Untersuchungen zum Proteom des Transkriptionsaktiven Chromosoms aus Spinatchloroplasten

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Die unten aufgelistete Publikation und zwei eingereichte Manuskripte sind Bestandteile der Dissertation. Zusätzlicher Bestandteil ist ein noch nicht eingereichtes Manuskript. Im nachfolgenden Einführungstext wird durch römische Nummerierung auf diese Arbeiten verwiesen.

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Abkürzungen

ATP	Adenosin-5‘-triphosphat
E. coli	*Escherichia coli*
GFP/RFP	das grün/ rot fluoreszierende Protein
HLP	das histonähnliche Protein
HU	*heat unstable*
LC	Flüssigchromatographie
MFP1	*matrix attachment region-binding filament-like protein 1*
MS	Massenspektrometrie
PPR	*pentatricopeptide repeat*
PEND	*plastid envelope DNA-binding protein*
ptNAP	das plastidäre nukleoidassoziierte Protein
ptDNA	plastidäre DNA
SWI/SNF	*switching defective/ sucrose non fermenting*
SiR	Sulfitreduktase
TAC	Transkriptionsaktives Chromosom
TPR	*tetratricopeptide repeat*
Why1	das Whirly1-Protein

Zusammenfassung

Plastiden besitzen eigene DNA (ptDNA), die zusammen mit einer Vielzahl an Proteinen in Strukturen verpackt ist, die den bakteriellen Chromosomen ähneln. Diese Nukleoproteinkomplexe werden als plastidäre Nukleoide oder Plastidenkerne bezeichnet. Im Unterschied zum Chromatin im Zellkern gibt es nur wenige Informationen über die Organisation und Dynamik der Nukleoide in den Plastiden. Zur Identifizierung von Proteinen des Transkriptionsaktiven Chromosoms (TAC) aus Spinatchloroplasten wurden Proteomanalysen durchgeführt. In einer vorangegangenen Proteomanalyse mit einer TAC-Fraktion aus Senfchloroplasten wurde das DNA-Bindeprotein Whirly1 identifiziert. Mit dem spezifischen Antikörper konnte hier gezeigt werden, dass das Protein in der konventionell präparierten TAC-I-Fraktion, aber nicht in der hochaufgereinigten TAC-II-Fraktion von Gerstenchloroplasten vorkommt. Durch eine Proteomanalyse der hochaufgereinigten TAC-II-Fraktion konnten darüber hinaus sechs neue DNA-Bindeproteine identifiziert werden. Sie wurden als plastidäre nukleoidassoziierte Proteine (ptNAP) benannt. Eines der neu identifizierten ptNAP ist das Protein AtSWIB-1 (*Arabidopsis thaliana SWIB domain-containing protein-1*). Das Protein ist in der TAC-II-Fraktion angereichert und bindet an plastidäre DNA. Dies deutet darauf hin, dass das SWIB-1-Protein im Unterschied zum Whirly1-Protein eine integrale Komponente des TAC ist. Durch Fusionen mit dem GFP-Protein konnte eine duale Lokalisation des AtSWIB-1-Proteins im Zellkern und in den Nukleoiden der Plastiden gezeigt werden. Datenbankrecherchen erlaubten die Identifizierung von drei weiteren SWIB-Domänenproteinen von *Arabidopsis thaliana,* deren vorhergesagte plastidäre Lokalisation durch GFP-Fusionen bestätigt werden konnte. Zusammen mit SWIB-1 gehören sie zu den ersten Chromatinmodellierungsfaktoren, die außerhalb des Kerns vorkommen. Aufgrund der Ergebnisse dieser Arbeit wird die Hypothese aufgestellt, dass Plastiden Chromatinmodellierungsproteine besitzen, die über Veränderungen in der Struktur der Nukleoide die Genexpression beeinflussen können.

Summary

Chloroplasts possess their own DNA (ptDNA), which is packaged with proteins into structures analogous to bacterial chromosomes, termed nucleoids or plastid nuclei. In contrast to nuclear chromatin, there is only limited information on the organization and dynamics of the plastid nucleoids. In order to investigate the protein composition of the transcriptionally active chromosome (TAC) fractions from chloroplasts and to identify new components of it, proteomic analyses were performed. One of the DNA-binding proteins identified in a previous study was the Whirly1 protein. Immunological experiments with an antibody specific for HvWhirly1 allowed the detection of the protein in a conventionally prepared TAC-I fraction from barley chloroplasts but not in a highly purified TAC-II fraction. By proteome analyses with the highly purified TAC-II fraction, six new DNA-binding proteins were identified. They were named plastid nucleoid-associated proteins (ptNAP). One of newly identified ptNAPs is the AtSWIB-1 (*Arabidopsis thaliana SWIB domain-containing protein-1*) protein. Immunological analyses with an antibody specific for AtSWIB-1 allowed the detection of the SWIB-1 protein in both the conventionally prepared TAC-I and the highly enriched TAC-II fraction from spinach chloroplasts indicating that SWIB-1 in contrast to Whirly1 is a core component of TAC. An AtSWIB-1:GFP fusion protein was shown to be dually located in the nucleus and in plastid nucleoids. Binding of the SWIB-1 protein to plastid DNA was confirmed by *Southwestern* analysis. Database searches revealed that SWIB-1 together with further five SWIB-domain proteins belong to a subgroup of the SWIB family being predicted to be targeted to organelles. Fusions with GFP protein confirmed a plastid location for three of them. Together with SWIB-1, these proteins are the first identified chromatin remodeling factors being imported into plastids. Based on these findings, it is hypothesized that plastids possess chromatin remodeling proteins in order to regulate the expression of their genes by introducing structural changes of the nucleoid.

1. Einleitung

1.1 Organisation der plastidären DNA in Nukleoiden von Höheren Pflanzen

Aufgrund ihres endosymbiontischen Ursprungs bildet die plastidäre DNA (ptDNA) zusammen mit einer Vielzahl an kern- und plastomkodierten Proteinen dicht gepackte Nukleoproteinkomplexe, welche den bakteriellen Nukleoiden ähneln. In diesen hochorganisierten Strukturen, die auch als Plastidenkerne bezeichnet werden (Briat et al., 1982; Kuroiwa, 1991), finden zahlreiche DNA- und RNA-abhängige Prozesse wie Transkription, Replikation und Rekombination statt (Sato et al., 2003; Sakai et al., 2004). Diese Nukleoproteinkomplexe werden daher als plastidäre Nukleoide bezeichnet und als Segregations- oder Erbeinheit betrachtet (Kuroiwa et al., 1994; Nagata et al., 1999). Das plastidäre Genom (Plastom) der Höheren Pflanzen hat je nach Pflanzenart eine Größe von ca. 120-160 kbp und kodiert für Proteine des Photosyntheseapparates, des eigenen Transkriptionsapparates, für ribosomale (rRNA) als auch Transfer-RNA (tRNA) sowie für Proteine des NADH-Dehydrogenasekomplexes. Die Anzahl der Nukleoide in den Plastiden Höherer Pflanzen variiert zwischen einem einzelnen Nukleoid in Proplastiden bis zu vierzig in differenzierten Chloroplasten (Miyamura et al., 1986). Während der Chloroplasten-entwicklung konnten nicht nur Änderungen in der Anzahl der Nukleoide, sondern auch in der Verteilung der Nukleoide innerhalb der Plastiden beobachtet werden (Miyamura et al., 1986; Sato et al., 1993, 1998). Während in Proplastiden die Nukleoide im Zentrum der Organellen lokalisiert sind, sind in jungen Plastiden die Nukleoide an der inneren Hüllmembran und in differenzierten Chloroplasten an den Thylakoidmembranen verankert. Die Verankerung der ptDNA an der inneren Hüllmembran der Chloroplasten wird durch das Protein PEND (*plastid envelope DNA-binding*) vermittelt (Sato et al., 1993, 1997; Sato & Ohta, 2001). Es wird angenommen, dass in früheren Entwicklungsstadien der Pflanze das PEND-Protein die Bindung der Nukleoide an die Hüllmembran ermöglicht und dadurch die DNA-Replikation und eine korrekte Aufteilung der DNA während der Teilung der Organellen maßgeblich beeinflusst (Sato et al., 2003). Das Protein MFP1 (*matrix attachment region-binding filament-like protein 1*) ist hingegen für die Bindung der

Nukleoide an die Thylakoidmembranen in reifen Chloroplasten verantwortlich (Meier et al., 1996; Jeong et al., 2003). Es wird angenommen, dass für die Funktion der Replikations- und Transkriptionskomplexe eine Bindung an feste Strukturen notwendig ist. Die Thylakoid- oder Hüllmembranen würden dann eine ähnliche Rolle haben wie die cytoplasmatische Membran der Bakterien oder die Matrix im Zellkern der Eukaryoten (Sato & Ohta, 2001). Der genaue Mechanismus der räumlichen Verteilung der Nukleoide innerhalb von Plastiden während der Entwicklung ist noch unbekannt.

Im Gegensatz zu Plastiden besitzen Bakterien nur ein einziges Nukleoid, in dem die DNA stark verpackt und hochorganisiert ist. Für die Verpackung der DNA in eukaryotischen Zellen sind die Histone verantwortlich. In Bakterien wird diese Aufgabe durch zahlreiche histonähnliche Proteine (HLP *histone-like protein*) übernommen (**Tabelle 1**). Da aber einige der HLPs weder eine ähnliche Struktur noch ähnliche Domänen wie die Histone besitzen, wurde vorgeschlagen, diese Proteingruppe als Nukleoidassoziierte Proteine (NAP) zu bezeichnen (Dillon & Dormann, 2010). Zu den bakteriellen NAP-Proteinen gehören das Protein HU (*heat unstable*) und mehrere andere HLPs wie z.B. die Proteine H-NS (*histone-like nucleoid-structuring protein*), FIS (*factor for inversion stimulation*), IHF (*integration host factor*), Lrp (*leucine-responsive regulatory protein*) und Dps (*DNA-binding protein from starved cells,* **Tabelle 1**, (Travers & Muskhelishvili, 2005)). NAPs binden an die DNA und verändern deren Verpackungsgrad durch Schleifen- oder Kurvenbildung. Diese Veränderungen in der Topologie der DNA beeinflussen maßgeblich die Prozesse der Transkription, Rekombination und Replikation.

Prokaryotische HLP-Proteine konnten in Plastiden von einigen Algenarten als auch in Dinoflagellaten und Apicomplexa (zwei Gruppen des Stamms Alveolata) identifiziert werden (Sato et al., 2003; Chan et al., 2006). In einigen Dinoflagellaten wurde nachgewiesen, dass im Zellkern Histone durch HLP-Proteine ersetzt wurden (Chan et al., 2006). Zu den HLP-Proteinen in den Algen gehören: das Protein HC (*histone-like protein of chloroplasts*) von *Cyanidioschyzon merolae* (Kobayashi et al., 2002), das Protein HlpA von *Guillardia theta* (Wang & Liu, 1991; Wu & Liu, 1997) und das HLP-Protein von *Chlamydomonas reinhardtii* (Karcher et al., 2009). Auch in Vertretern der parasitischen Apicomplexa findet man bakterielle HU-Proteine, wie

z.B. das PfHU-Protein von *Plasmodium falciparum* (Sasaki et al., 2009). Allen HLP- und HU-ähnlichen Proteinen gemeinsam ist das niedrige Molekulargewicht, der basische isoelektrische Punkt und die Fähigkeit zur Bindung an die DNA. Die Funktion von einigen HLPs konnte durch Komplementierung von *E. coli* Mutanten ohne HU bestätigt werden (Kobayashi et al., 2002; Sasaki et al., 2009).

Tabelle 1: Nukleoidassoziierte Proteine in Bakterien, Algen und Alveolata.

Gen	NAP-Protein	Identifikations-Nummer	Mw kD	pI	Lysin-Gehalt (%)	Referenzen
hupA *hupB*	HU, *heat unstable* Regulation der Transkription α- und β- Untereinheit, *E. coli*	AP_003818 AP_001090	9 9	10 10	12 10	Rouvière-Yaniv & Kjeldgaard, 1979
hupA	HC, histonähnliches Protein der Chloroplasten, *C. merolae*	BAB86889	13	10	11	Kobayashi et al., 2002
Avar 0300 6458	bakterielles, Nukleoid-DNA-Bindeprotein, *A. variabilis*	ZP_001577 91	10	10	9	*NCBI Microbial Genomes Annotation Project*
hlp	histonähnliches Protein *C. reinhardtii*	EDP01736	18	11	10	Merchant et al., 2007; Karcher et al., 2009
hlpA	histonähnliches Protein der Chloroplasten, *C. phi/ G. theta*	B41609	10	11	11	Stern et al., 1984; Wang & Liu, 1991; Wu & Liu, 1997
PfHU	bakterielles histonähnliches Protein, *P. falciparum*	XP_001351 921	22	9	14	Ram et al., 2008; Sasaki et al., 2009
Glom	histonähnliches Protein *P. polycephalum*	BAB86364	41	10	20	Sasaki et al., 2003
H-NS	*histone-like nucleoid-structuring protein*, *E. coli*	CAA47740	15	5	8	Danchin & Krin, 1995
FIS	*factor for inversion stimulation E. coli*	CAQ33587	11	9	7	Koch et al., 1988 Skoko et al., 2006
IHFα *IHFβ*	*integration host factor* α- und β-Untereinheit, *E. coli*	AP_002332	11 11	9 9	10 9	Swinger & Rice, 2004
Lrp	*leucine-responsive regulatory protein*, *E. coli*	AP_001519	19	9	6	de los Rios & Perona, 2007
Dps	*DNA-binding protein from starved cells*, *E.coli*	CAA49169	19	6	7	Almirón et al., 1992

Bisher konnten weder im plastidären Genom noch im Kerngenom von Höheren Pflanzen Gene für histonähnliche Proteine identifiziert werden (Sato et al., 2003). In den 80-er und 90-er Jahren gab es Berichte über den Nachweis von Proteinen mit Ähnlichkeit zu HU oder Histonen in den Nukleoiden von Spinat (Briat et al., 1984) und Erbse (Yurina et al., 1995). In den Nukleoiden aus Spinat konnte mit dem gegen das HU-Protein aus *E. coli* gerichteten Antikörper ein Protein in der Größe von ca. 17 kD nachgewiesen werden (Briat et al., 1984). Wenige Jahre später wurde von G. Crevel und Mitarbeitern (1989) berichtet, dass sie über eine Aufreinigung mittels Zellulose-Affinitätschromatographie ein 10 kD großes DNA-Bindeprotein - HC (*histone-like chloroplast*) - aus Spinatchloroplasten isolieren konnten. Dieses HC-Protein wurde aber durch den HU-Antikörper nicht erkannt (Crevel et al., 1989). Neueste Datenbankanalysen, in die die vollständig sequenzierten Genome von Pflanzenarten wie Arabidopsis, Reis oder Pappel einbezogen wurden, konnten die früheren Ergebnisse aber nicht bestätigen.

1.2 Das Transkriptionsaktive Chromosom (TAC)

Die Transkriptionsaktivität der Plastiden kann in zwei biochemisch unterschiedlichen Fraktionen nachgewiesen werden: in der löslichen Stroma-Fraktion und in der Membranfraktion, aus der das Transkriptionsaktive Chromosom (TAC) isoliert werden kann (Greenberg et al., 1984; Hallick et al., 1976). Während die RNA-Polymerase im TAC fest an die DNA bindet, kann die in der löslichen Stroma-Fraktion enthaltene RNA-Polymerase durch hohe Salzkonzentrationen oder Abwesenheit von Magnesiumionen von der DNA abgelöst werden. Im Unterschied zur löslichen Stroma-Fraktion können die TAC-Extrakte *in vitro* nach Zugabe von freien Nukleotiden RNA-Ketten verlängern, deren Initiation zuvor *in vivo* in der Pflanze stattgefunden hat.

Die ersten Transkriptionsaktiven Chromosomen wurden aus Chloroplasten von *Euglena gracilis* isoliert (Hallick et al., 1976). Danach folgten TAC-Präparationen aus Blättern von Spinat (Briat & Mache, 1980; Krause & Krupinska, 2000), Senf (Reiss & Link, 1985; Pfalz et al., 2006), Erbse (Tewari & Goel, 1983), Gerste (Falk, 1994; Suck, 1996), Mais (Müller, 2004) und Arabidopsis (Pfalz et al., 2006).

Die ersten Untersuchungen zur Charakterisierung von DNA-Bindeproteinen in TAC-Fraktionen erfolgten gemäß der von Bülow et al. (1987) etablierten *Southwestern* Methode. In diesem Verfahren werden die TAC-Proteine nach elektrophoretischer Auftrennung und Transfer auf eine Nitrocellulosemembran renaturiert und mit einer radioaktiv markierten DNA-Sonde inkubiert (Bülow et al., 1987). Auf diese Weise konnten acht Proteine im Molekulargewichtsbereich von 17-145 kD, die unterschiedliche Bindungsaffinitäten zu einzel- und doppelsträngiger DNA aufwiesen, in TAC-Fraktionen aus Senfchloroplasten nachgewiesen werden (Bülow et al., 1987). Nach dem gleichen Verfahren wurde in TAC-Extrakten aus Gerste ein 17 kD Protein identifiziert (Suck, 1996). Ähnliche Resultate wurden mit den TAC-Fraktionen aus Spinatchloroplasten erhalten (**Arbeit II**, Abb. 6). Hierbei wurden außer dem 17 kD Protein zwei weitere Proteine in der Größe von 14 und 19 kD detektiert.

Die Analyse der Proteinmuster der hochaufgereinigten TAC-Fraktion in einem SDS-Polyacrylamidgel ergab, dass die TAC-Fraktion aus ca. 30-40 Proteinen besteht (Krause & Krupinska, 2000). Immunologisch konnten in TAC-Extrakten die α- (Suck et al., 1996) und β- Untereinheiten (Krause & Krupinska, 2000) der PEP (*plastid encoded RNA-polymerase*), das MFP1- (*matrix attachment region-binding filament-like protein 1*, (Meier et al., 1996; Jeong et al., 2003)) Protein (Melonek et al., 2010), Why1 (*Whirly1*, (Melonek et al., 2010)), TCP34 (*tetratricopeptide-containing chloroplast protein of 34 kD*, (Weber et al., 2006)) und das ETCHED1-Protein (da Costa e Silva et al., 2004) nachgewiesen werden.

1.3 Identifizierung von DNA-Bindeproteinen durch Massenspektrometrie

Die geringen Mengen, in den die TAC-Proteine in den Plastiden vorliegen, verhinderten lange Zeit deren Identifikation durch Sequenzierung. Erst die neuesten Methoden der Massenspektrometrie erlaubten in den letzten Jahren eine Aufreinigung und Identifizierung neuer DNA- und nukleoidassoziierten Proteine.

1.3.1 Proteomanalyse von TAC-Extrakten

Die erste Proteomanalyse einer TAC-Fraktion wurde mit Extrakten aus Chloroplasten von Arabidopsis und Senf durchgeführt (Pfalz et al., 2006). Die einzelnen Schritte der Präparation sind in der **Abbildung 1** aufgeführt. Eine Anreicherung der mit der DNA

assoziierten Proteine im TAC wurde durch mehrere Gelfiltrations- und Ultra-zentrifugationsschritte erreicht. Zum Entfernen der Nukleinsäuren wurden die Proteine aus der TAC-Fraktion aus Arabidopsis mittels Phenol/Chloroform-Fällung angereichert und das resultierende Proteingemisch nach Spaltung mit Trypsin massenspektrometrisch analysiert. Auf diese Weise konnten 35 TAC-Proteine identifiziert werden. Davon waren 14 bereits als Proteine klassifiziert, die an Transkription, Translation, Replikation und Proteinmodifikationen beteiligt sind, wie z.B. α-, β-, β'-, β''-Untereinheiten der PEP, γ-Untereinheit der DNA-Polymerase 1 (POLGAMMA1), zwei Untereinheiten der DNA-Gyrase und der Elongationsfaktor EF-TU. In den transkriptionsaktiven Fraktionen aus Senf und Arabidopsis wurden insgesamt 18 neue TAC-Proteine identifiziert. Sie wurden als pTAC- (plastidäres Transkriptionsaktives Chromosom) Proteine bezeichnet. Acht von diesen pTAC-Proteinen besitzen DNA- oder RNA-Bindedomänen, wie PPR (*pentatricopeptide repeat*) und TPR (*tetratricopeptide repeat*), die Whirly-KGKAAL-Domäne, die SAP- (*SAF-A/B, Acinus, and PIAS*) und SET-Domänen (*Su(var)3-9, Enhancer-of-zeste, Trithorax*), das SMR- (*small MutS-related*) Motiv und die KOW- (*Kyrpides, Ouzounis, and Woese*) Domäne. Zu den neu identifizierten pTACs gehören auch Proteine, die an einzelsträngige DNA binden, wie zwei Whirly-Transkriptionsfaktoren, Whirly1 und 3 und das Protein OSB2 (*organellar single stranded DNA-binding protein 2*, (Zaegel et al., 2006)), das als pTAC9 bezeichnet wurde.

1.3.2 DNA-Bindeproteine einer Membranfraktion

Im Jahr 2005 haben Phinney und Thelen eine Proteomanalyse einer bei Triton-X-100 Behandlung unlöslichen Membranfraktion aus Erbsenchloroplasten durchgeführt. Das Ziel der Untersuchungen war es, die Proteine des Acetyl-CoA-Carboxylase-komplexes zu identifizieren (Phinney & Thelen, 2005). Die wichtigsten Schritte der Methode sind in der **Abbildung 1B** dargestellt. Interessanterweise konnte mit diesem Verfahren eine Fraktion der plastidären DNA zusammen mit 35 Proteinen, die mit der DNA assoziiert sein könnten, angereichert werden. Neben den α- und β-Untereinheiten der PEP wurden in der gegenüber Triton stabilen Fraktion andere bereits bekannte DNA-Bindeproteine der Chloroplasten identifiziert.

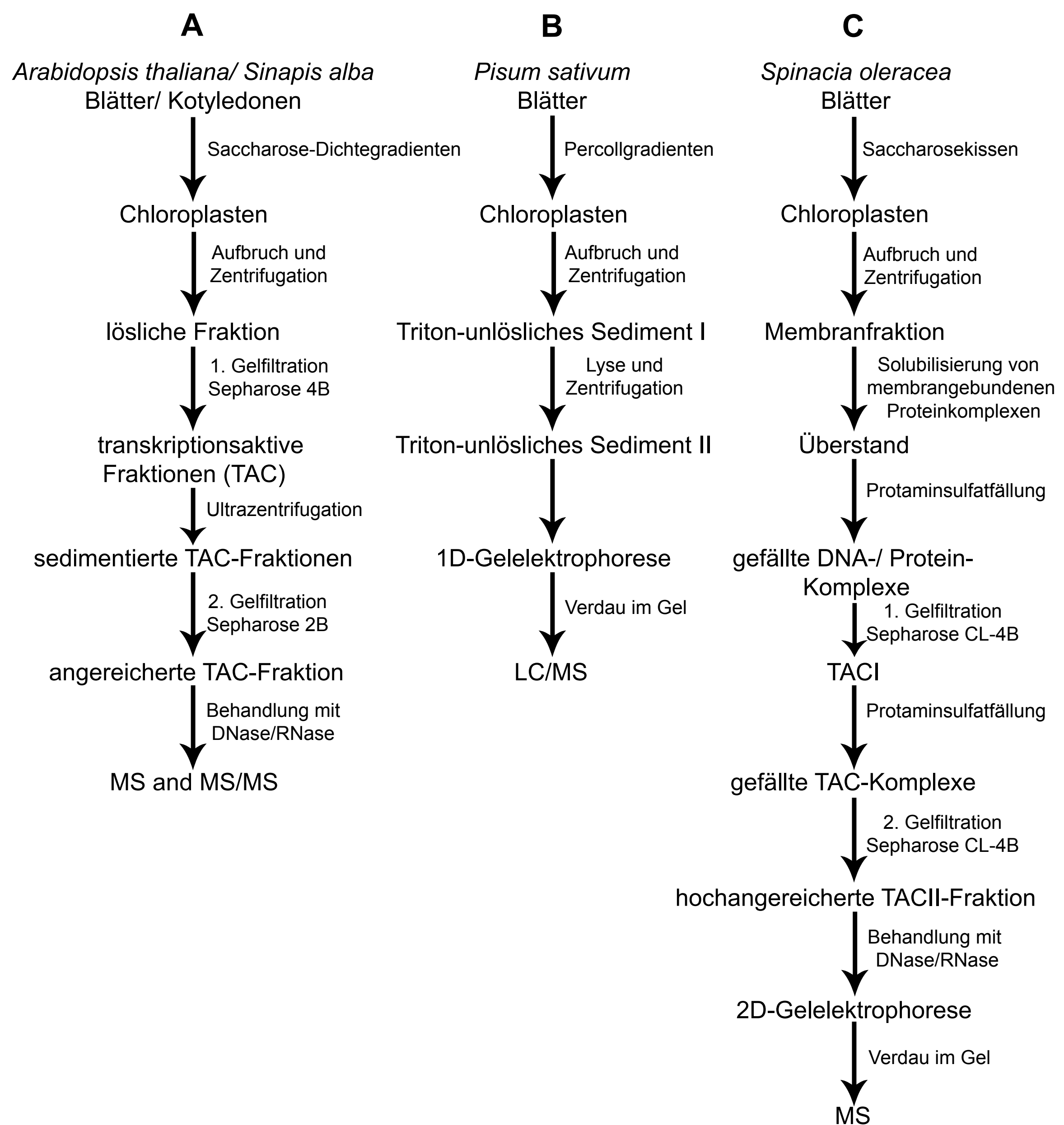


Abbildung 1: Der Vergleich von verschiedenen Aufreinigungsmethoden zur Gewinnung von TAC-Fraktionen (**A**: Pfalz et al., 2006 und **C**: Krause & Krupinska, 2000) und einer mit Triton-X-100 unlöslichen Membranfraktion (**B**: Phinney & Thelen, 2005) aus Chloroplasten.

Hierzu gehörte die Sulfitreduktase SiR (Sato et al., 2001; Sekine et al., 2002), das PEND-Protein (Sato et al., 2001), mehrere pTAC-Proteine, wie pTAC6, -12 und -14 (Pfalz et al., 2006), die A- und B-Untereinheiten einer DNA-Gyrase und eine RNA-Helikase. Darüber hinaus konnten auch die Histone H2B und H3 massenspektrometrisch identifiziert werden. Möglicherweise handelt es sich hierbei nur um Proteine, die histonähnliche Motive in ihrer Sequenz aufweisen und nicht die Histone selbst. Des Weiteren wurden in der gegenüber Triton stabilen Fraktion Proteine

identifiziert, die zu anderen multienzymatischen Komplexen gehören, wie zum Pyruvatdehydrogenasekomplex oder zum Calvin-Zyklus.

1.3.3 Plastidäre nukleoidassoziierte Proteine (ptNAP)

Die hier beschriebenen Präparationen von DNA-haltigen Fraktionen aus Chloroplasten ermöglichten die Identifizierung von mehreren neuen DNA-Bindeproteinen der Plastiden, die die Architektur der plastidären DNA modifizieren könnten (**Tabelle 2**). Sie werden im Folgenden als plastidäre nukleoidassoziierte Proteine (ptNAP) bezeichnet. Die im TAC-Proteom identifizierte Proteine pTAC2, -3 und -14 verfügen über DNA-Bindedomäne wie SMR/MutS, SAP und SET und sind im Zellkern an der Organisation der Chromosomen beteiligt und könnten in den Plastiden an der Modellierung der DNA-Topologie beteiligt sein (Aravind & Koonin, 2000; Dillon et al., 2005). Eines der neu identifizierten DNA-Bindeproteine in der gegenüber Triton stabilen Fraktion war das zu einem SWI/SNF-Komplex zugehörige Protein AtSWI3D (*Arabidopsis thaliana switching defective/ sucrose non fermenting 3D*), das im Zellkern als Chromatinmodellierungsfaktor funktioniert (Jerzmanowski, 2007). Das pTAC14-Protein, das in beiden hier beschriebenen Präparationen identifiziert wurde, verfügt über eine SET-Domäne, der im Zellkern eine Rolle in der Regulation der Chromatinstruktur und der Genexpression zugeschrieben wird (Dillon et al., 2005). Die identifizierten ptNAP-Proteine in den beiden Präparationen sind jedoch überraschend unterschiedlich. Wenn man die große Anzahl der nukleoid-assoziierten Proteine in Bakterien betrachtet (**Tabelle 1**), so ist zu vermuten, dass eine ähnlich große Anzahl an NAP-Proteinen auch in den Plastiden zu finden ist. Es könnte also sein, dass durch die verschiedenen Präparationen nur wenige Vertreter der mit den plastidären Nukleoiden assoziierten Proteine aufgereinigt wurden.

Tabelle 2: Massenspektrometrisch identifizierte ptNAP-Proteine, **A**: Pfalz et al. (2006) **B**: Phinney & Thelen (2005).

ptNAP-Proteine	**Identifikations-Nummer**	**Funktion**	**Proteom**	
			A	**B**
*plastid transcriptionally active*2 (pTAC2)	NP_177623	DNA–Bindung	x	
*plastid transcriptionally active*3 (pTAC3)	NP_187076	DNA–Bindung	x	
*plastid transcriptionally active*14 (pTAC14)	NP_193746	DNA–Bindung	x	x
DNA-Gyrase Untereinheit A (ATGYRA)	NP_187680	Einführung von negativen *supercoils*	x	x
DNA-Gyrase Untereinheit B (ATCPGYRB)	NP_187638	Einführung von negativen *supercoils*	x	x
DNA-Gyrase Untereinheit B (ATMGYRB)	NP_850762	Einführung von negativen *supercoils*		x
DNA/RNA-Helikase	AAN72199	ATP-abhängiges Abwickeln von DNA/RNA		x
RNA-Helikase	AAO42779	ATP-abhängiges Abwickeln von RNA		x
Arabidopsis thaliana switching defective/ sucrose non fermenting 3D (AtSWI3D)	AAL67003	Chromatinmodellierung		x
Sulfitreduktase (SiR)	BAC81658	DNA-Verpackung		x

In den Proteomanalysen mit TAC- und Membranfraktionen wurden Proteine sowohl prokaryotischen als auch eukaryotischen Ursprungs gefunden. Es wurde vorgeschlagen, dass im Laufe der Evolution Proteine, die primär eine Funktion im Zellkern erfüllten, eine neue zusätzliche Funktion in den Plastiden erhielten (Kodama, 2007).

2. Whirly1 als Komponente des Transkriptionsaktiven Chromosoms

Die Proteomanalyse der TAC-Extrakte aus Senf- und Arabidopsischloroplasten erlaubte die Identifizierung des AtWhy1-Proteins als Komponente des Transkriptions-aktiven Chromosoms (Pfalz et al., 2006). In früheren Arbeiten von Krause et al. (2005) konnte der zunächst ausschließlich im Zellkern lokalisierte AtWhy1-Transkriptionsfaktor (Desveaux et al., 2002) mittels Fusionen mit dem grün fluores-zierenden Protein (GFP) unerwartet in Chloroplasten nachgewiesen werden, wo das Protein eine ähnliche Verteilung wie die Nukleoide zeigte (Krause et al., 2005). Letztendlich konnte eine duale Lokalisation des Proteins im Zellkern und in Chloroplasten einer Zelle durch Immungoldanalysen mit dem gegen das Why1-Protein der Gerste gerichteten Antikörper nachgewiesen werden (Grabowski et al., 2008).

Unter Verwendung eines spezifischen HvWhy1-Antikörpers sollte nun untersucht werden, ob das Why1-Protein in den TAC-Fraktionen aus Gerste vorliegt, und zusätzlich sollte geklärt werden, ob das Protein an DNA oder RNA bindet. Um die Zugehörigkeit des Whirly1-Proteins zum TAC zu überprüfen, wurden im Folgenden zwei TAC-Fraktionen, die als TAC-I und TAC-II bezeichnet wurden, aus Gersten-keimlingen präpariert. TAC-II ist eine hochaufgereinigte Fraktion, die aus einer TAC-I-Fraktion durch zusätzliche Aufreinigungsschritte erhalten wird (Krause & Krupinska, 2000) und dadurch höhere spezifische Transkriptionsaktivitäten aufweist. Immunologische Analysen von Proteinfraktionen aus Chloroplasten mit dem HvWhy1-Antikörper ergaben, dass nur ein Teil des Whirly1-Proteins in der Membran-fraktion vorliegt. Der größte Anteil konnte in der Stroma-Fraktion nachgewiesen werden. Darüber hinaus wurde das Whirly1-Protein nur im TAC-I und nicht in der hochaufgereinigten TAC-II-Fraktion detektiert (**Arbeit I**, Abb. 3). Diese Ergebnisse deuten darauf hin, dass zum einem Why1 nur locker mit TAC assoziiert und dadurch keine integrale Komponente der Fraktion ist. Um zu prüfen, ob Why1 möglicherweise an RNA bindet, wurden RIP-Chip-Analysen in Kooperation mit Prof. Christian Schmitz-Linneweber (Humblodt-Universität Berlin) durchgeführt. Sie erlaubten die

Identifizierung von intronhaltigen RNA-Spezies als Liganden von Why1 (**Arbeit I**, Abb. 5). Die anschließende *Northern Blot* Analyse mit RNA aus transgenen Gerstenpflanzen mit einem *Knockdown* des *why1* Gens zeigte, dass die Prozessierung von ausgewählten Transkripten durch HvWhy1 beeinflusst wird (**Arbeit I**, Abb. 6).

Eine zusätzlich durchgeführte Immungoldanalyse zeigte, dass das Why1-Protein nur im Zellkern mit der DNA kolokalisiert ist, während es in den Chloroplasten weniger eng mit der DNA assoziiert zu sein scheint (**Arbeit I**, Abb. 1a, b). Um die Kolokalisierung von Whirly1 mit den Nukleoiden genau zu untersuchen, wurde das PEND-RFP- (RFP *red fluorescent protein*) Protein, das aufgrund seiner Bindung an die plastidäre DNA für die Visualisierung der Nukleoide geeignet ist (Terasawa & Sato, 2005), eingesetzt. Die Aufnahmen von Tabakprotoplasten mit dem konfokalen Mikroskop zeigten, dass die grüne Fluoreszenz des Why1:GFP-Fusionsproteins nicht mit allen durch PEND sichtbar gemachten Nukleoiden kolokalisiert (**Abbildung 2, Arbeit I,** Abb. 1c-e).

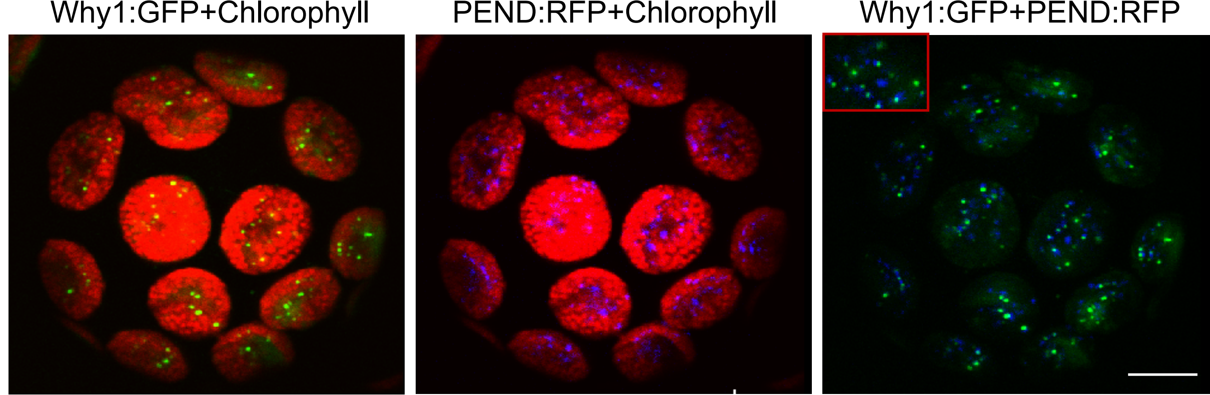


Abbildung 2: Kolokalisierung von Why1:GFP mit PEND:RFP-Protein in Tabakprotoplasten. Zur Erzielung eines besseren Kontrasts wurde die Fluoreszenz des PEND:RFP-Proteins in blauer Farbe dargestellt. Der angegebene Maßstab entspricht 10 µm.

Diese Ergebnisse deuten darauf hin, dass nicht alle Nukleoide in einem Chloroplast gleich sind und dass vielleicht nur einzelne Exemplare transkriptionsaktiv sind und daher RNA enthalten. Ein Teil der Why1:GFP-Signale wurde außerhalb der Nukleoide detektiert. Dies deutet auf eine zusätzliche Funktion des Proteins im Stroma hin.

3. Proteomanalyse der hochaufgereinigten TAC-Fraktion

Im Rahmen dieser Doktorarbeit sollte die Proteinzusammensetzung der konventionell präparierten TAC-I-Fraktion mit der Proteinzusammensetzung der hochaufgereinigten TAC-II-Fraktion (Krause & Krupinska, 2000) verglichen werden. Außerdem sollten die Proteine der TAC-II-Fraktion massenspektrometrisch identifiziert werden.

3.1 Aufreinigung und zweidimensionale Gelelektrophorese von TAC-II

Die TAC-I- und TAC-II-Fraktionen aus Spinatblättern wurden nach dem von Krause und Krupinska (2000) erarbeiteten Protokoll isoliert (**Abbildung 1C**). Nach der Präzipitation durch Chloroform/Methanol-Fällung (Wessel & Flügge, 1984) wurden die TAC-Proteine auf einem IPG- (*immobilized pH gradient*) Streifen fokussiert und anschließend auf einem SDS-Polyacrylamidgel aufgetrennt. Die Coomassie-Färbung der 2D-Gele zeigte, dass sich die TAC-I- und TAC-II-Fraktionen der Spinatchloroplasten sehr stark in der Proteinzusammensetzung und in den relativen Intensitäten der *spots* unterscheiden. Während auf dem 2D-Gel der TAC-I-Fraktion 132 *spots* sichtbar waren, konnten auf dem 2D-Gel der TAC-II-Fraktion nur 85 *spots* gezählt werden (**Abbildung 3**).

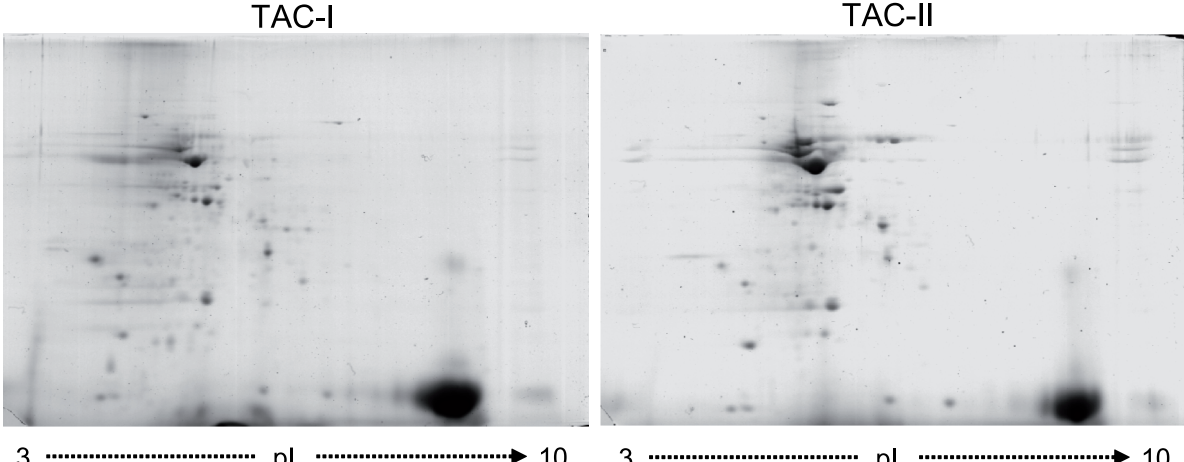


Abbildung 3: Vergleich der Proteinmuster vom TAC-I und TAC-II auf 2D-Gelen nach Coomassie-Färbung. Es konnten 123 Proteinspots in der TAC-I und 85 in der TAC-II Fraktion gezählt werden (**Arbeit II**).

Alle gefärbten *spots* wurden aus dem 2D-Gel der TAC-II-Fraktion ausgestochen und die nach einer tryptischen Spaltung erhaltenen Peptide wurden massenspektrometrisch analysiert. Die Identifizierung der Proteine erfolgte über Vergleiche der ermittelten Peptidmassen mit Massen von theoretisch gespaltenen Proteinen (**Arbeit II**).

3.2 Im TAC identifizierte Proteine

Durch Massenspektrometrie konnten 46 Proteine in der TAC-II-Fraktion aus Spinatchloroplasten identifiziert werden (**Arbeit II**, Anhang, Tabelle I). Aufgrund ihrer möglichen Funktionen können sie in drei Gruppen eingeteilt werden. Zur ersten Gruppe gehören Proteine, die an Transkription, Replikation, DNA-Erhaltung und Strukturierung beteiligt sind, wie z.B. die RpoC1 und RpoC2 Untereinheiten der plastomkodierten RNA-Polymerase (PEP). Sechs von diesen Proteinen besitzen bereits beschriebene RNA- oder DNA-Bindedomänen, wie MYB/SANT, PPR oder Motive, die typisch für Transkriptionsfaktoren oder Chromatinmodellierungsfaktoren sind (z.B. Armadillo oder SWIB). Die zweite Gruppe besteht aus Proteinen, die eine Funktion bei der Translation oder den posttranslationalen Prozessen erfüllen. Zu diesen zählen der EF-TU Elongationsfaktor und die Chaperone HSP70 und HSP40. Einige der identifizierten Proteine wurden als mögliche Kontaminationen eingeordnet (**Arbeit II**, Anhang, Tabelle I).

Sechs der neu identifizierten TAC-Proteine können in Anlehnung an die Nomenklatur für Bakterien (s. Kapitel 1.1) als plastidäre nukleoidassoziierte Proteine (ptNAP) bezeichnet werden. Zu den ptNAP-Proteinen im TAC-II zählen ein Protein vom Typ SMC (*Structural Maintenance of Chromosomes*), ein Armadillo/β-catenin Protein, zwei MYB/SANT (SWI3, ADA2, NCor, TFIIB) Transkriptionsfaktoren, ein SWIB-Protein und ein zu Rho ähnlicher Transkriptionsfaktor (**Arbeit II**, Tabelle 1).

3.3 Charakterisierung des SWIB-1-Proteins von *Arabidopsis thaliana*

Das ptNAP-Protein mit dem kleinsten Molekulargewicht im TAC ist das Protein SWIB-1 (*SWIB domain-containing protein-1*), das durch seine Sequenzhomologie mit dem SWIB-1-Protein von Arabidopsis (AtSWIB-1) charakterisiert werden konnte.

Durch *in silico* Analyse der Aminosäuresequenz des SWIB-1-Proteins aus Arabidopsis ließen sich drei Hauptbereiche in dem Protein unterscheiden: eine plastidäre Importsequenz am aminoterminalen Ende des Proteins, ein lysinreicher Bereich und eine SWIB-Domäne (**Arbeit II**, Abb. 2A). Sequenzvergleiche zeigten, dass der lysinreiche Bereich des AtSWIB-1-Proteins große Ähnlichkeit mit einem Teil der CTD-Domäne (*carboxyl-terminal domain*) des Histon H1 aus Tabak und mit einem lysinreichen Bereich des CND41- (*chloroplast nucleoid DNA binding protein*) Proteins aufweist (**Arbeit II**, Abb. 2C). Der genannte Bereich von CND41 wurde bereits als ein DNA-Bindemotiv charakterisiert (Nakano et al., 1997; Murakami et al., 2000). Es ließ sich also vermuten, dass dieser Teil von AtSWIB-1 eine DNA-Bindedomäne repräsentieren könnte. Die SWIB-Domäne kann für Protein-Protein Interaktionen verantwortlich sein und ist typisch für die Familie der SWI/SNF ATP-abhängigen Chromatinmodellierungsproteine, die im Zellkern an der Aktivierung der Transkription beteilig sind (Bennett-Lovsey et al., 2002).

3.3.1 Die subzelluläre Verteilung des AtSWIB-1:GFP-Proteins

Die Programme LocTree (Hua & Sun, 2001) und TargetP (Emanuelsson et al., 2007) sagten eine duale Lokalisation des AtSWIB-1-Proteins im Zellkern und in den Chloroplasten vorher. Zur Überprüfung der subzellulären Verteilung des AtSWIB-1-Proteins wurden sowohl *in vitro* Importexperimente mit isolierten Erbsenchloroplasten durchgeführt als auch *GFP*-Fusionskonstrukte transient in Zwiebelzellen exprimiert. Die Ergebnisse der Importexperimente zeigten, dass das SWIB-1-Protein in die Chloroplasten transportiert wird (**Arbeit II**, Abb. 2B). Darüber hinaus bestätigten die fluoreszenzmikroskopischen Aufnahmen von Zwiebelepidermiszellen und Tabak-protoplasten, die transient das *SWIB-1:GFP*-Konstrukt exprimierten, die duale Lokalisierung des Proteins im Zellkern und in Plastiden (**Arbeit II**, Abb. 3B, 4A). Interessanterweise, traten die GFP-Signale in den Tabakprotoplasten als grüne Punkte auf. Zur Überprüfung der Kolokalisation dieser Signale mit den Nukleoiden, wurden erneut Transformationsexperimente mit den *AtSWIB-1:GFP*- und *PEND:dsRED*-Konstrukten durchgeführt, in denen das PEND-Protein für die Visualisierung der Nukleoide eingesetzt wurde (s. Kapitel 2). Die Aufnahmen mit dem konfokalen Mikroskop zeigten, dass das SWIB-1-Protein in den Plastiden mit dem PEND-Protein in den Nukleoiden kolokalisierte (**Arbeit II**, Abb. 4).

3.3.2 Immunologischer Nachweis und DNA-Bindung des SWIB-1-Proteins

Der immunologische Nachweis des SWIB-1-Proteins in Fraktionen aus Arabidopsisblättern wurde mit dem AtSWIB-1-Antikörper durchgeführt. Die Ergebnisse des *Western Blots* bestätigten die Lokalisation des AtSWIB-1-Proteins im Zellkern und in den Plastiden (**Arbeit II**, Abb. 5). Im Vergleich zur plastidären Fraktion wurde in der Kern-Fraktion ein Protein mit einem höheren Molekulargewicht detektiert. In beiden Fraktionen lag zusätzlich ein Protein höheren Molekulargewichts vor, das höchstwahrscheinlich durch posttranslationale Protein-Modifikationen, wie Acetylierung oder Phosphorylierung erklärbar ist. Mit demselben Antikörper konnte in Proteinextrakten aus Spinatblättern ein Protein mit einem Molekulargewicht von etwa 19 kD detektiert werden (**Arbeit II**, Abb. 6). Der Antikörper erkannte auch ein Protein in den TAC-I- und TAC-II-Fraktionen. Dies zeigt, dass SWIB-1 eine integrale TAC-Komponente ist.

Die Untersuchungen zur DNA-Bindung des SWIB-1-Proteins erfolgten mit einer *Southwestern* Methode (s. Kapitel 1.2). Nach Inkubation der membrangebundenen TAC-Proteine mit der *16S rDNA* Sonde konnten im TAC drei DNA-Bindeproteine mit einem Molekulargewicht von 14, 17 und 19 kD detektiert werden. Wenn die TAC-Proteine auf der Membran mit dem anti-AtSWIB-1-Antikörper vorinkubiert wurden, nahm die Intensität der 19 kD-Bande nach der Inkubation mit der radioaktiven Sonde ab. Dies zeigt, dass das 19 kD-Protein von dem gegen Arabidopsis SWIB-1-Protein gerichteten Antikörper erkannt wird und damit das entsprechende Protein in Spinat ist (**Arbeit II**, Abb. 7).

4. SWIB-Domänenproteine

Das SWIB-1-Protein gehört zu den ersten Chromatinmodellierungsfaktoren, für den eine Lokalisation in Chloroplasten gezeigt werden konnte. Die Ähnlichkeit des SWIB-1-Proteins zu den histonähnlichen Proteinen deutet darauf hin, dass während der Evolution eukaryotische, kernkodierte Chromatinmodellierungsfaktoren in den Plastiden die Aufgaben der ursprünglichen bakteriellen HU-Proteine übernommen haben. SWIB-1 gehört zu einer Proteingruppe, die durch ihre duale Lokalisation im Zellkern und den Plastiden charakterisiert ist. Es ist zu vermuten, dass diese Proteine, zu denen auch Why1 und MFP1 gehören, wichtige Funktionen in der Kommunikation zwischen Organellen und dem Zellkern erfüllen (Krause & Krupinska, 2009; Krause et al., 2009).

4.1 Vorkommen und Funktion von SWIB-Domänenproteinen

SWIB-Domänenproteine gehören zur Familie des SWI/SNF-Chromatinmodellierungs-komplexes, der zuerst in Hefe entdeckt wurde (Peterson et al., 1994; Wang et al., 1996). Hefemutanten, die Defekte in dem Komplex aufwiesen, waren nicht mehr in der Lage, ihren Paarungstyp zu wechseln (SWI, *switching defective*) oder Saccharose zu fermentieren (SNF, *sucrose non fermenting*) (Sudarsanam & Winston, 2000). Im Zellkern nutzt der SWI/SNF-Komplex die durch Hydrolyse von ATP gewonnene Energie, um die DNA für interagierende Proteine zugänglicher zu machen. In Säugetieren ist die SWIB-Domäne ein konservierter Bereich, der in drei sogenannten BAF60- (*BRG-associated factors 60 kD*) Untereinheiten des Komplexes zu finden ist (Wang et al., 1996; Nomoto et al., 1997). Im Zellkern interagieren die BAF60-Proteine mit Transkriptionsfaktoren, die die spezifische Erkennung der DNA-Sequenzen ermöglichen. Nach der Bindung an die DNA vermitteln die BAF60-Proteine das Andocken des SWI/SNF-Chromatinmodellierungskomplexes an diese DNA-Region und leiten auf diese Weise den Transkriptionsstart ein (Lorès et al., 2010).

Da bis jetzt kein pflanzlicher Chromatinmodellierungskomplex als Ganzes aufgereinigt werden konnte, ist der aktuelle Wissensstand über die Proteine des SWI/SNF-Komplexes in Höheren Pflanzen sehr gering, und die Rolle dieser Proteine kann nur anhand ihrer Sequenzhomologie und der damit vermuteten funktionellen Ähnlichkeit der SWI/SNF-Untereinheiten der Hefe oder der Menschen beschrieben werden (Jerzmanowski, 2007). Zwei SWIB-Proteine wurden auch im Genom von Chlamydien gefunden (Stephens et al., 1998), die höchstwahrscheinlich die SWIB-Domäne von einem eukaryotischen Wirt erworben haben (Stephens et al., 1998; Bennett-Lovsey et al., 2002). Interessanterweise wurde in Höheren Pflanzen und in Chlamydien die SWIB-Domäne auch als ein eigenständiges Protein (*stand-alone*) und nicht nur als Teil eines größeren Proteins gefunden (Bennett-Lovsey et al., 2002, **Arbeit III**).

4.2 SWIB-Domänenproteine in *Arabidopsis thaliana*

Neben dem durch Proteomanalyse identifizierten SWIB-1-Protein ermöglichten Datenbanksequenzrecherchen die Identifizierung von 17 weiteren SWIB-Domänen-proteinen in *Arabidopsis thaliana* (s. Kapitel 3.2), die aufgrund ihrer Ähnlichkeit und enthaltenen zusätzlichen Domänen in vier Gruppen aufgeteilt werden konnten (**Arbeit III**, Abb. 1). Da für alle sechs Proteine der Gruppe 4 eine Lokalisation in den Organellen (TargetP) vorhergesagt wurde (**Arbeit III**, Tabelle 1), sollte deren subzelluläre Lokalisation mittels Fusionen mit dem GFP-Protein überprüft werden. Im Rahmen der Diplomarbeit von Mirl Trösch wurden *GFP*-Konstrukte von *AtSWIB-2* bis *-6* vorbereitet und transient in Zwiebelepidermiszellen und Tabakprotoplasten exprimiert. Das AtSWIB-1-Protein konnte in vorherigen Arbeiten in den Nukleoiden und im Zellkern lokalisiert werden (s. Kapitel 3.3.1). Die mikroskopischen Aufnahmen von Zwiebelepidermiszellen zeigten, dass AtSWIB-2 und -3:GFP-Proteine in den Leukoplasten und SWIB-4:GFP im Zytoplasma lokalisiert sind (**Arbeit III**, Abb. 2A). Die grünen Signale von AtSWIB-5:GFP und AtSWIB-6:GFP wurden in den Mitochondrien detektiert (**Arbeit III**, Abb. 2A). Die gleichen *GFP*-Konstrukte wurden für die transiente Transformation von Tabakprotoplasten eingesetzt, wodurch die Lokalisation der SWIB-Proteine bestätigt werden konnte (**Arbeit III**, Abb. 2A). Für AtSWIB-6 wurde hier allerdings eine zusätzliche Lokalisation in den Chloroplasten

beobachtet. Die Ergebnisse der Untersuchungen zur subzellulären Lokalisierung von allen sechs SWIB-Proteinen der Gruppe 4 sind in der **Tabelle 3** zusammengefasst.

Tabelle 3: Lokalisierung der SWIB-Domänenproteine in den Organellen mittels Fusionen mit GFP.

Gen	Protein	Lokalisierung der GFP-Fusionsproteine	
		Zwiebelepidermiszellen	Tabakprotoplasten
At3g03590	AtSWIB-1	Leukoplasten + Zellkern	Chloroplasten (Nukleoide) + Zellkern
At2g14880	AtSWIB-2	Leukoplasten	Chloroplasten (Nukleoide)
At4g34290	AtSWIB-3	Leukoplasten	Chloroplasten (Nukleoide)
At3g48600	AtSWIB-4	Cytosol	Cytosol
At1g31760	AtSWIB-5	Mitochondrien	Mitochondrien
At2g35605	AtSWIB-6	Mitochondrien	Chloroplasten (Nukleoide) + Mitochondrien

Um die Verteilung der AtSWIB-2:, ASWIB-3: und AtSWIB-6:GFP-Proteine innerhalb der Chloroplasten zu untersuchen, wurden hochauflösenden Aufnahmen von transformierten Tabakprotoplasten mit dem konfokalen Mikroskop durchgeführt. Die Fluoreszenzsignale von plastidären SWIB:GFP-Proteinen zeigten punktuelle Muster auf, die auf eine Kolokalisation mit den Nukleoiden hindeuteten (**Arbeit III**, Abb. 2B und 3). Die Lokalisation in den Nukleoiden konnte letztendlich durch Kolokalisation der Fluoreszenzsignale von den *AtSWIB-2:, AtSWIB-3: und AtSWIB-6:GFP*-Konstrukten und der Fluoreszenz eines *AtSWIB-1:RFP*-Konstrukts bestätigt werden (**Abbildung 4**, **Arbeit III**, Abb. 4).

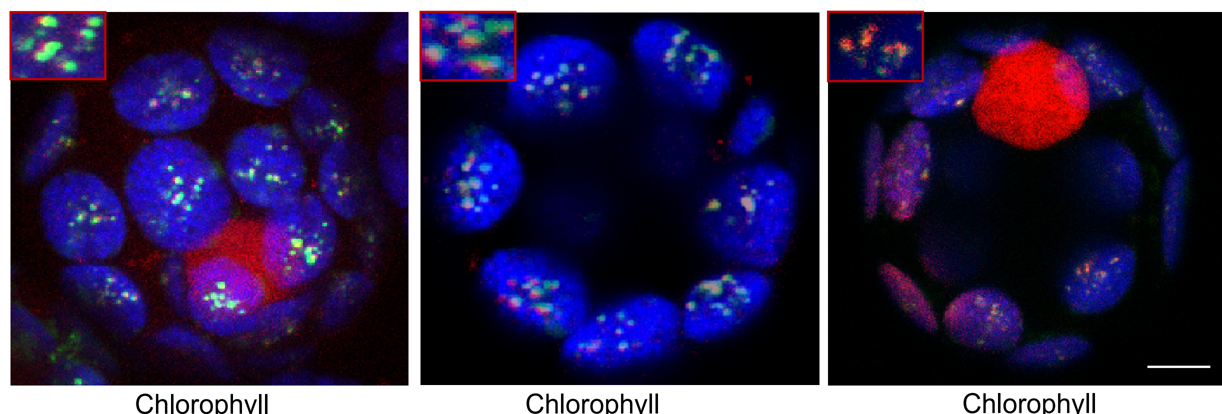


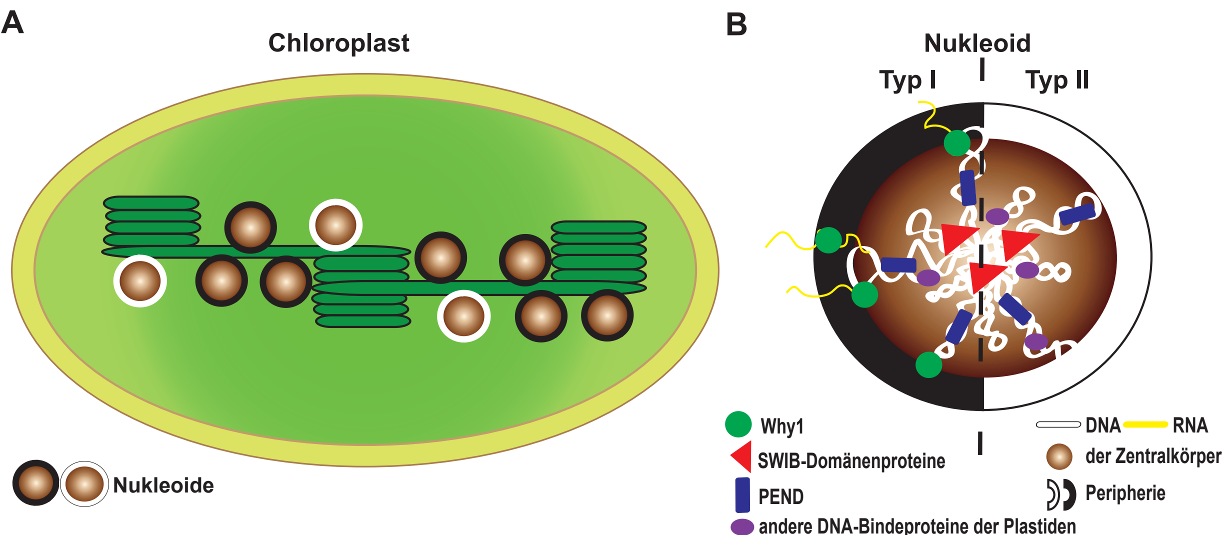
Abbildung 4: Kolokalisierung von AtSWIB-1:RFP mit den AtSWIB-2, -3 und -6:GFP-Proteinen in den Chloroplasten. Zur Erzielung eines besseren Kontrasts wurde die rote Fluoreszenz des Chlorophylls in blauer Farbe dargestellt. Der angegebene Maßstab entspricht 10 µm.

Die Überlagerung der Fluoreszenz der GFP- und RFP-Proteine zeigte jedoch, dass die Signale sehr oft nebeneinander lokalisiert waren **(Abbildung 4, Arbeit III**, Abb. 4B), was darauf hindeuten könnte, dass SWIB-1-Protein durch seine Histon H1-Domäne eine andere Funktion erfüllt und dadurch in einem anderen Bereich der Nukleoide als die AtSWIB-2-, -3- und -6-Proteine vorkommt.

Die Identifikation des SWIB-1-Proteins als TAC-Komponente ermöglichte die Entdeckung einer kleinen Familie von Chromatinmodellierungsfaktoren, die in den Organellen lokalisiert sind. Es ist zu erwarten, dass die Untersuchungen der Funktion von solchen Proteinen in den Organellen neue Hinweise auf die Regulation der Genexpression durch Veränderung der DNA-Topologie und Verpackung erbringen werden.

5. Heterogenität der plastidären Nukleoide

Untersuchungen zur Proteinzusammensetzung der plastidären Nukleoide ermöglichten die Identifizierung von neuen plastidären DNA-Bindeproteinen, die sowohl prokaryotischen als auch eukaryotischen Ursprungs waren (s. Kapitel 1.3, Tabelle 2, **Arbeit II** und **IV**). Eines der im TAC identifizierten eukaryotischen Proteine ist der Transkriptionsfaktor Whirly1 (Pfalz et al., 2006). Mittels Fusionen mit dem GFP-Protein konnte eine punktuelle Verteilung des Why1:GFP chimären Proteins in den Chloroplasten gezeigt werden (Krause et al., 2005). Die hochauflösenden mikroskopischen Aufnahmen, die aus den Kolokalisationsexperimenten stammten, zeigten, dass nur ein Teil der Whirly1:GFP-Signale mit den PEND:RFP-Fluoreszenzsignalen überlagerte (s. Kapitel 2, **Abbildung 2**, **Arbeit I,** Abb. 1). Dieses Ergebnis könnte darauf hindeuten, dass es unterschiedliche Typen von Nukleoiden in einem Chloroplast gibt (**Modell 1A**). Dies würde gut denkbar sein, da im Unterschied zu Bakterien, die nur ein Nukleoid besitzen, Chloroplasten über mehrere Nukleoide verfügen, deren Form, Anzahl und Verteilung sich innerhalb der Organellen während der Pflanzenentwicklung verändern (**Arbeit IV**).



Modell 1: Heterogenität der plastidären Nukleoide. (**A**) Heterogene Population der Nukleoide in einem Chloroplast. (**B**) Zwei Typen der Nukleoide, die unterschiedliche Proteinzusammensetzungen aufweisen.

Es könnte also möglich sein, dass die verschiedenen Nukleoide eines Chloroplasts im Hinblick auf Transkriptions-, Replikations- und Rekombinationsaktivität sowie DNA-Topologie und Proteinzusammensetzung unterschiedlich sind (**Modell 1A**). Untersuchungen zum Aufbau der Nukleoide von Hefezellen (*Saccharomyces ceravisiae*) zeigten, dass neben replizierenden auch nicht replizierende Nukleoide in einem Mitochondrium zu finden sind (Meeusen & Nunnari, 2003).

Die elektronenmikroskopischen Untersuchungen zum Aufbau der plastidären Nukleoide ergaben, dass sie aus einem Zentralkörper (*the central body*) und einer äußerem Schicht bestehen ((Briat et al., 1982), **Modell 1B**, **Arbeit IV**). Auch für den Aufbau der Nukleoide der humanen Zellen wurde eine zweischichtige Struktur (*layered structure*) vorgeschlagen, mit einem zentralen Kern als Ort der Transkription und Replikation und einer äußeren Schicht als Ort der Translation und Assemblierung der Komplexe (Bogenhagen et al., 2008; Shutt et al., 2010). Mit dem anti-HvWhy1-Antikörper konnte das Whirly1-Protein in der konventionell präparierten TAC-I-Fraktion und nicht in der hochaufgereinigten TAC-II-Fraktion nachgewiesen werden (**Arbeit I**, Abb. 3). Die Proteomanalysen der beiden TAC-Fraktionen zeigten, dass sie unterschiedliche Proteine enthalten (Krause & Krupinska, 2000, s. Kapitel 3.1, **Abbildung 3**, **Arbeit II**). Es ist anzunehmen, dass einige Proteine wie HvWhy1 während der Aufreinigung von TAC-I zu TAC-II vom TAC abgetrennt werden, da sie nur locker mit der DNA oder RNA assoziiert sind. Diese Proteine könnten in der Peripherie der Nukleoide, die in TAC-I noch enthalten sind, an posttranskriptionellen Prozessen beteiligt sein (**Modell 1**). Die Rolle des HvWhy1 Proteins in der Prozess-ierung der plastidären Transkripte konnte bereits in **Arbeit I** gezeigt werden.

Im Unterschied zu Whirly1 wurde das SWIB-1-Protein während der Aufreinigung der TAC-Fraktion angereichert (**Arbeit II**, Abb. 6A). Aus diesem Grund kann es als eine integrale Komponente der TAC-Fraktion bezeichnet werden, die wahrscheinlich an der Modellierung der plastidären DNA beteiligt ist (**Modell 1**). Ob SWIB-1 mit den anderen SWIB-Domänenproteinen der Plastiden kooperiert oder ob es vielleicht mehrere SWIB-Komplexe gibt, ist durch biochemische und zellbiologische Unter-suchungen zu klären.

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Lebenslauf

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Erklärung

Hiermit erkläre ich, dass die vorliegende Arbeit – abgesehen von der Beratung durch meine akademischen Lehrer – nach Inhalt und Form meine eigene Arbeit ist. Die Dissertation wurde bisher an keiner anderen Hochschule oder Universität vorgelegt. Ferner erkläre ich auch, dass ich noch keine früheren Promotionsversuche unternommen habe.

Kiel, den ....

Joanna Melonek

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ORIGINAL ARTICLE

Whirly1 in chloroplasts associates with intron containing RNAs and rarely co-localizes with nucleoids

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Abstract The nucleic acid binding protein Whirly1 of barley has been located to both chloroplasts and the nucleus of the same cell. Immunogold labelling furthermore showed that in vivo Whirly1 does not strictly co-localize with DNA in chloroplasts, while it is closely associated with DNA in the nucleus. High-resolution imaging of Whirly1-GFP and PEND-RFP fusion proteins revealed that only a minor part of Whirly1 co-localizes with nucleoids. The co-localization with nucleoids is in accordance with the detection of Whirly1 in a conventionally prepared fraction of the transcriptionally active chromosome (TAC). By further purification and enrichment of transcriptional activity Whirly1, however, was lost from the TAC fraction. Knockdown of Whirly1 in transgenic barley plants had neither impact on transcription of selected protein coding genes nor on genes coding for ribosomal RNAs or tRNAs. The results of RIP-chip experiments showed that barley Whirly1 as its maize orthologue associates with a set of intron containing plastid RNAs. Taken together, the results suggest that plastid-located Whirly1 functions primarily in RNA metabolism rather than as a DNA binding protein.

Keywords Whirly DNA binding proteins · Immunogold labelling · Barley RNAi knockdown plants · Plastid gene expression · RNA co-immunoprecipitation · Run-on transcription

Abbreviations

TAC	Transcriptionally active chromosome
RIP-chip	RNA co-immunoprecipitation and chip hybridization
sRNAP	Soluble RNA polymerase

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Introduction

The single stranded DNA binding factor Whirly1 belongs to a small family of plant-specific proteins with two members in most angiosperms and three members in *Arabidopsis thaliana* (Desveaux et al. 2005; Krause et al. 2005, 2009). By *in organello* import and localization of GFP fusion proteins, Whirly1 has been shown to be translocated into plastids (Krause et al. 2005). Moreover, immunological analyses revealed that the protein is located in chloroplasts and the nucleus of the same cell (Grabowski et al. 2008).

Crystallographic analysis revealed that Whirly1 of potato (StWhy1) has a cyclic quaternary structure inspiring

the name Whirly for this protein family. It has been proposed that StWhy1 binds as a tetramer to melted promoter regions, and might thus modulate transcription (Desveaux et al. 2002). In accordance with its binding to promoter sequences, in the nucleus Whirly1 has been shown to function as a transcription factor. Electrophoretic mobility shift assays indicated that nuclear Whirly1 in potato binds to the inverted repeat sequence of the elicitor response element (ERE) of the *PR10a* gene (Desveaux et al. 2000). Recently, chromatin immunoprecipitation revealed that the Whirly1 protein of *A. thaliana* together with Whirly3 in vivo can bind to the upstream region of the kinesin gene *AtKP1* and repress its transcription (Xiong et al. 2009). In another study it had been shown that Whirly1 in the nucleus is also involved in telomere maintenance (Yoo et al. 2007).

The attachment of the Whirly1 protein to DNA seems to be mediated by the KGKAAL domain (Desveaux et al. 2002, 2005). Bimolecular complementation assays with partial Whirly1-YFP fusion constructs have shown that in vivo Whirly1 interacts with itself when it is located in the nucleus (Grabowski et al. 2008). This result is in accordance with a function as a DNA binding tetramer. When the Whirly1-YFP fusion proteins were, however, delivered to plastids, no interaction of Whirly1 with itself was detectable (Grabowski et al. 2008). If tetramer formation would be a prerequisite for DNA binding, it is unlikely that Whirly1 functions as a DNA binding protein in plastids.

Plastid-located Whirly1 has been detected in the fraction of the transcriptionally active chromosome (TAC) (Pfalz et al. 2006) which suggested association of the Whirly protein to plastid DNA. Binding to plastid DNA was confirmed by nucleic acid co-immunoprecipitation with an antibody directed towards maize Whirly1 (Prikryl et al. 2008). However, binding to DNA was uniformly distributed over the entire plastid genome making it rather unlikely that Whirly1 in plastids is a gene-specific transcription factor. Transcriptional activities of plastid genes specific for ribosomal RNA and tRNA-G showed no difference between wild type and maize Why1 mutants, further speaking against an involvement in transcription (Prikryl et al. 2008). Recently, it has been suggested that plastid-localized Whirly proteins are involved in maintenance of plastid genome stability by preventing accumulation of illegitimate recombination products (Maréchal et al. 2009).

In this report, immunogold labelling and high-resolution imaging of Whirly1-GFP fusion proteins were used to re-examine the association of Whirly1 with plastid nucleoids. Both methods showed that the majority of Whirly1 in barley chloroplasts is not associated with DNA. Furthermore, the protein was shown to get lost from the transcriptionally active chromosome during purification. Transgenic barley plants with a knockdown of Whirly1 were shown to have a wild-type-like appearance, and did not show changes in transcription of a representative set of genes. We furthermore showed that HvWhy1 in chloroplasts associates with a set of intron containing RNAs. In contrast to the situation in maize, a knockdown of Whirly1 in barley had, however, no obvious effect on chloroplast development.

Materials and methods

Plant material

Barley (*Hordeum vulgare* L. cv. Steffi) seedlings used for preparation of TAC fractions were grown in a climate chamber for 7 days on vermiculite at 20°C in a light/dark regime of 16 h light and 8 h darkness. Transgenic barley plants (*Hordeum vulgare* L. cv. Golden Promise) with an RNAi knockdown of the *Whirly1* gene were prepared as described by Hensel et al. (2008). Lines E1 and E9 were shown to have one insertion of the RNAi cassette each, while line E6 had two insertions (Supplemental Fig. S1). In the T4 generation, lines E1 and E9 were found to be homozygous, whereas the E6 line was heterozygous. Plants of the T4 generation from the three lines E1, E6, E9 and of one mock line were cultivated in a glass-house for 21 days on soil under long day conditions (16 h light/8 h dark) at maximal 20°C. For transcription and protein analyses fully mature third and developing fourth leaves were used. For preparation of protoplasts, 5–6-week-old tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants grown on Murashige and Skoog medium were used.

Preparation of leaf segments and immunogold labelling

Small segments of barley leaves were fixed over night at 4°C in 4% (w/v) formaldehyde (freshly made from paraformaldehyde) in phosphate buffered saline solution (PBS), pH 7.4. After washing in PBS, the samples were dehydrated in a graded series of ice-cold ethanol and embedded in LR white resin (Plano, Marburg, Germany). Sections were cut with a diamond knife in a Leica Ultracut UCT ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany). Ultrathin sections were collected on pioloform-coated Au grids. After incubation with a primary antibody mixture of polyclonal anti-HvWhy1 (directed towards peptide P1, 1:10; Grabowski et al. 2008) from rabbit and monoclonal anti-DNA from mouse (1:50, clone AC-30-10, Chemicon/Millipore, Schwalbach, Germany) and washing, the sections were incubated with a mixture of the corresponding secondary antibodies (both diluted 1:50): anti-rabbit IgG, conjugated to 15 nm gold particles, and anti-mouse IgG, conjugated to 10 nm gold particles (British

BioCell International Ltd, Cardiff, UK). Finally, the sections were poststained with satured uranyl acetate in water and observed in a Philips CM10 transmission electron microscope (TEM, Philips Scientifics, Eindhoven, The Netherlands). The obtained pictures were further evaluated with the CELL* Imaging Software for Life Microscopy (Soft Imaging System GmbH, Münster, Germany). After measuring the area of chloroplasts and nuclei on printed images, the number of HvWhy1- and DNA-associated gold particles per 2 μm^2 section was calculated, and the distances between the HvWhy1- and DNA-associated particles were determined.

Transient expression of AtWhy1-GFP and PEND-RFP constructs in tobacco protoplasts

Protoplasts were isolated from tobacco leaves and were used for PEG-mediated transformation as described (Krause et al. 2005). AtWhy1-GFP and PEND-RFP constructs were already described by Krause et al. (2005) and Terasawa and Sato (2005), respectively. Distribution of GFP or RFP fusion proteins was inspected by confocal laser scanning microscopy and evaluated with the LAS FA SP5 software (Leica TCS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany).

Isolation of chloroplasts and preparation of stroma and thylakoid fractions

For immunological analysis, chloroplasts were prepared from primary foliage leaves of barley seedlings grown for 7 days by the procedure of Poulsen (1983). For fractionation of membranes and stroma, respectively, chloroplasts were osmotically lysed, and the membranes were enriched by centrifugation at 20,000×*g* for 10 min.

For run-on transcription assays, chloroplasts were prepared and purified on Percoll step gradients according to a standard procedure (Gruissem et al. 1986) modified as described (Krupinska 1992).

Isolation of transcriptionally active chromosome fractions (TAC-I and TAC-II)

The TAC-I fraction was prepared from barley chloroplasts by gel filtration on a Sepharose CL4B as described earlier (Suck et al. 1996). For further purification, the TAC-I fraction was subjected to precipitation by protamine sulphate and a second gel filtration on Sepharose CL4B column (Pharmacia, Freiburg, Germany) yielding TAC-II as described (Krause and Krupinska 2000). The transcriptional activity of TAC fractions was determined according to Krupinska and Falk (1994). One unit of transcriptional activity was defined as described by Suck et al. (1996) as 1 fmol radioactive-labelled UTP (α-^{32}P) incorporated into in vitro elongated RNA chains during 30 min at 30°C. Incorporation of nucleotides was measured with DE81 filters according to Suck et al. (1996).

Immunological analysis of protein levels

Total protein extracts prepared from barley leaves were isolated according to Dehesh et al. (1986). For immunological analyses, proteins in fractions eluting from Sepharose column during preparation of TAC-I were precipitated with methanol and chloroform according to the method of Wessel and Flügge (1984). Protein concentrations were determined according to (Bradford 1976) using Roti-Nanoquant according to the manufacturer's instructions (Roth, Karlsruhe, Germany).

Equal amounts of proteins were subjected to polyacrylamide gel electrophoresis employing a high concentration of Tris (Fling and Gregerson 1986), and were subsequently transferred onto nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Immunological analyses were carried out as described (Humbeck et al. 1996) using an ECL Western Blotting Detection Kit (GE Healthcare, Munich, Germany). For detection of HvWhy1 the antibody directed towards oligopeptide P2 (Grabowski et al. 2008) was used. Other antibodies used for immunological analysis were as follows: anti-MFP1-antibody (OSU91) directed towards nuclear-encoded MAR binding filament-like protein 1 (Jeong et al. 2003) and anti-TCP34 antibody directed towards *t*etratricopeptide-containing *c*hloroplast *p*rotein of *34* kDa (Weber et al. 2006). A specific antibody directed towards the α-subunit of plastid-encoded RNA polymerase (RpoA) had been previously provided by Dr. Hans Kössel (Suck et al. 1996; Krause and Krupinska 2000).

Determination of chlorophyll content

Pigments were extracted from barley leaves with 80% (v/v) acetone. Absorbance of chlorophylls was measured at 663 and 646 nm with a spectrophotometer (Shimadzu, UV-2501PC, Duisburg, Germany), and concentrations were calculated using the formula given by Lichtenthaler (1987).

Run-on transcription assays with isolated chloroplasts

Run-on transcription assays were performed with chloroplasts derived from barley third and fourth leaves as described (Krupinska and Apel 1989). DNA filters carrying 23 barley plastid genes blotted in a series of three dilutions: 320 (1×), 80 (4×) and 20 (16×) were prepared according to Krupinska (1992). After hybridization, the radioactively labelled filters were exposed to a phosphor imaging plate

(Fuji, Tokyo, Japan) and also to X-ray films (Hyperfilm MP, Amersham Biosciences, Freiburg, Germany). Radioactive signals measured by the image reader (FLA-5000, Fuji, Tokyo, Japan) were used for calculation of relative signal densities.

RNA co-immunoprecipitation assays

For chloroplast preparation and stroma extraction, 2-week-old barley plants were harvested and processed using conditions and procedures already established for maize (Schmitz-Linneweber et al. 2005). The antibody used to enrich HvWhy1 was described previously (directed against oligopeptide P2; Grabowski et al. 2008). Immunoprecipitation protocols, RNA labelling, hybridization of RNA on a maize chloroplast microarray and data analysis were carried out as reported previously (Schmitz-Linneweber et al. 2005). Control RIP-chip experiments were carried out using an antibody directed against the β-subunit of the chloroplast ATPase kindly provided by A. Barkan. Microarray probe positions and fluorescence data can be found in Supplemental Table S1.

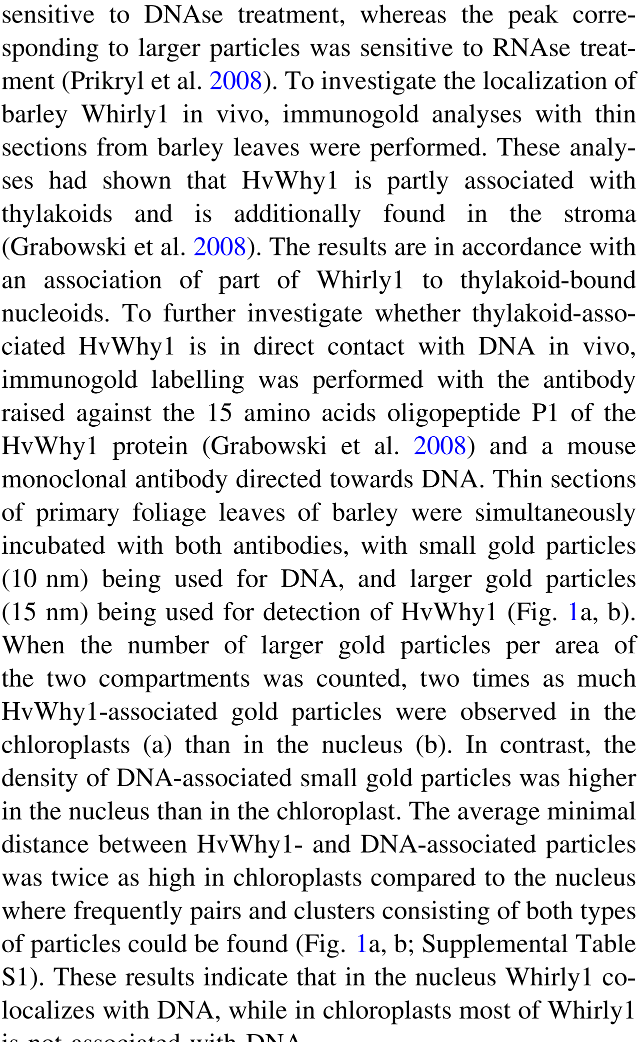
RNA gel blot analysis of selected plastid genes

RNA was extracted from third and fourth leaves of barley plants. Agarose gels and blotting onto nictrocellulose filters and hybridization were performed with standard procedures. Probes specific for selected barley plastid genes were amplified by PCR using following primer: *atpF* (exon2) 5′-GATTTATTAGATAATCGAAAACA-3′ and 5′-TTATCTCTTCCATTCCAGGG-3′; *atpF* (intron) 5′-AGGGAGTCTGTGCGAGTT-3′ and 5′-AATGAAAG TAGATTATTTTGTAAG-3′; *rpl16* (exon2) 5′-CTATA TCTCTACTATCGAATAAATT-3′ and 5′-AACCCCAAA AGAACCAGATT-3′; *rpl16* (intron) 5′-TCTCTAATTC CATAATATTTAAAA-3′ and 5′-CAACCTATTGCTTCG TATTG-3′. DNA fragments were radioactively labelled with α-^{32}PdCTP (Hartmann Analytic, Braunschweig, Germany) using the HexaLabel Kit (MBI Fermentas, St. Leon, USA). After hybridization, filters were exposed to X-ray films (Hyperfilm MP, Amersham Biosciences, Freiburg, Germany).

Results

The majority of chloroplast-located Whirly1 in vivo is not associated with DNA

ZmWhy1 was immunologically detected in two fractions of chloroplast stroma separated by sucrose density gradients centrifugation. The smaller of the two peaks was sensitive to DNAse treatment, whereas the peak corresponding to larger particles was sensitive to RNAse treatment (Prikryl et al. 2008). To investigate the localization of barley Whirly1 in vivo, immunogold analyses with thin sections from barley leaves were performed. These analyses had shown that HvWhy1 is partly associated with thylakoids and is additionally found in the stroma (Grabowski et al. 2008). The results are in accordance with an association of part of Whirly1 to thylakoid-bound nucleoids. To further investigate whether thylakoid-associated HvWhy1 is in direct contact with DNA in vivo, immunogold labelling was performed with the antibody raised against the 15 amino acids oligopeptide P1 of the HvWhy1 protein (Grabowski et al. 2008) and a mouse monoclonal antibody directed towards DNA. Thin sections of primary foliage leaves of barley were simultaneously incubated with both antibodies, with small gold particles (10 nm) being used for DNA, and larger gold particles (15 nm) being used for detection of HvWhy1 (Fig. 1a, b). When the number of larger gold particles per area of the two compartments was counted, two times as much HvWhy1-associated gold particles were observed in the chloroplasts (a) than in the nucleus (b). In contrast, the density of DNA-associated small gold particles was higher in the nucleus than in the chloroplast. The average minimal distance between HvWhy1- and DNA-associated particles was twice as high in chloroplasts compared to the nucleus where frequently pairs and clusters consisting of both types of particles could be found (Fig. 1a, b; Supplemental Table S1). These results indicate that in the nucleus Whirly1 co-localizes with DNA, while in chloroplasts most of Whirly1 is not associated with DNA.



The intraplastidic localization of Whirly1 fused to GFP was re-examined after transient transformation of protoplasts employing a high-resolution confocal microscope. As described previously (Krause et al. 2005) GFP was located to speckles throughout the chloroplast (Fig. 1c). Some of the speckles had a yellow colour indicating association with the red fluorescing thylakoids. Most of the speckles were, however, green. For comparison, a PEND-RFP construct was used for co-transformation with the Whirly1-GFP fusion construct. The *p*lastid *en*velope *D*NA binding (PEND) protein is known to bind to plastid DNA, and therefore, can be used for visualization of plastid nucleoids (Terasawa and Sato 2005). The signals obtained with the PEND-RFP protein showed similar speckled patterns as the GFP signals of the Whirly1-GFP protein. For better visualization, the red signals of RFP were converted into blue signals (Fig. 1d, e). In comparison to GFP, the RFP speckles were, however, more discrete and located in close proximity to the thylakoids (Fig. 1d). Merged images obtained for the two constructs revealed that most of the Whirly1-GFP fusion protein was

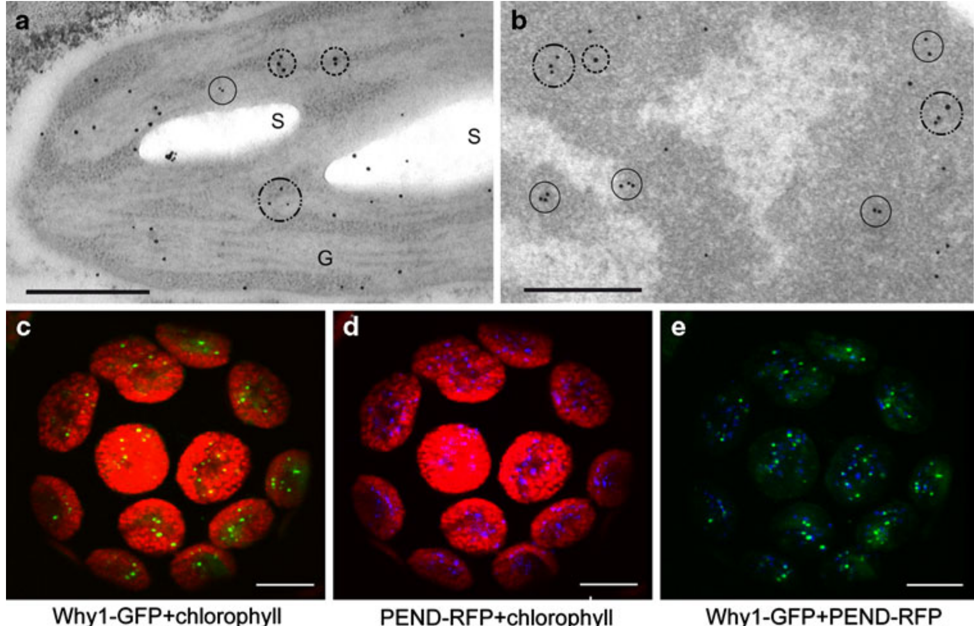


Fig. 1 Subcellular localization of the Whirly1 protein and DNA. Immunogold labelling of the HvWhy1 protein and DNA in chloroplasts (**a**) and nuclei (**b**) in ultrathin sections from barley leaves. Under these staining conditions the thylakoid membranes appear lighter than the stroma. HvWhy1-associated gold particles have a size of 15 nm, and DNA-associated particles have a size of 10 nm. Some clusters of particles are highlighted by *circles*. When they include only small particles, the *circle* is of a *full-line*; in case of clusters of large particles a *hatched line circle* and in case of clusters consisting of both types of particles a *circle of a complex line* was used. The number gold particles associated with Whirly1 and DNA, respectively, as wells as values for distances between HvWhy1- and DNA-associated gold particles are presented in Supplemental Table S1. *G* granum, *S* starch. *Scale bars* 500 nm. Distribution of the AtWhy1-GFP (**c**) and AtPEND-RFP (**d**) fluorescent signals in chloroplasts of tobacco protoplasts. The red fluorescence of the RFP protein was converted into a blue colour for better contrast. **e** Merged image of the fluorescent signals of the AtWhy1-GFP and AtPEND-RFP proteins. *Scale bar* 5.7 μm

not associated with the PEND-RFP fusion protein (Fig. 1e).

Whirly1 is a component of TAC-I, but not of highly purified TAC-II from barley chloroplasts

Plastid DNA of barley contains four elicitor response elements (ERE) which in the nucleus have been shown to be binding motifs for Whirly1 (Desveaux et al. 2000, 2002) and 12 similar elements with either one nucleotide exchanged or one nucleotide missing (Saski et al. 2007). Though the major fraction of Whirly1 in chloroplasts is not associated with DNA, a part of the protein pool could specifically bind to plastid DNA and might regulate transcription. Transcriptional activity in plastids can be found in a soluble fraction called soluble plastid RNA polymerase (sRNAP) and in a membrane-associated fraction called transcriptionally active chromosome (TAC) which is an elongation complex (Igloi and Kössel 1992). The TAC consists of both plastid DNA and nascent RNA chains as well as proteins attached to DNA or to RNA and being involved in posttranscriptional processes of plastid gene expression (Igloi and Kössel 1992; Lakhani et al. 1993; Krause and Krupinska 2000).

Whirly1 and 3 in *Arabidopsis thaliana* have been detected in a membrane derived fraction enriched in TAC (Pfalz et al. 2006). To examine whether Whirly1 in chloroplasts of barley is an intrinsic DNA binding protein of TAC, an antibody raised against barley Whirly1 (Grabowski et al. 2008) was used for Western blot assays with fractions collected during isolation of TAC-I by gel filtration. Immunological analysis of these fractions clearly showed that HvWhy1 co-elutes from the column with plastid DNA as shown by staining with ethidium bromide (Fig. 2).

Chloroplast membranes were used as a starting material for preparation of TAC (Hallick et al. 1976; Krause and Krupinska 2000). Accordingly, Whirly1 was immunologically detected in a chloroplast membrane fraction (Fig. 3). Similar to already identified TAC components such as RpoA (α-subunit of the plastome-encoded RNA polymerase; Suck et al. 1996) and TCP34 (*t*etratricopeptide-containing *c*hloroplast *p*rotein of *34* kDa, Weber et al. 2006), the abundance of Whirly1 was higher in the stroma fraction than in the membrane fraction (Fig. 3). Such a distribution of TAC proteins between membrane fraction and stroma is in accordance with the concept that TAC is a membrane-bound elongation complex, and the components

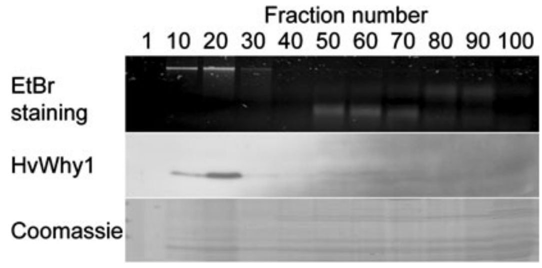


Fig. 2 HvWhy1 co-elutes from the Sepharose CL4B column with plastid DNA. After gel filtration 20 µl of every tenth fraction was loaded on an agarose gel containing ethidium bromide. The plastid DNA was present in fractions *10–30*. The staining revealed some additional bands in the fractions *50–90*, which could represent free plastid RNAs or tRNAs complexes. Additionally, proteins of the same fractions were analysed by polyacrylamide gel electrophoresis, transferred to nitrocellulose membrane and immunologically analysed with an antibody directed against the peptide P2 of barley Whirly1 protein. As a loading control, the Coomassie brilliant blue staining of proteins having molecular weights in the range from 20 to 40 kDa is shown

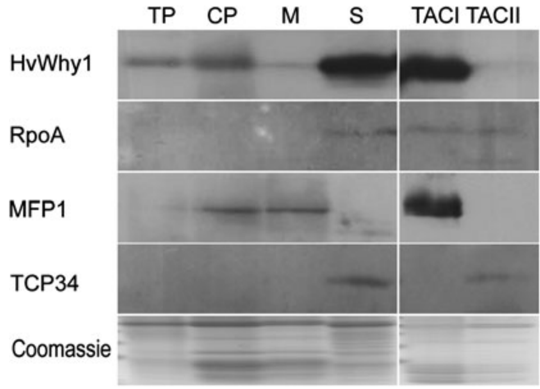


Fig. 3 Detection of the HvWhy1 protein in the TAC-I but not in the TAC-II fraction. SDS-PAGE was performed with 15 µg of total protein extract from barley leaves (*TP*), chloroplasts (*CP*), chloroplast membranes (*M*) and stroma (*S*), respectively. Aliquots of the transcriptionally active chromosome TAC-I and TAC-II fractions having the same transcriptional activity (17,000 U) corresponding to about 20 and 7 µg protein, respectively, were analysed in addition. Immunoreactions were performed with antibodies directed against HvWhy1, RpoA (α-subunit of plastome-encoded RNA polymerase), MFP1 (MAR binding filament-like protein 1) and TCP34 (tetratricopeptide-containing chloroplast protein of 34 kDa). As a loading control, Coomassie brilliant blue staining of the gel in the range of proteins with molecular weights from 20 to 50 kDa is shown

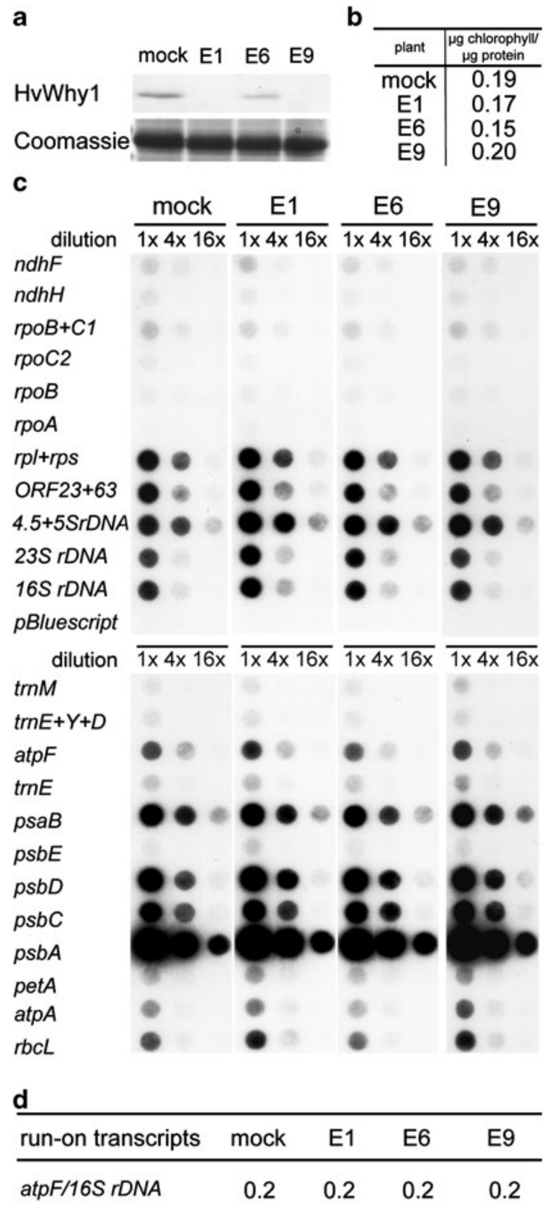
involved in transcription are also found in the soluble fraction of the plastid RNA polymerase (sRNAP) (Igloi and Kössel 1992).

To examine whether the presence of Whirly1 in the TAC fraction correlates with the transcriptional activity of the fraction, we compared its level in TAC-I with the level in the TAC-II extract which has been purified from TAC-I by precipitation and a second gel filtration (Krause and Krupinska 2000). Transcriptional activity based on protein content increased threefold during purification of TAC. Aliquots of TAC-I and TAC-II fractions having the same transcriptional activity but differing in protein content were loaded onto a gel. While the level of the α-subunit of RpoA was similar between TAC-I and TAC-II, the level of the HvWhy1 protein was clearly decreased in TAC-II compared to TAC-I (Fig. 3). For comparison, further proteins known to be TAC components were analysed for their distribution between TAC-I and TAC-II. The level of MAR binding filament-like protein 1 (MFP1), similar to the level of Whirly1, decreased during purification of TAC-I, while the level of TCP34 increased (Fig. 3). While the level of RpoA correlated with the transcriptional activity of TAC fractions, the levels of TCP34 and MFP1 did not correlate with transcriptional activity of the fractions. These results indicate that neither these two proteins nor Whirly1 in chloroplasts are part of the functional core of the transcriptional apparatus of plastids.

Plastid gene transcription is not affected in transgenic barley plants with a knockdown of Whirly1

To investigate whether Whirly has an effect on transcription in chloroplasts, run-on transcription assays were performed with chloroplasts from three independent barley Whirly1 RNAi knockdown lines and from a mock control line (Supplemental Fig. S1). To examine the expression level of the Whirly1 protein in the RNAi lines and in control plants, protein extracts prepared from leaf material were immunologically analysed. In the homozygous lines E1 and in E9 Whirly1 was almost undetectable, whereas in the heterozygous E6 line Whirly1 level was about 10–20% in comparison to the control plants (Fig. 4a). In contrast to the ivory and pale green phenotypes of Whirly1 maize mutants (Prikryl et al. 2008), leaves of barley Whirly1 knockdown showed a wild-type-like appearance as also reported for Arabidopsis Whirly1 T-DNA insertion mutants (Yoo et al. 2007; Maréchal et al. 2009). Accordingly, the chlorophyll content of the Whirly1 knockdown lines was similar to that of the mock line used as a control (Fig. 4b).

Run-on transcription assays were performed with mature third and fourth leaves. Radiolabelled run-on transcripts were hybridized with DNA dot-blot filters carrying a representative selection of 23 plastid gene-specific probes as reported earlier (Krupinska and Falk 1994). Visually, no obvious differences were observed between hybridization signal intensities obtained with the control line and the transgenic lines, respectively (Fig. 4c). Ratios of relative transcriptional activities of selected plastid genes calculated from values obtained by densitometric scanning of



plant	μg chlorophyll/ μg protein
mock	0.19
E1	0.17
E6	0.15
E9	0.20

run-on transcripts	mock	E1	E6	E9
atpF/16S rDNA	0.2	0.2	0.2	0.2
psbA/16S rDNA	10.1	10.7	11.3	13.1
16S rDNA/23S rDNA	1.3	1.0	1.3	1.0
rpl+rps/16S rDNA	2.0	2.0	2.0	2.5

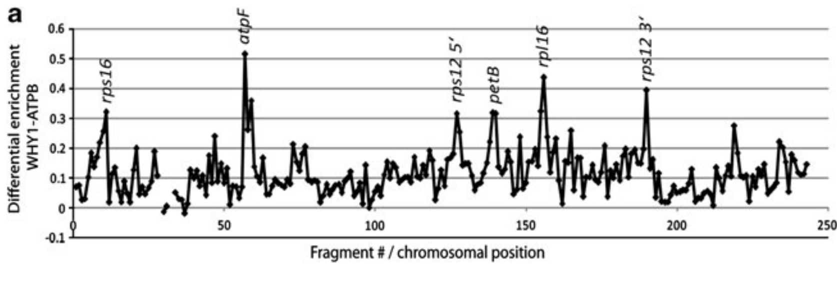
◀ **Fig. 4** Characterization of the barley Whirly1 RNAi lines. **a** Levels of the Whirly1 protein in the knockdown lines and in the mock plants. Equal amounts of protein from leaves from RNAi lines (*E1*, *E6* and *E9*) and mock plants were separated by polyacrylamide gel electrophoresis and after transfer immunologically analysed for the presence of the HvWhy1 protein. As a loading control, the Coomassie blue staining of the large subunit of Rubisco and two subunits of the ATPase having molecular weight of about 55 kDa are shown. **b** Chlorophyll content of chloroplasts prepared from leaves of the RNAi lines. **c** Hybridization patterns obtained with the run-on transcripts isolated from chloroplasts. ^{32}P-labelled transcripts were hybridized to identical blots containing DNA fragments representing 23 plastid genes and the control plasmid DNA pBluescript in three dilutions (1×, 4× and 16×). **d** Ratios of the relative transcriptional activities of selected plastid genes determined by run-on transcription assays with chloroplasts from barley Whirly1 RNAi lines (*E1*, *E6* and *E9*) and mock plants

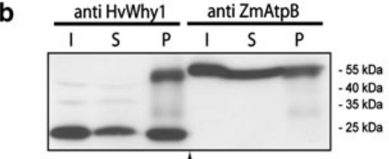
the autoradiograms as described previously (Krupinska and Falk 1994) were almost identical (Fig. 4d).

Barley Whirly1 associates with at least five chloroplast RNAs containing group II introns

To further examine whether HvWhy1 in like manner as ZmWhy1, might interact with plastid RNA and its association with TAC-I might be related to a function in posttranscriptional processes, the specific antibody raised towards the HvWhy1 protein was used for RIP-chip experiments with chloroplast stromal extracts isolated from barley leaves. To verify precipitation of the HvWhy1 protein, a control Western blot experiment was performed with aliquots from input, supernatant and pellet protein fractions obtained during the immunoprecipitation protocol (Fig. 5b). A protein of about 24 kDa detected in HvWhy1 immunoprecipitation pellets (IPs) corresponds in size to Why1 and is enriched in the pellet (P) fraction. A fraction of the protein remained in the supernatant (S). An additional band at the size of about 50 kDa appeared on the blot, and most probably represents some unspecific cross-reaction of the antibody.

Nucleic acids purified from the immunoprecipitation pellet were used as the experimental sample and labelled with the red fluorescing dye Cyanine 5 (Cy5), whereas nucleic acids prepared from the supernatant were used as a reference and labelled with the green fluorescing dye Cyanine 3 (Cy3). The labelled RNAs were then combined and hybridized to a microarray representing the complete plastid genome of maize (Schmitz-Linneweber et al. 2005). Only peaks three times above the median value of 0.10 of all differential signals on the array were considered further. By this approach, six prominent peaks corresponding to *atpF*, *rps16*, *rpl16*, *petB* and both parts of the trans-spliced *rps12* transcripts could be detected (Fig. 5a; Supplemental Table S2). With the exception of *rps16* and *petB*, these transcripts were also found to associate with the Whirly1 protein of maize (Prikryl et al. 2008). All transcripts co-immunoprecipitated with HvWhy1 have introns. In contrast to ZmWhy1, HvWhy1 did not co-precipitate with transcripts from *rpoC*, *rps14*, *petD*, *orf99/173*, *trnR* and *ndhA* genes (Fig. 5a). With the exception of *petD* and *ndhA*, these transcripts do not have introns. To investigate whether the association of HvWhy1 with RNA species containing introns has an impact on processing of the transcripts, Northern blot analyses were performed with RNA prepared from third





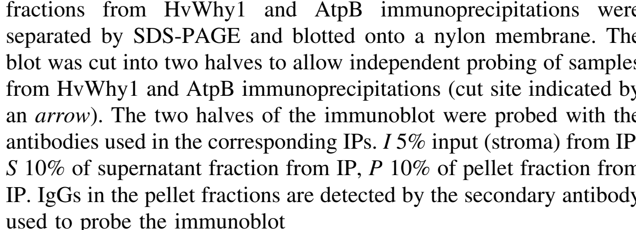


Fig. 5 Association of Whirly1 with chloroplast transcripts. **a** RIP-chip analysis of HvWhy1. The enrichment ratios (FCy5:FCy3) were normalized between two assays involving anti-HvWhy1 antibody and two assays involving AtpB antibody. The median normalized values for replicate spots from the AtpB data were subtracted from those from Whirly1 data and plotted according to fragment number. Fragments are numbered according to chromosomal position. The data used to generate this figure are provided in Supplemental Table S2. **b** Western analysis to verify precipitation of HvWhy1. Aliquots of fractions from HvWhy1 and AtpB immunoprecipitations were separated by SDS-PAGE and blotted onto a nylon membrane. The blot was cut into two halves to allow independent probing of samples from HvWhy1 and AtpB immunoprecipitations (cut site indicated by an *arrow*). The two halves of the immunoblot were probed with the antibodies used in the corresponding IPs. *I* 5% input (stroma) from IP, *S* 10% of supernatant fraction from IP, *P* 10% of pellet fraction from IP. IgGs in the pellet fractions are detected by the secondary antibody used to probe the immunoblot

and fourth leaves of transgenic barley lines showing a knockdown of Whirly1 (E1, E6 and E9) and from mock control plants. To confirm equal loading, the RNA separated by agarose gel electrophoresis was stained by ethidium bromide. In contrast to the ZmWhy1 mutants (Prikryl et al. 2008), but in accordance with the wild-type-like appearance of the barley Whirly1 knockdown plants (Maréchal et al. 2009), abundance of ribosomal RNA was not affected by the knockdown of Whirly1 (Fig. 6a). Hybridization with *atpF*-specific probes revealed that the abundance of *atpF* transcripts was increased in the Whirly1 knockdown plants compared to mock plants. A comparison between the hybridization pattern obtained with an intron-specific probe and an exon-specific probe, respectively, showed that specifically the abundance of spliced RNA was decreased in Whirly1 knockdown plants (Fig. 6a, b). In contrast, unspliced precursor transcripts overaccumulated in leaves of the transgenic plants in comparison to the wild type. This result is in accordance with the finding that in ZmWhy1 mutants the ratio of spliced to unspliced *atpF* transcripts is lower than in wild-type plants (Prikryl et al. 2008). The findings suggest that Whirly1 in barley as well as in maize affects the splicing of *atpF* transcripts. Another transcript immunoprecipitated by the antibody specific for HvWhy1 was *rpl16*. Northern blot analyses with probes specific for an exon and the intron, respectively, did not show significant differences in splicing (Fig. 6c, d). This result is also in accordance with the results obtained with RNA from ZmWhy1 mutants (Prikryl et al. 2008).

Discussion

Whirly proteins have been described as DNA binding proteins involved in regulation of transcription and telomere maintenance in the nucleus (Desveaux et al. 2005; Yoo et al. 2007; Krause et al. 2009). Very recently, it has been furthermore proposed that plastid-located Whirly1 and Whirly3 in *A. thaliana* are involved in maintenance of plastid genome stability (Maréchal et al. 2009). Such an organellar function is in accordance with the binding of Whirly proteins to DNA. Considering, however, that Whirly1 was shown to bind to DNA as a tetramer (Desveaux et al. 2002), it is unlikely that Whirly1 functions in chloroplasts as a DNA binding protein because bimolecular fluorescence complementation did not reveal interaction of Whirly1 with itself in plastids while the interaction in the nucleus was clearly detectable (Grabowski et al. 2008). In *Arabidopsis thaliana* plastidic Whirly proteins were found in the proteome of TAC (Pfalz et al. 2006), and in maize ZmWhy1 was shown to co-sediment with DNA complexes (Prikryl et al. 2008). Association of a protein with the TAC fraction does, however, not necessarily mean that the protein is a DNA binding protein. TAC has a rather complex protein composition and includes also proteins binding to RNA (Igloi and Kössel 1992; Lakhani et al. 1993; Krause and Krupinska 2000). Our new data on in vivo localization of barley Whirly1 together with the results on immunoprecipitation of Whirly1-associated RNA species suggest that plastid Whirly1 is rather an RNA binding protein than a DNA binding protein.

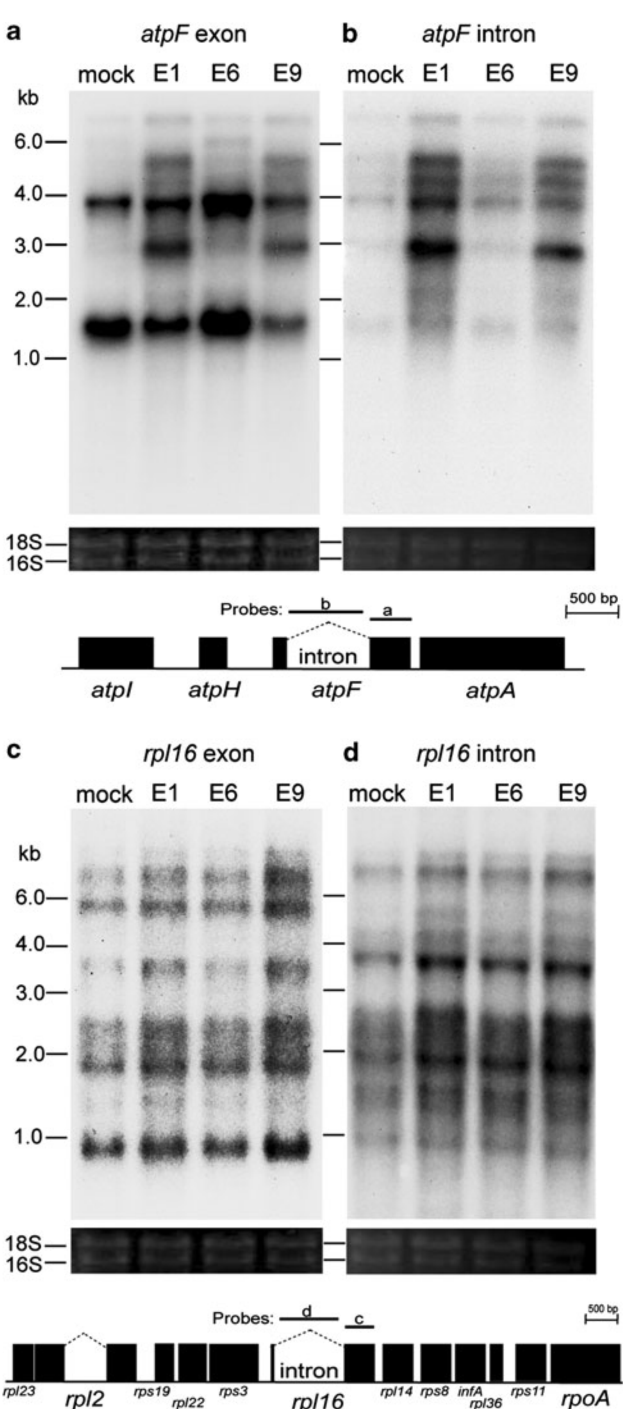


Fig. 6 Impact of barley Whirly1 on the abundance of the *atpF* and *rpl16* transcripts. RNA gel blot analysis of three RNAi lines and mock plants. Total RNA isolated from 4-week-old barley plants was loaded onto a denaturing agarose gel and after transfer onto a nitrocellulose membrane incubated with probes for **a** exon and **b** intron of the *atpF* gene, respectively and **c** exon and **d** intron of the *rpl16* gene, respectively. As a loading control, the ethidium bromide staining of the plastidic ribosomal 16S rRNA and the cytoplasmic 18S rRNA is shown. Maps of the polycistronic transcription units containing the barley *atpF* and *rpl16* genes are shown. Coding regions are indicated by *shaded boxes* and the introns by *dashed line*. The regions used for hybridization probes are marked

Co-localization with DNA in vivo was investigated by immunogold labelling with the Whirly1- and a DNA-specific antibody. In comparison to the nucleus, where Whirly1 was found in close contact with DNA, in chloroplasts rarely an association of Whirly1 with DNA was found. This result is in accordance with our previous findings that Whirly1 in the nucleus interacts with itself allowing the formation of DNA binding tetramers while in plastids no such interaction was detectable (Grabowski et al. 2008). The results on immunolocalization of Whirly1 in chloroplasts are somehow contradictory to our earlier attempts to localize Whirly1 in chloroplasts by transient transformation of protoplasts with a Whirly1-GFP fusion construct (Krause et al. 2005). GFP fusion proteins were detected in speckles throughout the chloroplasts, and these speckles were discussed to potentially represent nucleoids. We therefore re-examined the localization of Whirly1-GFP fusion proteins in comparison to PEND-RFP fusion proteins by high-resolution confocal laser scanning microscopy. By this approach we could demonstrate that only few of the previously observed Why1-GFP speckles might represent nucleoids which were now clearly visualized by the PEND-RFP fusion protein in the same protoplasts expressing the Whirly1-GFP construct (Fig. 1c, d).

By proteome analysis, Whirly1 of *Arabidopsis thaliana* was detected in a fraction containing the transcriptionally active chromosome of chloroplasts (Pfalz et al. 2006). By immunological analysis, the distribution of Whirly1 between stroma and membrane fractions was shown to be similar to that of other TAC components, e.g. RpoA and TCP34. Our immunological analysis furthermore confirmed that Whirly1 is contained in a conventionally prepared TAC fraction (Fig. 2). When, however, this TAC fraction (TAC-I) was further purified to give TAC-II which has a higher specific transcriptional activity based on protein content as described by Krause and Krupinska (2000), only traces of the protein could be detected (Fig. 3). In contrast to the α-subunit of RpoA, the abundance of Whirly1 therefore does not correlate with transcriptional activity of the fractions. This result suggests that Whirly1 in chloroplasts is not part of the functional core of the transcriptional apparatus.

To further examine whether Whirly1 might affect transcription in plastids, run-on transcription assays were performed with barley Whirly1-RNAi plants having reduced levels of Whirly1. In comparison to Prikryl et al. (2008) who tested transcription of two ribosomal RNA genes and one *trn* gene, we extended the analyses to genes from different functional groups including genes encoding proteins of the photosynthetic apparatus. Although Whirly1 was almost undetectable in the homozygous lines, no changes in relative transcriptional activities of a

representative set of genes were observed between transgenic lines and control lines.

Nucleoids of human mitochondria were shown to have a layered structure with a central core as the site of transcription and replication and a peripheral zone (Bogenhagen et al. 2008). The results of our study suggest that Whirly1 is not an intrinsic component of the plastid nucleoid core. During association with the periphery of the nucleoids it might, however, be involved in posttranscriptional processes. By RNA co-immunoprecipitation and chip hybridization, several RNA species were identified as binding partners of ZmWhy1 (Prikryl et al. 2008). Some of these such as *atpF* contain introns, and Northern blot analyses showed that the abundance of unspliced *atpF* transcripts was enhanced in Whirly1 mutants (Prikryl et al. 2008). Using the same methodological approach, we identified several of the plastid RNA species binding to ZmWhy1 also to be bound to HvWhy1, e.g. a*tpF* and *rpl16*. While HvWhy1 was found to bind to intron containing RNAs only, the ZmWhy1 protein was shown to bind also to transcripts devoid of introns such as *rpoC*, *rps14*, *trnR* and *orf99/173*. Furthermore, we showed here that the *rps16* and the *petB* mRNAs are putative barley-specific ligands of Whirly1. These differences in RNA ligands might reflect differences in the properties of the two Whirly1 proteins. However, we cannot rule out that usage of the maize microarray for analysis of barley transcripts might have prevented detection of certain transcripts.

The differences in RNA species identified as binding partners of Whirly1 in maize and barley could be responsible for the differences in the phenotype of Whirly1 mutants of maize and barley Whirly1 knockdown plants. While maize mutants are severely disturbed in chloroplast development, the transgenic barley plants lacking Whirly1 have a similar chlorophyll content as the control plants. The barley plants thereby resemble the Whirly1 T-DNA insertion mutants described for *Arabidopsis thaliana* (Yoo et al. 2007; Maréchal et al. 2009). RNA analyses moreover showed that the abundance of plastidic ribosomal RNAs was neither affected by the knockdown of Whirly1 in barley nor by the T-DNA insertion in the Arabidopsis Whirly1 (Maréchal et al. 2009). Among the transcripts precipitated in maize and not in barley is the *rpoC* transcript. It is well known that plants with disrupted *rpoB/C* operon are disturbed in chloroplast development (De Santis-Maciossek et al. 1999). Because the *rpoB/C* operon has no intron, binding of Whirly1 to the transcript could be related to other processing events which might be affected in maize Whirly1 mutants.

Taken together, our analyses showed that Whirly1 predominantly is a stromal protein. Only a minor fraction was found to be loosely associated with the transcriptionally active chromosome where it could be involved in posttranscriptionally processes as suggested also by binding of HvWhy1 to plastid RNA. Our data demonstrate that Whirly1 in barley is not involved in maturation and abundance of ribosomal RNA. Similarly as in maize it was, however, shown to associate with a subset of intron containing plastid RNAs such as *atpF* and *rpl16*. Though in case of the *atpF* transcript splicing might be affected by Whirly1, this is not the case with the *rpl16* transcript, both in barley and in maize (Prikryl et al. 2008). This suggests that binding of Whirly1 to intron containing RNA species not necessarily has consequences for splicing. At least in maize Whirly1 was shown to bind also to transcripts not having introns such as *rpoC*, *rps14*, *orf99/173* and *trnR-ACG/orf23*. This suggests that Whirly1 could also play a role in other processing events which maybe affected in the Whirly1 maize mutants.

As already discussed by Maréchal et al. (2009) plastidic Whirly proteins fulfil a variety of functions depending on the cellular context and even species. As the expression of the Whirly1 gene in barley is under developmental control (Krupinska et al., unpublished), it is likely that the function of Whirly1 in plastids depends on the developmental stage of the organelle. As long as the developmental stage of the plastids investigated is not precisely defined, it is hard to compare results from studies with different plant material. Taking advantage of the developmental gradient of cells in a monocot leaf (Mullet 1988; Krupinska and Falk 1994), in future studies the intraplastidic distribution and functionality of plastidic Whirly1 at different stages of development will be investigated.

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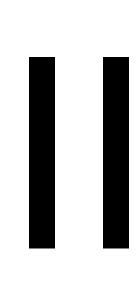

A truncated form of the nuclear SWIB-1 protein is a component of higher plant chloroplast nucleoids

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Abstract

Plastid nucleoid-associated proteins (ptNAP) of a highly purified fraction of the transcriptionally active chromosome (TAC) from chloroplasts of spinach (*Spinacia oleracea* L.) were analysed by two-dimensional gel electrophoresis (2-DE) and mass spectrometry. Among the 85 protein spots detected six spots were predicted to represent DNA binding proteins. The smallest of these proteins showed homology with Arabidopsis proteins containing a SWIB domain. Out of these candidates we selected SWIB-1 for functional characterization. By fusion with GFP this SWIB-1 (SWIB domain-containing protein-1) protein was shown to be dually targeted to plastids and to the nucleus. In chloroplasts SWIB-1 was shown to co-localise with nucleoids. Immunoblot analyses showed that the unprocessed form of the SWIB-1 protein having an apparent molecular mass of 19 kDa is present in nuclei while a 17 kDa form is enriched in fractions of chloroplast membranes and TAC, respectively. Binding of the SWIB-1 protein to plastid DNA was shown by Southwestern analysis.

Introduction

Cells of green plants typically contain thousands of plastid genomes (ptDNA) in form of circular 120-180 kbp molecules organised in aggregates which in analogy to the bacterial nucleoid were named plastid nucleoids (Kuroiwa et al, 1998; Sato et al, 2003). The plastid nucleoid-associated proteins (ptNAPs) required for the development dependent dynamics of nucleoid morphology and organisation within the plastids have not yet been identified (Sakai et al, 2004; Sato et al, 2003). In chloroplasts nucleoids are attached to the thylakoids. When thylakoid membranes were treated with the non-ionic detergent Triton X-100 a fraction named transcriptionally active chromosome (TAC) was obtained (Igloi & Kossel, 1992). TAC fractions have been prepared from chloroplasts of *Euglena gracilis* (Hallick et al, 1976), mustard (Bülow et al, 1987), barley (Krupinska & Falk, 1994; Suck et al, 1996), spinach (Krause & Krupinska, 2000) and recently also from *Arabidopsis thaliana* (Pfalz et al, 2006). The conventionally prepared TAC fraction (TAC-I) has a rather complex protein composition and was further purified by precipitation with protamine sulfate and re-solubilisation in the presence of heparin before it was subjected to a second round of gel filtration (Krause & Krupinska, 2000). Thereby a TAC-II fraction with a higher transcriptional activity was obtained. By one-dimensional gel electrophoresis 30-40 proteins were resolved (Krause & Krupinska, 2000). By immunological analyses subunits of the plastid encoded RNA polymerase (PEP) were identified in TAC fractions from barley (Suck et al, 1996) and spinach (Krause & Krupinska, 2000).

Apart from the proteins of the transcriptional apparatus the TAC fraction is expected to contain a number of nucleoid-associated proteins with DNA-binding properties which might be involved in replication, recombination and repair of DNA as well as in structuring of nucleoids (Sakai et al, 2004). In bacteria small molecular weight proteins such as the 9 kDa HU proteins and HU-like proteins (HLP) are responsible for the formation of nucleosome like structures (Dillon & Dorman, 2010) and also affect DNA-associated processes such as replication and transcription (Dillon & Dorman, 2010; Dormann & Deighan, 2003; Kamashev et al, 2008). Genes encoding potential counterparts of the bacterial nucleoid-associated proteins have, however, not been found in the genomes of higher plants (Riechmann et al, 2000; Sato et al, 2003). Among the few structural proteins of plastid nucleoids thus far identified in higher plants are two bifunctional proteins, SiR (sulfite reductase) and the protease CND41 (chloroplast nucleoid DNA-binding protein) (Sakai et al, 2004). SiR was shown to induce compaction of plastid nucleoids coinciding with a repression of

transcriptional activity (Sekine et al, 2002). Another DNA-binding protein of plastid nucleoids is PEND (plastid envelope DNA-binding protein) which was proposed to anchor nucleoids to plastid membranes (Sato et al, 1993). It has been isolated together with PD1 (plastid DNA binding 1) and PD3 (plastid DNA binding 3) (Sato et al, 1997) which are AT-hook proteins related to the nuclear chromatin remodelling HMG proteins (Grasser, 1995). It has been recently hypothesized that plastids during evolution have recruited nuclear proteins with eukaryotic DNA-binding domains for structural remodelling of their nucleoids (Kodama, 2007).

Though their sequences have not yet been identified, several DNA-binding proteins have been biochemically detected in thylakoids and nucleoid fractions (Baumgartner & Mullet, 1991; Briat et al, 1984; Bülow et al, 1987; Nemoto et al, 1988b; Yurina et al, 1988) and could have functionally replaced the bacterial histone-like HU proteins. Among these proteins are several low molecular weight proteins (10-20 kDa) which have been detected by cross-reaction with antibodies directed towards the bacterial HU proteins (Briat et al, 1984), by binding to plastid DNA (Baumgartner & Mullet, 1991; Bülow et al, 1987) or by their biochemical properties which placed them among the DNA-binding proteins (Nemoto et al, 1988b).

Proteome analyses performed with TAC fractions from mustard and *A. thaliana* did not identify DNA binding proteins with molecular weight below 20 kDa. Nevertheless, among the proteins of higher molecular weights, several novel TAC-associated proteins (PTAC) with DNA/RNA-binding domains were identified (Pfalz et al, 2006). PTAC1 is identical with the dual targeted Whirly1 (Grabowski et al, 2008) and was shown to function as a transcription factor in the nucleus (Desveaux et al, 2002; Desveaux et al, 2005; Krause et al, 2009) while its plastidic form was shown to bind to a subset of intron-containing RNA species (Melonek et al, 2010; Prikryl et al, 2008). The PTAC11 protein (Pfalz et al, 2006) is identical with Whirly3 which recently was described to function together with Whirly1 in plastids as antirecombination protein (Maréchal et al, 2009). PTAC14 from *A. thaliana* was identified as a SET-domain containing protein. Such ptNAP proteins belong to those acquired from the nucleus where they are known to be involved in chromatin remodelling (Jacob et al, 2009). It is striking that several of these proteins such as Whirly1 and also the PEND protein (Terasawa & Sato, 2009) might have kept their original location in the nucleus while becoming ptNAP proteins.

Although several DNA-binding proteins have been previously identified in plastids with biochemical methods, the small molecular weight proteins predicted to function as HU like

proteins have not been identified. Here we report on the identification of a 17 kDa SWIB domain-containing DNA-binding protein in a highly purified TAC fraction from spinach chloroplasts. A 19 kDa form of the protein was shown to be located in the nucleus by immunoblot analyses. This is the first chromatin remodelling associated protein described to be located in plastids and the nucleus.

Results

Identification of the SWIB-1 protein in the proteome of the TAC-II fraction

The protein compositions of TAC-I and of highly purified TAC-II from spinach chloroplasts were analysed by 2-DE. For isoelectric focussing (IEF) pH gradients from 3 to 10 were chosen. After second dimension denaturing gel electrophoresis, proteins were visualised by Coomassie blue staining. The protein compositions of TAC-I and TAC-II were observed to differ in number and relative intensities of protein spots (Fig. 1A, B). Visual inspection of the protein pattern of TAC-I allowed the identification of 132 spots on the 2-D-gel of TAC-I whereas on the 2-D-gel of TAC-II only 85 protein spots were discernible. This indicates that the complexity of TAC fractions is much higher than the Coomassie blue stained one-dimensional gels suggested (Krause & Krupinska, 2000). The protein spots identified in TAC-I and in TAC-II, respectively, were compared by Progenesis PG240 software to identify those proteins which were enriched in TAC-II. The proteins detected only in the TAC-I fraction were not used for further analyses.

All 85 protein spots of TAC-II were manually excised from the gel and digested with trypsin (Supplementary Figure 1). By this approach 47 TAC-II proteins with scores of 60 or more could be identified (Supplemental Table 1). 17 of them were identified by homology with protein sequences of *Arabidopsis thaliana*. Twelve proteins were identified by homologies with protein sequences from rice and nine by homology with sequences of spinach (*Spinacia oleracea*). Single proteins were identified by homologies with proteins of *Chenopodium quinoa, Medicago truncatula, Phytolacca americana, Chlamydomonas reinhardtii, Syntrophobacter fumaroxidans MPOB, Cryptosporidium hominis TU502* and two of *Salix gilgiana.*

According to their putative functions the identified 47 TAC-II proteins (Supplementary Table 1) can be grouped in three major categories. In the first group all proteins with putative roles in transcription, replication and maintenance and structuring of DNA are gathered. As

expected, subunits of the plastid-encoded RNA polymerase (PEP) namely RpoC1 (spot 13) and RpoC2 (spot 16) were detected in TAC-II. The RpoA subunit was identified with a score under 60 and therefore was not included in Supplementary Table I. The second group contains proteins putatively involved in protein translation and metabolism, e.g. EF-Tu plastid elongation factor (spot 17) and the two chaperones HSP70 and HSP40 (spots 2 and 3). Other proteins identified in the TAC-II fraction might be contaminants, although a functional role cannot be excluded (Supplementary Table I). For four unknown proteins, which are related to hypothetical rice proteins, neither a function could be assigned nor a homologue in the Arabidopsis databases could be identified (spots 20, 35, 36, 41).

Seven of the identified TAC proteins could be novel plastid nucleoid-associated proteins (ptNAPs) with DNA-binding properties which are able to alter the DNA compaction through bending, wrapping or bridging it (Dillon & Dorman, 2010). Since many of them were first classified as histone-like proteins, many of the bacterial NAP proteins have a low molecular weight, high isoelectric point and high lysine content (Dillon and Dorman, 2010). Among the plastid NAPs identified in this work are proteins possessing domains and motifs known to be directly involved in binding to DNA, as MYB/SANT, or having motifs typical for chromatin remodelling such as the armadillo motif and the SWIB domain (Table I). One of the putative ptNAPs (spots 11) represent a SMC (Structural Maintenance of Chromosomes) protein. For a second putative SMC protein a score of 52 was determined. The SMC proteins belong to a ubiquitous protein family present in almost all prokaryotic and eukaryotic organisms and have functions in chromosome condensation, segregation, cohesion, and DNA recombination and repair (Graumann & Knust, 2009). In *Escherichia coli*, SMC proteins were shown to induce negative supercoiling of DNA *in vivo*, thereby involving them in organisation and compaction of nucleoids (Graumann & Knust, 2009). Another SMC protein (AtSMC1) was identified in the proteome of chloroplasts from *Arabidopsis thaliana* (Kleffmann et al, 2004).

Another putative ptNAP (spot 15) has similarity in sequence with the Arabidopsis armadillo/β-catenin protein. Armadillo (Arm) repeat proteins form a large group of 108 proteins in Arabidopsis. One of these was recently shown to be involved in nuclear DNA replication and transcription (Masuda et al, 2008). Another armadillo β-catenin protein was identified in the chloroplast proteome of *Arabidopsis thaliana* (Zybailov et al, 2008).

Two further putative ptNAPs identified in TAC-II belong to the family of the MYB/SANT domain transcription factors having a HTH (helix turn helix) DNA-binding domain (spots 32, 46). Animal and fungal proteins containing SANT domains (shared by <u>S</u>WI3, <u>A</u>DA2, <u>N</u>-Cor,

TFIIB proteins) were found to be associated with histone modifying enzymes and ATP-dependent chromatin remodelling proteins (Boyer et al, 2004; Jerzmanowski, 2007).

Nucleoids were shown to possess low molecular weight DNA-binding proteins, which are candidates for functional replacement of the missing HU-like proteins in plastid nucleoids. The smallest putative NAP identified in the TAC-II fraction (spot 31) has a SWIB domain. The masses determined for the spinach protein matched with a sequence of *Arabidopsis thaliana* having high homology to a 15 kDa SWIB domain containing protein encoded by At3g03590. Recent database searches identified further SWIB domain proteins such the one encoded by the At2g35605 gene that is also predicted to be organelle targeted (Supplementary Figure 2).

SWIB domain-containing proteins are subunits of the SWI/SNF ATP-dependent chromatin-remodelling complexes that facilitate transcriptional activation. The SWIB domain is a conserved region found within the B group of mammalian BAF60 proteins which are present in alternative forms of the originally identified SWI/SNF complex (Bennett-Lovsey et al, 2002). SWIB domain-containing proteins were also found in proteome of TAC fractions isolated from barley plastids (data not shown) and therefore seem to be essential components of plastid nucleoids. In a proteome study of rice (*Oryza sativa*) etioplasts, a low molecular weight homologue of Arabidopsis SWIB proteins was identified (von Zychlinski et al, 2005).

With the aim to elucidate the functional role of the first annotated putative plastid targeted SWIB domain protein of *Arabidopsis thaliana*, SWIB-1, its subcellular localisation and DNA-binding properties were investigated.

Structure and subcellular localisation of the AtSWIB-1 protein

The complete sequence of the AtSWIB-1 protein is composed of 143 amino acids and the theoretical molecular weight of the precursor protein is 15.3 kDa. After cleavage of the predicted organelle targeting sequence (TargetP) consisting of 85 amino acids (Figure 2A), the mature protein is expected to have a molecular weight of 6.5 kDa. To investigate whether the AtSWB-1 protein is indeed targeted to plastids, *in vitro* import assays were performed. For this purpose the AtSWIB-1 protein was synthesised from the full-length cDNA by coupled *in vitro* transcription/translation. After separation by SDS-PAGE, the *in vitro* translation product had a molecular weight of 17 kDa (Figure 2B), which was higher than the predicted molecular mass. A reduced electrophoretic mobility was already reported for the

AtSWI3B protein, which is another component of the nuclear SWI/SNF complex in Arabidopsis (Sarnowski et al, 2002). The low electrophoretic mobility is likely due to the high content of basic amino acids in SWIB-1 resulting in a high pI of 10. Similar properties were described for histones and for the chloroplast encoded HU-like protein in red algae (Kobayashi et al, 2002)

For *in vitro* import assays the radioactively labelled *in vitro* translation product was incubated with isolated pea chloroplasts. By treatment with thermolysin, the amount of the precursor protein decreased while the processed form with a molecular mass of 15 kDa was protected from degradation (Figure 2B). This showed that the cleavage site of SWIB-1 predicted by TargetP yielding a mature protein with a theoretical molecular weight of 6.5 kDa was not correct.

Additionally, a region of low compositional complexity (amino acids 31-59, Figure 2A and 2C) at the N-terminus of the SWIB-1 protein was identified. This region is highly similar to a part of the C-terminal domain (CTD domain) of the histone H1 form tobacco and pea (Figure 2C). Moreover, *in silico* analysis revealed that this lysine-rich region of the AtSWIB-1 is highly similar to a region found at the N-terminus of the CND41 (<u>c</u>hloroplast <u>n</u>ucleoid <u>D</u>NA binding protein <u>41</u> kDa) protein (Figure 2C), which was described as DNA-binding domain (Murakami et al, 2000; Nakano et al, 1997). This part of the SWIB-1 protein might thus represent the DNA-binding domain of the protein. SWIB domain-containing proteins are expected to function in chromatin remodelling in the nucleus. Whereas no nuclear localisation signal could be found for the TAC-associated SWIB-1, a nuclear export sequence (NES, AA 115-121) in the C-terminal part of the protein (Figure 2A) was predicted by the NetNES programme (La Cour et al, 2004). This suggests that the SWIB-1 protein might belong to a group of proteins, which are dually located in the nucleus and in plastids (Krause & Krupinska, 2009).

The major part of the AtSWIB-1 protein is made up by the SWIB domain, which is composed of four alpha helices separated by two beta sheets (Figure 2A) as predicted by Protein Structure Prediction Server (PSIPRED) (McGuffin et al, 2000). When the amino acid sequence of the entire Arabidopsis SWIB-1 protein was compared with the sequence of a homologous protein of rapeseed (*Brassica napus*) a high similarity (76%) was found (Figure 2D) suggesting that apart from the SWIB domain other parts of the SWIB-1 protein are conserved among the Brassicaceae. The sequence of the SWIB domain is highly similar not only within dicots (rapeseed, grape) but also monocot plants like barley and maize (Figure

2D) and is most probably involved in protein-protein interactions. The phylogenetic analyses revealed moreover, that the SWIB domain is highly conserved not only in all eukaryotic organisms but can be also found in viruses and in Chlamydia (Supplementary Figure 3).

To further investigate the subcellular localisation of the AtSWIB-1 protein, the complete coding sequence of the *At3g03590* gene was fused at either the C-terminus or the N-terminus, respectively, with the sequence encoding the green fluorescence protein (GFP) under the control of the 35S cauliflower mosaic virus promoter. As a control, a construct encoding the GFP protein alone was employed (Figure 3A). Additionally, constructs of the AtSWIB-1 protein lacking either the first 14 or 28 amino acids (AtSWIB-1, Δ14 or Δ28 AA) were prepared (Figure 3A). The constructs were used for transient transformation of onion epidermal cells by particle bombardment. By microscopic analysis GFP fluorescence of the AtSWIB-1:GFP chimeric protein was detected in plastids as well as in the nucleus of onion epidermal cells (Figure 3B). When the GFP protein was fused at the N-terminus of the SWIB-1 protein the GFP fluorescing signals could be observed only in the nucleus (Figure 3B). While a construct lacking 14 amino acids at the N-terminus was still imported into plastids, a construct truncated by 28 amino acids was not imported in the plastids anymore and the green fluorescence signals could be observed exclusively in the nucleus (Figure 3B). These results indicate that for plastid targeting less than 28 amino acids at the N-terminus of SWIB-1 are required. It is likely that the cleavage site in the transit sequence of the SWIB-1 is located between the amino acids VSA (residues 27, 28, 29), which were reported to form a conserved cleavage site motif (Gavel & Heijne, 1990; Zybailov et al, 2008).

In order to more precisely analyze the distribution of the GFP signals in chloroplasts, protoplasts were prepared from tobacco leaves and were transformed with SWIB-1:GFP and PEND:GFP constructs in the presence of PEG. Microscopic analysis showed that the GFP signals were associated with discrete speckles, which are easily discernible in the large chloroplasts of mesophyll protoplasts (Figure 4A). The signals obtained with the PEND:GFP chimeric protein showed a rather similar distribution in chloroplasts (Figure 4A). Since the PEND protein is known to bind to plastid DNA and therefore can be used for visualisation of plastid nucleoids (Sato et al. 2005) these results indicate an association of SWIB-1 protein with DNA in chloroplasts. To further examine binding of SWIB-1 to DNA constructs specific for SWIB-1:GFP as well as PEND:dsRED proteins were co-transformed. Fluorescence microscopy showed that the distribution of the green and red fluorescing signals within the

chloroplasts is rather similar indicating a co-localisation of the two proteins with DNA (Figure 4B).

When a construct encoding GFP only was used for transformation, fluorescence was distributed uniformly throughout the cytoplasm and nucleus of the cell (Figure 4A) as already shown by (Köhler, 1998). When proteins extracted from transformed protoplasts were immunologically analyzed using a antibody specific for GFP, four immunoreactive protein bands were detected (Supplementary Figure 4A). A similar result was obtained when tobacco leaves were infiltrated with Agrobacteria transformed with the AtSWIB-1:GFP construct (Supplementary Figure 4B). These results indicated that the protein might be processed and/or might be posttranslationally modified.

To investigate the subcellular distribution of the endogenous AtSWIB-1 protein a polyclonal antibody was raised towards the peptide sequence KSDSPAKKTPRSTG representing the residues 50 to 63 of the At3g03590 protein (Figure 2A). The sequence of the peptide chosen for production of the SWIB-1 antibody is highly conserved among the dicot species and does not overlap with the SWIB domain (Figure 2A) in order to prevent detection of other SWIB domain proteins. Protein fractions derived from nuclei and chloroplasts of Arabidopsis and spinach leaves were analysed by immunoblotting. Immunoreactive proteins were detected both in chloroplasts and in the nuclear protein fraction (Figure 5). The major protein form in the nucleus was estimated to have a molecular weight of 19 kDa. In addition, a protein of a lower molecular weight (18 kDa) was detected. In comparison, the two closely migrating proteins detected in the plastid protein fractions had lower molecular weights of 17 kDa and16 kDa (Figure 5). The results of these immunological analyses are in accordance with the dual localisation of AtSWIB-1:GFP fusion proteins (Figure 4A). The results, moreover, suggest that the smallest nuclear form of the SWIB-1 protein has a higher molecular weight than the *in vitro* translation product indicating that in the nucleus SWIB-1 might be posttranslationally modified. In both compartments a second protein with a slightly higher molecular mass, respectively, can be detected in addition to the 16 kD plastid form and the 18 kD nuclear form of the protein. The results are in accordance with the detection of four protein bands in protoplasts and leaf tissue after transformation with a AtSWIB-1:GFP construct (Supplementary Figure 4). The high molecular weight forms of both the nucleus and the chloroplast located SWIB-1 could be the results of posttranslational modifications such as phosphorylation or acetylation. Indeed the H1 motif with its high content of lysine residues would be an ideal target for acetylation (Figure 2A) as it is predicted by the PAIL (prediction

of acetylation on internal lysines) server (Li et al, 2006). Prediction values for 14 putatively acetylated lysine residues are listed in Supplementary Table II. Additionally, N-terminal acetylation of the SWIB-1 protein was predicted by the NetAcet 1.0 server (Kiemer et al, 2005). It has been suggested that N-acetylation together with phosphorylation of some chloroplast precursor proteins in the cytoplasm is necessary for import and accumulation of the mature proteins inside the chloroplasts (Pesaresi et al, 2003; Waegemann & Soll, 1996).

Immunological detection of the SWIB-1 protein in plastid and TAC fractions from spinach

In order to examine the presence of the native SWIB-1 domain containing protein in spinach chloroplasts and in TAC-I and TAC-II fractions, the antibody prepared towards an Arabidopsis SWIB-1 peptide was used for immunoblot analyses. The anti-AtSWIB-1 antibody specifically recognized a protein of similar molecular weight in both chloroplasts (C) and a fraction enriched in thylakoid membranes (M) (Figure 6A). Similarly as in Arabidopsis chloroplasts, in chloroplasts of spinach two forms of the SWIB-1 protein were detected indicating a similar posttranslational modification of the protein. Intriguingly, only the lower molecular weight form of the protein was detected in TAC-I and TAC-II fractions (Figure 6A). When TAC-I and TAC-II fractions having the same amount of protein were analysed, the level of the immunoreactive protein detected by the SWIB-1 antibody was higher in TAC-II than TAC-I indicating an enrichment of the SWIB-1 protein during purification of TAC. It is feasible that SWIB-1 binds to DNA when it is not altered by posttranslational modification. Also in case of the plastid DNA binding MFP1 (MAR-filament binding like protein 1) protein, posttranslational phosphorylation was shown to decrease the DNA binding ability of the protein (Samaniego et al, 2006).

Apart from the shift in molecular weight by putative posttranslational modifications the nuclear form of SWIB-1 has a higher molecular weight than the plastidic form. To examine whether the plastidic and the nuclear form of SWIB-1, respectively, could be encoded from two different transcripts, a Northern blot analysis was performed with polyA$^+$-mRNA prepared from rosettes of Arabidopsis plants. By hybridization with a 182 bp DNA probe derived from the coding sequence of the *swib-1 gene* a single transcript of 929 nucleotides was detected (Supplementary Figure 5).

DNA-binding of spinach SWIB-1 protein

In order to investigate the DNA-binding properties of the spinach SWIB-1 protein, a Southwestern analysis with the TAC-II protein fraction was performed. For this purpose TAC-II proteins after transfer onto nitrocellulose membranes were renaturated. By incubation with a radiolabelled *16S rDNA* probe three low molecular weight protein bands were detected. When the membrane prior to incubation with DNA was incubated with the anti-SWIB-1 antibody, the intensity of the band corresponding to a DNA-binding protein of approximately 17 kDa was diminished in comparison to the other proteins. This indicates that the 17 kDa spinach protein of the TAC fraction has a similar sequence as the Arabidopsis SWIB-1 protein.

DISCUSSION

The presented work aimed at identification and characterisation of DNA-binding proteins associated with the transcriptionally active chromosome from spinach chloroplasts. Using a proteome approach, 85 protein spots enriched in the highly purified TAC-II fraction were detected and 47 of them could be identified by mass spectrometry. Among them seven were predicted to be novel DNA-binding proteins. While two proteins seemingly are of prokaryotic origin (one SMC protein and one Rho transcription-termination factor), four have eukaryotic DNA-binding domains. The SWIB domain-containing protein-1 (SWIB-1) was shown to be located in both chloroplast nucleoids and nucleus by transient transformation with GFP fusion constructs as well as by immunological analyses.

DNA-binding proteins in the proteome of highly purified TAC-II from spinach chloroplasts

Considering the high number of nucleoid associated proteins (NAP) in bacteria (Dillon & Dorman, 2010) the number of already identified proteins associated with higher plant plastid nucleoids is rather low. This might explain why none of the newly identified DNA-binding proteins in the TAC-II fraction from spinach chloroplasts was detected in the TAC fraction from mustard/*A. thaliana* and *vice versa*. In addition, the nucleoid-associated proteins, which were identified in the Triton-X-100 insoluble fraction from pea chloroplasts including SiR (Phinney & Thelen, 2005), were not found in the two TAC proteomes. It seems plausible that

the different preparation procedures enrich different parts of the nucleoids. While the TAC fraction from *A. thaliana* was directly subjected to mass spectrometry, proteins of spinach TAC-II were separated by two-dimensional gel electrophoresis and only those proteins enriched in TAC-II compared to the conventionally prepared TAC-I were analysed by mass spectrometry. During preparation of the TAC-II fraction heparin was used for resolubilisation of the complex. Heparin was shown to compete with DNA for binding of DNA-binding proteins (Sekine et al, 2002). Thereby several peripheral DNA-binding proteins are expected to be lost while core component of TAC might get enriched. A heterogeneous organisation of nucleoids with a central core active in transcription and a peripheral layer with further activities related to gene expression was also proposed for mitochondrial nucleoids (Shutt et al, 2010). It is possible that TAC-I contains peripheral proteins involved in posttranscriptional processes of gene expression such as Whirly1 which was shown to be an RNA-binding protein affecting the processing of intron-containing plastid RNA species (Melonek et al, 2010; Prikryl et al, 2008).

Although we were able to enrich some of the core DNA-binding proteins of nucleoids, many more proteins might have escaped detection. DNA-binding proteins usually are highly basic resulting in high isoelectric points, which might have prevented their focusing in a pH gradient ranging from pH 3 to 10. For example the SWIB-1 protein has been detected at a position corresponding to a pI of 8 although its theoretical pI after cleavage of the chloroplast targeting sequence is 10. Migration of the protein during 2D gel electrophoresis could have been disturbed by binding to DNA. Silver staining of the 2D gels has shown that much more proteins are contained in TAC-II than those detected by Coomassie staining. Therefore the DNA-binding proteins here described are certainly the most abundant proteins and might have functions in structuring of the nucleoid.

One of newly identified NAP is a SMC (structural maintenance of chromosomes) protein belonging to a ubiquitous protein family present in almost all prokaryotic and eukaryotic organisms and having functions in chromosome condensation, segregation, cohesion, and DNA recombination and repair (Graumann & Knust, 2009). Investigations on SMC proteins of *Escherichia coli* suggest that they are involved in negative supercoiling of DNA *in vivo*, and thereby play roles in organisation and compaction of nucleoids (Graumann & Knust, 2009). In addition to these newly identified SMC proteins of TAC-II, chloroplasts possess at least one other SMC protein (AtSMC1) which was identified in the proteome of *Arabidopsis thaliana* chloroplasts (Kleffmann et al., 2004). Four of the newly identified ptNAPs of TAC-

II possess eukaryotic DNA binding domains such as MYB/SANT. *In silico* analysis predicted plastid localisation for four proteins belonging to the MYB family of transcription factors and seven MYB-related transcription factors in rice *(Oryza sativa)*. Some of these proteins are predicted to be dually targeted to the nucleus (Schwacke et al, 2007). Another MYB/SANT protein had been previously identified in the proteome of Arabidopsis chloroplasts (Kleffmann et al, 2004).
During evolution, plastids most probably have acquired nuclear proteins possessing eukaryotic DNA-binding motifs for remodelling of their own DNA as it was proposed recently (Kodama, 2007). DNA-binding proteins with eukaryotic domains supposed to play roles in chromatin remodelling have been also identified in a TAC fraction prepared from chloroplasts of mustard and *Arabidopsis thaliana* (Pfalz et al, 2006) and in the proteome of a Triton-X-100 insoluble fraction from pea chloroplasts (Phinney & Thelen, 2005). These include the Whirly1 and 3 proteins, SAP and SET domain proteins (Pfalz et al, 2006) and a SWI3D protein (Phinney & Thelen, 2005).

Characterisation of SWIB-1

Proteome analyses with the TAC-II fraction from spinach chloroplasts enabled us to identify a low molecular weight DNA-binding protein, which was named SWIB-1 due to its SWIB domain. The mature SWIB-1 protein has a theoretical molecular weight of 15 kDa and has a high percentage of lysine (14%). In addition to the SWIB domain it has a histone H1-like domain which could be responsible for its binding to DNA. The SWIB domain itself is unlikely to be involved in DNA-binding but rather functions as a protein-protein interaction domain. Very recent database searches revealed that the protein SWIB-1 originally chosen for further analyses, belongs to a family of 18 SWIB domain proteins in *Arabidopsis thaliana.* Six low molecular weight proteins (12 – 21 kD) including SWIB-1 were predicted to be located in either plastids or mitochondria (Supplementary Figure 2). At the time of proteome analyses database searches with the peptide masses of the spinach protein spot 31 identified an unknown sequence of *Arabidopsis thaliana* having high homology to the sequence of the SWIB-1 protein. Recent database searches with our initial MS data from the spinach protein spot identified further putative SWIB domain containing organelle proteins of *Arabidopsis thaliana*. This suggests that chloroplast nucleoids might contain several SWIB domain proteins. The peptide chosen for production of the antibody specific for SWIB-1 did not include parts of the SWIB domain and therefore did not detect further SWIB domain proteins, neither in plastids nor in the nucleus. The two protein bands detected in plastid

fractions represent the mature form of the protein and a posttranslationally modified form thereof. Only these two forms and two additional nuclear forms were detected with a GFP specific antibody when protoplasts were transiently transformed with a AtSWIB-1:GFP construct.

In bacteria, nucleoid architecture and function depends on a group of proteins often referred to as histone-like proteins (HLP), which were recently named bacterial nucleoid-associated proteins (NAP, Dillon and Dorman, 2010). These groups of proteins have in common a high basicity, non-specific binding to DNA and a low molecular weight such as the 9 kDa HU protein (Dillon & Dorman, 2010; Luijsterburg et al, 2006). While some bacteria have one HU protein functioning as a homodimer, *E. coli* has two different HU proteins forming HU heterodimers (α and ß) (Luijsterburg et al, 2006). Analyses of higher plants genomes revealed that these don't possess genes which could encode corresponding nucleoid-associated proteins (Riechmann et al, 2000). This suggests that during evolution of plants the prokaryotic HU-like proteins might have been functionally replaced by novel proteins. In previous biochemical investigations putative candidate proteins which could have replaced the HU-like proteins having molecular weights in the range of 5 to 25 kDa were identified (Baumgartner & Mullet, 1991; Briat et al, 1984; Nemoto et al, 1988a).

As already emphasised by Bogorad (1991) the structure of plastid nucleoids and DNA topology might have important consequences for gene expression (Bogorad, 1991; Salvador et al, 1998). Plastid gene expression in barley and tobacco was shown to change during chloroplast development (Baumgartner et al, 1989; Krupinska & Falk, 1994; Nemoto et al, 1989; Nemoto et al, 1988b) and might be paralleled by changes in the structure of the nucleoids and in protein composition (Hansmann et al, 1985). It is possible that SWIB-1 and other SWIB domain proteins might be involved in structural reorganisation of nucleoids resulting in modulation of plastid gene expression during development. Nuclear SWIB domain proteins have been reported to be involved in eviction of histones during transcription elongation (Schwabish & Struhl, 2007). RNA polymerase II undergoes a dynamic cycle of nucleosome eviction and deposition permitting passage of the enzyme and restoration of normal chromatin structure (Schwabish & Struhl, 2007). Transcript elongation also is a key function of TAC, and the low molecular SWIB proteins might play roles related to this function.

SWIB domain containing proteins were also found in viruses and in Chlamydia, bacterial obligate intracellular parasites of humans and animals that undergo a biphasic developmental

cycle (Supplementary Figure 3). In transcriptionally inert elementary bodies (EB) the nucleoid was shown to be densely compact whereas in metabolically active reticulate bodies (RB) the nucleoid was observed to be loosely organised (Barry et al, 1992). Condensation of the nucleoid depends on the presence of a protein homologous to eukaryotic histone H1 (Barry et al, 1992). The gene encoding this H1-like protein was found to be expressed only at the late stage of chlamydial life cycle concomitant with the reorganisation of reticulate bodies into elementary bodies having a condensed nucleoid structure (Hackstadt et al, 1991).

It is expected that in addition to the SWIB proteins described here other SWI/SNF components are present in chloroplasts. *In silico* analysis indicated two rice proteins (Os05g15890 and Os06g01320) belonging to the SNF2 family, a large group of helicase-like proteins (Flaus et al, 2006) to have putative plastid localisation sequences (Schwacke et al, 2007). Many of the well-studied members of this family are core motor polypeptides within ATP-dependent chromatin remodelling complexes (Flaus et al, 2006). The presence of SWI/SNF like complexes in plastid nucleoids is further supported by the identification of a protein with similarity to an actin-interacting protein. Actin related proteins (ARP) were shown to be included in all yeast and animal SWI/SNF complexes (Clapier & Cairns, 2009; Jerzmanowski, 2007; Olave et al, 2002).

Chromatin remodelling factors in plastids

SWIB-1 has a sequence at the N-terminus (KKPAAKPKAKAKPKPKAKSDSPAK), which is highly similar to a part of the histone H1 protein from different plant species. Interestingly, the same peptide was found to be very similar to the histone-H1-like protein (Hc1) from *Chlamydophila felis.* This coincidence suggests that SWIB-1 and histone H1 function together in remodelling of nucleoids. Recently, the chlamydial histone H1-like proteins were shown to interact with a SET domain protein functioning as a histone methyltransferase (Murata et al, 2007).

In recent years evidence has accumulated that counterparts of proteins known to be involved in histone modification in the nucleus do also occur in plastids. The Arabidopsis SET domain-containing Trithorax-related protein 5 (ATXR5) by fusion with GFP protein was found to be dually located to both plastids and the nucleus (Raynaud et al., 2006). ATXR5 together with another SET domain-containing protein (At5g14260) was detected in the proteome of chloroplast from *Arabidopsis thaliana* (Zybailov et al, 2008). ATRX5 acts as H3K27 monomethyltransferase in nucleus (Jacob et al, 2009). It could be shown that mutations in the

atxr5 gene lead to transcriptional activation of repressed heterochromatic elements (Jacob et al, 2009). ATXR1, another ATXR protein, was predicted to be targeted to chloroplasts (Schwacke et al, 2007). Recently, a chloroplast localisation of two histone deacetylases (OdHDAC10 and 6) in rice was shown (Chung et al, 2009). Considering that SWIB-1 has a high probability for acetylation and occurs in several forms it is likely to be a target of such plastid localised deacetylases. It is possible that *vice versa* acetylation of SWIB-1 occurs in the organelle, because evidence for acetylation in plastids was obtained recently. The N-termini of O-acetylserine (thiol) lyase (OAS-TL) proteins were shown to be acetylated after cleavage of the chloroplast targeting sequence (Wirtz et al, 2010).

On the other hand, the double protein bands detected in the chloroplast fraction with the anti-SWIB-1 antibody could be a result of floppy cleavage of the chloroplast transit peptide of the native SWIB-1 protein by the stromal processing peptidase (SPP), since the SPP recognizes the cleavage site by its physicochemical properties than by specific amino acid residues in the sequence (Rudhe C., 2004). This however does not explain the origin of the double band in the nuclear protein fraction detected with the antibody.

DNA-binding properties of SWIB-1

The lysine-rich region of the SWIB-1 protein, which is highly similar to a part of the C-terminal domain of the histone H1, represents most probably the DNA-binding domain of the SWIB-1 protein, therefore we think that SWIB-1 binds to DNA in a rather sequence non-specific manner. By *in vitro* assays it had been shown that a very similar lysine-rich region at the N-termini of the CND41 protein is involved in binding to DNA (Nakano et al., 1997). Basic regions were also found at the N-terminal ends of the PD1 (plastid DNA-binding 1) and PD3 (plastid DNA-binding 3) proteins (Kodama, 2007). The occurrence of basic regions at the N-termini could be a key structure during conversion of a nuclear transcription factor to a plastid-located DNA-binding protein (Kodama, 2007).

Materials and methods

Plant material

Spinach (*Spinacia oleracea* L. cv. Deutscher Frisee) for chloroplast preparation and spring onions (*Allium fistulosum L.*) for transient transformation were purchased from the local market. Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants used for protoplast transformation were grown for 5-6 weeks on sterilized Murashige and Skoog medium at 24°C with a 16 hour photoperiod. Pea (*Pisum sativum* L. cv. Kleine Rheinländerin) plants used for preparation of chloroplasts for *in vitro* import assays were grown on Vermiculite for 10-14 days with a 16 hour photoperiod, too.
Arabidopsis thaliana wild type Columbia (Col-0) plants were cultivated at 22°C in soil under long day conditions (16 h light/8 h dark). Rossette leaves were used for isolation of protein extracts and RNA.

Isolation of transcriptionally active chromosome (TAC) fractions

For isolation of TAC fractions, chloroplasts were prepared from spinach leaves according to the procedure of (Douce et al, 1973). TAC extracts from spinach chloroplasts were prepared as described previously (Krause & Krupinska, 2000). To examine the transcriptional activity aliquots of TAC fractions were incubated at 30°C with radioactively labelled α-^{32}UTP (Hartmann Analytic, Braunschweig, Germany) as described (Krupinska & Falk, 1994).

Two-dimensional separation of TAC proteins and mass spectrometry

Preparation of TAC protein samples and two-dimensional gel electrophoresis (2-DE) were performed with some modifications as previously described (Amme et al, 2006; Schlesier et al, 2006). Prior to isoelectric focusing (IEF) TAC-fractions were treated with DNase I and RNase A (MBI Fermentas, St. Leon, USA) and precipitated with chloroform/methanol following the protocol of Wessel and Flügge (1984). Protein concentration was estimated with the Plus One 2D-Quant Kit (GE Healthcare, Uppsala, Sweden). 50 µg of TAC proteins were loaded on IPG dry gel strips (7 cm, pH gradient 3-10, GE Healthcare, Uppsala, Sweden). The second dimension was performed on 11.25 % (w/v) polyacrylamide gels containing 0.1 % (w/v) of SDS. Separated proteins were stained with colloidal Coomassie Brilliant Blue (Pierce Gel Code Blue, Pierce, Bonn, Germany).

Cloning of GFP-fusion constructs

For the preparation of a AtSWIB-1:GFP constructs, the complete coding sequence of the At3g03590 gene was amplified from Arabidopsis total cDNA by the iProof High-Fidelity DNA Polymerase (Bio-Rad Laboratories GmbH, Munich, Germany) using specific forward and reverse primers (5`-CACC atg tct tcc gtt gca-3`; 3´-agc agt ctt cac aaa gtg c-5`), respectively. The forward primer contained a CACC overhang for cloning into pENTR/D/TOPO vector (Invitrogen, GmbH, Karlsruhe, Germany). pENTR:AtSWIB-1 plasmids were used for site-specific recombination into binary Gateway expression vector pB7FWG2,0 (Invitrogen GmbH, Karlsruhe, Germany, http://www.psb.ugent.be/gateway/) or pEARLYGATE104 (Earley et al, 2006). The PEND:GFP and PEND:dsRED constructs were kindly provided by René Lorbiecke (Hamburg University) and were designed as described before (Terasawa et al, 2005; Terasawa & Sato, 2005). As a control the coding sequence for the green fluorescent protein was cloned into the pBluescript vector. All constructs were put under the control of the 35S cauliflower mosaic virus promoter.

Transient transformation assays

Transient transformation assays of *GFP* fusion constructs were performed with onion epidermal cells and with protoplasts, respectively. Transformation of onion epidermal cells was done as described (Krause et al, 2005). The tissue was analysed for GFP fluorescence with a Zeiss AxioPhot fluorescence microscope (Zeiss, West Germany, Oberkochen) equipped with a CCD camera, with excitation set at 450-490 nm and emission set at 515-565 nm wavelengths.

Tobacco protoplasts used for transient transformation assay were prepared as described (Nagy & P., 1976). Polyethylenglycol (PEG) - mediated transformation was performed as described (Negrutiu et al, 1987). Briefly, 50 µg of plasmid DNA were gently mixed with 2x 10^6 protoplasts and incubated in 40% (w/v) PEG 1500 (Merck, Hohenbrunn, Germany). In co-localisation experiments protoplasts were transformed with two plasmids simultaneously. After 24 hours incubation, protoplasts were inspected by fluorescence microscopy (Leica TCS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany). For detection of GFP fluorescence and chlorophyll autofluorescence the samples have been excited by the 488 and 458 nm lines of an argon laser, respectively.

In vitro import assays with pea chloroplasts

For *in vitro* import assays, the complete coding sequence of the At3g03590 gene was cloned behind the T7 RNA polymerase promoter into a modified pBluescript vector containing a sequence encoding a methionine/ serine tag (MSMSMS) at C-terminal end of the protein. Import assays were performed with isolated pea chloroplasts as described (Krause et al, 2005).

Protein isolation and immunoblot analysis

For extraction of total proteins, Arabidopsis rosette leaves were ground in liquid nitrogen. Nuclei were isolated from Arabidopsis rosette leaves as described (Desveaux et al, 2004). Protein concentrations were determined using Roti-Nanoquant according to the manufacturer's instructions (Roth, Karlsruhe, Germany) modified and measured according to (Bradford, 1976).

For SDS-polyacrylamide gel electrophoresis the buffer system of Laemmli (Laemmli, 1979) was used. For an efficient separation of proteins having low molecular weights, 16 % (w/v) polyacrylamide gels were used and proteins were blotted onto nitrocellulose (Schleicher & Schuell, Dassel, Germany). For Western Blot analysis, the polyclonal anti AtSWIB-1 antiserum was used in dilution of 1:1000 in TBS. The antibody was directed towards the peptide KSDSPKKTPRSTG (residues 50-63 of the protein) and was raised in rabbits by a company (Biogenes, Berlin, Germany). As controls, immunoblot assays with antibodies directed towards the cytochrome b_{559} apoprotein A of 9.5 kD (Vallon et al, 1987) as well as the polymerase I subunit A12.2 from rabbit (Agrisera AB, Vännäs, Schweden), were used. Immunoblotting and detection was done as described (Grabowski et al, 2008).

Southwestern analysis of TAC proteins

DNA-binding assays with TAC proteins were carried out as described (Bulow et al, 1987) with minor modifications. About 5 µg of total TAC-proteins from spinach were separated under denaturing conditions in 14% (w/v) polyacrylamide gels (Laemmli, 1979) and subsequently transferred onto a nitrocellulose membrane employing a transfer buffer consisting of 50 mM NaCl, 2 mM EDTA, 0.1 mM DTT and 10 mM Tris/HCl, pH 7.0. Renaturation of the proteins took place overnight in a buffer consisting of mM EDTA, 10 mM Tris/HCl pH 7.0, 0.02% BSA, 1% (w/v) low non-fat milk powder and 0.02% (w/v) polyvinylpyrrolidon. The membrane was cut into two halves and one half was incubated with

the SWIB-1 specific antibody diluted 1:50 in phosphate buffer (PBS). The other half of the membrane was incubated with PBS only. After washing in transfer buffer, the membrane was incubated with an α-^{32}P-labelled *16SrDNA* probe in renaturation buffer. After hybridisation the membranes were washed with renaturation buffer.

Prediction servers

TargetP (http://www.cbs.dtu.dk/services/TargetP/), NetNES (http://www.cbs.dtu.dk), NetAcet (http://www.cbs.dtu.dk/services/NetAcet/), PhosPhAt (http://phosphat.mpimp-golm.mpg.de/)

Supplementary data

Supplementary data have been submitted.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Figure legends

Figure 1 Two dimensional separation of TAC-I and TAC-II proteins. Proteins were separated subsequently by isoelectric focussing in a pH gradient from 3-10 and by gel electrophoresis. Preparation and treatment of samples was as described in the materials and methods section. After staining with colloidal Coomassie, 132 spots could be detected in case of TAC-I and 85 protein spots could be detected in case of TAC-II.

Figure 2 Structure of the AtSWIB-1 protein. **(A)** The AtSWIB-1 protein sequence has a length of 143 amino acids. The chloroplast targeting peptide (cTP) is marked by small italic letters. The arrowhead indicates the position of the putative target peptide cleavage site. The lysine-rich region similar to the part of histone H1 depicted in white letters. The sequence of the oligopeptide used for antibody production is presented in bold letters. Putative acetylation sites are indicated with asterisks (*), and an NES (nuclear export sequence) is shown by a box. The SWIB domain of AtSWIB-1 protein is underlined and consists of four alpha helices and two beta sheets, which are indicated by grey boxes. **(B)** The translocation of the AtSWIB-1 protein into chloroplasts was shown by *in vitro* import of the radiolabelled *in vitro* translation product with isolated pea chloroplasts. While the precursor protein was sensitive towards thermolysin, the mature SWIB-1 protein of 15 kDa molecular mass was protected from thermolysin treatment (+). **(C)** Alignment of the lysine-rich region of AtSWIB-1 with partial sequence of histone H1 from tobacco and an analogous lysine-rich region of the CND41 protein. **(D)** Comparison of the amino acid sequences of AtSWIB-1 orthologues from rapeseed *(Brassica napus*) TC70632, grape (*Vitis vinifera*) CAN68050, barley (*Hordeum vulgare*) TC157411 and maize (*Zea mays*) ACG31364. The SWIB-1 domain is underlined. The alignment was generated in ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html).

Figure 3 Subcellular distribution of AtSWIB-1:GFP chimeric proteins. The AtSWIB-1 protein was fused with GFP at its N- or C-terminus (1, 2), respectively, and transiently expressed in onion epidermal cells. For comparison, constructs with N-terminal deletions of either 14 amino acids or 28 amino acids (3, 4) were used. As a control the coding sequence of the GFP protein alone was used (5). Constructs were used for transformation of onion epidermal cells as described in material and methods. Scale bars: 7.5 µm.

Figure 4 (A) Localisation of AtSWIB-1:GFP fusion protein in chloroplast nucleoids and nucleus of protoplasts isolated from tobacco leaves. As controls PEND:GFP and GFP were

visualized. Scale bars: 7.5 μm. **(B)** Co-localisation of the AtSWIB-1:GFP protein with the PEND:dsRED protein in the nucleoids. Scale bar: 2 μm

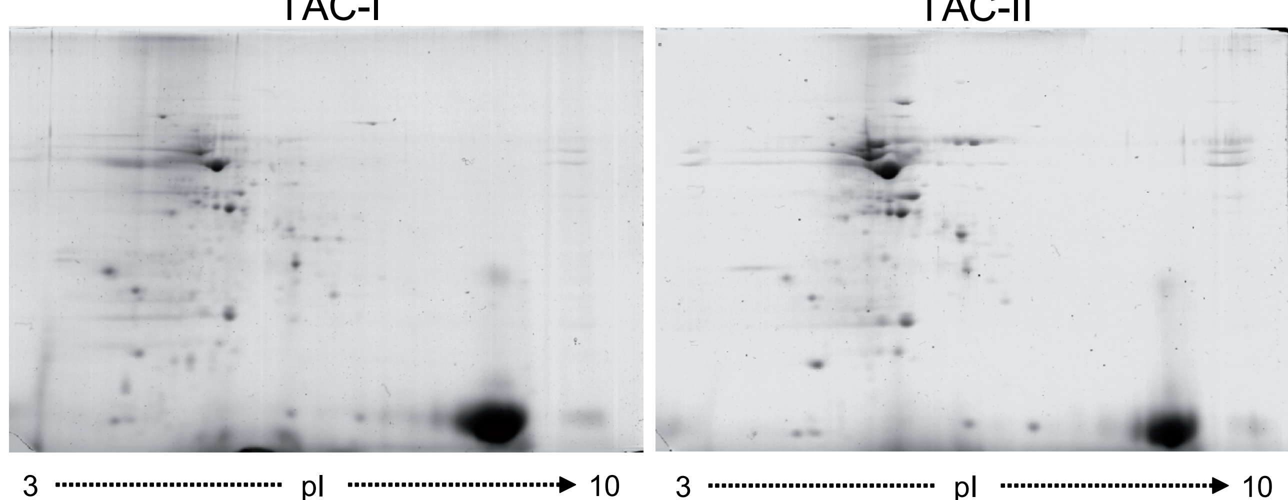
Figure 5 Immunological detection of AtSWIB-1 protein in fractions isolated from Arabidopsis rosette leaves: total leaf protein (TP), nuclei (N), chloroplast (C), membranes (M) and stroma (S). The peptide antibody directed towards the AtSWIB-1 protein recognised two proteins of 19 and 20 kDa in the nuclei fraction and two of 17 and 18 kDa molecular weights in chloroplast fractions. For control of the protein fractions' purity immunoassays were performed with antibodies directed towards cytochrome b_{559} and the subunit A12.2 of nuclear RNA polymerase I. Equal loading of the fractions is shown by staining with Coomassie Brilliant Blue (CBB).

Figure 6 Immunological detection of the spinach protein homologous to AtSWIB-1 and DNA-binding analysis **(A)**. Immunoreactions were performed with total proteins (TP), chloroplast proteins (C), membranes (M) and stroma (S) proteins extracted from spinach leaves. In addition TAC-I and TAC-II fraction with the same amount of protein were analysed.
(B) For Southwestern analysis proteins of TAC-II were electophoretically separated and blotted onto a nitrocellulose membrane. Proteins were renatured and incubated with radioactively labelled *16S rDNA* probe. Whereas the sample shown in lane 1 was directly incubated with DNA, the samples shown in lanes 2 and 3were incubated with the anti-SWIB-1 antibody or with BSA, respectively, prior to incubation with DNA The protein band prevented to bind to DNA by the SWIB-1 antibody is indicated by an arrow. For comparison Silver staining of the fraction is shown in lane 4.

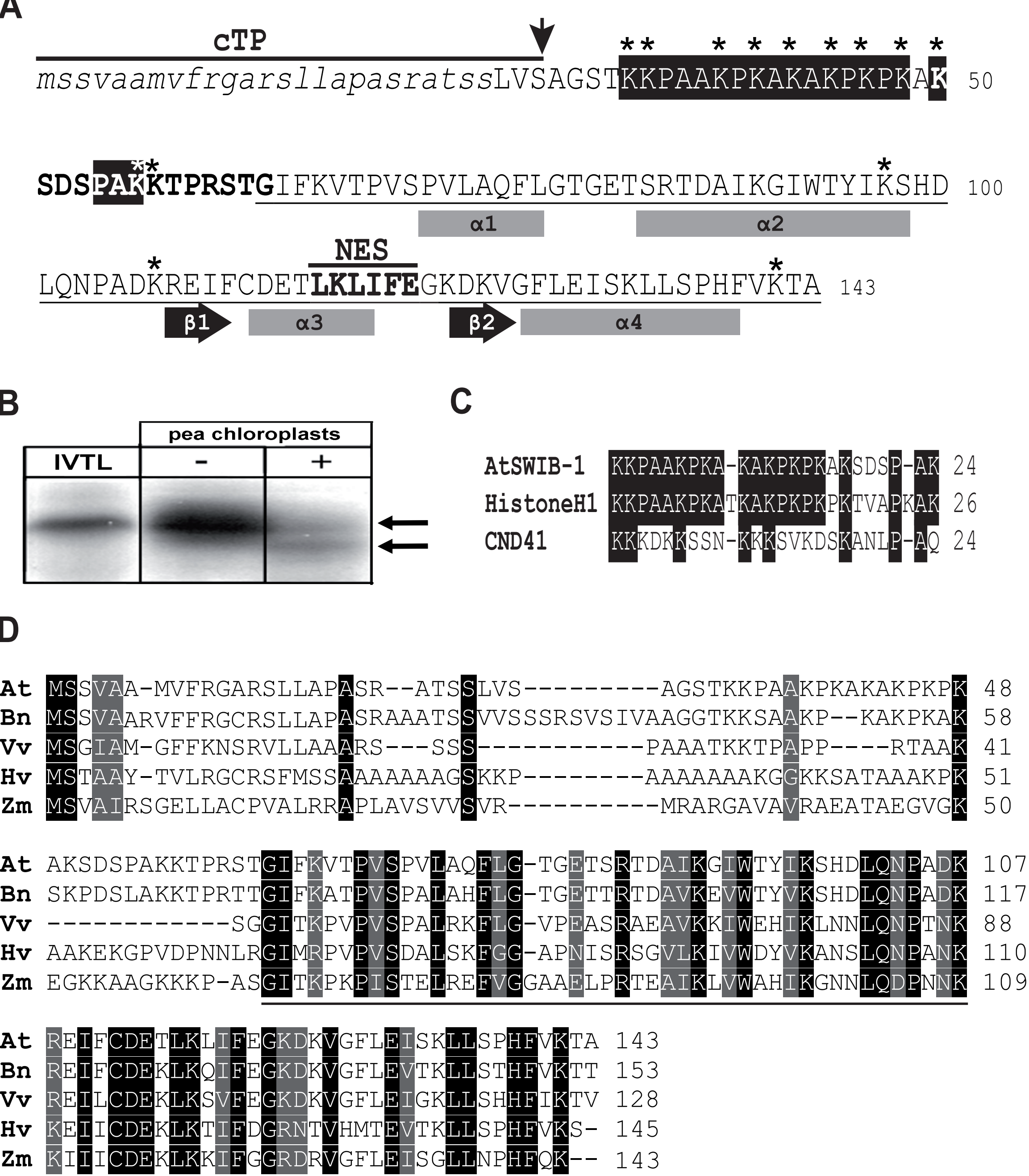
Table I. Identified putative plastid nucleoid associated proteins in the TAC-II-fraction

no. (spot no.*)	identified NAP proteins	accession number	conserved domains/motives/ regions	function	Mw (kD)	pI	lysine content
1. (31)	SWIB complex BAF60b domain-containing protein	At3g03590	SWIB/MDM2 domain, low complexity region	chromatin remodelling complexes	15	11	14%
2. (39)	hypothetical protein, transcription termination factor Rho	gi|53791664	RNA-binding region RNP-1, PRK12678	transcription termination	22	12	1 %
3. (32)	myb-related transcription factor SANT- superfamily	At2g02060	N-CoR and TFIIIB DNA-binding domains	DNA-binding, regulation of transcription	29	7	6 %
4. (15)	hypothetical protein, armadillo/beta-catenin repeat family protein	At2g27430	armadillo-type fold, armadillo-like helical	nucleic acid-binding/ protein binding	49	8	8 %
5. (46)	myb-related transcription factor SANT- superfamily	At5g02320	N-CoR and TFIIIB DNA- binding domains	DNA-binding, regulation of transcription	62	8	10 %
6. (10)	unknown protein, RecF/ RecN/ SMC-N terminal domain	At5g36780	RecF/RecN/SMC N- terminal domain	chromatin and DNA dynamics/ DNA metabolism and recombination	66	5	11 %

* spot number on the TAC-II 2D-gel (Supplementary Table I)



TAC-I
TAC-II
3
pI
10
3
pI
10



A
cTP
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SDSPAKKTPRSTGIFKVTPVSPVLAQFLGTGETSRTDAIKGIWTYIKSHD 100
α1
α2
NES
LQNPADKREIFCDETLKLIFEGKDKVGFLEISKLLSPHFVKTA 143
β1
α3
β2
α4
B
IVTL
pea chloroplasts
-
+
C
AtSWIB-1 KKPAAKPKA-KAKPKPKAKSDSP-AK 24
HistoneH1 KKPAAKPKATKAKPKPKPKTVAPKAK 26
CND41 KKKDKKSSN-KKKSVKDSKANLP-AQ 24
D
At MSSVAA-MVFRGARSLLAPASR--ATSSLVS---------AGSTKKPAAKPKAKAKPKPK 48
Bn MSSVAARVFFRGCRSLLAPASRAAATSSVVSSSRSVSIVAAGGTKKSAAKP--KAKPKAK 58
Vv MSGIAM-GFFKNSRVLLAAARS---SSS-----------PAAATKKTPAPP----RTAAK 41
Hv MSTAAY-TVLRGCRSFMSSAAAAAAAGSKKP--------AAAAAAAKGGKKSATAAAKPK 51
Zm MSVAIRSGELLACPVALRRAPLAVSVVSVR----------MRARGAVAVRAEATAEGVGK 50
At AKSDSPAKKTPRSTGIFKVTPVSPVLAQFLG-TGETSRTDAIKGIWTYIKSHDLQNPADK 107
Bn SKPDSLAKKTPRTTGIFKATPVSPALAHFLG-TGETTRTDAVKEVWTYVKSHDLQNPADK 117
Vv ------------SGGITKPVPVSPALRKFLG-VPEASRAEAVKKIWEHIKLNNLQNPTNK 88
Hv AAKEKGPVDPNNLRGIMRPVPVSDALSKFGG-APNISRSGVLKKIVWDYVKANSLQNPANK 110
Zm EGKKAAGKKKP-ASGITKPKPISTELREFVGGAAELPRTEAIKLVWAHIKGNNLQDPNNK 109
At REIFCDETLKLIFEGKDKVGFLEISKLLSPHFVKTA 143
Bn REIFCDEKLKQIFEGKDKVGFLEVTKLLSTHFVKTT 153
Vv REILCDEKLKSVFEGKDKVGFLEIGKLLSHHFIKTV 128
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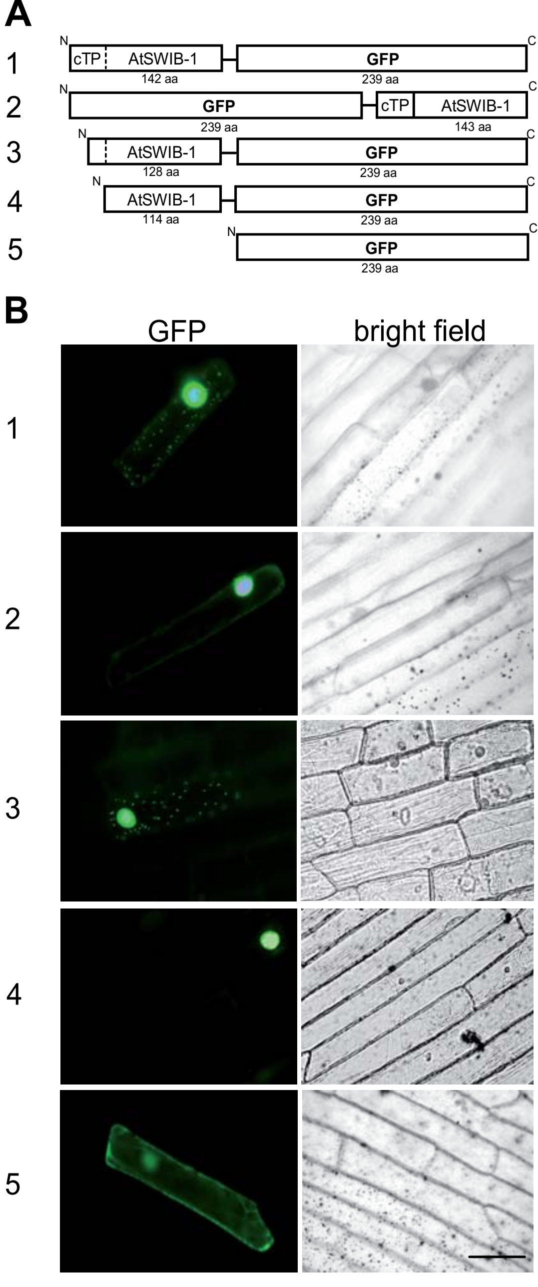
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GFP
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2
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239 aa
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C
B
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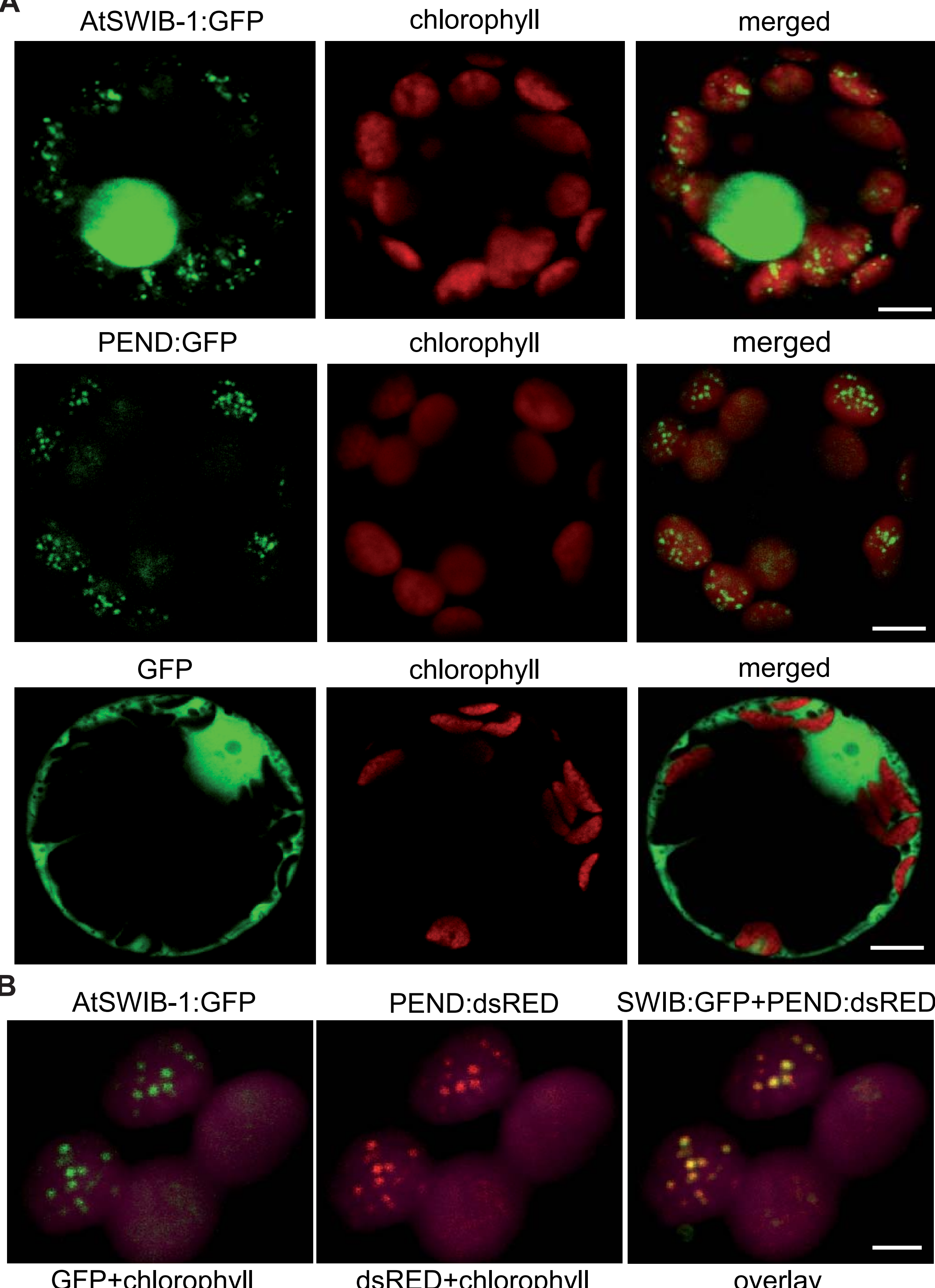
bright field
1
2
3
4
5


A
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chlorophyll
merged
PEND:GFP
chlorophyll
merged
GFP
chlorophyll
merged
B
AtSWIB-1:GFP
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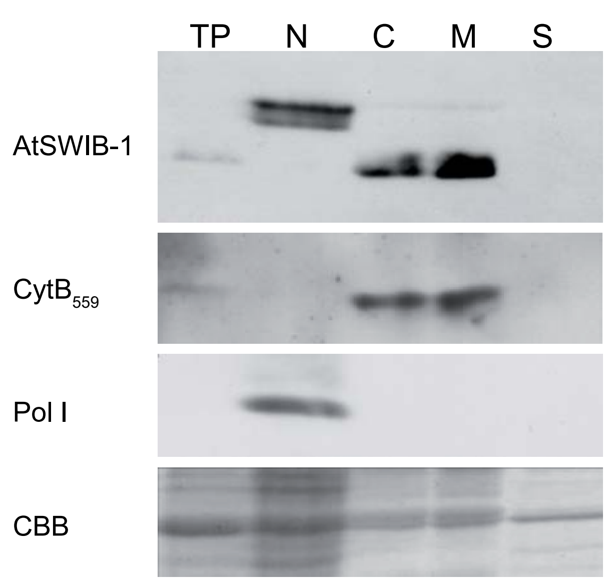

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M
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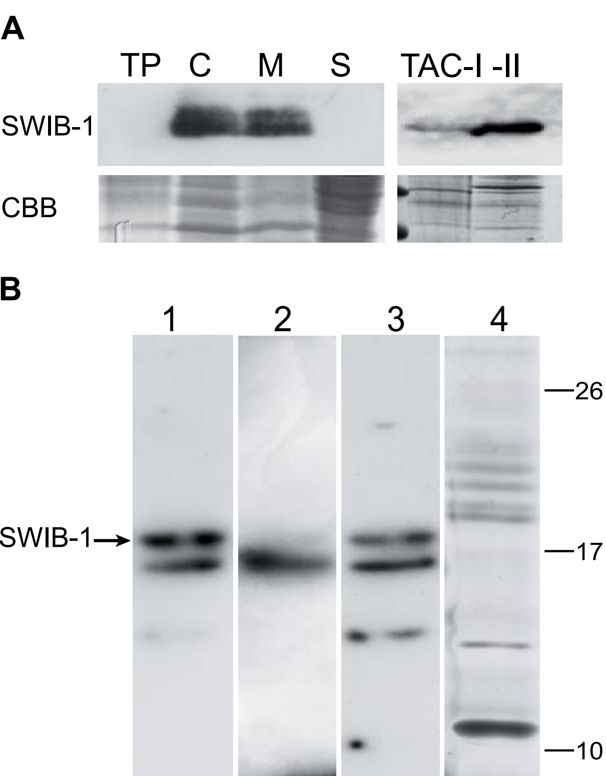
CytB559
Pol I
CBB


A
TP
C
M
S
TAC-I -II
SWIB-1
CBB
B
1
2
3
4
26
17
10
SWIB-1

Supplementary Table 1. Proteins identified on the TAC-II 2D-gel.

Spot no.	protein name	accession number	species	score	no. of identified peptides	theoretical mass	location	pI
1.	ClpC protease	gi|4105131	*S. oleracea*	174	18	99417	CHLOROPLAST	7
2.	heat shock 70 protein HSP70, molecular chaperone DnaK	gi|2654208	*S. oleracea*	137	13	76094	CHLOROPLAST	5
3.	heat shock 40 protein HSP40, molecular chaperone DnaJ	gi|116059922	*O. sativa*	60	8	41437	CHLOROPLAST	9
4.	similar to Athila ORF1	gi|6623974	*A. thaliana*	64	9	53944	unclear	6
5.	ATP synthase CF1 subunit alpha	gi|114527	*S. oleracea*	135	18	55417	CHLOROPLAST	5
6.	aminotransferase class IV family protein	gi|22330856	*A. thaliana*	62	9	62173	unclear	7
7.	Polygalacturonase glyco- hydro28-superfamily	gi|6714524	*S. gilgiana*	73	8	41827	unclear	9
8.	hypothectical protein, similar to glutaredoxins	gi|3377810	*A. thaliana*	60	8	67121	CHLOROPLAST	5

9.	hypothetical protein CHLREDRAFT_80366 similar to At3g61860	gi|159475431	*C. reinhardtii*	73	8	20328	unclear	9
10.	unknown protein, similar to RecF/ RecN/ SMC_N terminal domain protein	gi|18421573	*A. thaliana*	62	12	66410	unclear	6
11.	ATP synthase CF1 subunit beta	gi|733448	*S. oleracea*	117	19	53711	CHLOROPLAST	5
12.	male sterility MS5-like protein, tetratricopeptide repeat (TPR) protein	gi|6562280	*A. thaliana*	61	9	47947	mitochondrion	9
13.	RNA polymerase, beta' subunit RPOC1	gi|116748985	*S. fumaroxidans MPOB*	65	8	150748	CHLOROPLAST	9
14.	unknown protein, pentatricopeptide repeat (PPR) protein	gi|26449475	*A. thaliana*	62	12	63781	unclear	9
15.	hypothetical protein, similar to armadillo/beta-catenin repeat family protein	gi|4314385	*A. thaliana*	63	9	48733	chloroplast	9
16.	RNA polymerase beta chain, RPOC2	gi|90403713	*P. americana*	66	8	98846	chloroplast	10
17.	AtRABE1b/AtRab8D (Arabidopsis Rab GTPase homolog E1b) chloroplast translation elongation factor EF-Tu precursor	gi|15237059	*A. thaliana*	61	10	51598	chloroplast	7

18.	actin interacting protein, FAD linked oxidase family	gi|4006920	*A. thaliana*	61	8	57424	chloroplast	7
19.	hypothetical protein OsJ_002267, similar to AT5G47100	gi|125570927	*O.sativa*	61	6	29842	unclear	8
20.	hypothetical protein	gi|50509425	*O. sativa*	66	6	20976	chloroplast	12
21.	rubisco activase precursor	gi|170129	*S. oleracea*	248	28	51452	chloroplast	7
22.	rubisco activase	gi|21950712	*C. quinoa*	72	7	47811	chloroplast	7
23.	ATP synthase gamma subunit	gi|755801	*S. oleracea*	109	10	36442	chloroplast	6
24.	sedoheptulose-1,7-bisphosphatase, chloroplast precursor	gi|3914940	*S. oleracea*	132	14	42054	chloroplast	7
25.	disease resistance protein (TIR-NBS-LRR)	gi|15241561	*A. thaliana*	64	13	113050	mitochondrion	7
26.	similar to putative U1 small nuclear ribonucleoprotein 70 kDa	gi|2289001	*A. thaliana*	66	8	33500	nucleus	9
27.	expressed protein Os03g01730, similar to At2g17695	gi|108705752	*O. sativa*	61	5	28178	unclear	9
28.	hypothetical protein Os10g0517400 aldo/keto reductase family, similar to At5g53580	gi|115482950	*O. sativa*	64	7	41115	chloroplast	9

29.	hypothetical protein Os05g0430300, similar to At1g34320	gi|115464047	*O. sativa*	68	13	71065	unclear	9
30.	hypothetical protein OsJ_009702, similar to At5g05000	gi|125585555	*O. sativa*	65	9	80699	chloroplast	9
31.	unknown protein, SWIB complex BAF60b domain-containing protein similar to At2g35605 and At3g03590	gi|18403898	*A. thaliana*	60	6	11943	chloroplast/ mitochnodrion	10
32.	DNA/ binding transcription factor, SANT- superfamily, similar to At2g02060	gi|15233562	*A. thaliana*	66	5	19204	chloroplast	7
33.	RNA-directed DNA-polymerase (Reverse transcriptase)	gi|124360558	*M. truncatula*	62	15	113620	secretory pathway	9
34.	Os01g0721800, disease resistance protein (NBS-LRR class)	gi|115439635	*O. sativa*	70	10	58412	unclear	9
35.	hypothetical protein OsI_022219	gi|125555381	*O. sativa*	62	5	14599	unclear	8
36.	Os06g0625000	gi|115469020	*O.sativa*	62	5	22377	mitochondrion	10
37.	ATP synthase subunit B’ chain, chloroplast subunit	gi|461595	*S. oleracea*	88	8	24444	chloroplast	6
38.	ATP synthase CF1 epsilon chain	gi|11497534	*S. oleracea*	117	10	14691	chloroplast	7

39.	hypothetical protein, transcription termination factor Rho	gi|53791664	*O. sativa*	74	8	22247	chloroplast	12
40.	ribonuclease pancreatic (RNaseA)	gi|54039260	*B. taurus*	64	7	13698	exogenously added	8
41.	hypothetical protein OsI_022219	gi|125555381	*O. sativa*	65	6	14590	unclear	8
42.	putative UV-damaged DNA binding factor	gi|18377609	*A. thaliana*	67	8	121305	nucleus	6
43.	WRKY15, transcription factor	gi|42570895	*A. thaliana*	68	7	28481	chloroplast/ nucleus	9
44.	polygalacturonase glyco- hydro28-superfamily	gi|6714524	*S. gilgiana*	63	6	41827	unclear	9
45.	hydroxyproline-rich glycoprotein MCB17	gi|9293881	*A. thaliana*	62	10	59192	unclear	7
46.	myb-related transcription factor, SANT- superfamily, similar to At5g02320	gi|67592395	*C. hominis TU502*	77	7	19732	unclear	8

Protein localisation prediction - TargetP

Mass spectrometry

Protein spots were manually excised and trypsin digested (Porcine Sequencing Grade, Promega, Mannheim, Germany) as described (Brumbarova et al, 2008). Peptide masses obtained after analysis with MALDI-TOF MS were used for searches with the MASCOT search engine (Matrix Science, http://www.matrixscience.com) against the *Viridiplantae* index of the NCBI non-redundant protein sequence database and the Arabidopsis EST gene index of the TIGR database. Parameters for mass spectrometry analysis and database search were as described in supplementary Table I.

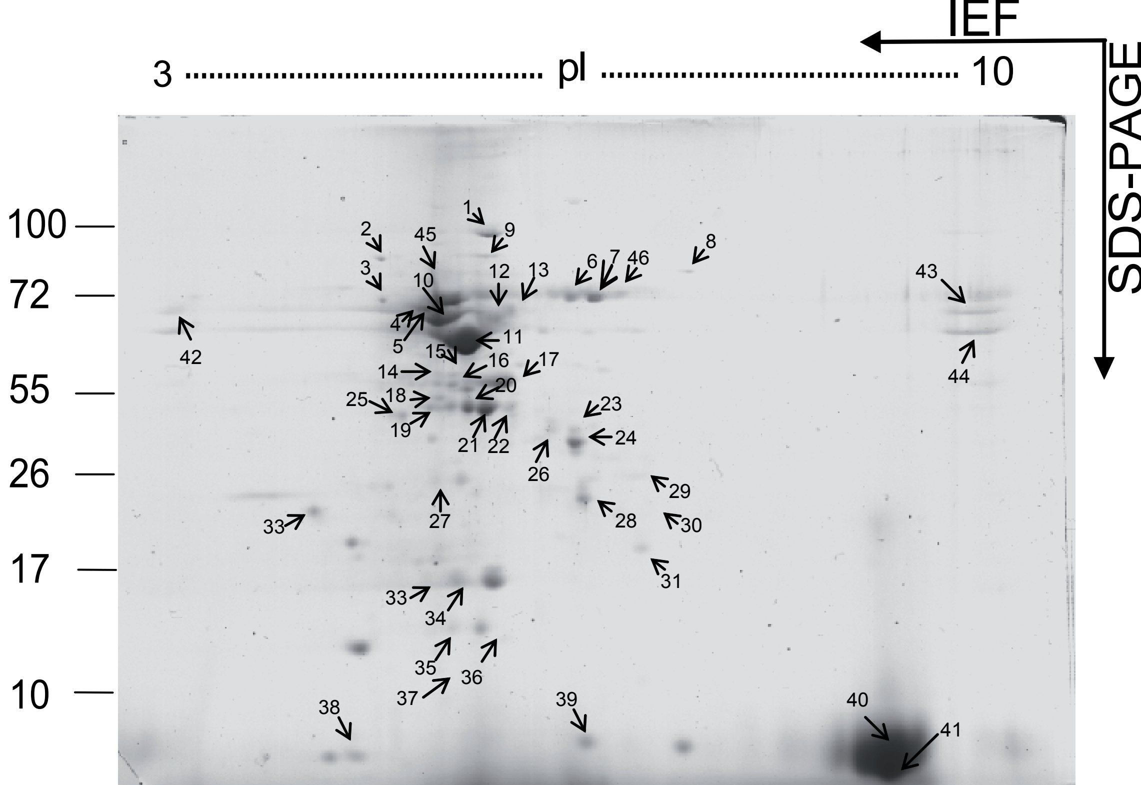
Acquisition of Peptide Mass Fingerprint (PMF) data was performed on a REFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in reflector mode as described in Brumbarova et al. (2008). The spectra were calibrated using external calibration and subsequent internal mass correction. For databank search Biotools 3.0 software (Bruker Daltonics) with the implemented MASCOT search engine (Matrix Science, http://www.matrixscience.com) was utilized, searching for *Viridiplantae* in the non-redundant NCBI database, and the Arabidopsis EST gene index of the TIGR database. Parameters for the search were: monoisotopic mass accuracy; 100 ppm tolerance; missed cleavages 1; and the allowed variable modifications: oxidation (Met), propionamide (Cys) and carbamidomethyl (Cys).

Supplementary Table II. Prediction scores of acetylation on internal lysines of AtSWIB-1.

peptide	position (residue)	score	threshold
VSAGSTKKPAAKP	33	1.12	0.5
SAGSTKKPAAKPK	34	2.17	0.5
TKKPAAKPKAKAK	38	3.00	0.5
KPAAKPKAKAKPK	40	2.44	0.5
AAKPKAKAKPKPK	42	2.89	0.5
KPKAKAKPKPKAK	44	2.99	0.5
KAKAKPKPKAKSD	46	3.26	0.5
KAKPKPKAKSDSP	48	2.21	0.5
KPKPKAKSDSPAK	50	2.70	0.5
KSDSPAKKTPRST	56	1.32	0.5
SDSPAKKTPRSTG	57	1.64	0.5
GIWTYIKSHDLQN	97	0.85	0.5
LQNPADKREIFCD	107	0.79	0.5
LSPHFVKTA****	141	7.14	0.5

PAIL: Prediction of acetylation on internal lysines (http://bdmpail.biocuckoo.org/)

Li et al., 2006



Supplementary Figure 1 Two-dimensional separation of the highly purified transcriptionally active chromosome (TAC-II) isolated form spinach leaves. After separation Coomassie staining revealed a protein pattern consisting of 85 spots. All visualised spots on the polyacrylamide gel were cut out. After trypsin digestion the corresponding peptides were analysed with MALDI-TOF. Proteins belonging to 46 spots could be identified and are listed in Supplementary Table I.

Mass spectrometry

Protein spots were manually excised and trypsin digested (Porcine Sequencing Grade, Promega, Mannheim, Germany) as described (Brumbarova et al, 2008). Acquisition of Peptide Mass Fingerprint (PMF) data was performed on a REFLEX III MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) operating in reflector mode as described in Brumbarova et al. (2008). The spectra were calibrated using external calibration and subsequent internal mass correction. For databank search Biotools 3.0 software (Bruker Daltonics) with the implemented MASCOT search engine (Matrix Science, http://www.matrixscience.com) was utilized, searching for Viridiplantae in the non-redundant NCBI database, and the Arabidopsis EST gene index of the TIGR database. Parameters for the search were: monoisotopic mass accuracy; 100 ppm tolerance; missed cleavages 1; and the allowed variable modifications: oxidation (Met), propionamide (Cys) and carbamidomethyl (Cys).

```
AtSWIB-1 --------------------------mssvaamvfrgarsllapasratsslvsAGSTKK  34
AtSWIB-2 malssgifsttflcvdtAPLRSSMLSPSSLRLSPNHPTNLRMVRAVTSAAAASSDPT---  57
AtSWIB-3 mavssgtfs-tflcikttsFRNPSTPLSSLRFASHPAAKLRLVRAVTSATE-SSEPT---  55
AtSWIB-4 --------------------------mspis-kilggcrtlmakavtnaTATGAG-----  28
AtSWIB-5 --------------------------magis-rvlggfrtlmskaatveaVTVAG-----  28
AtSWIB-6 -----------------------------ms-rvfgacrvlmakaasstgAKTGR-----  25

AtSWIB-1 PAAKPKAKAKPKPKAKSDSPAKKTPRSTGIFKVTPVSPVLAQFLGTGETSRTDAIKGIWT  94
AtSWIB-2 -------------------TTTKTREPRGIMKPRPVSQAMQDVVGVPEIPRTQALKRIWA  98
AtSWIB-3 -------------------ATNK-RVPRGIMKPRPVSPEMQDIVELPEIARTQALKRIWA  95
AtSWIB-4 -----------------------VDRRGGIHKIFPVSESLARFVGQSEVSFSTAMEKVEQ  65
AtSWIB-5 -----------------------EGK--GIFKTRPVSQQLASFAGERELTRGSALKKVWE  63
AtSWIB-6 -----------------------QGT--GILKVVPVSKPLANFIGENEVSRTTAVKKIWE  60

AtSWIB-1 YIKSHDLQNPADKREIFCDETLKLIFEGKDK-VGFLEISKLLSPHFV---------KTA- 143
AtSWIB-2 YIKEHDLQDPQNKRDILCDEKLKKIFEGKER-VGFLEIAKLIGPHFL------------- 144
AtSWIB-3 YIKEHDLQDPQNKREILCDEKLKKIFEGRDR-VGFLEIAKLIGPHFL------------- 141
AtSWIB-4 YTDDHNLWNPENIEEILCDDNLKTIFDGQDKVVGVREMTELLLRHFPNVRTMSAKVKATG 125
AtSWIB-5 YVKLHNLQNPANKKEIHCDDKLKTIFDGKDK-VGITEIMKLLSPHFP---------KSV- 112
AtSWIB-6 YIKLNNLQNPVNKREILCDEQLKTIFSGKDT-VGFLEISKLLSQHFP---------KSA- 109

AtSWIB-1 ----------------------------------------------------------   143
AtSWIB-2 ----------------------------------------------------------   144
AtSWIB-3 ----------------------------------------------------------   141
AtSWIB-4 GGTIEKWGFNDIVKVSEPLARFVGQSEISFDAALRKLLDYAFDHKLVDEGVTLLEFPW   183
AtSWIB-5 ----------------------------------------------------------   112
AtSWIB-6 ----------------------------------------------------------   109
```

B

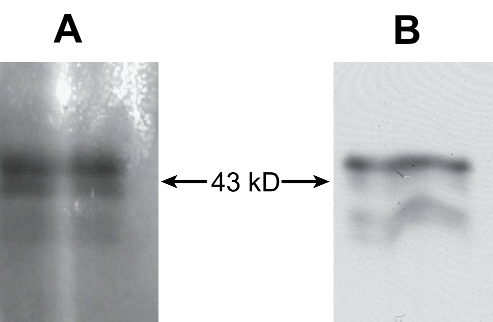
gene locus	**protein**	**Mw kD**	**TargetP**				
			score			predicted localization	TP length (aa)
			cTP	mTP	others		
At3g03590	AtSWIB-1	15,3	0.790	0.004	0.313	chloroplast	85
At2g14880	AtSWIB-2	15,9	0.982	0.011	0.081	chloroplast	43
At4g34290	AtSWIB-3	15,9	0.966	0.017	0.036	chloroplast	75
At3g48600	AtSWIB-4	20,3	0.165	0.287	0.423	mitochondrion	117
At1g31760	AtSWIB-5	12,3	0.073	0.436	0.265	mitochondrion	43
At2g35605	AtSWIB-6	11,9	0.149	0.372	0.205	mitochondrion	15

Supplementary Figure 2 Subfamily of organellar SWIB domain-containing proteins in *Arabidopsis thaliana*. (**A**) Aligment of six stand alone SWIB domain-containing proteins predicted to be targeted to organells. The alignment was performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and depicted using The Sequence Manipulation suite (http://www.bioinformatics.org/sms2/).
(**B**) Predicted localisation of the proteins by TargetP.

```
       plant  IFKVTPVSPVLAQFLGTG---ETSRTDAIKGIWTYIKSHDLQNPADKREI 47
        moss  LTRAIQVSPTLKKFLGVG---ECSRPESMKRIWDYIKDQKLQNPQNKREI 47
 Apicomplexa  ----------LATFMGKT---EASRVEVVKHIWDYIKRHNLQSPENKRMI 37
       yeast  SVRKVLLSAPLQKFLGSE---ELPRTQVVKMIWQYIKEHDLQNPKDRREI 47
       fungi  -VRKVLLSPKLQEFLGET---ELPRTQVVKKVWDYIKEHDLQNPDDRREI 46
     viruses  ------VSKELAEFLGIGPDEKIARTTVVSKLNEYITTHGLQNPEHKIEV 44
    bacteria  FMQPVNVSADLAAIVGAG---PMPRTEIIKKMWDYIKKNGLQDPTNKRNI 47
       human  QPPQYKLDPR ARLLGVH---TQTRAAIMQALWLYIKHNQLQDGHEREYI 47

       plant  FCDETLKLIFEG---KDKVGFLEISKLLSPHFVKTA   80
        moss  LCDEKLKPVLGG---KDKVGFTEIAKLLSEHFP---   77
 Apicomplexa  NADSTLRPLFQ----KDQVSMFELNKLLSKF-----   64
       yeast  LCDEKMEPIFG-----KKMTMFSMNKLLTKHLFN--   76
       fungi  LCDEKMQPIFG-----KKMTMFSLNKILANHLFN--   75
     viruses  LLDEPLRKLLNPPEDFGKVTYFNLCKLVGSHFP---   77
    bacteria  NPDDKLAKVFGT---EKPIDMFQMTKMVSQHIIK--   78
       human  NCNRYFRQIFS---- GRLRFSEIPMKLAGLLQ---   76
```

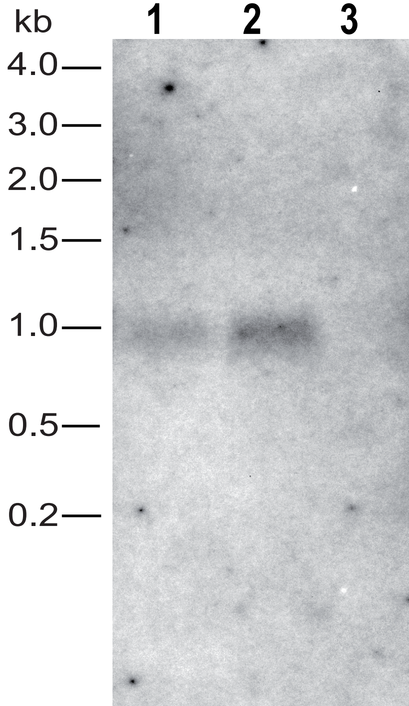
Supplementary Figure 3 Alignment of SWIB-domain protein representatives of a plant (*Arabidopsis thaliana*, AAO39929), a moss (*Physcomitrella patens*, EDQ68916), Apicomlexa (T*oxoplasma gondi*, EEA98853), yeast (*Saccharomyces ceravisiae*, CAA90204), fungi (*Vanderwaltozyma polyspora*, ED018829), viruses (*Feldmannia species virus,* ACH46789), bacteria (*Chlamydia trachomatis*, NP_219973), human (Homo sapiens Q92925).The alignment was performed with ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) and depicted using The Sequence Manipulation suite (http://www.bioinformatics.org/sms2/).



Supplementary Figure 4 Immunological detection of GFP fusion proteins of AtSWIB-1 in protein extracts from tobacco protoplasts (**A**) or tobacco leaves (**B**) used used for transient transformation assays. Protoplast transformation was performed as described in the Materials and methods section. Tobacco leaves were infiltrated with Agrobacteria which has been transformed with a AtSWIB-1:GFP fusion construct.

Agroinfiltration of tobacco leaves in planta

Agrobacterium-mediated transformation was performed with fully expanded tobacco leaves of 4 weeks old plants. For this purpose 10 ng of the plasmid carrying the AtSWIB-1:GFP construct were transformed into *Agrobacterium tumefaciens* GV3101 cells by electroporation with Bio-Rad Gene Pulser (BioRad, München, Germany). After three days of growth at 28°C the bacteria were resuspended in a solution consisting of 1/2 MS-salts, 5%(w/v) sucrose, 0.44 µM BAP (Duchefa Biochemie BV, Haarlem, The Netherlands) and 0.005% SILWET (Leu+Gygax, Birmenstorf AG, Switzerland). Intracellular spaces of intact leaves were infiltrated by a plastic syringe without needle. After 2-3 days proteins were extracted and immunologically analysed.



Supplementary Figure 5 Northern blot analysis. 2 µg (lane 1) and 6 µg (lane 2) of the purified mRNA or 30 µg of total RNA (lane 3) isolated form Arabidopsis leaves were separated on denaturing 1.5 % agarose gel and transferred onto a nitrocellulose membrane. After hybridisation with the radioactively labelled (α-^{32}P-dCTP)*16S rDNA* probe the filters were exposed to X-ray film. Only one band could be observed on the gel.

Four low molecular weight SWIB domain proteins of *Arabidopsis thaliana* are targeted to plastid nucleoids

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Abstract

Proteome analysis of a highly purified transcriptionally active chromosome (TAC) from spinach chloroplasts identified a 17 kD SWIB domain protein named AtSWIB-1. Database searches revealed that this protein belongs to a subgroup of six putatively organelle targeted low molecular weight proteins within the family of 18 SWIB domain proteins of *Arabidopsis thaliana*. Transient transformation of onion epidermal cells and tobacco protoplasts with GFP fusion constructs of all six low molecular weight proteins showed that besides AtSWIB-1 also AtSWIB-2 and -3 are targeted to plastids, whereas AtSWIB-6 was shown to be dually targeted to plastids and mitochondria. Co-localization studies with the nucleoid specific PEND protein showed that besides AtSWIB-1 also AtSWIB-2, -3 and -6 are targeted to plastid nucleoids. Co-localization of AtSWIB-1:RFP with either AtSWIB-2:GFP, AtSWIB-3:GFP or AtSWIB-6:GFP revealed that the different SWIB proteins are closely located to each other but are not parts of one complex. It is hypothesized that the plastidic SWIB domain containing proteins are involved in restructuring of nucleoids.

Introduction

Plastid DNA is organized in compact structures resembling nucleoids of bacteria (Kuroiwa 1991;Sakai et al. 2004). Plastid nucleoids are membrane-bound and are sites of replication, transcription and posttranscriptional control of gene expression. With respect to their transcriptional activity they have been also named transcriptionally active chromosome (TAC) (Igloi and Kossel 1992;Sakai et al. 2004). The protein composition of nucleoids is highly complex and changes during chloroplast development (Falk 1994;Nemoto et al. 1989;Nemoto et al. 1988) and in response to environmental cues. Microscopic and biochemical data suggest that nucleoids consist of a transcriptionally active core with densely packed DNA and a peripheral layer where posttranscriptional processes take place (Krupinska et al., submitted).

Isolated nucleoid fractions contain proteins involved in replication, transcription and posttranscriptional processes of gene expression as well as structural proteins involved in alterations in ptDNA conformation, in remodelling of the nucleoid structure and in the attachment of nucleoids to plastid membranes (Sakai et al. 2004). Recent proteome analyses with TAC fractions from *Arabidopsis thaliana* enabled the identification of several novel DNA/RNA binding proteins with typical eukaryotic motifs that are related to chromatin remodelling proteins functioning in the nucleus such as the SAP (SAF A/B, Acinus and PIAS) domain (Pfalz et al. 2006). By our recent proteome analysis of a highly purified TAC fraction from spinach chloroplasts further proteins with eukaryotic domains such as SWIB domain proteins were identified (Melonek et al., submitted). The SWIB domain is a conserved region in BAF60b proteins which are components of SWI/SNF complexes which were originally identified in yeast (Burns and Peterson 1997). SWI/SNF complexes are involved in ATP-dependent chromatin-remodelling. They might be targeted to a subset of genes by direct interactions with gene-specific transcriptional activators (Yudkovsky et al. 1999). The SWIB domain proteins identified in chloroplasts were shown to have rather low molecular weights and to have a high isoelectric point. Such basic low molecular weight proteins could have replaced the bacterial histone like proteins such as HU which have been found to form nucleosome-like structures in bacterial chromosomes(Rouviere-Yaniv and Gross 1975). Histone-like proteins in bacteria are involved in different processes associated with DNA such as recombination and repair (Dormann and Deighan 2003;Kamashev et al. 2008). A study with a mutated form of the protein showed that HU mediated structural reorganization of the bacterial nucleoid might result in dramatic changes in transcription and in cellular morphology and physiology (Kar et al. 2005).

SWI/SNF complexes are conserved in all eukaryotic organisms, but are not found in bacteria except Chlamydia (Stephens et al. 1998) that are obligate intracellular parasites of humans

and animals. Chlamydia undergo a biphasic developmental cycle: In transcriptionally inert elementary bodies (EB) the nucleoid was shown to be densely compacted whereas in metabolically active reticulate bodies (RB) the nucleoid was observed to be loosely organized (Barry et al. 1992).

In a previous paper we have presented evidence for dual localization of the low molecular weight protein SWIB-1 in nucleoids of chloroplasts and the nucleus (Melonek et al., submitted). In this paper we report on the targeting of three further low molecular weight SWIB domain proteins to plastid nucleoids. It is hypothesized that the plastidic SWIB proteins play a role in restructuring of the nucleoids during plastid differentiation and/or in response to environmental changes.

Results

Sequence analyses

Proteome analysis identified SWIB-1 as a component of the plastid transcriptionally active chromosome (TAC) (Melonek et al., submitted). TAIR (The Arabidopsis Information Resource, www.arabidopsis.org) database analyses allowed the identification of 18 proteins with a SWIB domain in *Arabidopsis thaliana* (Figure 1A). ClustalW alignments (Larkin et al. 2007) showed that based on their amino acid sequences the Arabidopsis SWIB proteins can be subdivided into four major groups (Figure 1A). Most proteins belonging to group 1 and 4 are stand-alone SWIB domain proteins and are predicted to be dually located to either mitochondria or chloroplasts and nucleus, whereas proteins of groups 2 and 3 have domains in addition to the SWIB domain. These proteins are predicted to be targeted to the nucleus, only.

The two group 1 proteins having a molecular weight of about 50 kD have high similarity with the yeast SWP73 protein (Jerzmanowski 2007) which is a component of the SWI/SNF complex with a functional role in transcriptional activation by modulation of the chromatin structure (Cairns et al. 1996). Both proteins are predicted to be targeted to the nucleus by SubLoc (Hua et al. 2001) but to plastids by TargetP and WoLFSORT (Emanuelsson et al. 2000;Horton et al. 2007). The At5g14170 gene encoding the protein CHC1 was identified by screening for genes involved in Agrobacterium mediated transformation (Crane and Gelvin 2007).

Group 2 consists of six proteins of higher molecular weight ranging form 60 to 195 kD and being predicted by SubLoc to localise exclusively to the nucleus (Hua and Sun 2001) (Figure 1A). The members of this group possess additional domains such as RING, Zinc-finger,

Plus-3 and GYF (glycine-tyrosine-phenylalanine*).* All members of this group are predicted to be involved in histone modification and regulation of transcription (TAIR,(Swarbreck et al. 2008)). Three members of group 3 having molecular weights of 40-50 kD are predicted by SubLoc to be targeted to the nucleus, whereas the 12 kD At4g26810 protein is predicted to localize to mitochondria (Hua and Sun 2001) (Fig. 1A). Interestingly, the three larger proteins possess each a DEK domain at their C-terminal ends. DEK domain proteins having structure homologous to E2F/DP transcription factors (Devany et al. 2008) were shown to modify the structure of DNA by introducing supercoils (Kappes et al. 2004).

The fourth group has six members of low molecular weights (12-20 kD). The amino acid sequences of their SWIB domains show high similarity (Fig. 1B). All of these proteins are predicted to be targeted to either plastids or mitochondria by the TargetP prediction program (Emanuelsson et al. 2007) (Table1). Additionally, a nuclear localization was predicted for AtSWIB-1, AtSWIB-2, AtSWIB-3 and AtSWIB-6 when the SubLoc prediction programme was used (Hua and Sun 2001) (Table1). Indeed AtSWIB-1 was shown to be dually targeted to plastids and to the nucleus. The dual localization was further confirmed by immunological analyses with a specific antibody directed towards the protein (Melonek et al., submitted). To further investigate the subcellular localization of the other five members of the low molecular weight group of Arabidopsis SWIB proteins, *GFP* fusion constructs were employed for transient transformation assays.

Subcellular localization of AtSWIB-GFP fusion proteins

For construction of GFP fusion proteins the cDNA sequences of *AtSWIB* genes of group 4 except *AtSWIB-1* were fused at the 3' end with the *GFP* gene sequence. The constructs were tested for expression by particle bombardment of onion epidermal cells. These analyses showed that in case of SWIB-2 and SWIB-3 constructs, GFP fluorescence was associated with the plastids (Fig. 2A). The green fluorescence of the SWIB-4:GFP chimeric protein was observed in the cytoplasm, while the SWIB-5: and SWIB-6:GFP signals were dispersed in many small speckles of different sizes throughout the cytoplasm which could indicate a localization in both plastids and mitochondria (Fig. 2) . The control construct giving rise to GFP alone showed fluorescence in both the cytoplasm and the nucleus (Fig. 2A). The nuclear localization of GFP has been frequently observed (Fischer-Kilbienski et al. 2010;Köhler 1998).

The *AtSWIB-GFP* constructs were further used for transient transformation of protoplasts prepared from tobacco leaves (Fig. 2B). These assays confirmed targeting of AtSWIB-2 and AtSWIB-3 to plastids. After transformation with the AtSWIB-4 construct, fluorescence in protoplasts was detected in the cytoplasm confirming the result obtained with onion epidermal cells (Fig. 2). In protoplasts it became obvious that GFP fluorescence of the *AtSWIB-5* con-

struct was associated with small tubular compartments resembling mitochondria, whereas the AtSWIB-6 showed fluorescence in mitochondria like structures as well as in chloroplasts (Fig. 2B).

Since most of the protoplasts were derived from mesophyll cells having large chloroplasts, the fluorescence signals at higher magnification gave information not only on organellar localization of the proteins, but enabled investigations on the distribution of the signals within chloroplasts. In case of the *AtSWIB-2* construct, green fluorescence signals were distributed in speckles throughout the chloroplasts (Fig. 3A). Similarly as AtSWIB-2:GFP, the AtSWIB-3:GFP and AtSWIB-6:GFP fusion proteins were detected in speckles inside chloroplasts. A comparison with the *PEND:GFP* construct which can be used for visualization of nucleoids (Terasawa and Sato 2005b) and with the *AtSWIB-1:GFP* construct (Melonek et al., submitted) indicated that these speckles were indeed nucleoids. In particular in case of AtSWIB-6 fluorescence in chloroplasts was yellowish indicating a localization in close association with the chlorophyll containing thylakoid membrane.

In order to analyze the processing of the chloroplast targeting sequences of the AtSWIB-1, -2 and -3 proteins*,* the *AtSWIB:GFP* constructs were used for *Agrobacterium* mediated transformation of tobacco leaves. Two days after infiltration, proteins were extracted from the transformed parts of the leaves and used for immunoblot analysis with an antibody directed towards GFP. While in case of the dually located SWIB-1 protein, where the nuclear and the plastid isoforms have different molecular weights (Melonek et al., submitted), two GFP fusion proteins having molecular weights of 45-44 kD and 42-40 kD were detected, protoplasts transformed with AtSWIB-2 and AtSWIB-3 fusions proteins contained only one immunoreactive protein having molecular weights of 43 kD, respectively (Fig. 3B). These molecular weights were expected after cleavage of N-terminal target peptides from the GFP-fusions proteins. These results indicate that AtSWIB-2 and AtSWIB-3 in contrast to AtSWIB-1, which is dually located to chloroplasts and nucleus, are exclusively targeted to plastids. In protoplasts transformed with an *AtSWIB-6* construct at least two immunoreactive bands were detected. This result is in accordance with a dual localization in plastids and mitochondria.

Co-localization of SWIB proteins in chloroplast nucleoids

To investigate whether the SWIB proteins associated with chloroplast nucleoids are different subunits of single complex, co-localization of the SWIB-1 protein with either the SWIB-2, the SWIB-3 or the SWIB-6 protein was analyzed by co-transformation of protoplasts from tobacco leaves. For this purpose the cDNA of the *AtSWIB-1* gene was fused with RFP. This allowed the simultaneous detection of AtSWIB-1 and one of the other SWIB proteins fused to GFP in protoplasts. The assays clearly confirmed that AtSWIB-2, AtSWIB-3 and AtSWIB-6

locate to nucleoids as AtSWIB-1. Overlay of the fluorescence signals showed however that the RFP and GFP signals don't overlap although they are closely located to each other. This indicates that the AtSWIB-2, -3 and -6 proteins don't belong to the same complex as AtSWIB-1. It is likely that the low molecular weight SWIB proteins are distributed among different parts of the nucleoids (Krupinska et al., submitted). Since SWIB-1 did perfectly co-localize with the PEND protein and was identified as a core component of TAC (Melonek et al., submitted) it is likely that the other SWIB proteins belong to the peripheral layer of nucleoids (Krupinska et al., submitted).

Discussion

By proteome analyses with a highly purified TAC fraction from spinach chloroplasts SWIB domain proteins were identified. Database searches showed that *Arabidopsis thaliana* has 18 genes for SWIB domain proteins. Members of groups 1-3 of the family were predicted to be targeted to the nucleus where they could act as subunits of transcriptional activator SWI/SNF complexes (Yudkovsky et al. 1999). The six members of group 4 having low molecular weights (10-20kD) were predicted to be targeted to either plastids or mitochondria (Table 1). By transient transformation with *GFP* fusion constructs the predicted plastid targeting could be confirmed for AtSWIB-1 (Melonek et al. submitted) as well as for AtSWIB-2, At-SWIB-3 and AtSWIB-6. Co-localization studies with RFP and GFP fusion proteins revealed that SWIB-1 has another position in nucleoids than the other three SWIB domain proteins. Since SWIB-1 was identified as an intrinsic component of TAC and shown to co-localize with the PEND protein it is likely to be associated with DNA in the core of nucleoids (Krupinska et al., submitted). In comparison to the other plastidic SWIB domain proteins of group 4, SWIB-1 has a 24 amino acid-long peptide (KKPAAKPKAKAKPKPKAKSDSPAK) at the N-terminal end of the mature protein, which is highly similar to a part of the histone H1 protein from different plant species (Melonek et al., submitted). The same peptide was found to be very similar to the histone-H1-like protein (Hc1) from *Chlamydophila felis* (Melonek et al., submitted). Intriguingly, Chlamydia are the only bacteria described to possess SWIB domain proteins, and both the SWIB domain proteins and Hc1 are involved in condensation/decondensation of chlamydial nucleoids (Barry et al. 1992). The presence of eukaryotic proteins containing chromatin-associated domains in Chlamydia (Stephens et al. 1998) suggests mechanistic similarities between chlamydial nucleoid condensation/ decondensation and eukaryotic chromatin remodelling. Recently, Chlamydial histone H1-like proteins were shown to interact with a SET domain protein. Nuclear proteins with a SET domain were shown to function as histone methyltransferases (Murata et al. 2007).

Genome sequencing of *Chlamydia trachomatis* revealed that besides a stand-alone SWIB protein (CT460) a second diverged SWIB domain copy is fused to the COOH-terminus of topoisomerase I (Stephens et al. 1998). This suggests that SWIB domains might have impact on the activity of topoisomerases involved in the regulation of DNA topology. The genome of *Arabidopsis thaliana* encodes five type I DNA topoisomerases. By different prediction programmes (TargetP, MultiLoc and WoLFPSORT), three of them were predicted to be targeted to organelles. Additionally, three genes for type II topoisomerases could be found in the Arabidopsis genome. Three of them are predicted to be targeted to organelles by TargetP (Emanuelsson et al. 2000). In the proteome of the highly purified transcriptionally active chromosome (TAC-II) from spinach one of these (At4g23890) has been already identified (Melonek, unpublished). One of the type I topoisomerases (At4g31210) has been recently identified in the proteome of spinach chloroplasts (Baginsky et al. 2007). Subunits A and B of gyrase, a bacterial type of topoisomerase I, which introduce negative supercoils to DNA, were found in the proteome of a TAC fraction prepared from chloroplasts of *Arabidopsis thaliana* (Pfalz et al. 2006).

It is likely that SWIB domain proteins possibly together with topoisomerases and gyrases in plastids and mitochondria are involved in structural reorganization of nucleoids and DNA topology. Chloroplast nucleoids of Chlamydomonas were shown to undergo changes in DNA topology during their diurnal life cycle (Salvador et al. 1998). Fluctuations of DNA superhelicity correlated with changes in the rate of plastid gene transcription suggesting a contribution of DNA conformation in control of plastid gene expression (Salvador et al. 1998). In particular negative supercoiling favored the transcription of genes encoding ß- and ε- subunits of maize plastid coupling factor 1 (*cf1*BE) (Stirdivant et al. 1985).

It is expected that in addition to the SWIB proteins here described other SWI/SNF components are present in chloroplasts. *In silico* analysis indicated that two rice proteins (Os05g15890 and Os06g01320) belonging to the SNF2 family of helicase-like proteins (Flaus et al. 2006) have putative plastid localization sequences (Schwacke et al. 2007). Many of the well-studied members of this family are essential motor polypeptides within ATP-dependent chromatin remodelling complexes (Flaus et al. 2006).

Elucidation of the exact function of low molecular weight SWIB domain proteins in nucleoid/TAC fractions from chloroplasts requires characterization of mutant plants. Mutants impaired in nucleus located SWI proteins were observed to have defects in developmental processes such as flowering (Sarnowski et al. 2005). Mutants impaired in plastidic low molecular weight SWIB proteins will be in particular characterized for changes in nucleoid structure, DNA topology and plastid gene expression.

Materials and Methods

Sequence analysis and construction of the phylogenetic tree

All Arabidopsis SWIB-domain containing proteins were found in The Arabidopsis Information Resource database (TAIR, www. arabidopsis.org). The sequence analysis and comparisons were preformed with "The Sequence Manipulation Suite" (http://www.bioinformatics.org/sms/) and ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) programmes. The phylogenetic tree was drawn by the means of the TreeView program (Version 1.6.6).

Plant material and growth conditions

Tobacco (*Nicotiana tabacum* L. cv. Xanthi) plants used for protoplast transformation were grown for 5-6 weeks on sterilized Murashige and Skoog medium at 24°C with a 16 hours photoperiod, whereas plants used for microinjection were grown on soil in a growth chamber at 24°C with 16 hours light. Spring onions (*Allium fistulosum* L.) used for transient transformation were purchased from the local market.

SWIB:GFP fusion constructs

For preparation of GFP fusion proteins the complete coding sequences of group 4 *AtSWIB* genes without the stop codons were amplified from total Arabidopsis cDNA and first cloned into the pENTR/D/TOPO-Vector of the Gateway™ Cloning System (Invitrogen, GmbH, Karlsruhe, Germany). The LR recombination reaction was performed with pBAT-TL-BG (Schwacke et al. 2007) or pB7FWG2,0 (Karimi et al. 2002) destination vectors and the positive pENTR-clones according to the manufacturer's protocol (Invitrogen GmbH, Karlsruhe, Germany). The resulting *AtSWIB:GFP* constructs were transformed into cells of the *E. coli* DH5α strain. As a control the *PEND:GFP* construct (Terasawa and Sato 2005a) and *GFP* alone (Stratagene, La Jolla-CA, USA) were used. All constructs were under the control of the 35S cauliflower mosaic virus promoter.

Transient transformation assays

Transformation of onion epidermal cells with the *GFP* fusion constructs was performed as described (Krause et al. 2005). The GFP fluorescence in the transformed cells was analysed with either a Zeiss Axiophot fluorescence microscope (Zeiss, West Germany, Oberkochen) or with a confocal laser scanning microscope (Leica TCS SP5, Leica Microsystems CMS GmbH, Wetzlar, Germany). The preparation of tobacco protoplasts used for transient transformation in the presence of PEG was performed as described (Krause et al., 2005). For

detection of GFP fluorescence and chlorophyll autofluorescence, the samples were excited either by the 488 nm line of an argon laser or by the 633 nm line of an He-Ne laser.

In planta agroinfiltration of tobacco leaves

Agrobacterium tumefaciens GV3101 cells carrying the AtSWIB:GFP constructs were resuspended in infiltration medium containing 1/2 MS-salts, 5% (w/v) sucrose, 0.44 µM BAP (Duchefa Biochemie BV, Haarlem, The Netherlands), 0.005% w/v SILWET (Leu+Gygax, Birmenstorf AG, Switzerland) and injected with a plastic syringe without needle into intracellular spaces of the intact leaves of 4 weeks-old tobacco plants. After 2 days, proteins were extracted from the infiltrated leaf sections and separated on 16% (w/v) polyacrylamide gel according containing SDS and the buffer system according to Laemmli (Laemmli 1979). For immunological analysis a monoclonal antibody specific for GFP (Clontech, Heidelberg, Germany) and a second antibody directed towards mouse IgG (Sigma-Aldrich Chemie GmbH, Taufkirchen, Deutschland) were used.

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Table 1 SWIB proteins of group 4: Molecular weights and prediction of their subcellular localization by TargetP or SubLoc; respectively, P=plastid; M=mitochondrium; N=nucleus; Cyt=cytoplasm.

gene locus	protein	Mw kD	TargetP				Sub Loc	Net NES
			score		predicted localization	TP length (aa)		
			cTP	mTP				
At3g03590	AtSWIB-1	15	0.790	0.004	P	85	N	+
At2g14880	AtSWIB-2	16	0.982	0.011	P	43	N	+
At4g34290	AtSWIB-3	16	0.966	0.017	P	75	N	+
At3g48600	AtSWIB-4	20	0.165	0.287	M	117	Cyt	-
At1g31760	AtSWIB-5	12	0.073	0.436	M	43	M	-
At2g35605	AtSWIB-6	12	0.149	0.372	M	15	N	-

* prediction made with the NetNES program (http://www.cbs.dtu.dk/services/NetNES/)

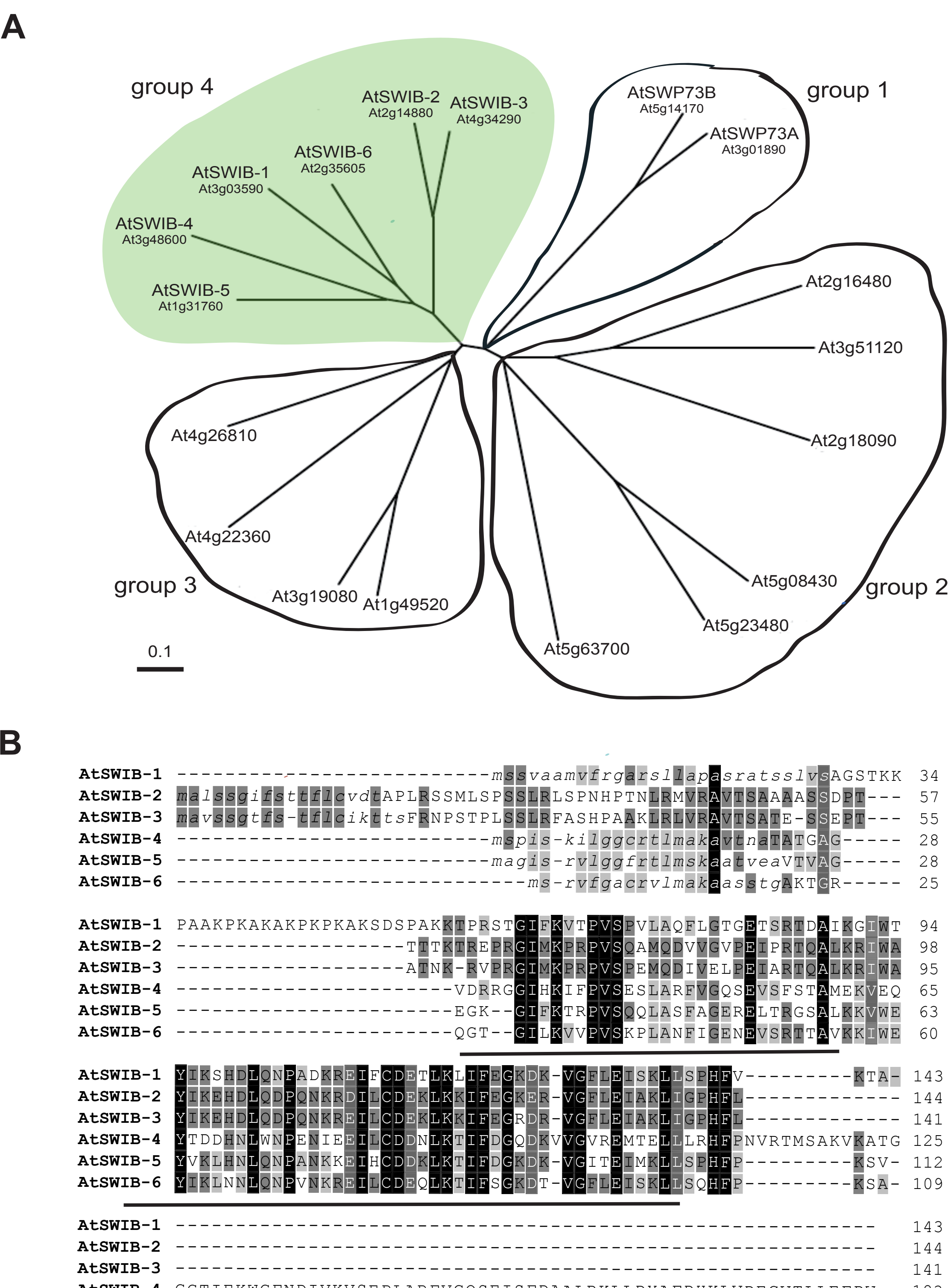

Fig. 1 Phylogenetic analysis of the 18 SWIB domain proteins of *Arabidopsis thaliana* and sequence comparison of the putative organellar SWIB proteins. (**A**) Protein sequences were aligned using the Clustal W programme. The unrooted tree was constructed using the neighbour joining method (Saitou and Nei 1987). The proteins could be subdivided in four groups. Members of the group four have molecular weight ranging from 10 to 20 kD. (**B**) Sequence comparison of the SWIB members belonging to group 4 and being predicted to be targeted to the organelles. Putative targeting sequences are written in italics. The SWIB domain is underlined.
Identical amino acids are depicted in white letters on black ground, the grey letters mark amino acids of similar type.

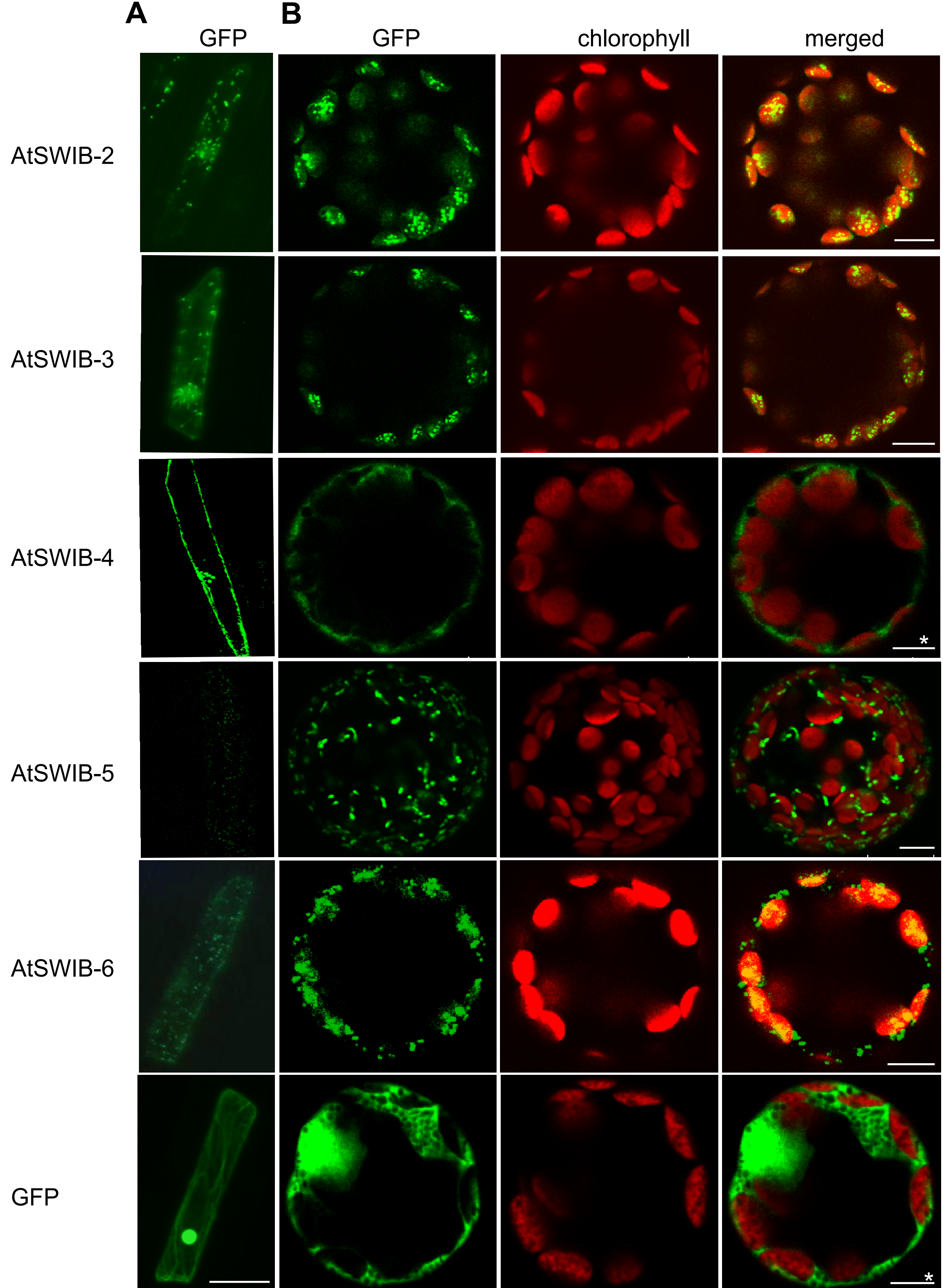

Fig. 2 Subcellular distribution of AtSWIB:GFP chimeric proteins. (**A**) The AtSWIB proteins were fused with GFP at their C-termini and were transiently expressed in onion epidermal cells. As a control the coding sequence of the GFP protein alone was used. Scale bars: 100 µm (**B**) Transient transformation of tobacco protoplasts. Scale bars: 7,5 µm or 4 µm (marked by asterisks). As control GFP not fused to another protein was shown.

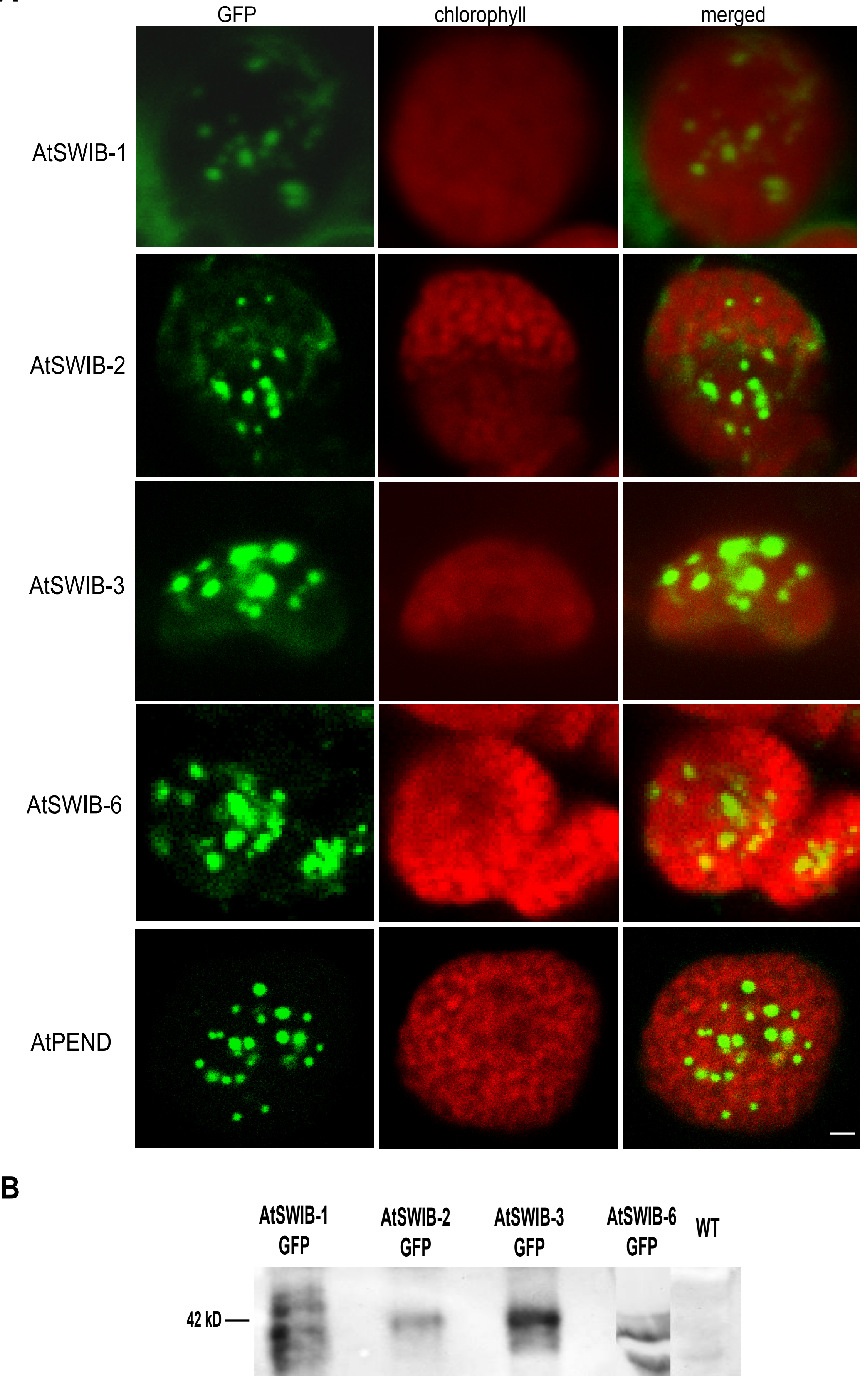

Fig. 3 (**A**) High resolution microscopy of individual chloroplasts of protoplasts transformed with C-terminal GFP fusion constructs of AtSWIB-1, -2, -3 and -6. For comparison the fluorescence signals of the PEND:GFP protein are shown. Scale bars: 600 nm (**B**) Immunological detection of GFP fusion proteins of SWIB-1, SWIB-2, SWIB-3 and SWIB-6 proteins in extracts from tobacco leaves after infitration with Agrobacterium tumefaciens.

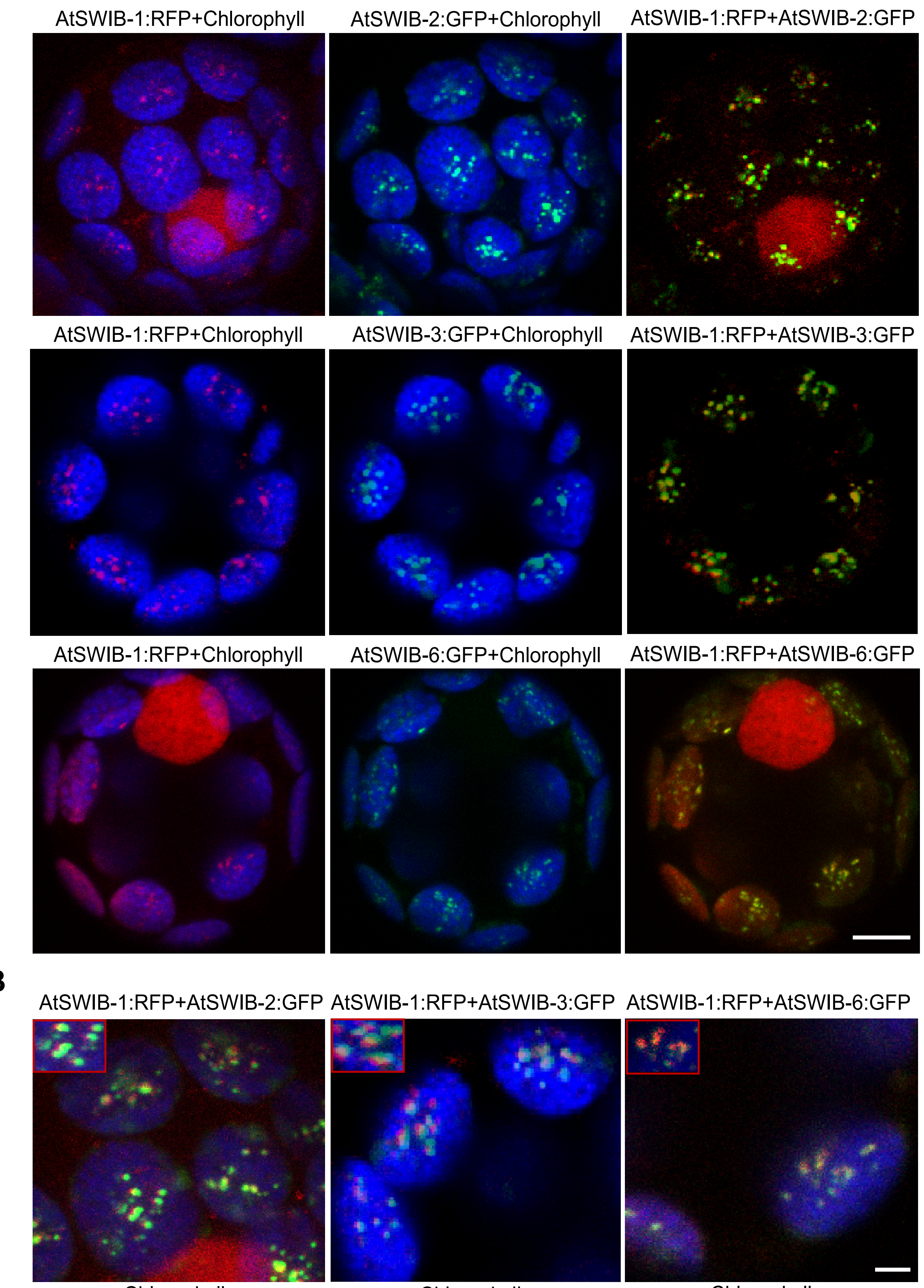

Fig. 4 Co-localization of AtSWIB-1 protein with other plastidic SWIB proteins in chloroplast nucleoids. Transformation of protoplasts was performed with an AtSWIB-1:RFP construct and simultaneously with either AtSWIB-2:GFP, AtSWIB-3:GFP or AtSWIB-6:GFP constructs. The red fluorescence of chlorophyll was converted into blue colour for better contrast. (**A**) An overview of the whole protoplast. Scale bar: 7.5 µm (**B**) A detailed view of the distribution of the fluorescent signals within chloroplasts. Scale bar: 1.4 µm

IV

New insights into plastid nucleoid structure and functionality

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Keywords

Nucleoid-associated proteins, Plastid nucleoid structure, Plastid gene expression

Abbreviations

NAP, Nucleoid-associated protein; NEP, Nuclear-encoded plastid RNA polymerase; PEP, Plastid-encoded plastid RNA polymerase; ptDNA, Plastid DNA; TAC, Transcriptionally active chromosomes

Abstract

Investigations over many decades have revealed that the nucleoids of higher plant plastids are highly dynamic with regard to their number, their organization and protein composition. Membrane attachment and environmental cues seem to determine the activity and functionality within the nucleoids and point to a highly regulated structure-function relationship. The heterogeneous composition and the many functions that are seemingly associated with the plastid nucleoids could be related to the high number of chromosomes per plastid. Recent proteomic studies have brought novel nucleoid-associated proteins into the spotlight and indicate that the plastid nucleoid is an evolutionary hybrid of prokaryotic nucleoid components and eukaryotic (nuclear) chromatin features. These recent advances will provide the basis for future studies on the relationship between nucleoid structure, plastid gene expression and plastid-nucleus communication.

Introduction

Plastid DNA is assembled in complex structures of high molecular weight that are attached to intraplastidial membranes and contain proteins as well as RNA. These structures, termed plastid nucleoids or plastid nuclei (Sakai et al. 2004), are associated with numerous enzymatic activities such as DNA repair, DNA replication, recombination, transcription and posttranscriptional control of gene expression. Microscopic studies showed that the sub-plastidial localization and composition of the complexes is dynamic and seems to undergo age-dependent changes that could be coupled to changes in the overall activity and specificity of RNA polymerases and components of other regulatory levels of plastid gene expression.

For a long time, knowledge about the protein components of the plastid nucleoid was limited to information from biochemical studies, which were summarized in excellent reviews by Sato and coworkers as well as Sakai, Takano and Kuroiwa (Sakai et al. 2004;Sato et al. 2003). The improvement of bioinformatic targeting

predictions, GFP-based analysis of subcellular protein localization and last but not least the increasing sensitivity of proteome analyses have led to a recent sharp increase in our knowledge of the composition of the plastid nucleoids. Among the newly identified proteins are DNA binding proteins with eukaryotic motifs for which a dual targeting activity to the plastids and the nucleus has either been demonstrated or is postulated. The current review will concentrate mainly on these recent advances and will refer to older knowledge only when necessary for the integrated picture of the whole nucleoid. We therefore apologize to those whose work could not be cited due to space limitations.

Organization of the plastid multicopy genome in higher plants

Having evolved from bacteria, plastids and mitochondria of plant cells have not only inherited a genome that consists of a circular DNA molecule but have also kept many features of its structural organization. Plastid nucleoid structure has been under investigation ever since the concept of organellar nucleoids was introduced (Kuroiwa et al. 1982) and has been reviewed in much detail (Sakai et al. 2004).

The plastid genome of higher plants occurs in high copy numbers which differ in various organs, cell types and also depend on the developmental stage of plastids (Boffey et al. 1979;Mullet 1993). In mesophyll cells of green leaves, numbers in the range from 2.000 to 50.000 genomes per cell were estimated (Bendich 1987;Coleman and Nerozzi 1999;Kuroiwa et al. 1982). On average, 10-20 copies of the plastid genome are organized into a variable number of structures that originally were named plastid nucleoids due to their resemblance to their bacterial counterparts (Kuroiwa 1991). The fundamental difference between genome organization in plastids versus that in bacteria is that the plastids are multi-nucleated as opposed to the single-nucleated organization of their prokaryotic counterparts (Sakai et al. 2004).

Dynamic changes in number, shape and distribution of plastid nucleoids

Significant differences in nucleoid number, shape and distribution have been found to parallel developmental changes in structure and function of plastids and are reviewed elsewhere (Sato et al. 2003). Recently, differences in nucleoid number and morphology were also reported to occur in different sectors of variegated leaves (Sakamoto et al. 2009) and during plastid division. While plastids of wild-type *Arabidopsis thaliana* contain a filamentous network of multiple nucleoids during chloroplast division (Terasawa and Sato 2005b), overexpression or knockdown of the chloroplast division protein AtYLMG1-1 protein caused the formation of an irregular network of chloroplast nucleoids or aggregation of the nucleoids as larger structures, respectively (Kabeya et al. 2010). Immunofluorescence studies revealed further that AtYLMG1-1 is located in a punctuate pattern with the speckles always being in close proximity to thylakoid membranes and possibly co-localizing with nucleoids (Kabeya et al. 2010).

Sub-nucleoid domains

A functional diversification in different zones was reported for nucleoids of mitochondria (Shutt et al. 2010) and bacteria (Dillon and Dorman 2010) as well as for the chromatin in the nucleus (Matera et al. 2009;Shaw and Brown 2004). The benefit of these sub-compartmental structures is an accumulation of DNA-binding proteins and DNA-regions to certain areas, which might enhance the rates of certain biochemical reactions associated with gene expression and might confer an evolutionary advantage over a random chromatin architecture (Matera et al. 2009).

Evidence for discernible sub-domains with different specific functions have also been found within plastid nucleoids. Initial reports from electron microscopic studies, which showed that the deproteinized spinach plastid chromosome is organized in a folded form around a central body composed of proteins which are not extractable by usual procedures (Herrmann et al. 1974;Yoshida et al. 1975),

were soon followed by biochemical data that confirmed the observation that proteins are either firmly bound to DNA in a central region or are more loosely bound to the peripheral DNA fibrils. These observations led to the proposal of a layered structure of plastid nucleoids (Briat et al. 1982;Hansmann et al. 1985) (reviewed by (Sakai et al. 2004)) (see Fig. 1). An estimated 30-50% of the ptDNA is incorporated in the central body. Surprisingly, ultrastructural studies revealed that the DNA loops protruding from the core of chloroplast nucleoids are lacking in nucleoids from chromoplasts of *Narcissus pseudonarcissus* (Hansmann et al. 1985). This would lead to the hypothesis that the layered structure is dynamic and under developmental control. Two of the many open questions are what determines the relative proportions and degrees of compaction in the two layers of plastid nucleoids and whether this has functional consequences.

The central body was by some authors compared with eukaryotic heterochromatin and the more dispersed DNA-protein complexes in the periphery with eukaryotic euchromatin where transcription is active (Sato et al. 2001;Sato and Ohta 2001). However, this contrasts with the observation that even highly condensed plastid DNA can be actively transcribed (Kuroiwa et al. 1990). This concept would be in line with the current model of mitochondrial nucleoid architecture in which replication and transcription were suggested to occur in the central core whereas translation and complex assembly occur in peripheral regions (Bogenhagen et al. 2008). Sakai and coworkers in fact suggested that the central body is involved in membrane binding and that this is a prerequisite for active transcription (Sakai et al. 2004). On the other hand, at least one type of plastid RNA polymerase, namely the NEP encoded by the *RPOTmp* gene seems to be sensitive to membrane binding and is switched off when it is brought into close proximity of the thylakoid membranes. This sensitivity is conferred by a thylakoid membrane-embedded RING protein, NIP1 (NEP interacting protein 1), that showed specific interaction with RpoTmp (Azevedo et al. 2008), reflecting the complex interplay between structure, distribution and activity.

Considering that the two plastid nucleoid layers or sub-domains have different functionality, they are expected to also have different sets of proteins. Whether they also attract different chromosomal regions, has not been answered conclusively but there is some evidence for this. For example, the analysis of transcripts synthesized by highly purified transcriptionally active chromosomes (TAC) that resemble the central body, revealed that ribosomal RNA transcripts were enriched (Krause and Krupinska 2000). This might indicate a spatial organization of different transcription foci similar to what has been found in bacteria.

Heterogeneity of plastid nucleoids within the same plastid

A feature that distinguishes plastid nucleoid architecture from that of its bacterial equivalents is its multi-nucleate nature. With respect to the tremendous differences in plastid nucleoid morphology in different tissues and between different plant lineages (Kuroiwa 1991;Kuroiwa et al. 1981) structural and functional variance among the multiple nucleoids of a single chloroplast is likely. Recently, transformation of tobacco protoplasts with a construct encoding a GFP:Whirly1 fusion protein showed that Whirly1 is associated only to a sub-population of nucleoids (Melonek et al. 2010). This is a first evidence for heterogeneity of nucleoids in a given mesophyll cell. Size and position of different nucleoid sub-populations could very well reflect the different requirements for enzymatic activities within the plastid nucleoids during plastid biogenesis.

Structural and functional heterogeneity has been also reported for mitochondrial nucleoids of human and yeast. By localization of GFP fusions of nucleoid proteins with DNA it became evident that in yeast cells at least two populations of nucleoids exist within the same mitochondrion. An actively replicating type can be distinguished by its association with a proteinaceous structure spanning the inner and the outer membrane (Meeusen and Nunnari 2003).

Analysis of the composition of plastid nucleoid-associated proteins (NAPs)

Compact nucleoids and **transcriptionally active chromosomes** (so-called **TACs**) are the two membrane bound fractions that have been predominantly isolated in order to elucidate the composition of NAPs in general and of the transcriptional apparatus in particular (see reviews of (Gruissem and Tonkyn 1993;Igloi and Kössel 1992;Sakai et al. 2004) and references therein). The TAC fraction is prepared from plastid membranes by release of the nucleoids with detergents and by subsequent Sepharose gel filtration and other purification steps (Krause and Krupinska 2000). Recent proteomic analysis of these (Melonek et al., manuscript submitted) and other sub-nucleoidal fractions (Pfalz et al. 2006;Phinney and Thelen 2005) have revealed that several DNA binding proteins with eukaryotic motifs are components of plastid nucleoids. On the other hand, plastid proteins homologous to the abundant prokaryotic HU protein were not detected in higher plants which stands in contrast to their identification in some algal plastids, in the apicoplasts of apicomplexans (Sato et al. 2003) as well as in some dinoflagellates (Chan et al. 2006). This obvious tendency towards using alternative proteins with eukaryotic DNA binding motifs could mirror a high interdependency between chloroplasts and the nucleus in higher plants that is less pronounced in the evolutionarily more primitive algae.

NAPs involved in gene expression

A number of functional activities have been associated with the nucleoids of plastids. Primarily, these include the maintenance and replication of the plastid genome as well as the expression of plastid genes. Accordingly, subunits of the plastid-encoded RNA polymerase, PEP, have been identified in several of the proteomic studies of plastid nucleoid fractions of Arabidopsis (Pfalz et al. 2006), mustard (Pfalz et al. 2006) and spinach (Melonek et al., manuscript submitted). In addition, several other proteins potentially involved in the regulation of ptDNA transcription were identified, among them a protein with similarity to the transcription termination factor Rho (Melonek et al., manuscript submitted).

Besides PEP, a nuclear-encoded plastid RNA polymerase, NEP, has been postulated to be involved in plastid gene transcription (for a review see Börner et al. 1999). So far, this NEP has neither been identified in the transcriptionally active chromosome fraction nor in the soluble protein fraction (Pfannschmidt and Link 1994; Krause and Krupinska 2000) that has recently be subjected to proteomic analysis (Schröter et al. 2010) nor in any of the nucleoid or TAC proteomes. It is, therefore, likely that its amount is still below the detection limit of the current analytical methods.

Several PPR proteins were also identified in the nucleoid or TAC proteomes. Since the majority of PPR proteins has been associated with organellar RNA processing and translation (Schmitz-Linneweber and Small 2008), this indicates that posttranscriptional levels of gene expression such as RNA stability and turnover are at least in part closely connected to the nucleoids. This is in line with the notion that ribosomes seem to be quite closely associated with the nucleoids of plastids (Pfalz et al. 2006;Phinney and Thelen 2005) and also those of mitochondria (Nosek et al. 2006).

NAPs involved in DNA replication and repair

Nucleoids have been shown to be active in replication of DNA (Heinhorst and Cannon 1993;Kuroiwa 1991) and some candidates for these activities have been identified in the newly published nucleoid proteomes but so far very few reports have analysed these activities more carefully. Among the candidates that are involved in these activities are members of the Rec family. One protein with a RecF/RecN/SMC domain has been identified in the spinach TAC proteome (Melonek et al. 2010, manuscript submitted). In rice, two RecQ-like helicases, OsRecQ1 and OsRecQsim, were found to be targeted to the plastids and were discussed as components of a plastid-specific DNA-repair system (Saotome et al. 2006). Recently, it has been reported, that the two plastid Whirly proteins AtWhy1 and AtWhy3 that have been identified in TAC proteomes (Pfalz et al. 2006) function as antirecombination proteins contributing to safeguard plastid

genome integrity (Marechal et al. 2009). Whirly proteins can bind to single stranded DNA and RNA (Desveaux et al. 2000;Prikryl et al. 2008) and seem to assume a number of important functions in nucleoid metabolism. Like the Whirly group of proteins, the organellar single stranded DNA binding proteins (OSBs) are a small family of proteins involved in ptDNA stability and recombination surveillance (Marechal and Brisson 2010). OSB2 alias pTAC9 was identified in the Arabidosis TAC proteome (Pfalz et al. 2006).

Architectural NAPs

While bacterial nucleoids are associated with the cytoskeleton (Travers and Muskhelishvili 2005), organellar nucleoids are attached to intraplastidial membranes that were proposed to serve as a platform for DNA synthesis and transcription (Sakai et al. 2004). Consequently, many of the architectural nucleoid proteins that have so far been identified in plastids are involved in anchoring the nucleoids to either envelope or thylakoid membranes.

PEND

The plastid envelope DNA binding protein (PEND) is a 70 kD membrane-spanning protein with a basic region plus leucine zipper (bZIP) domain that *in vivo* forms dimers which tether the nucleoids to the inner plastid envelope (Sato et al. 1993;Sato et al. 1998). The PEND protein was initially discovered in developing pea chloroplasts (Sato et al. 1993). Homologs were later detected in other angiosperms (e.g. in *Brassica napus* (Waldmuller et al. 1996)) while functional homologs in algae and in non-flowering plants are still not known (Terasawa and Sato 2005a). The cbZIP domain of PEND was shown to bind selectively to AT-rich regions of plastidic DNA containing the canonical sequence TAAGAAGT (Sato and Ohta 2001). Interestingly, both the PEND protein from pea and its rapeseed homolog, GSBF1, were found to repress the expression of nuclear genes (Waldmuller et al. 1996;Wycliffe et al. 2005). That PEND indeed fulfils a direct role in the nucleus was supported by the recent observation that a PEND:GFP fusion protein is targeted to the nucleus when the N-terminal

presequence is deleted (Terasawa and Sato 2009). It has been proposed that the PEND protein might be first targeted to plastids where the N-terminal presequence is cleaved and that it might be relocated to the nucleus when the chloroplast envelope is degraded (Terasawa and Sato 2009).

PD1 and PD3

PD1 (plastid DNA binding 1) having a molecular mass of 50 kD and PD3 (plastid DNA binding 3) with a molecular mass of 130 kD were detected during research on PEND (Sato et al. 1995). They both possess AT-hook motifs which were originally found in the high mobility group I (HMG-I) of nuclear proteins and could be shown to interact with the minor groove AT-rich regions of nuclear DNA (Grasser 1995). In addition to the two or five AT-hooks of PD1 and PD3, respectively, PD3 possess eight CxxC motifs, which are presumed to be metal binding sites of the protein (Sato et al. 1995). PD3 also has a jmjC domain at the C-terminus which is proposed to be involved in chromatin remodelling (Kodama 2007). Structural information about the proteins suggests that these plastid DNA binding proteins have evolved from nuclear AT-hook containing transcription factors (Kodama 2007). The localization of the PD1 and PD3 proteins to the plastid nucleoids was shown by immunological analysis with specific antibodies. In contrast to PEND which was shown to be distributed between membrane and stroma fractions, PD1 and PD3 were exclusively found in membrane fractions containing the nucleoids (Sato et al. 1995).

MFP1

The MAR binding filament-like protein 1 (MFP1) mediates the attachment of nucleoids to thylakoid membranes. MFP1 was first described in tomato as a nuclear DNA binding protein that connects chromatin with the nuclear envelope via matrix attachment regions (MAR) (Meier et al. 1996). MFP1, as an anchor protein, has an additional N-terminal hydrophobic membrane-spanning domain (Meier et al. 1996). Its targeting to the thylakoid membranes of chloroplasts was detected some years later and MFP1 is now known to be involved in anchoring

the nucleoids to the thylakoid membranes with the C-terminal DNA binding domain orientated towards the stroma (Jeong et al. 2003) (see model in Fig. 1).

SiR

SiR (sulfite reductase) is one of the most abundant proteins of nucleoids with a molecular weight of 70 kD. It was shown to repress the transcriptional activity in isolated nucleoids by compacting their DNA (Sekine et al. 2002). In contrast to the PEND protein its binding to DNA is apparently sequence-independent. The 68 kD protein DCP68 (Cannon et al. 1998) that suppresses ptDNA replication was found to correspond to SiR, broadening the spectrum of functional roles that this protein plays. SiR, which initially has been identified as a sulfite reductase is therefore not a bifunctional (Sato et al. 2001) but rather a multifunctional protein. It has been shown that binding to DNA does not influence the sulfite reductase activity of the bifunctional protein (Sekine et al. 2007) but, *vice versa*, it is not known whether the enzymatic activity modulates the DNA compacting activity. Such an interplay would be of particular interest as it would potentially link gene expression to chloroplast metabolism. SiR has been detected in the proteome of a Triton X-100 insoluble plastid fraction that is enriched in NAPs but devoid of membranes (Phinney and Thelen 2005).

CND41

The chloroplast nucleoid DNA binding protein (CND41) is another protein that binds non-specifically to plastid DNA via a helix-turn-helix motif and a putative zinc finger motif. It was identified initially in nucleoids isolated from cultured tobacco cells (Nakano et al. 1993). CND41 has been regarded as a negative regulator of transcription because its abundance in various tissues negatively correlates with the levels of certain plastid mRNAs (Nakano et al. 1997). Its sequence is similar to those of aspartic proteases and it exhibits strong proteolytic activity at an acidic pH (Murakami et al. 2000). In senescent leaves of barley its expression was shown to be down-regulated (Parrott et al. 2007). This

suggests that its activity is associated to the maintenance of chloroplast function and not to dismantling of chloroplasts.

Swi/SNF proteins

Among the 85 proteins found to be enriched in highly purified TAC-II fractions from spinach chloroplasts is SWIB-1 (Melonek et al., manuscript submitted). SWIB-1 has a SWIB domain which is a conserved region in all subunits of BAF60 proteins that are known components of SWI/SNF chromatin remodelling complexes originally identified in yeast (Burns and Peterson 1997). Sequences for 18 SWIB domain-containing proteins could be found in the genome of *Arabidopsis thaliana* but only the SWIB-1 protein has a histone like sequence motif (KKPAAKPKAKAKPKPKAKSDSPAK). A SWIB-1:GFP fusion protein was located in plastids and the nucleus (Melonek et al., submitted), placing SWIB-1 alongside several other dually targeted nucleoid proteins like PEND, MFP1, Whirly1 and others (Krause and Krupinska 2009). Immunological analyses showed, moreover, that the nuclear SWIB-1 protein has a higher molecular weight than the plastid form. The exact function of this protein still awaits further investigations.

Bifunctionality and dual targeting of plastid NAPs

An intriguing aspect is the association of metabolic/biosynthetic chloroplast functions with nucleoid fractions (see Table 1). This discovery suggests that nucleoids could be closely associated with functional complexes of plastids and that some of the nucleoid-associated proteins are even bifunctional. Such proteins could be involved in the linkage of metabolism and gene expression. It is rather unlikely that the bifunctional nucleoid components fulfilling a second metabolic function are purification artefacts because a considerable number of these proteins are consistently being detected with different purification approaches (Table 1). Moreover, the well-documented association of yeast and human mitochondrial nucleoids with metabolic enzymes whose occurrence seems to be highly conserved between different fungal and mammalian species

(Nosek et al. 2006, Kucej and Butow 2007)) makes it feasible that these types of bifunctional proteins play an important functional role in the nucleoids. Interestingly, many putative bifunctional proteins are shared by mitochondria and plastids. It has been proposed that such activities could mediate signal transduction from metabolic stimuli to the nucleoids and invoke structural changes (Kucej and Butow 2007).

Another common trait seems to be the close association of ribosomal subunits with nucleoids. Proteins involved in translation and RNA binding proteins have been found in all plastid nucleoid fractions containing subunits of PEP (Phinney and Thelen 2005, Pfalz et al. 2006, Melonek et al. 2010, manuscript submitted) and were also reported for yeast mitochondria (Kucej and Butow 2007, Nosek et al. 2006) and references cited within) (Table 1). Their systematic occurrence in diverse preparations and organisms suggests that posttranscriptional processing steps and translational activity are probably closely associated with the nucleoids. This is in accordance with reports from bacterial nucleoids (Gowrishankar and Harinarayanan 2004).

Several of the plastid DNA binding proteins that were experimentally identified possess motifs that are typically found in nuclear transcription factors (Kodama 2007). Examples are PEND, CND41, PD1, PD3 (Kodama 2007) as well as Whirly1 (Krause et al. 2005) and the newly identified NAP SWIB-1 (Melonek et al., manuscript submitted). It has been suggested that these proteins have changed their subcellular localization during evolution and became plastid proteins. For some of these proteins experimental evidence for a dual localization in plastids and in the nucleus has already been reported (Table 2). Two *in silico* studies performed on eukaryotic transcription factors have revealed that a considerable number of transcription factors with a nuclear localization sequence also have an N-terminal plastid targeting sequence, increasing the number of putative dually targeted DNA binding proteins significantly (Schwacke et al. 2007;Wagner and Pfannschmidt 2006). Among the DNA binding proteins predicted to be dually located, some were initially described to have functions in

nuclear chromatin remodeling. It is feasible that proteins like SWIB-1 (Melonek et al., manuscript submitted) have replaced the HU like proteins which still can be found in algae. This occurrence of chromatin remodelling proteins with functions in the nucleus and plastids could imply that communication between plastids and the nucleus occurs by dynamic changes in both plastid nucleoid structure and nuclear chromatin architecture. Whether a differential distribution of the proteins between the two compartments could induce changes in structure and gene expression awaits further investigations.

Conclusions and prospects

Understanding how genomes function is one of the central questions in the post-genomic era. Due to the polyploid nature of the plastids and the multi-nucleoid and heterogeneous organization of their genetic material, analyses of composition, structure and function of the nucleoids have been slowed down compared to, for example, the analysis of the nuclear chromatin. The recent new insights into plastid NAP composition was overdue and opens up for a new level of investigations of plastid genome functionality. Particularly, systematic investigations of the ptNAP inventory during plastid development and in response to changes of the environment remain to be performed.

Environmental impact on nucleoid morphology represents another intriguing open question. Chloroplasts can be regarded as sensors of environmental changes (Bouvier et al. 2009) that are perceived as imbalances in electron flow resulting in changes in the redox state of the plastoquinone pool and other redox systems of chloroplasts (Pfannschmidt et al. 2009). These imbalances are known to alter the transcription of a number of plastid genes (Pfannschmidt et al. 1999). Considering the tight association of nucleoids and thylakoids, a direct perception of redox changes in the photosynthetic apparatus by redox sensitive proteins in the nucleoids as also reported for a soluble RNA polymerase preparation from chloroplasts (Schröter et al. 2010) must be expected.

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Tables

Table 1: Examples for bifunctional ptNAPs in plastids.

Primary function	Plastid NAPs
NADP-dependent reductase	NmrA-like (1)
Iron superoxide dismutase	Fe-SOD1 (2) Fe-SOD3 (2)
Thioredoxin	TRX P (2)
ferredoxin:sulfite reductase precursor	SiR (1,3)
Mur ligase	MurE (2)
Rubisco activase	Rca (4)
Ribosomal subunits	Rpl4 (1) Rpl12-1 (2) Rps3 (2) Rpl29 (2) RaiA (1)
Translation elongation	EF-Tu (2, 4)
Telomere binding protein	Whirly1 (2) Whirly3 (2)
Pyruvate-dehydrogenase	OdpA (1)
Heat shock proteins	HSP70 (4) HSP40 (4)
Proteases	ClpC protease (4)
ATPase	ATPase α, β,γ and ε subunit (4)
amino acid biosynthesis	T12H1_16 (At3g05190) (4)

1= Phinney and Thelen 2005; 2= Pfalz et al. 2006; 3= Sato et al. 2007; 4= Melonek et al. 2010, manuscript submitted

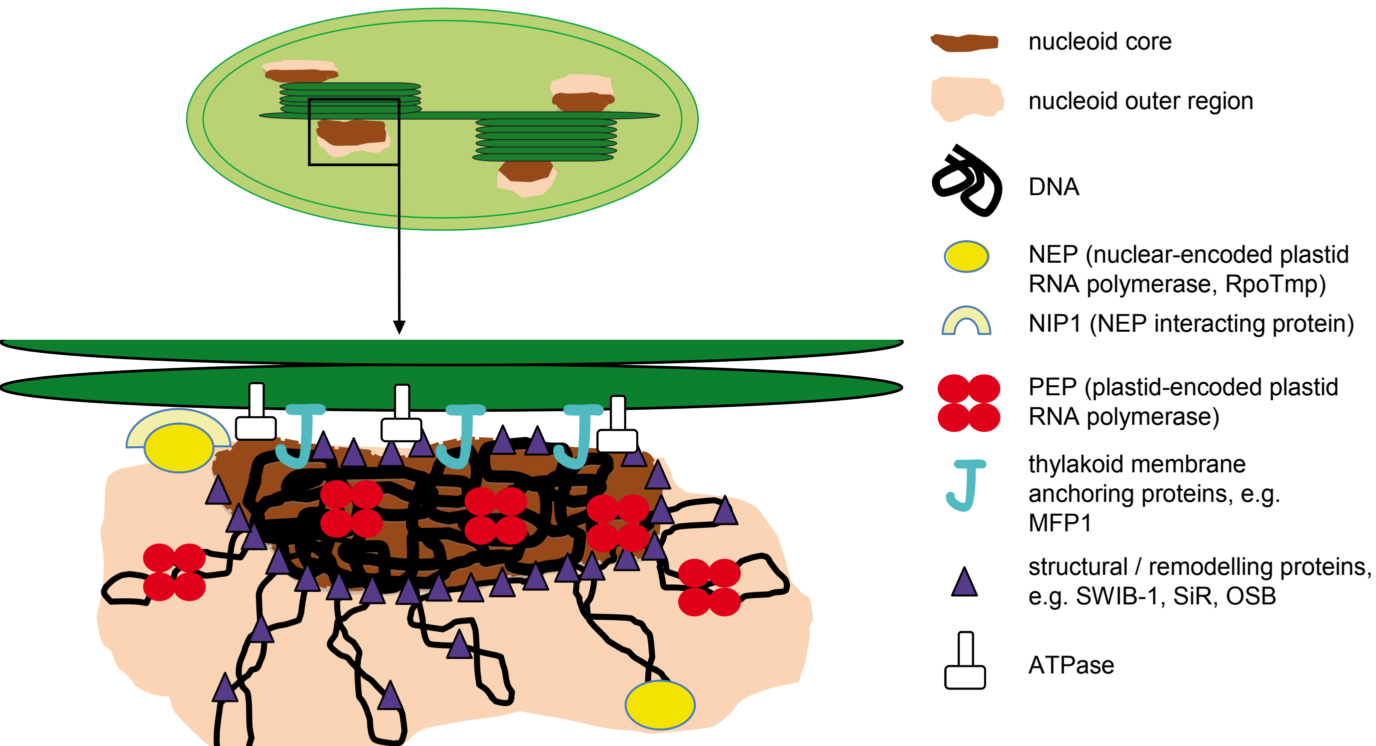
Table 2: Examples for identified ptNAPs that are dually targeted to plastids and the nucleus.

NAP	plastid function	nuclear function
PEND	attachment to envelope membranes (Sato et al. 1993, 2001)	unknown (Terasawa and Sato 2009)
MFP1	attachment to thylakoid membranes (Jeong et al. 2003)	chromatin attachment to the nuclear envelope (Meier et al. 1996)
Whirly1	ssDNA and RNA binding; recombination (Pfalz et al. 2006, Prikryl et al. 2008, Grabowski et al. 2008, Melonek et al. 2010, Maréchal et al. 2010)	transcription factor and telomere homeostasis (Desveaux et al. 2000, Yoo et al. 2007)
SWIB-1	unknown (Melonek et al. manuscript submitted)	component of SNF/SWI transcriptional activation complexes

Figure legends

Figure 1: Model for the layered structure of plastid nucleoids.

Plastid nucleoids consist of a dense layer, or nucleoid core, where protein-DNA interactions are insensitive to salt or detergent treatments (see text) and where transcriptional activity is particularly high (Sakai et al. 2004). Several membrane anchor proteins in this layer can mediate the attachment of the DNA to plastidial membranes. The nucleoid core is surrounded by a second layer where DNA-protein interactions and possibly DNA compaction are less tight. Switching between the „core DNA“ conformation and the „surrounding DNA“ conformation could be mediated by chromatin remodelling proteins like SWIB-1 that are depicted here exemplarily at the interphase between the two layers. Other membrane-associated proteins like the NEP interacting protein NIP can selectively control the activity of the NEP polymerase by preventing its association with plastid DNA (Azevedo et al. 2008).


nucleoid core
nucleoid outer region
DNA
NEP (nuclear-encoded plastid RNA polymerase, RpoTmp)
NIP1 (NEP interacting protein)
PEP (plastid-encoded plastid RNA polymerase)
thylakoid membrane anchoring proteins, e.g. MFP1
structural / remodelling proteins, e.g. SWIB-1, SiR, OSB
ATPase