparatus was done with brefeldin A (BFA).

BFA is an inhibitor of the Golgi-mediated vesicular transport (Ritzenthaler et al. 2002). BFA targets GTP-exchange factors (GEFs) and thus inhibits the activation of Arf1p, which recruits coat proteins for transport vesicles to the Golgi (Nebenführ et al. 2002). This inhibition results in a disorganization of the Golgi-stacks, as can be nicely seen in Figure 24 K, where the fluorescent signal of ST-GFP shows the organization of the so called brefeldin A compartment.

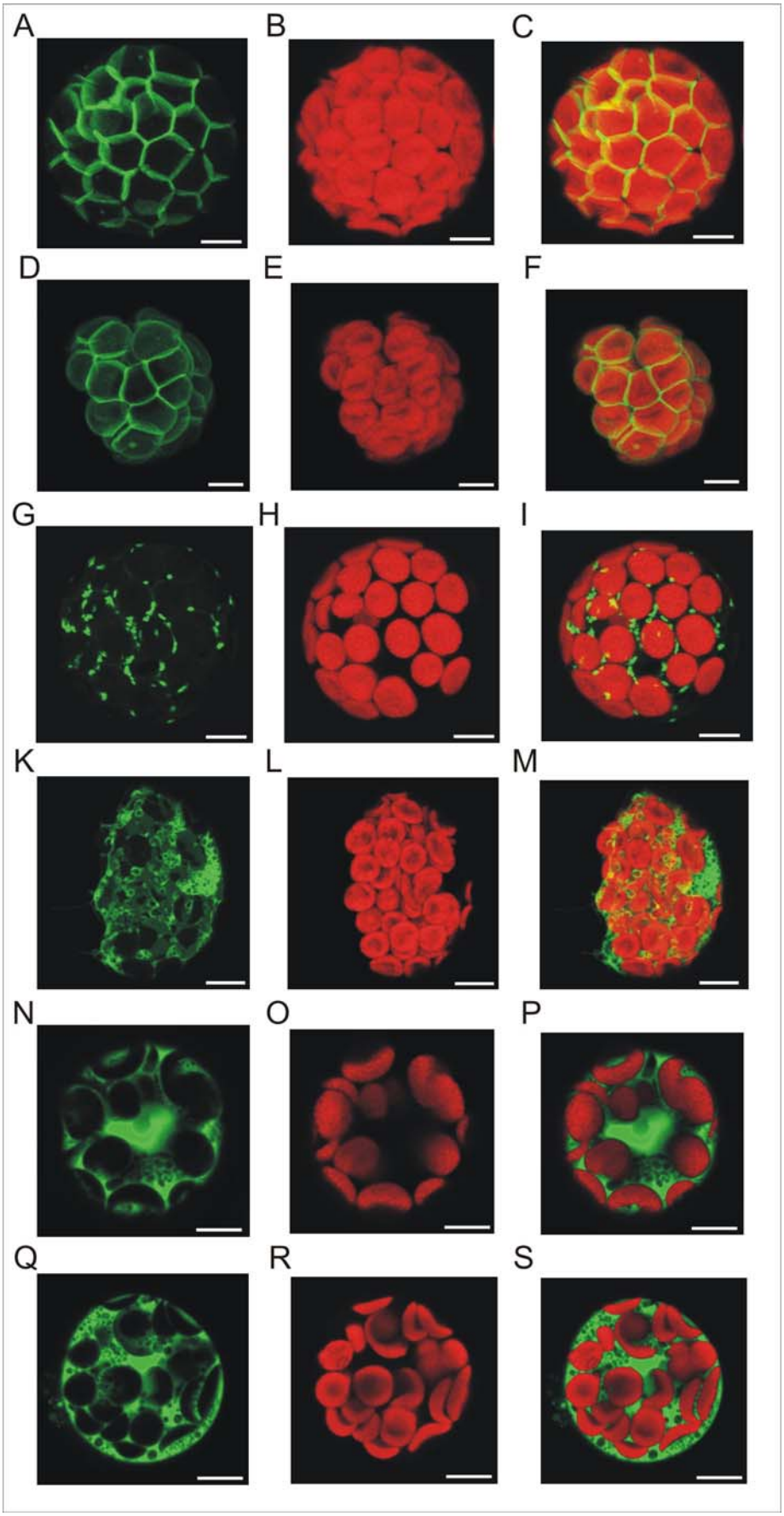

Figure 24 Influence of BFA on the localization of Chup1-GFP. Left panel: GFP fluorescence, middle panel: chlorophyll autofluorescence, right panel: overlay picture. Chup1-GFP (A-F), ST-GFP (G-M) and GFP (N-S)

were transformed into *A.thaliana* protoplasts without treatment with BFA (A-C, G-I, N-P) or with BFA-treatment (D-F, K-M, Q-S). Bar = 10 µm.

In contrast, Chup1 targeting to chloroplasts was not impaired by addition of BFA (Figure 24 A vs D). As expected, the distribution of the GFP control is also not affected by BFA (Figure 24 N vs Q). This is of course due to the cytoplasmic and nuclear localization of GFP, which is not dependent on a distribution through the secretory pathway.

4.5.4 Targeting of Chup1 to the chloroplast membrane in vitro

To further assess the localization of Chup1 at the chloroplast surface, and to support the *in vivo* GFP localisation studies (see 4.5), *in vitro* binding of Chup1 to purified outer envelope of chloroplasts was tested. For the experiment Chup1 protein was in vitro translated in wheat germ extract (Figure 26 A) and used in further experiments as no translation product was obtained from reticulocyte lysate (Figure 26 C).

The binding of Chup1 to the outer envelope could be confirmed, as Chup1 was coprecipitated with the outer envelope (Figure 25 A, lane 2) but did not sediment in the absence of OE (lane 4).

This interaction is mediated by the N-terminal domain of Chup1, as the Chup1-NT fragment comprising the N-terminal residues of the Chup1 protein likewise cosediments in an outer envelope dependent manner (Figure 25, lane 4). These results confirm the above established conclusion that Chup1 is indeed localized at the outer envelope.

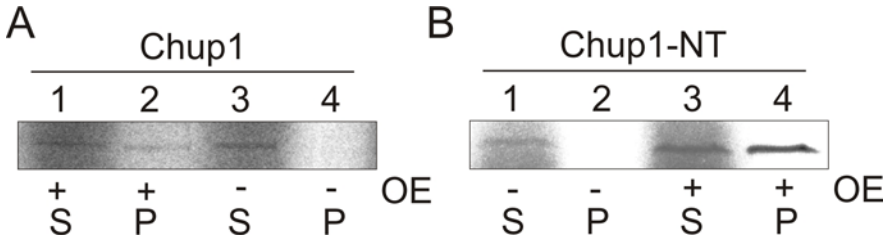

Figure 25 Cosedimentation of Chup1/Chup1-NT and OE. A Radioactively labelled Chup1 (lanes 1-4) was incubated with OE (lanes 1 and 2), supernatant and pellet fractions were subjected to SDS-PAGE and autoradiography after centrifugation. **B** Radioactively labelled Chup1-NT (lanes 1-4) was incubated with OE (lanes 3 and 4), supernatant and pellet fractions were subjected to SDS-PAGE and autoradiography after centrifugation.

4.6 Chup1 interaction with actin

4.6.1 Expression of Chup1

To characterize interactions of the Chup1 protein, binding studies with heterologous expressed protein were required. All attempts to express full-length Chup1 protein failed however. In the *E. coli* system, different strains (BL21(DE3), TOP10, JM101) as well as strains containing helper plasmids (pLysS, pMICO, Rosetta) were used to enable tight expression and/or expression of plant genes with rare codons. Furthermore expression with a GST-tag to enhance solubility and to avoid potentially lethal aggregation was tested but proved to be not successful. A change to a eukaryotic expression system and expression of CHUP1 with a HA-tag in yeast was not yielding any protein. An attempt to express CHUP1 with a secretion signal in yeast to allow the immediate export from the cytoplasm and thus avoid aggregation of misfolded protein that could result in cell death was also not successful. An expression of full-length CHUP1 was only possible in the *in vitro* system. But interestingly, *in vitro* expression also failed when CHUP1 was translated in reticolucyte lysate rather than in wheat germ extract (Figure 26 C). This points to an expression mechanism that is requiring factors only present in plant systems for the expression of functional full-length protein (probably specialized chaperones). This is conceivable as Chup1 has no equivalent in prokaryotes or animals. Therefore, radioactively translated full-length Chup1 was gained from *in vitro* expression in wheat germ extract (Figure 26 A). The N-terminal part of Chup1, as well as the middle part comprising the coiled-coil and the actin binding domain (see Figure 20) could be expressed as insoluble inclusion bodies in *E. coli*. The lack to properly fold in *E. coli* is probably due to the hydrophobic domain on one hand and the large coiled-coil which can cause aggregation on the other hand. A large fraction of the protein was however successfully refolded to gain soluble protein by the method of rapid dilution (data not shown). The C-terminus of Chup1 was obtained in a soluble form and purified over Ni-NTA column (Figure 26 B). With the proteins in hand, *in vitro* binding experiments could be initiated.

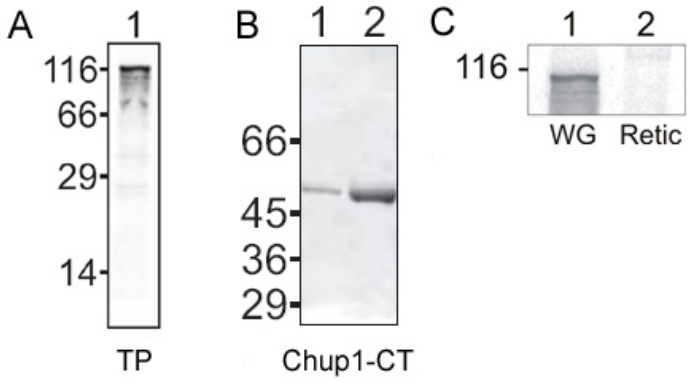

Figure 26 Expression of Chup1 A: radioactively [^{35}S]Met labelled translation product (TP) from in-vitro translation in wheat germ with CHUP1-pDest as template, autoradiography from SDS-PAGE gel. B: Coomassie stained SDS-PAGE gel from elution fractions of Chup1-CT expressed in *E. coli* and purified over Ni-NTA sepharose (lane 1 0.8 µg and lane 2 4 µg protein loaded). C: Translation product of CHUP1 pDEST17 in wheat germ extract (WG, lane 1) or reticulocyte lysate (lane 2), subjected to SDS-PAGE and autoradiography.

4.6.2 Purification of plant actin

For interactions studies of actin with the chloroplast and Chup1, actin had to be purified from endogenous plant sources. Actin can readily be gained from muscle tissue of different species. Protocols for the isolation of actin from plant tissue were limited and not satisfying (Diaz-Camino and Villanueva 1999). The problems for actin purification from plants consisted in the lower concentration of actin and high protease content in plant tissue preparations. Following the protocol from Diaz-Camino and Villanueva published in 1999 and adapting it from *Phaseolus vulgaris* to *Pisum sativum* plants (see 3.2.4.2), a sufficient amount of actin could be prepared for experiments.

For the purpose of purification DNaseI was coupled to sepharose for affinity chromatography of actin from plant extracts. Figure 27 (lane 1) shows the actin purified from pea compared to actin from chicken obtained from Sigma (lane 2). In lane 3 (Figure 27), the specificity of the anti actin antibody from sigma against pea actin is shown. Also, no cross-reactivity of the actin-antibody with antigenes from the outer envelope was observed during incubation of the outer envelope vesicles with pea actin

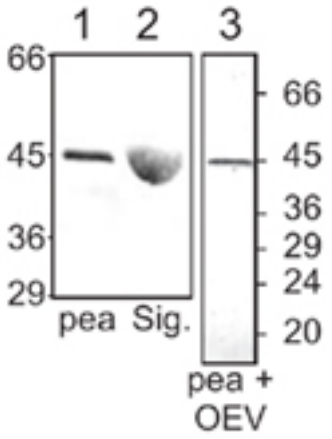

Figure 27 Actin purified from pea, specificity of actin antibody. Pea actin (lane 1), actin from Sigma (lane 2) and pea actin pelleted with outer envelope vesicles (OEV, lane 3) were immunodetected with actin antibody

4.6.3 Interaction of actin with the outer envelope of chloroplasts

As a first measure to characterize the molecular events in chloroplast movement, the participation of actin - which was proposed to be involved by inhibition experiments (see 2.3) - had to be confirmed. Therefore, an interaction of chloroplasts and actin either directly or through linker complexes is indispensable for a participation of actin in chloroplast movement in this respect.

In Figure 28 pea actin was incubated with outer envelope vesicles from pea at conditions omitting the polymerization of G-actin. A co-sedimentation of actin together with the OE can be detected (Figure 28, lane 2 vs. lane 4). Interestingly, a minor fraction of actin is also found in the envelope fraction (lane 2) prior to the addition of actin, which is removed by protease treatment (lane 8). The association of actin with the OE is a proteinaceous interaction, as a treatment of the OE with trypsin, to digest protein domains not protected by the membrane, results in a loss of interaction (lane 6 vs. lane 4). The occurrence of actin in the supernatant after incubation with protease treated membranes (lane 5) proves the efficient removal of protease activity by trypsin inhibitors. Without the addition of OE, G-actin is not found to pellet, which demonstrates that monomeric actin was present (lane 9, 10) and the precipitation reflected specific binding.

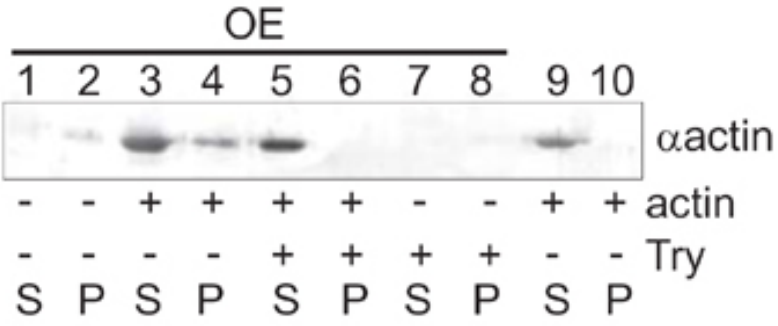

Figure 28 Interaction of actin with the outer envelope of chloroplasts. Outer envelope membranes (lane 1-8) were incubated with G-actin (lane 3-6) or without G-actin (lane 1, 2). Lanes 5-8 were treated with trypsin. For control G-actin alone was processed (lane 9, 10) Supernatant (S) and membrane fraction (P) were separated by centrifugation.

To assess the binding properties of actin to the OE, a kinetic of the association was conducted (Figure 29). Already at 0 min, actin can be found in the pellet (lane 2), which might reflect endogenous actin bound to the membrane fraction (see Figure 28, lane 2) or a rapid interaction. When comparing lane 4 vs. lane 6 (Figure 29), it can be observed that the binding of actin to the outer membrane fraction of chloroplasts is rapid and saturated already after 10 min under the conditions used.

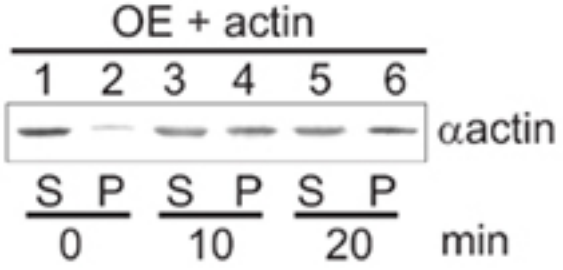

Figure 29 Interaction of actin with outer envelope, time scale. Outer envelope membranes were incubated with G-actin (lane 1-6) for 10 (lane 3, 4) or 20 min (lane 5, 6) and supernatant (S) and membrane fraction (P) were separated by centrifugation. In lane 1, 2 samples were centrifuged directly after addition of actin. Equal amounts of pelleted membrane fraction and supernatant subjected to SDS-PAGE, transferred and stained with actin antibodies are shown.

4.6.4 Complex formation by Chup1 and actin

To challenge the proposal that Chup1 is the OE receptor recognizing actin, based on the motif search (4.4.2), F-actin was incubated with *in-vitro* translated Chup1 protein. F-actin is a high molecular weight complex and is sedimented at accelerations of 300 000g for 1h, which can be used to study the interaction of factors. A specific interaction of Chup1 with F-actin can be observed, because Chup1 sediments after addition of F-actin (Figure 30). It does not sediment significantly in the absence of actin (lane 2) or in the presence of G-actin (lane 8).

Furthermore, F-actin is not able to precipitate Toc34 (lane 6) – an outer envelope protein - confining the specificity of the Chup1-actin interaction.

Figure 30 Cosedimentation of Chup1 and F-actin. Radioactively labelled Chup1 (lane 1-4, 7, 8) or Toc34 (lane 5, 6) were incubated with F-actin (lane 3-6) or G-actin (lane 7, 8) and supernatant (S) and pellet fraction (P) were separated by centrifugation, subjected to SDS-PAGE and autoradiography.

The interaction of Chup1 with actin, however, is not dependent on the filamentous state of actin. Chup1 can be specifically immunoprecipitated by antibodies against actin after addition of G-actin (Figure 31). Chup1 is retained on the column by G-actin and only eluted in low pH (lane 5), whereas it is not retained by the control (lane 4 and 6).

With Chup1 being present in the outer envelope (Figure 21, Figure 25) and actin associated with the outer envelope (Figure 28) and additionally interacting with Chup1 (Figure 30, Figure 31), Chup1 might well be one interaction partner of actin present at the outer envelope membrane.

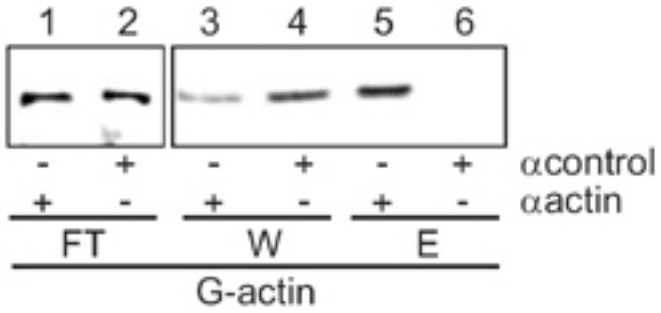

Figure 31 Immunoprecipitation of radioactively labelled Chup1 with G-actin. Chup1 (lane 1, 2; FT, flow through) incubated with G-actin was immunoprecipitated by anti-actin (lane 5; E elution) or control antibodies (lane 6, E elution). The wash of the column is shown in lane 3, 4 (W, wash). Fractions were submitted to SDS-PAGE and autoradiography.

4.7 Chup1 profilin interaction

From sequence information, for Chup1 a profilin binding motif PRM1 (Holt and Koffer 2001) can be classified from the proline-rich region (Figure 16). A binding of Chup1 to profilin is

conceivable, as profilin is a multifunctional actin binding protein. Thus, a function of profilin in chloroplast movement can be considered (see Discussion). To test this hypothesis, binding studies were conducted to prove an interaction of Chup1 and profilin.

4.7.1 Profilin purification and confirmation of functionality

Profilin was purified from *P.sativum* or *A. thaliana* with the use of poly-L-proline affinity chromatography by the method of Vidali et al. (1995). Profilin has a high affinity to PLP as it naturally binds to proline-rich proteins in vivo. Profilin was recovered in high quantities and in high purity (Figure 32).

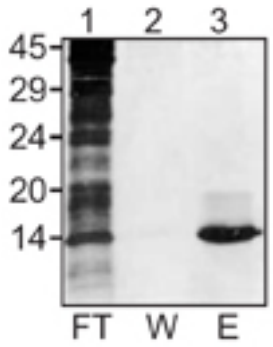

Figure 32 Profilin purification. Profilin isolated from pea extract by poly-L-proline affinity chromatography. Flow through (lane 1), wash (lane 2) and elution fraction (lane 3) were subjected to SDS-PAGE and silver stained.

To test whether profilin isolated from plant extracts by the described method is functional, its ability to bind actin was assayed. Profilin was initially described as an actin binding protein, interacting with actin in a 1:1 complex (Carlsson et al. 1976). Therefore actin binding to the isolated plant profilin was tested. The fluorescence quenching of tryptophan in proteins results from excited state encounters of the tryptophan with the functional groups of the amino acids in the surrounding protein (Harris and Hudson 1990). This is also true for the formation of the profilin-actin complex, which is accompanied by a change in tryptophan fluorescence. Perelroizen and colleagues (1994) observed that the fluorescence intensity of the tryptophans for a mixture of actin and profilin was lower than the sum of the intensities measured for actin or profilin alone. The fluorescence quenching results from the quenching of fluorescence of the tryptophans upon interaction, which are present in the profilin binding pocket of actin.

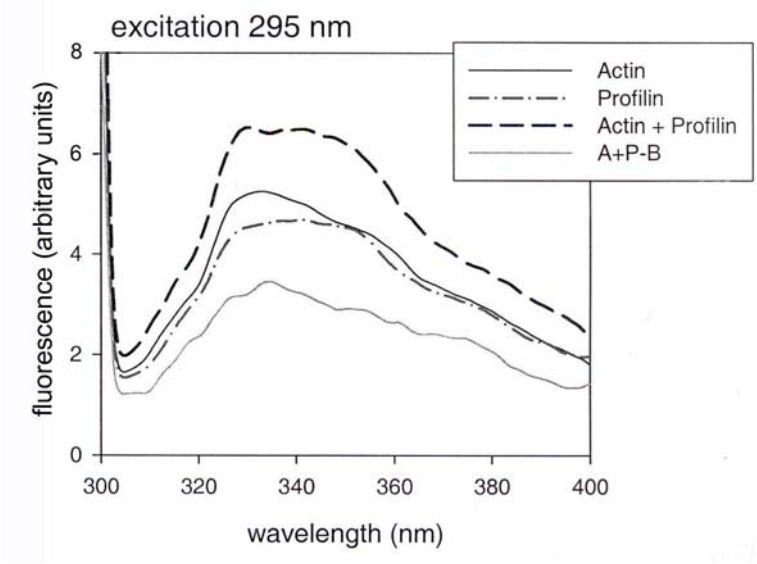

Figure 33 Fluorescence emission spectrum of profilin and actin. Tryptophan fluorescence of actin (solid black line), profilin (dashed-dotted line), actin and profilin together (B) at the same concentrations as before (dashed line) and difference spectrum of the added single fluorescence spectra of actin and profilin subtracted by the spectrum of actin and profilin together (A +P –B, solid grey line) at an excitation wavelength of 295 nm.

The purification process yielded functional profilin (Figure 33). The fluorescence emission spectra of actin, profilin and the actin-profilin complex are shown. The difference spectrum (A + P- B) (Figure 33, grey line) has a lower fluorescence emission than the single spectra of actin or profilin alone and thus displays the tryptophan quenching of the actin-profilin complex. Therefore it can be assumed that the binding properties of profilin were conserved during the purification process and that profilin is active.

4.7.2 Interaction of Chup1 and profilin

To determine, if an interaction of Chup1 and profilin can occur, the purified profilin was spotted on nitrocellulose membrane. The immobilized profilin was incubated with *in-vitro* translated radioactively labelled Chup1 protein (Figure 26 A). A specific concentration-dependent binding of Chup1 to profilin was observed (Figure 34 A, lane 2). No association of Chup1 to the likewise spotted control protein BSA was detected (Figure 34 A, lane1). This result demonstrates that Chup1 is interacting with profilin.

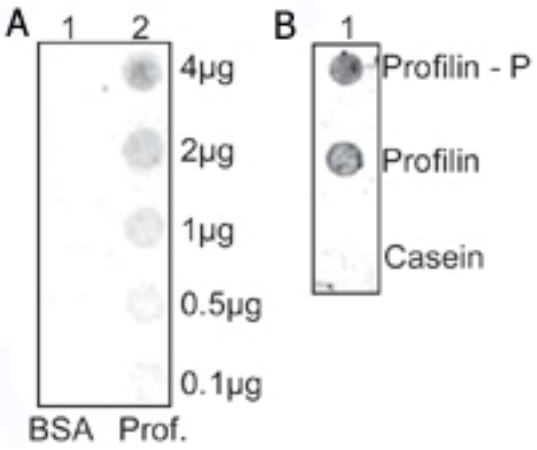

Figure 34 Binding of Chup1 and profilin. A *In-vitro* translated Chup1 was incubated with indicated amounts of BSA (lane 1) or profilin (lane 2) on an affinity matrix. The binding was visualized after extensive wash steps by autoradiography. **B** Chup1 *in-vitro* translation product was incubated with an affinity matrix coated with 20µg of casein or profilin treated with phosphatase (Profilin – P) or without treatment (Profilin). The binding was visualized after extensive wash steps by autoradiography.

4.7.3 Interaction of Chup1 and profilin occurs independent of phosphorylation

Plant profilin can be phosphorylated in vivo (Guillen et al. 1999, Limmongkon et al. 2004). Phosphorylation is thought to alter the specificity of profilin for different proline-rich domain containing proteins (Aparicio-Fabre et al. 2006). It was demonstrated by (Sathish et al. 2004) that the affinity of phosphorylated profilin to poly-L-proline is higher than the affinity of the unphosphorylated form. Thereby an enrichment of the phosphorylated profilin through the purification process using the affinity chromatography on PLP sepharose is likely. To test whether the phosphorylation of profilin affects the binding to Chup1, profilin was phosphatase treated. Chup1 then was incubated with profilin or the phosphatase treated profilin and casein as control protein immobilized on nitrocellulose membrane. However, no alteration of the binding efficiency of Chup1 towards profilin was observed, because the association was not altered by phosphatase treatment of profilin (Figure 34B).

4.7.4 Interaction of Chup1 and profilin in vivo

A remarkable observation was made, when ΔN-Chup1-GFP and profilin-RFP were co-expressed in the same protoplast. When profilin-RFP is expressed in the absence of ΔN-Chup1-GFP, it is distributed in the cytoplasm (Figure 35 E) and in part in the nucleus (not easily seen in Figure 35). This localisation is consistent with the function of profilin as a multifunctional actin filament regulating protein. The localisation of profilin in the nucleus

has been explained with a function in inhibiting actin filamentation in the nucleus (Stüven et al. 2003) and/or with a role of profilin in pre-mRNA splicing (Skare et al. 2003).

When profilin is now co-expressed with ΔN-Chup1, this localization changes to adopt a punctuate pattern like that observed from ΔN-Chup1 (Figure 23 B). Indeed, a co-localization of profilin-RFP and ΔN-Chup1-GFP can be observed (Figure 35 B, C). The overexpression of ΔN-Chup1-GFP directs profilin-RFP to the sites of ΔN-Chup1-GFP accumulation. The punctuate pattern formed by profilin-RFP is dependent on the presence of ΔN-Chup1-GFP.

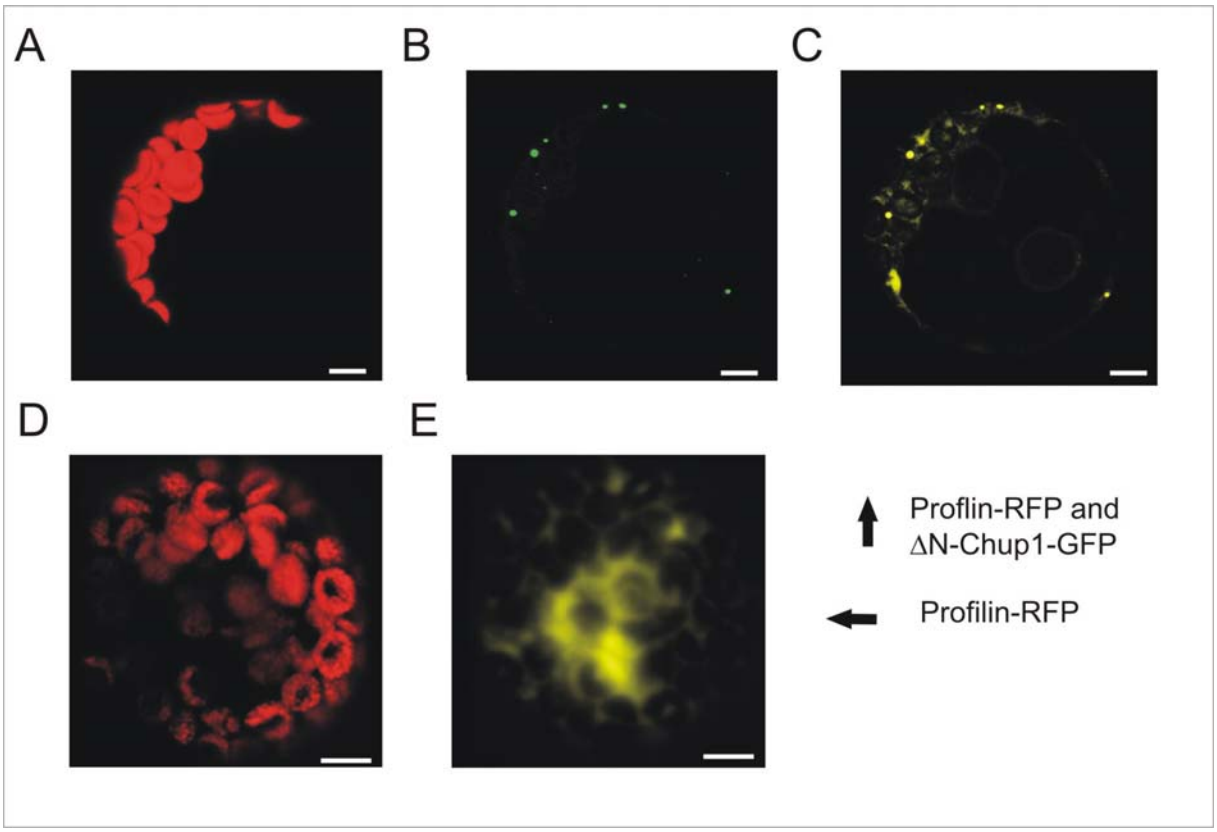

Figure 35 Interaction of ΔN-Chup1-GFP and Profilin-RFP. A-C Co-expression of ΔN-Chup1-RFP and profilin-RFP in *A.thaliana* protoplasts, autofluorescence (left), GFP (middle), RFP (right). D-E expression of profilin-RFP, autofluorescence (left), RFP (middle)

4.7.5 Delimitation of the profilin binding domain in Chup1

To test the prediction of the profilin binding motif present in the C-terminal part of Chup1 and to delimit the binding activity to the PRM1 motif in Chup1, a construct of the C-terminal part of Chup1 (Chup1-CT) was used (Figure 26). Chup1-CT includes the PRM1 motif but lacks other predicted functional domains (except a short coiled-coil Figure 20). The interaction between Chup1 and profilin is indeed mediated by the proline-rich domain, because the C-

terminal part of Chup1 including the PRM1 motif efficiently interacts with profilin (Figure 36). A concentration dependent binding of profilin to Chup1-CT can be detected by staining with profilin antibody. A cross-reaction of the antibody with Chup1-CT can be excluded from Figure 36 (upper panel). The casein control protein shows no interaction with profilin.

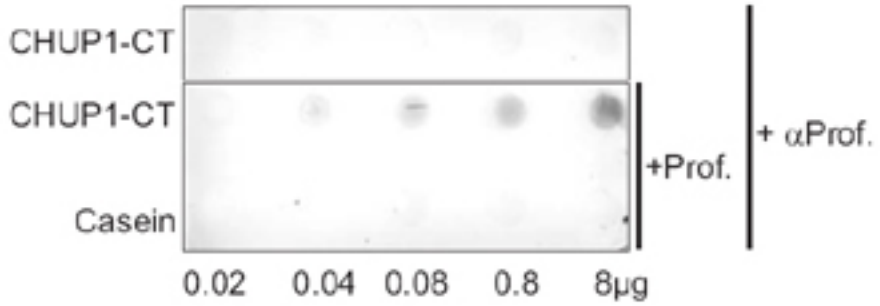

Figure 36 Binding of Chup1-CT to profilin. Chup1-CT (or casein as control) was spotted in indicated amounts on the affinity matrix and incubated with profilin (lower panel) or without profilin (upper panel), and immunostained with anti-profilin.

4.8 Interplay of actin, profilin and Chup1

It was now important to explore the combined interaction between actin, profilin and Chup1. Fluorescent labelling of profilin with dansylchloride [5 (dimethylamino) naphtalene-1-sulfonyl chloride] (Weber 1952) was used to measure interaction of profilin to Chup1. The dansyl group covalently interacts with the primary amine in lysines present in the protein (e.g. Haugland 2003). To analyze whether Chup1 modulates the interaction between profilin and actin, the fluorescent emission spectrum of dansylated profilin, incubated with actin or Chup1-CT was measured. An increase of the dansyl fluorescence (e.g. Enguita et al.1996) is expected upon interaction. Indeed, the expected increase in fluorescence was obtained (Figure 37A); the fluorescence spectrum of dansyl-profilin incubated with actin or Chup1-CT exceeded the fluorescence of the dansyl-profilin/BSA control or that of dansyl-profilin alone. The fluorescence increased in a concentration dependent manner, when Chup1-CT was titrated to dansyl-profilin (Figure 37 B).

To determine whether Chup1-CT influences the interaction of actin and profilin, Chup1-CT was titrated to a mixture of profilin and actin. The observed fluorescence was corrected for the fluorescence induced by profilin-Chup1-CT and the remaining signal was normalized to the initial fluorescence induced by the actin-profilin interaction itself. A Chup1-CT

concentration dependent increase of the fluorescence was obtained (Figure 37 C), indicating an interaction of the three proteins.

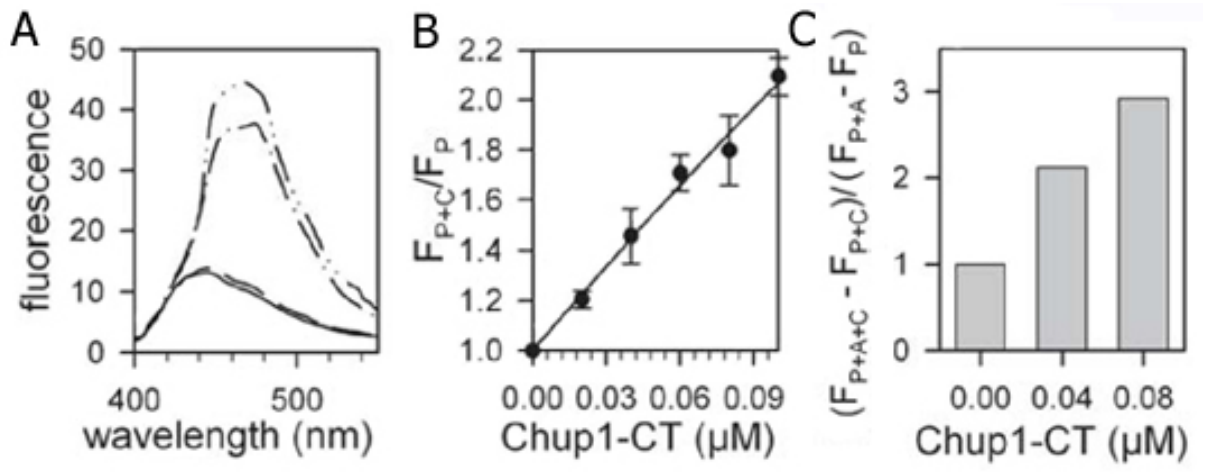

Figure 37 Fluorescence measurements of dansyl-profilin. A Profilin labelled with dansyl (0.4µM, solid line) was incubated with 0.25µM BSA (dashed line), 0.1µM actin (dashed – dotted line) or 0.1µM Chup1-CT (dashed - double dotted line) and the fluorescence was determined exciting with 337nm. **B** The fluorescence increase of dansyl-profilin (0.4µM) at 450nm at different Chup1-CT concentrations was determined. The line shows the least square fit to $F_{max}*c_{Chup1\text{-}CT}/(K_D + c_{Chup1\text{-}CT})$. **C** 0.4µM dansyl-profilin was incubated with 0.2µM actin and fluorescence was determined in the presence of increasing amounts of Chup1-CT. Shown is the difference of the fluorescence of dansyl-profilin in the presence of actin and in the absence of actin normalized to the initial fluorescence of dansyl-profilin bound to actin in the absence of Chup1-CT

5 Discussion

Chloroplast movement is of high importance for chloroplast integrity and the plant's light gathering ability. The hazardous impact of light on the photosystems as well as the crucial gathering of light for energy is a double-edged sword for the chloroplast. A tight balance has to be kept to ensure optimal conditions for the chloroplast in different light fluence rates. Chloroplast movement is a major part of the system ensuring the right balance. The involvement of Chup1 in chloroplast movement has been demonstrated in phenotypic observations. A biochemical analysis of Chup1 however was lacking. As light is the trigger for regulation of chloroplast movement, the global gene expression in response to light was analysed by expression profiling.

Expression profiling of changes induced by blue light and the *chup1* mutation

From a global expression profile of wild type plants in response to blue light stimulus the expression of blue light sensitive genes was tested. After 5 min of blue light treatment only 3 genes were found to be regulated. A chloroplast localized superoxide dismutase was found with an enhanced transcript level that is possibly regulated by a microRNA. The regulation of transcript levels by blue light regulated miRNAs would point to a novel blue light regulating pathway in plants. Furthermore, the level of reactive oxygen species - that were found to be involved in signalling to the actin cytoskeleton (Choi et al 2008) - could apply regulatory force for the chloroplast in signalling to actin.

The analysis of the obtained data for the genes directly involved in chloroplast movement, namely Phot1/2, Jac1, Pmi1/2/15, leads to the conclusion that chloroplast movement is not causally regulated by gene expression, as no drastic change in expression was detected. Chloroplast movement has been shown to be insensitive to the loss of the nucleus in fern (Wada, 1988). From the conducted microarray experiments, the conclusion can now be drawn that chloroplast movement in higher plants is likewise not transcriptionally regulated. This might, however, not be the case for components of the signal cascade upstream of the mentioned targets, as many light regulated kinases were found.

In this study the chloroplast unusual positioning phenotype of Chup1 discovered by Kasahara et al. (2002) was confirmed with an independent knock-out mutation in the *CHUP1* gene. The chloroplasts in this mutant are unable to react to strong light with avoidance movement (Figure 11). This manifests Chup1 as indispensable for chloroplast movement.

From expression analysis, a differential regulation of genes responding to high light treatment was found for the wild type and the Δ*chup1* mutant (Figure 12). Strikingly, most of the genes altered in their expression in response to the deletion of CHUP1 are found to be up-regulated in wild type plants in response to enhanced light conditions. This can be explained by a shift in the sensitivity to light in Δ*chup1* as an adaptation mechanism to strong light caused by the lack of protection for the chloroplasts by the defect in chloroplast movement (Kasahara et al. 2002). Furthermore, most of the regulated genes were involved in stress response. This may reflect adaptation to environmental conditions of the mutant and might give a link of the signal cascades of stress induced changes and light stimulus.

psChup1 is a putative functional homologue of atChup1

From RACE experiments, a homologue of atChup1 in pea was discovered (Figure 14, Figure 15, Figure 16, Figure 18). The high overall sequence homology as well as the high conservation of the functional Chup1 domains, the N-terminal hydrophobic domain, the coiled-coil domain, the actin-binding domain and the PRM1 profilin binding domain, makes psChup1 a very likely candidate for a Chup1 functional homologue in pea. In this screen no other isoforms were detected, as is the case for *Physcomitrella patens* Chup1, where two isoforms Chup1a and Chup1b have been submitted to the database.

Several sequences from a range of plant species were found with a high similarity to the C-terminus of atChup1, which were not included in this study as it can not be judged at this time whether they have an implication on chloroplast movement. Nonetheless, these proteins highlight the conserved structure of the C-terminus, which might be interesting for future studies. For example in a BLAST search a protein from *Medicago* was found to share high similarity with the C-terminus. This protein is designated a phosphoinositide-binding (clathrin adaptor). A function in phosphoinositide binding could be important for regulatory action with respect to profilin (see below).

Chup1 targeting and localization

In a previous study (Oikawa et al. 2003), the localization of Chup1 had been determined solely via a 25 aa fragment of the N-terminus of the protein. In this study, the aim was to discover the actual site for targeting information and the inherent targeting information of the full-length protein. It could be observed, that the full length Chup1-GFP protein is targeted to the chloroplast (Figure 21). Thus, no other targeting information is conveyed by the full-length protein in comparison to the N-terminus alone (Oikawa et al. 2003). The chloroplasts

are in most cases evenly surrounded by Chup1-GFP fluorescence, but in a number of cases the fluorescence was forming a pattern in all observed protoplasts of the sample (Figure 22 A). Here, Chup1-GFP is apparently localized at the contact sites between chloroplasts. The conditions under which such a localisation appeared were not determined, but they could reflect a more specific structuring of Chup1 under certain conditions (e.g. light intensity, calcium concentration etc.). The observed more intense regions of fluorescence at certain parts on the chloroplast (Figure 22 D, G, I) could display regions where contact to interaction partners (e.g. actin sites) is maintained and a higher protein concentration is favourable. They could however also be caused by artificial overexpression under a strong promoter. The observation of vesicular structures in some cases (Figure 22 G, H) remains however more elusive. Whether they reflect a physiological localization –which is unlikely as only rarely observed - or result from overexpression artefacts can not be judged by this experiment.

The influence of the N-terminal hydrophobic domain on the targeting of Chup1 was investigated, as it was proposed, that it could function as a membrane anchor for Chup1 at the chloroplast envelope (Oikawa et al. 2003). Indeed, the N-terminal hydrophobic domain could be identified as the explicit signal for targeting. A deletion of the hydrophobic domain led to abolition of the targeting (Figure 23). This proves, that the N-terminus is not only sufficient (Oikawa et al. 2003), but also essential and is the only targeting information for targeting to the chloroplast surface.

To determine, where the mis-targeting of ΔN-Chup1-GFP in the cell was directed to, localization to mitochondria or the Golgi apparatus was assayed, which can form a similar punctuate pattern in the cell. ΔN-Chup1-GFP was shown to neither localize to mitochondria (Figure 23 D) or the Golgi (not shown). Thus the most plausible explanation is that the ΔN-Chup1-GFP construct aggregates in the cytoplasm possibly caused by the lack of being able to target to its proper destination.

To determine, whether ΔN-Chup1-GFP can be directed to the actin cytoskeleton by means of the actin binding domain, a co-expression with the actin marker fABD2-RFP was conducted. An association of the ΔN-Chup1-GFP signal with the RFP signal from actin was however not determined. The ΔN-Chup1-GFP protein thus may be in a (unfolded) state not allowing association of the actin binding domain with actin.

An additional observation could be made in the process. By staining protoplasts with the actin marker fABD2-GFP, an intact cytoskeleton was observed, with fine structures being visible. The actin filaments were detected in the vicinity of the chloroplasts, surrounding them

closely. This confirms the observation of the basket-like structures, detected by Kandasamy and Meagher (1999) and Kwok and Hanson (2004).

As several aspects were pointing to a possible dual localisation of Chup1, this had to be excluded experimentally. A dual localisation was discovered for the light receptor Phot2, which is a crucial factor in chloroplast movement and relocalizes to the Golgi. Also, a targeting for Chup1 to the secretory pathway and glycosylation was predicted (by prediction programs) and recent research discovered protein transit from the Golgi to the chloroplast (Villarejo et al. 2005).
An alternate localisation for Chup1 was, however, not observed under the presence of the secretion- and vacuolar protein transport inhibitor BFA (Figure 24). Targeting of Chup1 to the chloroplast via the secretory pathway can thus be excluded. This is also in line with the observation, that light-induced chloroplast movement was observed even in the presence of BFA (Kong et al. 2006). Chup1 is thus targeted to the chloroplast via the classical translocation pathway (e.g. Soll and Schleiff 2004) mediated by the N-terminal domain and not through vesicle transport.
An alternative pattern of Chup1 localization at the chloroplast envelope through different environmental stimulus, however, is conceivable (Figure 22 A).

Chup1 and actin both interact with the outer envelope

Chup1 localization to the chloroplast has been confirmed by GFP studies (Figure 21). A more accurate experiment was needed, to determine the localization of the protein and to exclude localization in the inner envelope or the inter-membrane space, which can not be differentiated by GFP fluorescence from localization at the outer envelope. As expected, Chup1 as well as Chup1-NT indeed bind to isolated outer envelope from chloroplasts (Figure 25). This is also again evidence for the N-terminus functioning as a membrane anchor for Chup1. Furthermore, actin isolated from pea was co-precipitated with the outer envelope (Figure 28). This interaction was protease sensitive (Figure 28, lane 6), and is thus dependent on a proteinaceous component residing in the outer envelope, which was not removed by the isolation process for the outer envelope and is thus an integral membrane protein on the outer periphery.
Some time after the experiments for this study were conducted, Kumatani et al. (2006) could show the interaction of F-actin with chloroplasts from spinach. This experiment nicely confirms the results shown here, that actin interacts with the chloroplast. In addition to this, it

is now clear that the chloroplast is able to bind G-actin (Figure 31) as well as F-actin, which adds up to our knowledge.

In a time scale experiment a fast interaction of actin with the outer envelope was demonstrated (Figure 29). The quick interaction points to a high affinity actin binding site in Chup1. The actinin-like actin binding domain that is modified a trifle in Chup1 might therefore still be able to mediate efficient binding. An exact relation of the proposed actin binding site to the actin binding function and exclusion of further sequence motif influences have to be tested in future. A rapid binding of actin to the chloroplast is essential for chloroplast movement - if a timely reaction to environmental stimulus is considered a precondition. In line, a light sensitive rapid rearrangement of the actin cytoskeleton in preparation for chloroplast movement at a time scale of 10 min was observed by Sakurai et al. (2005).

To a small extent, endogenous actin was found to be present in outer envelope fractions (Figure 28). In the chloroplast preparations from Kumatani et al. 2006, no actin was found to be present any more on the chloroplasts after the isolation. Explanations for this might either be a more sensitive detection in this study, a different isolation protocol and spinach/pea diversities or different environmental conditions (e.g. light conditions) that favour anchoring to actin.

Chup1 interacts with G- and F-actin

As both actin and Chup1 can bind to the outer envelope it was logical to assume that Chup1 interacts with actin at the chloroplast by means of its actin binding domain. Already Oikawa et al. (2003) detected the actinin-like actin binding domain in Chup1 (see chapter 4.4.2.2). In the work of his group, the actin binding domain of Chup1 was expressed with a GST-tag and co-immunoprecipitated with F-actin. In this work the aim was now to assay the properties of the native full length protein in the interaction with actin. Indeed, Chup1 was found to be co-sedimented with F-actin (Figure 30, lane 4). An interaction of Chup1 and F-actin can thus take place under the given conditions *in-vitro*. Even more, as Chup1 does not induce sedimentation of G-actin (Figure 30, lane 8), a direct function of Chup1 in polymerization of actin can be excluded. In a co-immunoprecipitation experiment, Chup1 was found to interact also with G-actin (Figure 31). Interestingly, Chup1 thus is able to interact with both G- and F-actin (Figure 30, Figure 31). As stated before, dual binding of actin was also found for the proteinaceous component on the chloroplast that interacts with actin. Only a few actin binding proteins have been reported to bind G- as well as F-actin, such as for instance gelsolin and

calponin (Ferjani et al. 2006). The ability to bind both G- and F-actin can bestow regulating properties. This was proposed for calponin, where a turnover of the actin cytoskeleton is thought to be sensed by calponin which as a result could perform a signalling function (Ferjani et al. 2006).

The conclusion can now be made, that Chup1 is the interaction partner of actin present in the outer envelope (Figure 21, Figure 25, Figure 30, Figure 31) and thus the true "missing link" that ties chloroplasts to the actin cytoskeleton and is responsible for chloroplast movement. As this is indeed very conceivable, the binding of Chup1 to G- and F-actin would imply now a regulatory function for Chup1. It is imaginable, that Chup1 is bound to G-actin (and maybe also functions as monomeric actin storage pool) when a temporary release from the F-actin filaments is necessary upon movement, and that a tethering to F-actin filaments is favoured under conditions when no movement action is required. In this case the F-actin binding ability of Chup1 may be decisive.
A binding of Chup1 to G-actin could be initiated by increasing amounts of G-actin in the surrounding medium by actin sequestering proteins and thus lead to a release of the chloroplast from actin filaments by a change in binding of Chup1 to G-actin rather than F-actin. (The anchoring of the chloroplast would thus be abolished, but a re-binding to the filament could set in at a later moment, when the (stronger) actin tracks for movement have been re-built). Under which conditions an interaction of Chup1 with G-actin or F-actin is favoured has to be elucidated in future studies. Apart from competition in binding, also a change in light conditions, in interaction with different modulator proteins/second messengers or in ionic conditions in the medium is conceivable.

Discovery of the Movement Complex?

For interaction studies, functional profilin could be purified from pea leaf tissue. The binding properties of profilin were retained, as was determined by tryptophan quenching in complex with actin (Figure 33).
An interaction of Chup1 and profilin could be proven by different binding experiments (Figure 34 and Figure 36). The involvement of an interaction domain present in the C-terminal part of Chup1 was shown with recombinantly expressed Chup1-CT (Figure 36). The interaction site of profilin and Chup1 has thus to be located in the C-terminal part of Chup1. This is strong evidence for the PRM1 motif to act as the binding domain for profilin.

It remains to be unravelled by site-directed mutagenesis, whether the conserved proline structure "positive-PPP-positive" in the Chup1 orthologs is also important for mediating interaction (see 4.4.2.3).

In co-expression experiments with ΔN-Chup1-GFP and profilin-RFP, a striking observation was made (Figure 35 B, C). Profilin-RFP is targeted to the sites of ΔN-Chup1-GFP agglomeration in the cell. Such a pattern of localisation is not observed when profilin-RFP is expressed without presence of ΔN-Chup1-GFP. In this case, profilin is rather evenly distributed in the protoplast (Figure 35 E). This observation can be interpreted as an interaction of Chup1 and profilin in a cellular environment. An in-vivo interaction of profilin and ΔN-Chup1 in the cell thus is likely. It can not be determined, however, whether ΔN-Chup1-GFP is in an active and properly folded state. An unspecific co-aggregation of profilin-RFP with aggregated ΔN-Chup1 can thus not be entirely excluded, but is unlikely in the light of the observed Chup1-profilin interaction in the previously shown experiments.

Still more evidence for the interaction of Chup1 and profilin was gained from fluorescence experiments with dansyl-profilin. The interaction of Chup1-CT and profilin could be confirmed. An increase in fluorescence was observed when Chup1-CT was mixed with dansyl-profilin (Figure 37 A); this increase was of a comparable level as the increase observed with actin and dansyl-profilin. This leads to the assumption that the binding efficiency of Chup1 to profilin also is at a comparable level. The interaction of Chup1-CT and dansyl-profilin was demonstrated to be concentration-dependent (Figure 37 B).
Furthermore, a concentration dependent increase in fluorescence was observed, when the fluorescence of Chup1 profilin and actin corrected for the fluorescence of Chup1 and profilin was determined (Figure 37 C).

From these results it can be concluded, that Chup1-CT, actin and profilin can interact as a trimeric complex. Only if Chup1 interacts with a dansyl-profilin-actin complex an increase of fluorescence is conceivable, as profilin interacts with actin in a 1:1 complex.
As it is assumed, that the C-terminus of Chup1 is not directly interacting with actin (as the actin binding domain is not present in Chup1-CT) an alternating complex between Chup1-profilin and Chup1-actin would in any event be unlikely. Thus, as a competition in binding would not take place, a trimeric complex is the logical conclusion. In the given case, in the

trimeric complex, actin is interacting with Chup1-CT through profilin. The results suggest that the trimeric complex is more stable than the individual binary complexes.
A constellation with Chup1 binding both actin and profilin singularly with the respective binding domains is not tested by this procedure. It is nonetheless likely and conceivable in the context of chloroplast anchoring through Chup1 as discussed beforehand, and might have high relevance *in vivo*. This other trimeric complex thus awaits still its experimental proof.

Is the discovered trimeric complex of Chup1, actin and profilin now acting as the sought after "movement complex" at the chloroplast outer envelope? This complex would at least have all the required functions necessary to initiate movement. In this scenario, Chup1, situated at the outer envelope, functions as the membrane anchor with its hydrophobic domain and interacts with G- and F-actin. The modulating action of polymerizing and depolymerizing actin filaments is done by profilin, which can be recruited to the chloroplast by Chup1 (due to its PRM1 motif). Profilin in complex with actin (profilactin) can deliver actin monomers to the vicinity of the chloroplast, where polymerization action is needed.
As profilin is likely to play a key part in chloroplast movement, our attention should be directed to profilin in the next section.

Profilin, in fact, is a key regulator of the actin cytoskeleton (Theriot and Mitchison 1993, e.g. Sohn and Goldschmidt-Clermont 1994, Staiger et al. 1997). Profilin's regulation of actin and its involvement in signalling to the cytoskeleton and regulatory functions in the cell is a well researched on topic. This is due to the fact that profilin is an abundant multipotent protein that has a multitude of interactors and serves as a hub to control complex interaction networks and whose versatile activities are essential for cell viability (e.g. Sohn and Goldschmidt-Clermont 1994, Witke 2004, Yarmola and Bubb 2006). A cue to the importance of profilin in the cell is seen by its sheer abundance in the cell as profilin content can reach up to an amount of 0.3% of total protein in *Arabidopsis* leaves (Chaudhry et al. 2007). Plants have large multigene families encoding profilins, and different tissues or cells can express multiple profilin isoforms. In *Arabidopsis* five profilin isoforms are encoded: constitutive profilin (PRF1-3) expressed in leaf tissues (Jeong et al. 2006) and pollen specific profilin (PRF4, 5, Kandasamy et al. 2002). The profilin isoform to interact with Chup1 in chloroplast movement has to be identified in future.
Importantly, the interaction of profilin with proline-rich proteins is thought to direct profilin to sites of rapid actin assembly and is involved in regulating profilin activity (e.g. Gibbon et

al. 1998, Paavilainen et al. 2004). This now places Chup1 again in a more central role in the regulation of chloroplast movement.

Profilin has to be considered a global player for many actin-filament dependent cellular functions including the positioning of chloroplasts. Adding to profilin's role as an important factor in chloroplast movement, profilin action could be regulated by signalling pathways, similar to the ones discovered in mammals, which will be discussed in the following chapter. But still, a quest for other components of the "movement complex" is desirable.

Chup1 and profilin and the network of movement regulation

To fit Chup1 and profilin in to the regulatory network, the signalling to the chloroplast has to be understood. On the basis of the experimental evidence gained from this study and evidence from literature, a model for a signalling cascade for light induced chloroplast movement was composed (Figure 38), which was published in Schmidt von Braun and Schleiff (2007). A lot of information has been gained in the last few years about the mechanism of light perception of the phototropins. What is not understood today is the signalling cascade downstream of the phototropin activation. But from the participating proteins and related signal cascades a conclusive model can be obtained (Figure 38).

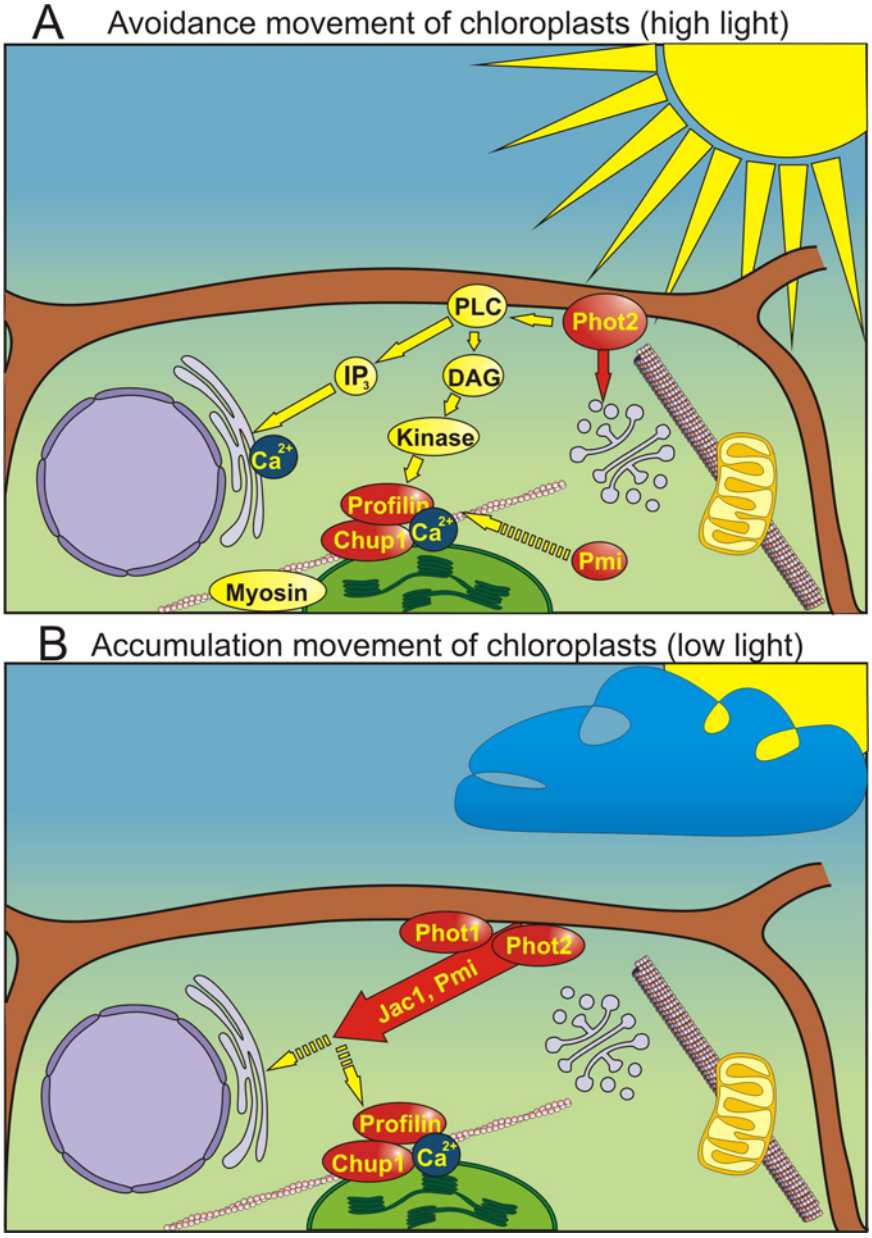

Figure 38 The regulation of chloroplast movement Shown is a model for the signalling cascade initiating the avoidance movement of chloroplast under high fluence rates of light (A) and the accumulation movement of chloroplasts under low fluence rates of light (B). Proteins depicted in red were identified to be involved in chloroplast movement, whereas components in yellow are hypothesized to be present. The release of calcium ions is marked in blue. Arrows give the direction of the signal cascade. Dashed arrows either indicate unsecured participations or directions. Further details and discussion are given in the text. From Schmidt von Braun and Schleiff 2007

Phototropins undergo autophosphorylation upon light activation (see 2.2.1). It has been supposed that other targets can be phosphorylated by the phototropins. A likely candidate is phospholipase C (PLC), as it has been shown that in the presence of PLC inhibitors calcium flux in reaction to blue light is reduced (Harada et al. 2003).

Therefore, a signalling cascade for the avoidance movement can be envisioned emanating from PLC and leading to the phosphoinositide pathway which is well described in mammalian

systems (e.g. Sohn and Goldschmidt-Clermont 1994). The plant counterpart PLC-II could take this part when activated by phosphorylation through Phot2. Mammalian PLC can hydrolyse phosphatidylinositol(4,5)bisphosphate (PIP2) to produce inositol 1,4,5-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). IP3 is known to bind to receptors on intracellular calcium stores and trigger a calcium release (e.g. reviewed in Xia and Yang 2005, Mikoshiba 2007). A calcium increase is known to occur in plants activated by blue-light. This calcium increase is supposed to be a crucial signal for chloroplast movement.

Support for this model also comes from two other observations. The first observation is that plant PLC-II in turn can also be activated by calcium (Drøbak et al. 1994) which could be a feed back regulation for chloroplast movement. In another observation, an inhibitor of phosphoinositide-3-kinases (wortmannin) was able to inhibit chloroplast responses to blue light in Lemna triscula (Grabalska and Malec 2004).

When following the cascade further, an interesting relation to profilin can now be considered. In mammals, DAG activates protein kinases like protein kinase C, which in turn can phosphorylate profilin (Guillen et al. 1999, Vemuri and Singh 2001). Furthermore, profilin is known to interact with PLC and can thus protect PIP2 from hydrolysis (Drøbak et al. 1994). In plants also an interaction of profilin with multiple molecules of PIP2 was observed (Drøbak et al. 1994). Thus a “back-loop” regulation of the signal cascade by profilin is possible. Phosphorylation of profilin was shown to have no effect on Chup1 binding (Figure 34 B), but it might well influence regulatory properties of profilin with respect to the signal cascade. It was shown for instance in *Phaseolus vulgaris*, that phosphorylation of profilin in the binding region for poly-L-proline inhibits binding to phosphoinositide 3-kinase (Aparicio-Fabre et al. 2006).

However, in plants no real homologue to protein kinase C is identified yet (but might be soon, compare 4.1.1.2). Hence, phosphatidic acid derived from DAG might be the important second messenger by activation of for instance MAP kinases (e.g. Laxalt and Munnik 2002, Wang et al. 2004). This would be a plausible option, as the phosphorylation of plant profilin by a MAP kinase was reported (Limmongkon et al. 2004). This closes the signal cascade from Phot2 to profilin. The mode of action and regulation of the Pmi proteins in this cascade has to be illuminated in future, as little is known about them yet.

Another aspect to consider is the fact that Phot2 relocalizes to the Golgi apparatus (Kong et al. 2006) and possibly also to the chloroplast (Harada and Shimazaki 2007; Weber, Düsseldorf personal communication). IP3 induced calcium release from the Golgi was reported (Pinton et al. 1998). Thus, Phot2 could also directly be involved in the regulation of calcium flux from the Golgi or even from the chloroplast which could induce specific calcium patterns. It is tempting to speculate that the chloroplast itself takes part in regulating the movement An involvement of the chloroplast in signalling has been suggested based on microbeam observations, where single chloroplasts are able to specifically move in reaction to the beam although the signal for the avoidance response can not be transported long-distance (Wada et al. 2003). A direct participation in signalling could be assigned due to the fact that chloroplasts exhibit a large stromal calcium spike at the transition from light to dark, induced by the release of calcium from the thylakoid lumen and subsequently to the cytosol (Sai et al. 2002). Additionally, it was observed that DCMU, an inhibitor of photosynthesis, reduced the motility of plastids in Vallisneria gigantea (Dong et al. 1996) and suppressed the rearrangement of actin filaments (Sakai et al. 2005) upon light treatment. This speaks for a signal released from chloroplasts that causes actin filament rearrangement.

Further support for the model comes from a Yeast Two Hybrid analysis conducted with Chup1 as bait (see Suppl. Table 1). A phosphatidylinositol phosphatase was found to interact with Chup1 and poses a direct link to the signal cascade. The same holds true for the calmodulin-binding protein found in the screen. This could mark another contact point for calcium to influence the activity of the proteins of the movement complex besides the above discussed interactions. Additionally, a putative myosin heavy chain was found in the screen, which can be an important clue for the detection of the molecular motor involved in chloroplast movement. Evidence of myosins involved in chloroplast movement is strong (see 2.4). Furthermore, a clathrin-binding γ-adaptin was found in the screen, which functions in vesicle-mediated transport from the Golgi or plasma membrane (Schledzewski et al. 1999). This is interesting with respect to Jac1 which possesses a domain possibly also involved in clathrin binding (see 2.5.2).

The regulation of the accumulation movement is even less understood (Figure 38 B). Parts of the puzzle that could be stuck together for the avoidance movement are missing for the low-light event. In accumulation movement, Phot1 and Jac1 seem to be major players as their mutants display the most severe defects here.

Profilin and the regulation of chloroplast movement

Profilin emerges now to probably be a potent key-regulator in chloroplast movement. Profilin is a well known modulator of the actin cytoskeleton, as it is able to induce polymerisation and depolymerisation (Yarmola and Bubb 2006). Profilin even is considered the major link through which the actin cytoskeleton can communicate with signalling pathways (Sohn and Goldschmidt-Clermont 1994), which has also been implicated for plant systems (Drøbak et al. 1994, Guillen et al. 1999). Profilin can recruit actin to sites of actin polymerisation with the help of proline-rich proteins (Paavilainen et al. 2004). In chloroplast movement, this of course would be the role of the proline-rich protein Chup1. The profilactin complex would be recruited to the chloroplast to initiate actin polymerisation to prepare the tracks for movement or for anchoring the chloroplast. Interestingly, the movement of Listeria (see 2.4) also depends on a profilin dependent polymerisation of actin for movement (Geese et al. 2000).

The interesting part is that also actin depolymerisation can be regulated by profilin. Depending on the activities of effectors or the presence of actin capping proteins, profilin is able to rapidly sequester actin filaments. In fact profilin was initially only realized to be an actin sequestering protein (Carlsson et al. 1977, Pollard and Cooper 1984).

The initiation of movement involves the sequestering of the actin baskets that anchor the chloroplasts to allow unhindered movement. A dual role for profilin in chloroplast movement in building up new filaments or sequestering filaments would involve precise regulation. Several modes of profilin regulation have been reported. The activity of profilin can be regulated by phosphorylation, through proline-rich proteins, phosphoinositides and calcium concentration. The sequestering activity of profilin has been shown previously to be dependent on calcium concentration (Kovar et al. 2000), which would be in line with the calcium dependence of chloroplast movement. The actin cytoskeleton status is also sensitive to calcium concentrations (Reddy 2001, Wasteneys and Galway 2003)

To summarize, a dual activity of profilin in regulating chloroplast movement would be the most elegant way (Figure 39). Dependent on its phosphorylation status, the calcium concentration or regulation of activity through protein ligands, profilin could act in polymerisation or depolymerisation of actin. A role of different profilin isoforms (Kovar et al. 2000) has to be considered as well.

Much speculation is still involved in these models, but future studies may be able to orient on it to prove matters.

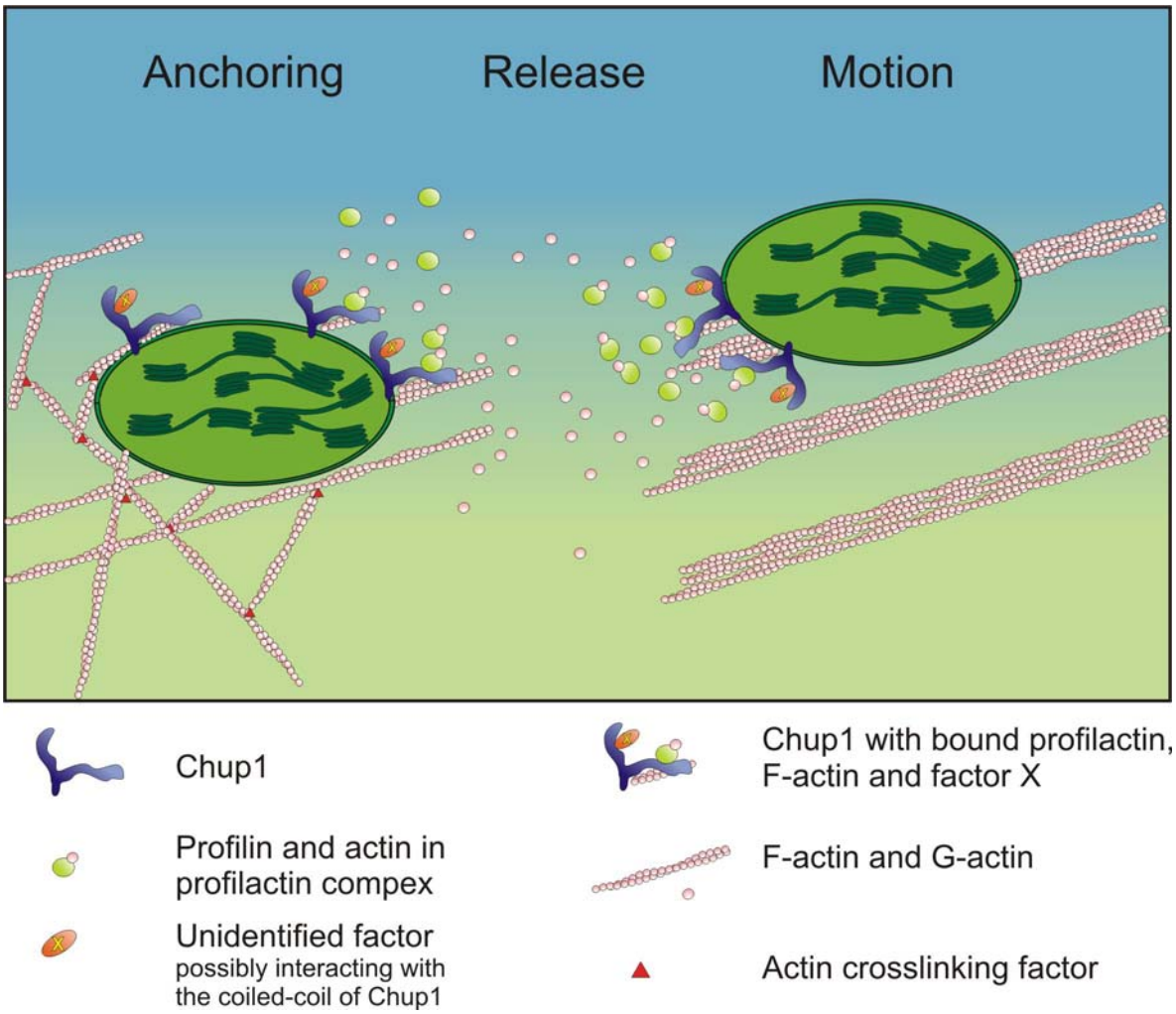

Figure 39 Model for Chloroplast movement involving the interaction of Chup1 profilin and actin. The model depicts the action of Chup1 at the surface of the chloroplast, recruiting profilin to initiate depolymerisation of actin baskets to release the chloroplast from the actin anchor. After the release, Chup1 recruits profilactin to initiate polymerisation of actin bundles which provide the tracks for the movement of the chloroplast to its destination. In this model, the process is regulated by the signal cascade described in the text and by additional unidentified factors interacting with the coiled-coil domain of Chup1.

5.1 Future perspectives

For the future, it will be exiting to explore the interaction of Chup1 to profilin more specifically. Is there for instance sensitivity in the binding activity to calcium concentration? Where exactly does the signal cascade have its point of contact, is it the interaction of profilin with different factors (possibly dependent on phosphorylation) or the regulation of Chup1 or actin? And to this respect, are there further factors interacting with Chup1, possibly through the coiled-coil domain? A major task will be to unravel the interplay of the other factors (Jac, Pmi), involved in chloroplast movement, with the signal cascade. The signal pathway itself has to be experimentally addressed. Furthermore the identification of the myosin that is involved in chloroplast accumulation will be important, as well as the nature of force

generation through actin in the avoidance movement. And eventually, the identification of the role of the chloroplast itself in movement, taking part in the regulation of movement possibly through calcium efflux, will be fascinating to discover.

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Curriculum Vitae

Name:	Serena Schmidt von Braun, née Steinhübl
Date of birth:	24.10.1977 in Munich
Nationality:	German

Education

09/88 - 07/97	Gymnasium Weilheim Mathematics and Science Branch, graduated with Abitur

University

11/97 - 07/98	Study of Chemistry at the Technical University Munich
11/98 - 10/03	Study of Biology at the Ludwig Maximilians University Munich, Diploma degree 10/2003
01/03 - 10/03	Diploma thesis at GSF Neuherberg, division radiation biology and DNA repair
01/04 - now	Ph. D. at the LMU Munich Department I Biochemistry and Plant Physiology

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Ehrenwörtliche Versicherung

Die vorliegende Dissertation wurde von der Verfasserin selbständig und ohne unerlaubte Hilfe angefertigt. Die Verfasserin hat zuvor nicht versucht, anderweitig eine Dissertation einzureichen oder sich einer Doktorprüfung zu unterziehen. Die Dissertation wurde keiner weiteren Prüfungskommission weder in Teilen noch als Ganzes vorgelegt.

Wessobrunn, den 08.05.08 Serena Schmidt von Braun

Abbreviations

aa	amino acid
Al	Aluminium
ATP	adenosine-5'-triphosphate
bp	base pair
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
Chup1	chloroplast unusual positioning1
CnBr	cyanogen bromide
CT	carboxy terminus
DAG	1,2-diacylglycerol
DCMU	dichlorophenyl dimethylurea
DMSO	dimethyl sulfoxyde
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
GFP	green fluorecent protein
His	histidine
IP3	inositol 1,4,5-trisphosphate
IPTG	isopropylthiogalactoside
Jac1	J domain protein required for chloroplast accumulation response
kD	kilo Dalton
MAP kinase	mitogen-activated protein kinase
µmol	measure of fluence rate of light in mols of photons, short for µmol $m^{-1}s^{-1}$
Met	methionine
MS	Murashige and Skoog
NBT	nitroblue tetrazolium, 4-nitrotetrazoliumchlorid-blue-hydrate
NT	amino terminus
OD	optical density
PCR	polymerase chain reaction
Phot	Phototropin
pI	isoelectric point
PIP2	phosphatidylinositol(4,5)bisphosphate
PLC	phospholipase C
PLP	poly-L-proline
Pmi	plastid movement impaired
PMSF	phenylmethane sulfonyl fluoride
LOV	light, oxygen or voltage
RACE	rapid amplification of cDNA ends
RFP	red fluorecent protein
ROS	reactive oxygen species
SDS PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
WT	wild type
YTH	Yeast Two Hybrid

Supplements

DNA Sequence of *Pisum sativum* Chup1 discovered by RACE

ATGATAGTCAGGTTAGGACTCATTGTTGCTGCTTCATTAGCAGCTTTTACAGTTAAGCAGCTCAATC
TTGGAAACTCTAAATCAGATCATGGTCAAGAAAGGTCTCAAAAGCATCAAGACGAAGACACCGAA
CAAGAGCAGGTCACTAGTATTACAGATGATTCTCATCATCAAAGGAATGATACTGAGGAGGAAGA
AGAGGAGAAAGAGGAGGTCAAGTTAATTAGCAGCATAATTAATAGAGCTAATAATTTCGAAGATG
ATGATATTCTACCAGAATTTGAAGACCTTTTATCCGGAGAGATTGAGTTATCATTTCCTAGCGATGA
TAATAAGGATGAGAAAGAAAGAGTTTATGAGATAGAGATGGCATACAATGACAGCGAGTTAGAAC
GACTGCGGCAGCTAGTGAAGGAATTGGAGGAAAGGGAAGTGAAACTTGAAGGAGAATTGCTTGAG
TACTATGGTTTAAAGGAGCAGGAATCAGACATTGTAGAGTTACAAAGGCAGCTGAAAATTAAGAC
GGTGGAAATAGATATGCTTAATATTACGATTAACTCGTTACAGGCCGAGAGGAAGAAGCTTCAAG
AAGAACTCACAAATGGAGCTTCAGCAAAGAGAGATCTTGAGTTGGCTAGAAACAAGATAAAGGAG
CTACAAAGGCAAATGCAGCTTGAGGCTAACCAAACAAAAGGCCAACTTTTGTTGCTTAAACAGCA
AGTTTCTGGTCTACAGGTGAAAGAAGAAGTGGGTGCCAGAAATGATGCTGAGATTGAAAAGAAAT
TGAAAGCTGTGAATGACTTAGAGGTTAATGTTGTGGAGCTTAGGAGGAAAAATAAAGAACTTCAG
TACGAGAAGCGAGAGTTAACTGTTAAACTCAATGCTGCTGAATCTAGAGTAGCAGAGCTCTCCAAC
ATGACAGAGAGTGAAATGGTTGCCAAGGTCAAAGAGGAGGTCAGCAACCGAGAGACACGCAAAG
AATCAAAGCAAGTGGAAGGACTTCAAATGAATAGGTTTAGTGAAGTGGAAGAGCTCGTATACCTT
CGTTGGGTCAATGCATGTTTGAGGTATGAGCTAAAGAATCAGCAGGCACCCTCGGGAAAATATCGG
CGCGCGACCTCAGCAAGAACCTTAGCCCAATCACAAGCGAGAGCAAAGCAGCTGATGTTAGAATA
CGCTGGATCGGAACGAGGTCAAGGGGACACAGATCTCGATAGCAATTTCTCTCATCCCTCTTCACC
AGGAAGTGAAGATTTTGACAATGCTTCTATTGATAGCTTTAGTAGCAAATATAGTAGTATTAGCAA
GAAAACTAGCATAATCCAAAAATTGAAGAAATGGGGCAAACTCAAAGATGATTCTAGTGCTCTTTC
ATCACCATCAAGATCATTTTCAGGAAGTTCTCCAAAAAGGATGAGTATGAGTGTTAAATCTAGGGG
TCCACTCGAAAGCTTGATGATAAGGAATGCCAGTGATAGTGTGGCCATCACCACCTTTGGTCAAGG
GGATCTAGAATCTTCTTATTCTCCTGAAACTTCAACTCCTGCTAGTGCTGATCTTAGAAAAATCCCA
TCTACCGACTCACTAAATTCTGTTGCTACTTCATTCCATTTGATGTCCAAGTCATCTGTTGATGCGTC
TGTGGACGAAAAGTACCCTGCATATAAATATCGCCATAAATTGGCCATGGCTAGAGAGAGTGATCT
AAAAGATAAGGCGGAGAAAGCAAGAGTGCAGAAGTTTGGTAATCATTCAAATTTGAATATGATCA
AGACTGAAAGAGAGAGGCCTCATATATCTTTGCCACCTAAACTTTCTCAAATAAAGGAGAAGCCAA
TTGTTCCTGATAGTCCAAATGACCAATCTGAGGATGGAAAGAATGTTGAAAACCAAAACATTAGCA
AGATGAAGCTTGCCGACATTGAGAAAAGGCCTACTCGGGTGCCTAGGCCGCCTCCTAAACCATCAG
GTGGTGGTTCTGTTAGCACAAATTCAAATCCTGCGAATGGAATACCATCTGCTCCATCCATTCCTCC
TCCCCCTCCTCGTCCACCAGGAGGACCGCCTCCTCCACCTCCTCCACCAAGAGGTCTATCAAGAGG
GGCAATGGATGACGACAAAGTTCACCGAGCTCCACAGTTAGTTGAGTTTTATCAGTCATTGATGAA
ACGGGAGGCAAAGAAGGATACTACTCCGTTACTAGTCTCTTCAACCGGTAACGCATCTGATGCCAG
AAGCAACATGATTGGGGAAATTGAGAATAGATCAACATTCCTCTTAGCAGTGAAAGCTGATGTAG
AAACACAAGGTGATTTTGTCACATCCTTGGCAACTGAAGTTAGAGCATCCTCCTTTTCAGATGTCAA
TGACTTGGTTGCCTTTGTGAACTGGCTAGATGAAGAACTTTCCTTCTTGGTTGATGAACGAGCTGTC
CTGAAGCACTTTGATTGGCCTGAGGGGAAAGCAGATGCACTAAGGGAAGCAGCTTTTGAATATCA
AGATCTTATGAAATTGGAGAAGCAAGTCTCTACCTTCATTGATGATCCAAAGCTCTCGTGTGATGCT
GCTCTCAAGAAAATGTATTCCTTGCTTGAAAAAGTAGAGCAAAGCGTATATGCACTGTTGCGAACA
AGAGATATGGCTATTTCACGATACAAGGAATTCGGAATCCCAATAAACTGGCTACAAGATTCAGGA
GTTGTGGGCAAGATAAAGCTTTCTTCTGTACAACTAGCAAGGAAGTATATGAAACGTGTTGCATCT
GAACTTGATGCATTATCTGGACCTGAAAAGGAACCAGCTAGAGAGTTCTTGATTCTGCAAGGCGTG
CGTTTTGCTTTCCGCGTCCATCAGTTTGCAGGAGGCTTTGACGCAGAGAGCATGAAGGCTTTCGAA
GACCTAAGGAGCCGCATCCAAACCCCTCAAATTGGTGGAGAAGAGAGTAAACCAGAATCATAG

Amino Acid sequence of *Pisum sativum* Chup1

MIVRLGLIVAASLAAFTVKQLNLGNSKSDHGQERSQKHQDEDTEQEQVTSITDDSHHQRNDTEEEEEEK
EEVKLISSIINRANNFEDDDILPEFEDLLSGEIELSFPSDDNKDEKERVYEIEMAYNDSELERLRQLVKELE
EREVKLEGELLEYYGLKEQESDIVELQRQLKIKTVEIDMLNITINSLQAERKKLQEELTNGASAKRDLEL
ARNKIKELQRQMQLEANQTKGQLLLLKQQVSGLQVKEEVGARNDAEIEKKLKAVNDLEVNVVELRRK
NKELQYEKRELTVKLNAAESRVAELSNMTESEMVAKVKEEVSNRETRKESKQVEGLQMNRFSEVEEL
VYLRWVNACLRYELKNQQAPSGKYRRATSARTLAQSQARAKQLMLEYAGSERGQGDTDLDSNFSHPS
SPGSEDFDNASIDSFSSKYSSISKKTSIIQKLKKWGKLKDDSSALSSPSRSFSGSSPKRMSMSVKSRGPLES
LMIRNASDSVAITTFGQGDLESSYSPETSTPASADLRKIPSTDSLNSVATSFHLMSKSSVDASVDEKYPAY

KYRHKLAMARESDLKDKAEKARVQKFGNHSNLNMIKTERERPHISLPPKLSQIKEKPIVPDSPNDQSED
GKNVENQNISKMKLADIEKRPTRVPRPPPKPSGGGSVSTNSNPANGIPSAPSIPPPPPRPPGGPPPPPPPPRG
LSRGAMDDDKVHRAPQLVEFYQSLMKREAKKDTTPLLVSSTGNASDARSNMIGEIENRSTFLLAVKAD
VETQGDFVTSLATEVRASSFSDVNDLVAFVNWLDEELSFLVDERAVLKHFDWPEGKADALREAAFEY
QDLMKLEKQVSTFIDDPKLSCDAALKKMYSLLEKVEQSVYALLRTRDMAISRYKEFGIPINWLQDSGV
VGKIKLSSVQLARKYMKRVASELDALSGPEKEPAREFLILQGVRFAFRVHQFAGGFDAESMKAFEDLRS
RIQTPQIGGEESKPES

Supporting Table 1 Yeast Two Hybrid analysis with Chup1 as bait.

Yeast two hybrid screen results with Chup1 as bait. Given is the AGI number of the gene and the name of the protein and/or function. Yeast Two Hybrid Analysis was performed by Hybrigenics (Paris, France) with a *CHUP1* template provided in the pOL GFP vector as bait.

AT2G24270.1	ALDH11A3 aldehyde dehydrogenase/ oxidoreductase
AT5G13000	ATGSL12 1,3-beta-glucan synthase/ transferase, transferring glycosyl groups
AT5G23450	ATLCBK1 LONG-CHAIN BASE (LCB) KINASE 1, diacylglycerol kinase
AT5G35970	ATP binding / ATP-dependent helicase/ DNAbinding
AT4G20360	ATP binding / GTP binding / translation elongationfactor
AT2G29940	ATPase, coupled to transmembrane movement of substances
AT1G22190	AP2 domain containing transcription factor /DNA binding
AT3G04400	EMB2171 (EMBRYO DEFECTIVE 2171); structural constituent of ribosome
AT2G41430.1	ERD15 (EARLY RESPONSIVE TO DEHYDRATION 15)
AT4G25100.2	FSD1 iron superoxide dismutase
AT1G23900.2	GAMMA-ADAPTIN 1; clathrin binding
AT5G23120	HCF136 (high chlorophyll fluorescence 136) stability and/or assembly factor of photosystem II
AT4G13940.1	HOG1 HOMOLOGY-DEPENDENT GENE SILENCING 1; adenosylhomocysteinase
AT4G20380.2	LSD1 LESION SIMULATING DISEASE
AT3G51600	LTP5 LIPID TRANSFER PROTEIN 5 lipid transporter
AT4G24190.1	SHD SHEPHERD , ATP binding / unfolded protein binding
AT1G14610	TWN2 TWIN 2, ATP binding / tRNA ligase/ valine-tRNA ligase
AT3G15350.2	acetylglucosaminyltransferase
AT2G05710	aconitate hydratase, cytoplasmic, putative / citrate hydro-lyase/aconitase,putative
AT5G56360	calmodulinbinding, similar to protein kinase C substrate
AT1G31550.2	carboxylic ester hydrolase/ hydrolase, acting on ester bonds /lipase
AT5G48010	pentacyclic triterpene synthase, putative
AT4G16190	cysteine-type endopeptidase/ cysteine-type peptidase
AT3G46180	galactosetransporter
AT5G54390	AHL (HAL2-LIKE); 3'(2'),5'-bisphosphate nucleotidase/ inositol or phosphatidylinositol phosphatase
AT5G38410	ribulose-bisphosphatecarboxylase
AT3G61790	seven in absentia (SINA) familyprotein
AT3G47420	sugar porter/transporter
AT1G70090	transferase, transferring glycosyl groups / transferase, transferring hexosylgroups
AT5G18630.3	triacylglycerollipase
AT1G04820	tubulin alpha-2/alpha-4 chain (TUA4)
AT4G22500	unknown, similarity to glycine-rich cell wall protein precursor
AT2G07707	ATP synthase protein YMF19 (Mitochondrial protein YMF19)
AT2G32240	putative myosin heavy chain
AT1G79040	PSII-R (photosystem II subunit R)
AT1G16810.1	unknown protein
AT3G20380	meprin and TRAF homology domain-containing pr. / MATH domain-containing pr.
AT1G31330	PSI subunit III (PsaF, photosystem I subunit F)

Supporting Table 2. Genes regulated in wild type background upon illumination with blue light.

Given are the genes found to be categorized in the experiments using wild type. The first column gives the time of illumination with blue light in minutes (T), the second column the category of expression (C) as of Figure 7; the third column the AGI code of the gene, the fourth column the name of the protein, the fifth column indicates whether the genes is found to be higher (u) or lower (d) expressed in non treated plants in comparison to plants treated with 10µmol blue light (NL), the sixth column gives number of spots identified as positive by the new classification (SP); the seventh column the number of spots with signal intensities supporting the classification (NP); the eighth column gives the (putative) function of the encoded protein; the ninth column gives the (putative) localization; the tenth column gives a relevant reference.

T	C	AGI code	Name	NL	SN	NP	function	localization	Ref.
5	2	AT5G53450	-	-	11	9	protein kinase[a]	chloroplasts[a]	Kang et al., 2003
	4	AT1G12520	CCS1	-	9	7	copper chaperone for superoxide dismutase	chlorolast or secretion	Chu et al., 2005
		AT2G28190	CSD2	-	6	5	superoxide dismutase	chloroplast	Kliebenstein et al., 1998
30	2	AT1G36370	-	-	10	8	glycine hydroxymethyltransferase[a]	cytosolic[a]	-
		AT3G30720	-	-	10	8	unknown	unknown	-
		AT4G04610	atAPR1	-	11	10	phosphoadenosine phosphosulfate sulfotransferase [a]	chloroplasts[a]	Setya et al., 1996
	4	AT1G15100	RHA2A	-	11	9	Ring H2 finger protein [a]	secretion[a]	-
		AT1G17100	-	-	11	9	SOUL heme-binding protein [a]	secretion[a]	-
		AT1G68550	-	-	11	9	AP2 domain cont. transcription factor[a]	nucleus[a]	-
		AT1G76240	-	d	11	10	unknown	unknown	-
		AT2G44940	-	d	11	9	AP2 domain cont. transcription factor[a]	nucleus[a]	-
		AT3G44450	-	d	11	11	unknown	unknown	-
		AT3G56290	-	-	11	9	unknown	unknown	-
		AT5G45820	AtPKS18	d	11	10	SNF1-related protein kinase	cytosolic	Gong et al., 2003
	5	AT1G01560	atMPK11	u	11	10	mitogen-activated protein kinase 11	unknown	Hamel et al., 2006
		AT1G02660	-	u	11	9	putative triacylglycerol lipase[a]	unknown	-
		AT1G05575	-	u	11	10	unknown	unknown	-
		AT1G15010	-	u	11	9	unknown	unknown	-
		AT1G19380	-	u	11	10	unknown	unknown	-
		AT1G19770	atPUP14	u	11	9	purine permease 14	unknown	Gillissen et al., 2000
		AT1G21010	-	u	11	10	unknown	unknown	-
		AT1G22890	-	-	9	7	unknown	unknown	-

		AT1G25550	-	-	10	9	myb family transcription factor[a]	unknown	-
		AT1G27730	atZAT10	u	11	10	salt-tolerance zinc fingertranscription factor	nucleus	Rossel et al. 2007
		AT1G28330	atDRM1	-	9	7	dormancy-associated protein	unknown	Gonzali et al. 2006
		AT1G49500	-	-	11	9	unknown	secretion[a]	-
		AT1G52200	atPCR8	u	7	6	putative plant cadmium resistance protein	unknown	Song et al. 2004
		AT1G53170	atERF8	-	11	10	ERF transcription factor 8	nucleus[a]	Yang et al., 2005
		AT1G66090	-	u	11	11	Putative disease resistance protein	chloroplasts[a]	-
		AT1G69490	atNAP	-	11	10	NAC-type transcription factor	nucleus	Gou and Gan, 2006
		AT1G69890	-	u	10	8	unknown	unknown	-
		AT1G70290	atTPS8	-	11	9	rehalose-6-phosphate synthase 8	unknown	Leyman et al. 2001
		AT1G70740	-	u	11	11	putative receptor-like protein kinase[a]	unknown	-
		AT1G73500	atMKK9	-	11	9	MAP kinase kinase 9		Hamel et al., 2006
		AT1G74450	-	u	11	9	unknown	unknown	-
		AT1G74930	-	u	11	10	putative AP2 domain-containing transcription factor[a]	nucleus[a]	-
		AT1G76650	-	u	11	9	calcium-binding EF hand family protein	unknown	-
		AT2G15960	-	-	10	9	unknown	unknown	-
		AT2G18700	atTPS11	-	11	11	trehalose-phosphatase protein	unknown	Chary et al. 2008
		AT2G22500	atPUMP5	u	11	9	Mitochondrial uncoupling protein	mitochondria	Borecky et al., 2006
		AT2G22880	-	u	11	11	unknown	unknown	-
		AT2G24550	-	u	11	9	unknown	unknown	-
		AT2G24600	-	u	11	9	ankyrin repeat family protein[a]	unknown	-
		AT2G25735	-	u	11	10	unknown	unknown	-
		AT2G26190	-	u	10	8	calmodulin-binding family protein[a]	unknown	-
		AT2G26530	-	u	11	10	calmodulin-binding family protein[a]	unknown	-
		AT2G26560	atPLP2	u	9	7	lipid acyl hydrolase	unknown	La Camera et al., 2005
		AT2G27830	-	u	11	9	Putative pentatricopeptide (PPR) repeat-containing protein	unknown	-
		AT2G31880	-	u	11	10	putative receptor-like protein kinase[a]	secretion[a]	-
		AT2G35930	-	u	11	10	U-box domain-containing protein[a]	unknown	-
		AT2G38790	-	u	11	9	unknown	unknown	-
		AT2G40000	-	u	11	10	unknown	unknown	-
		AT2G40140	ZFAR1	u	11	10	zinc finger transcription factor	unknown	AbuQamar et al., 2006
		AT2G41100	atTCH3	u	11	9	environmental stimuli-responsive Ca2+ binding protein		Sistrunk et al., 1993

		AT2G44500	-	u	11	9	unknown	unknown	-
		AT3G04640	-	u	11	9	unknown	secretion[a]	-
		AT3G05200	ATL6	u	11	9	putative RING-H2 zinc finger protein[a]	secretion[a]	Salinas-Mondragón et al., 1999
		AT3G06070	-	-	10	10	unknown	unknown	-
		AT3G06500	-	u	9	7	putative beta-fructofuranosidase[a]	chloroplasts[a]	-
		AT3G07350	-	u	11	10	unknown	unknown	-
		AT3G10020	-	u	11	10	unknown	unknown	-
		AT3G10985	atSAG20	u	10	9	Wound induced protein 12	-	Miller et al., 1999
		AT3G15630	-	u	11	10	unknown	unknown	-
		AT3G19580	AZF2	u	11	11	zinc finger (C2H2 type) protein 2	nucleus	Sakamoto et al., 2004
		AT3G28340	-	-	11	9	putative galactinol synthase[a]	secretion[a]	-
		AT3G44260	-	-	11	10	Putative CCR4-NOT transcription complex protein[a]	unknown	-
		AT3G46620	-	u	11	10	RING-domain protein of unknown function[a]	unknown	-
		AT3G49530	ANAC062	u	9	8	Arabidopsis NAC domain containing protein 62	membrane-associated	Riechmann et al., 2000
		AT3G49940	LBD38	-	10	8	LOB domain protein 38	unknown	-
		AT3G50260	ATERF#011	u	11	9	AP2 domain-containing transcription factor[a]	nucleus	Riechmann et al., 2000
		AT3G52400	SYNTAXIN 122	-	10	8	vesicle transport syntaxin-type t-SNARE protein	plasma membrane	Sanderfoot et al., 2000
		AT3G55980	F27K19.160	u	11	11	zinc finger (CCCH-type) family protein	unknown	Riechmann et al. 2000
		AT3G57450	-	u	11	9	unknown	unknown	-
		AT3G61060	ATPP2-A13	-	10	9	similar to ATPP2-A12 (Phloem protein 2-A12)	unknown	Dinant et al., 2000
		AT3G62950	GRXC11	-	11	9	glutaredoxin family protein	endomembrane system	-
		AT4G02380	AtLEA5	-	9	7	late embryogenesis abundant like protein	unknown	Weaver et al., 1998
		AT4G05070	C17L7.2	-	11	9	similar to wound induced protein-like	chloroplast[a]	
		AT4G14365	-	u	7	6	zinc finger (C3HC4-type RING finger) family protein	unknown	-
		AT4G17230	SCL13	u	11	10	scarecrow-like protein	unknown	Bolle et al., 2000
		AT4G17490	AtERF6	u	11	11	ethylene-responsive element binding factor	nucleus	Riechmann et al., 1998
		AT4G17900	-	-	7	6	zinc-binding family protein	unknown	-
		AT4G18880	HSF21	u	11	9	heat shock transcription factor 21	unknown	Riechmann et al., 2000
		AT4G20860	-	u	11	9	FAD-binding domain-containing protein	endomembrane system	-
		AT4G23180	CRK10	u	10	9	receptor-like protein kinase	secretory[a]	Du et al., 2000
		AT4G23220	-	u	9	7	receptor-like protein kinase[a]	unknown	-

		AT4G24380	-	u	10	8	serine hydrolase[a]	unknown	-
		AT4G24570	AtDIC2	u	11	9	mitochondrial dicarboxylate carrier[a]	Mitochondria[a]	-
		AT4G27280	-	u	11	10	calcium-binding EF hand family protein	unknown	-
		AT4G28140	-	u	11	10	AP2 domain-containing transcription factor[a]	nucleus[a]	Riechmann et al., 2000
		AT4G29190	-	-	11	11	zinc finger (CCCH-type) family protein	unknown	Riechmann et al., 2000
		AT4G29780	-	u	11	11	unknown	unknown	-
		AT4G33050	EDA39	u	10	9	calmodulin binding	unknown	Pagnussat et al., 2005
		AT4G34150	-	u	10	8	C2 domain-containing protein	unknown	Kawamura et al., 2003
		AT4G35480	RHA3b	-	11	10	RING-H2 finger protein [a]	unknown	Jensen et al., 1998
		AT4G36040	J11	-	11	9	DNAJ heat shock N-terminal domain-containing protein	chloroplast[a]	-
		AT4G36500	-	-	10	10	unknown	mitochondria[a]	-
		AT4G37260	MYB73	-	10	10	myb DNA-binding protein	nucleus[a]	Kranz et al., 1998
		AT5G04340	C2H2	u	11	10	zinc finger (C2H2 type) protein[a]	unknown	Chrispeels et al., 2000
		AT5G06320	NHL3	u	11	10	harpin-responsive protein[a]	plasma membrane[a]	Varet et al., 2002
		AT5G06860	PGIP1	-	11	9	polygalacturonase inhibiting protein 1	secretory[a]	Kobe et al. 2001
		AT5G10695	-	u	11	9	unknown	unknown	-
		AT5G11070	-	u	11	10	unknown	unknown	-
		AT5G20230	BCB	u	10	10	blue copper-binding protein	secretory[a]	van Gysel et al., 1993
		AT5G22270	-	-	11	9	unknown	unknown	-
		AT5G24590	TIP	-	11	9	NAC-type transcription factor	nucleus[a]	Ren et al. 2000
		AT5G25440	-	u	7	6	receptor-like protein kinase	cytoplasm/nucleus[a]	-
		AT5G26920	-	u	11	9	calmodulin binding	unknow	-
		AT5G27420	-	u	11	9	zinc finger (C3HC4-type RING finger) family protein[a]	secretory[a]	-
		AT5G28770	BZO2H3	-	9	8	bZIP protein	nucleus[a]	Riechmann et al., 2000
		AT5G39580	-	u	9	7	peroxidase[a]	secretory[a]	-
		AT5G47070	-	u	10	9	receptor-like protein kinase[a]	unknown	-
		AT5G52050	-	u	11	11	MATE related efflux carrier[a]	membrane[a]	-
		AT5G57560	XTH22	u	11	9	xyloglucan endotransglucosylase-hydrolase	cell wall	Xu et al., 1995
		AT5G58430	EXO70B1	-	11	9	exocyst subunit EXO70[a]	exocyst[a]	-
		AT5G59080	-	-	9	8	unknown	chloroplast	-
		AT5G59550	-	-	11	10	zinc finger (C3HC4-type RING finger) family protein	unknown	-

		AT5G59820	RHL41	u	11	9	RESPONSIVE TO HIGH LIGHT 41	unknown	Riechmann et al., 2000
		AT5G60680	-	u	11	11	unknown	unknown	-
		AT5G66070	-	u	10	8	similar to zinc finger (C3HC4-type RING finger) family protein[a]	secretory[a]	-
	6	AT1G48630	-	-	10	8	activated protein kinase C receptor[a]	cytosolic[a]	-
	7	AT1G68840	RAV2	-	11	9	DNA-binding protein RAV2 (RAV2)	-	-
		AT2G20670	-	-	11	11	unknown	unknown	-
		AT4G37610	atBT5	u	11	10	transcription regulator BT5	nucleus	Du and Poovaiah, 2004
		AT5G19120	-	u	11	10	unknown	secretion[a]	-
	8	AT4G16780	atHB2	u	10	8	Homeobox-leucine zipper protein HAT4	nucleus[a]	Carabelli et al. 1993
	9	AT1G17380	-	u	10	9	unknown	unknown	-
		AT1G56600	atGOLS2	u	10	9	galactinol synthase 2	unknown	Taji et al. 2002
		AT1G61890	atDTX37	u	9	7	MATE efflux family protein[a]	unknown	Li et al., 2002
		AT2G34600	-	u	10	8	unknown	unknown	-
		AT3G25780	atAOC3	u	10	8	allene oxide cyclase 3	Plasma membrane	He et al., 2002; Dunkley et al., 2006
		AT4G27410	atRD26	u	11	11	NAC-type transcription factor	nucleus	Lee et al., 2006
		AT4G34410	-	u	11	9	putative AP2 domain-containing transcription factor	unknown	-

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