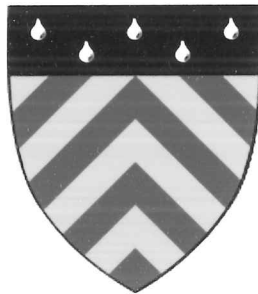


PhD. 29859

Characterisation of an *Arabidopsis* Mutant with Altered Greening Characteristics



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Clare Hall

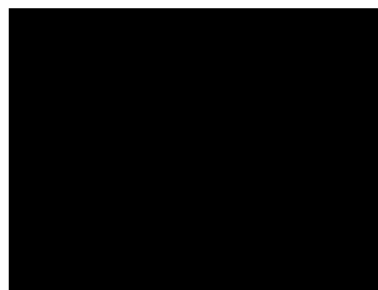


A dissertation submitted to the University of Cambridge
for the degree of Doctor of Philosophy

June 2006

Disclaimer

The research presented in this dissertation was carried out in the laboratory of Professor John C. Gray in the Department of Plant Sciences, University of Cambridge, between January 2003 and May 2006. The results described here are all my own work and the dissertation includes nothing that is the outcome of work done in collaboration, except as specified in the text. No part of this work had been submitted for any degree at any university. The work presented is the subject of a manuscript in preparation.



Acknowledgements

I would like to thank my supervisor Professor John C. Gray for all his guidance, support and encouragement. I also thank other members in Molecular Biology and Department of Plant Sciences, both past and present, who have offered me invaluable help and advice. Thanks are extended to the Gates Cambridge Trust and the Overseas Research Students Awards Scheme, for their generous sponsorship for the whole duration of my study. Special thanks to Clare Hall for its pastoral support and the friendship that I earned.

Most of all, this thesis is dedicated to my family in Malaysia, in particular my parents for their unfailing love and support, and Siew who has put up with me throughout the time.

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To my family

Abstract

Two plastid-to-nucleus signalling pathways had previously been identified by studies on *genomes uncoupled* (*gun*) mutants of *Arabidopsis*. A pathway responding to the state of plastid protein synthesis was disrupted in *gun1* mutants. Unlike the wild type, the mutants show expression of nuclear photosynthesis genes in the presence of norflurazon, which results in chloroplast photooxidation, or in the presence of lincomycin, an inhibitor of plastid translation. The aim of this study was to understand the role of plastid protein synthesis in plastid-to-nucleus signalling.

Five putative *gun1*-like mutants from a new collection of *Arabidopsis gun* mutants with a green fluorescent protein (GFP) reporter gene under the control of a tobacco *RbcS* (encoding ribulose-1,5-bisphosphate carboxylase small subunit) promoter were examined further. One of the mutant lines, PR48.2N, showed two-fold higher transcript abundance of nuclear photosynthesis genes, *RBCS* and *LHCBI* (encoding light-harvesting chlorophyll *a/b*-binding protein 1), compared to wild type with or without treatments of norflurazon or lincomycin.

Pigment analysis of PR48.2N seedlings illuminated for 16 hours after being subjected to various lengths of dark treatment demonstrated that the mutant line accumulated less chlorophyll than wild type after short periods of darkness (2-4 days) but showed an enhanced ability to green after prolonged dark treatments (5-10 days). Consistent with the enhanced greening ability, transcript abundance of nuclear photosynthesis genes was higher and there was more thylakoid membrane in chloroplasts in greened PR48.2N seedlings after prolonged darkness compared to the wild type.

Microarray analysis indicated that a group of transcripts encoding seed storage proteins, oleosins and late embryogenesis abundant proteins showed very low abundance in PR48.2N seedlings. The promoter regions of the genes shared some *cis*-elements possibly involved in regulation by abscisic acid (ABA). However, the ABA content of PR48.2N seedlings was not significantly different to wild type, although the

germination of mutant seeds was more sensitive to inhibition by ABA than the wild type.

Expression of the GFP reporter gene in the presence of lincomycin and the enhanced greening ability of PR48.2N were shown to be inherited in a recessive manner by examining the segregation of these phenotypes in the F2 progeny of a PR48.2N (Ws) x wild type (*Ler*) cross. Genetic analysis using F2 individuals from a *gun1-1* x PR48.2N cross showed that the mutated genes were not allelic but might be interacting because putative double mutants with much higher GFP expression were observed. Preliminary data from PCR-based mapping methods using a small F2 mapping population suggest that the locus providing enhanced greening ability in PR48.2N may be in a region on chromosome V. PR48.2N phenotypes such as expression of the GFP reporter gene in the presence of norflurazon or lincomycin, enhanced greening ability, lower transcript levels of seed protein genes, and hypersensitivity to the ABA inhibitory effect on germination appeared to co-segregate in the mapping population.

Although PR48.2N mutant was initially identified in a screen for *gun1*-like mutants, the evidence presented in this dissertation suggests that the product encoded by the gene mutated in PR48.2N may have a regulatory role in the transition from heterotrophic to photoautotrophic growth during early seedling development.

Abbreviations

ABA	abscisic acid
ABRE	ABA response elements
ACCase	acetyl-CoA carboxylase
AFLP	amplified fragment-length polymorphism
ARMS	amplification refractory mutation system
ATP	adenosine-5'-triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair
BSA	bovine serum albumin
CAPS	cleaved amplified polymorphic sequences
cDNA	complementary DNA
CE	coupling element
Clp	caseinolytic protease
CoA	coenzyme A
Col	Columbia
cpm	counts per minute
cRNA	complementary RNA
DAF	days after flowering
dATP	2'-deoxyadenosine-5'-triphosphate
DCMU	3-(3,4-dichlorophenyl)-1,1-dimethylurea
dCTP	2'-deoxycytosine-5'-triphosphate
dGTP	2'-deoxyguanosine-5'-triphosphate
dNTP	2'-deoxynucleoside-5'-triphosphate
dTTP	2'-deoxythymidine-5'-triphosphate
DEPC	diethyl pyrocarbonate
DMBIB	2,5-dibromo-3-methyl-6-isopropyl- <i>p</i> -benzoquinone
DMF	<i>N,N</i> -dimethylformamide
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methanesulphonate
GFP	green fluorescent protein

GO	gene ontology
GUS	β -glucuronidase
h	hour
kbp	kilobase pair
LB	Luria-Bertani
LEA	late embryogenesis abundant
<i>Ler</i>	Landsberg <i>erecta</i>
LRE	light-responsive elements
MgPPMe	Mg-protoporphyrin monomethyl ester
Mg-ProtoIX	Mg-protoporphyrin IX
min	minute
MOPS	3-morpholin-4-ylpropane-1-sulfonic acid
mRNA	messenger RNA
MS	Murashige and Skoog
NADH	nicotinamide adenine dinucleotide
NEP	nuclear-encoded RNA polymerase
NPTII	neomycin phosphotransferase II
nt	nucleotide
ORF	open reading frame
PBS	phosphate buffered saline
PCI	phenol/chloroform/isoamyl alcohol
Pchl _{id}	protochlorophyllide
PCR	polymerase chain reaction
PEP	plastid-encoded RNA polymerase
PIPES	2-[4-(2-sulfoethyl)piperazin-1-yl]ethanesulfonic acid
PS	photosystem
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
rRNA	ribosomal RNA
Rubisco	ribulose-1,5-bisphosphate carboxylase/oxygenase

S	Svedberg
s	second
SDS	sodium dodecylsulphate
SEM	standard error of the mean
SNP	single nucleotide polymorphism
SSC	saline sodium citrate
SSLP	simple sequence length polymorphism
TBE	Tris-borate-EDTA
TE	Tris-EDTA
TEM	Transmission electron microscopy
Tris	tris(hydroxymethyl)aminomethane
tRNA	transfer RNA
Tween 20	polyoxyethylene-20-sorbitan monolaurate
UTR	untranslated region
UV	ultraviolet
vol	volume
v/v	volume per volume
Ws	Wassilewskaja
w/v	weight per volume
w/w	weight per weight

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Chapter 1

Introduction

1. Introduction

Plant cells emerged through an endosymbiotic event between a protoeukaryote and a cyanobacterium over 1000 million years ago (Dyall *et al.*, 2004). The cyanobacterium then evolved into the plastid, one of the organelles in plant cells essential for photosynthesis, storage of products and biosynthesis of many classes of molecules. Plastids are double-membrane-bound, cytoplasmic organelles present in all plant cells multiplying by binary division of existing organelles (López-Juez and Pyke, 2005). In this chapter, various aspects of plastids especially the retrograde signalling pathway to the nucleus will be introduced.

1.1 Origin of plastids

It has been long proposed that plastids in modern plant cells evolved from free-living, photosynthetic prokaryotic organisms via an ancient endosymbiotic event (Mereschkowsky, 1905). This notion was uncertain until work by Bonen and Doolittle (1975) provided the first quantitative and molecular measure of similarities between 16S rRNA of *Porphyridium* chloroplasts and 16S rRNA of bacteria and cyanobacteria. However when the genome size of a cyanobacterium, for example *Synechocystis* PCC6803 (genome size: 3573 kbp; hypothetical proteins: 3168) (Kaneko *et al.*, 1996), is compared to the plastome size of a higher plant, for example *Arabidopsis thaliana* (plastome size: 154 kbp; hypothetical proteins: 87) (Sato *et al.*, 1999), a huge portion of cyanobacterial genes appears to have been lost during the course of evolution (Abdallah *et al.*, 2000). By comparing the nucleome of *Arabidopsis thaliana* and the genome of *Synechocystis* PCC6803, it has been suggested that a considerable amount of genetic information has been transferred to the nucleus, as there is a significant percentage of genes in the *Arabidopsis* nucleome found to be of cyanobacterial origin (Abdallah *et al.*, 2000; Rujan and Martin, 2001). Homologues of 44 different genes of plastid-encoded proteins have been identified as functional nuclear genes of chloroplast origin through an analysis using 210 protein-coding genes in chloroplast genomes of a glaucocystophyte, a rhodophyte, a diatom, a euglenophyte and five land plants, providing further evidence for endosymbiotic gene transfer to the nucleus in plants (Martin *et al.*, 1998).

Weeden (1981) formulated the “product specificity corollary” or “gene transfer corollary” proposing that the majority of genes for proteins integral to organellar metabolism were transferred to the nucleus, where they integrated into the regulatory hierarchy of the nucleus and acquired a transit peptide sequence, so that the gene products could be relocated into the organelle of their origin. This theory was supported by some work (Martin and Cerff, 1986; Baldauf and Palmer, 1990; Brennicke *et al.*, 1993) but challenged by others. Evidence derived from analyses on chloroplast or cytosolic isoenzymes of Calvin cycle enzymes showed that the cellular localisation of the isoenzymes does not correlate with the endosymbiotic ancestry of the organelle. The products of genes transferred from cyanobacteria have often localised in the cytosol, replacing pre-existing functions of the nuclear lineage, and in some cases mitochondrial enzymes have replaced the chloroplast homologues (Martin *et al.*, 1990, 1993; Martin and Schnarrenberger, 1997; Martin, 1998).

It is still unclear why this transfer of genetic material from plastid to the nucleus took place during the course of evolution. One possibility is that asexual propagation adopted by the symbionts due to the separation from their free-living counterparts appears to increase the accumulation of deleterious mutations and therefore the transfer of genes to the nuclear genome could increase the rate of genetic recombination and reduce the genetic load (Martin and Herrmann, 1998; Martin *et al.*, 1998). The possibilities why there were genes retained in the chloroplast genome include the need for rapid redox regulation of gene expression (Allen, 2003) and the difficulty of importing hydrophobic membrane proteins (López-Juez and Pyke, 2005). The evolutionary processes by which the transferred genes gained appropriate regulatory elements and protein targeting sequences are also yet to be elucidated.

1.2 Plastid types

All plastid types derive from small, colourless, undifferentiated and, in most cases, maternally inherited proplastids, which are usually found in meristematic cells of shoots and roots. The differentiation of the plastids depends on the type of cell in which they reside and environmental clues, such as light for chloroplast formation (López-Juez and Pyke, 2005). The specialised plastids can be categorised into two groups based on their photosynthetic competence (Mullet, 1988). Non-photosynthetic plastids consist of

the precursory proplastids, etioplasts (chloroplast precursors in dark-grown plants), chromoplasts (carotenoid-containing plastids in petals and fruits), amyloplasts (starch-storing plastids in roots and tubers), elaioplasts (lipid-storing plastids in oil glands and fruits) and leucoplasts (non-green plastids located in roots and non-photosynthetic tissues of plants that may become specialised for storage of starch, lipid or protein) (Schnepf, 1980), whereas chloroplasts, which are probably the most abundant and complex plastids, contain the site of photosynthetic activities. In addition, plastids are also important sites for a number of metabolic pathways (McFadden, 2001). Chloroplasts have been demonstrated to show variation in composition and function. For instance, chloroplasts in mesophyll cells of C₄ plants are deficient in CO₂ fixation proteins and chloroplasts in bundle sheath cells of C₄ plants are deficient in proteins involved in photosystem (PS) II (Mullet, 1988). Since plastid types are products of differentiation and, are in some cases, interconvertible (Herrmann, *et al.*, 1992; López-Juez and Pyke, 2005), they may be described as a continuous spectrum of types rather than discrete categorisation (Pyke, 1999).

Vestigial plastids known as apicoplasts are also found in malarial, toxoplasmodial, and related parasites (McFadden and Waller, 1997) suggesting that parasites obtained their plastids in an ancient evolutionary event that also created some major algal groups (McFadden, 2000). Trypanosomatid parasites have been shown to harbour a number of genes sharing apparent common ancestry with plants and/or bacteria and therefore may have been involved in a lateral gene transfer from some plastid-like organism(s) during evolution (Waller *et al.*, 2004).

1.3 The plastid genome

The presence of DNA in the chloroplasts was widely accepted after Sager and Ishida (1963) discovered a satellite DNA with a lower buoyant density in a caesium chloride density gradient than the nuclear DNA in isolated *Chlamydomonas* chloroplasts. The DNA constitutes the plastid genome or plastome, a double-stranded circular DNA molecule, typically ranging in size from 120 to 160 kbp (Palmer, 1985) with more than 100 functional genes encoding ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) and proteins (Sugiura, 1992, 1995). The studies of reassociation kinetics of denatured chloroplast DNA of *Chlamydomonas* have revealed that the chloroplast

genome is present in multiple copies (Bastia *et al.*, 1971; Wells and Sager, 1971). In contrast to the usual plastid genome organisation of a single large circle, plasmid-like DNA, which seems to be additional to the main chloroplast genome, has been observed in plastids of some green algae (La Claire and Wang, 2000). Dinoflagellate plastid genes are located on small circles of 2-3 kbp (Howe *et al.*, 2003). Most circles contain a single gene, some contain two genes, and some contain none. It has also been proposed that long, polyploid linear and branched molecules of plastid DNA undergoing replication are abundant (Bendich, 2004).

The chloroplast genomes of most plants contain a pair of inverted repeat sequences of 6 to 76 kbp separating the genomes into small and large single-copy regions of average sizes 20 kbp and 80 kbp, respectively (Palmer, 1985). Most of the variation in size of the angiosperm chloroplast genomes can be accounted for by changes in the size of their inverted repeats (Palmer, 1985). Nonetheless, the chloroplast genomes in some species of legumes and conifers lack the inverted repeat (Palmer, 1985; Strauss *et al.*, 1988). It has been proposed that the inverted repeat was present in the chloroplast genomes of the common ancestors in land plants and one copy of the inverted repeat was lost in some legumes and conifers during the course of evolution (Palmer *et al.*, 1987b; Strauss *et al.*, 1988). The loss of the inverted repeat may compromise stability of the chloroplast genomes by lessening constraints on rearrangement (Strauss *et al.*, 1988). Chloroplast genomes in some algal species, such as *Acetabularia mediterranea* and *Euglena gracilis*, contain tandem repeats, whereas chloroplast genomes in some *Chlamydomonas* species, subclover (*Trifolium subterraneum*) and geranium (*Pelargonium hortorum*) contain families of dispersed repeats (Palmer, 1985; Palmer *et al.*, 1987a, b).

The plastid genomes of about 60 species have been completely sequenced and the sequences have been deposited in the Chloroplast Genome Database (ChloroplastDB) (<http://chloroplast.cbio.psu.edu/>) (Cui *et al.*, 2006). The genes of the chloroplast genomes of higher plants can be broadly categorised into two major groups according to their biological functions (Sugiura, 1992). The first group of chloroplast genes encode components of the plastid genetic system involved in plastid transcription and translation. Plastid genes categorised in this group include genes encoding ribosomal proteins and rRNAs for the plastid 70S ribosomes, tRNAs, subunits of a

plastid-encoded bacterial-like RNA polymerase, translation initiation factor 1 (IF1), and the proteolytic subunit of the ATP-dependent Clp protease (Gray *et al.*, 1990; Maurizi *et al.*, 1990). There is also a *sprA* gene encoding a small plastid RNA possibly involved in the maturation of 16S rRNA (Vera and Sugiura, 1994). The second group encompasses the genes that encode proteins of the photosynthetic apparatus. They are those genes encoding the large subunits of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) responsible for carbon fixation (Ellis, 1979), components of PSI and II involved in light harvesting and energy transfer, components of the cytochrome *b₆f* complex involved in photosynthetic electron transfer between PSI and II, components of the ATP synthase complex, and components of the plastid NADH dehydrogenase complex possibly involved in cyclic electron flow around the PSI complex in the light and in a chloroplast respiratory chain in the dark (Burrows *et al.*, 1998). Plastid genomes also contain the *accD* gene encoding a subunit of a key enzyme, acetyl-CoA carboxylase (ACCase), providing malonyl-CoA for fatty acid biosynthesis in plants (Li and Cronan, 1992; Sasaki *et al.*, 1993). Interestingly, the plastid genome of the non-photosynthetic parasitic plants *Epifagus virginiana* lacks genes for the photosynthetic system found in other plastid genomes of photosynthetic plants (Wolfe *et al.*, 1992).

There are a number of open reading frames (ORFs) with unknown functions found in the plastid genomes. Some of these ORFs are conserved in size, location and sequence among the sequenced plastid genomes (Shimada and Sugiura, 1991; Sugiura, 1992) and are designated *ycfs* (hypothetical chloroplast open reading frames) (Hallick and Bairoch, 1994). The products of some of these *ycf* genes, such as *ycf1* and *ycf2*, are essential for cell survival (Drescher *et al.*, 2000). The functions of the products of some of these *ycf* genes have been elucidated. The *ycf10* gene (also known as *cemA*) encodes a chloroplast membrane protein involved in CO₂ transport (Rolland *et al.*, 1997), *ycf6* (also known as *petN*) encodes a subunit of the cytochrome *b₆f* complex (Hager *et al.*, 1999), *ycf9* encodes a PSII core subunit, PsbZ (Swiatek *et al.*, 2001), and products of *ycf3* and *ycf4* are required for PSI assembly (Boudreau *et al.*, 1997; Ruf *et al.*, 1997; Naver *et al.*, 2001).

1.4 Plastid gene expression

The gene expression system of plastids shows many similarities to that of prokaryotes. For instance, plastids possess 70S ribosomes, rRNAs and tRNAs with similar sequences to their counterparts in *Escherichia coli*, and uncapped mRNA without poly(A) tails (Sugita and Sugiura, 1996). Plastid gene expression is regulated at many different levels such as transcription initiation, mRNA processing, mRNA stability, mRNA translation and post-translational processing (Gruissem, 1989; Gruissem and Tonkyn, 1993; Mayfield *et al.*, 1995).

Like prokaryotes, most chloroplast genes are transcribed in dicistronic or polycistronic transcription units of related functions, such as *atpB-atpE*, or unrelated, such as *clpP-5'rps12-rpl20*. However there are a number of genes, such as *psbA*, *rbcL* and most of the tRNA genes, are transcribed in monocistronic transcription units (Sugita and Sugiura, 1996). The plastid genome encodes *E. coli*-like RNA polymerase α , β , β' and β'' subunits (encoded by *rpoA*, *rpoB*, *rpoC1* and *rpoC2*, respectively) in two polycistronic transcription units, which form the core of a bacterial-like plastid-encoded RNA polymerase (PEP) (Sugita and Sugiura, 1996; Maliga, 1998). The promoter recognition specificity of PEP is conferred by different sigma factors associated with the core of the RNA polymerase and encoded by the nuclear genome (Allison, 2000). Plastid gene promoters contain bacterial-like -35 (TTGACA) and -10 (TATAAT) regulatory sequences although some chloroplast tRNA genes lacking the elements are still efficiently transcribed (Gruissem *et al.*, 1986; Gruissem, 1989). From the observations that a subset of plastid genes are transcribed in *Epiphagus virginiana* lacking functional PEP subunit genes (Morden *et al.*, 1991) and ribosome-deficient plant mutants lacking plastid translation products including PEP (Han *et al.*, 1993; Hess *et al.*, 1993), it had been proposed that plastid genes of higher plants are transcribed by two types of RNA polymerases that are encoded by nuclear and plastid genomes (Maliga, 1998).

A distinct nuclear-encoded polymerase (NEP), which requires none of the RNA polymerase subunits encoded in the plastid genome, is involved in plastid transcription (Lerbs-Mache, 1993; Morden *et al.*, 1991; Chiba *et al.*, 1996; Allison *et al.*, 1996; Serino and Maliga, 1998). The existence of the NEP has been confirmed after the

identification of *Arabidopsis* genes encoding phage-type RNA polymerases targeted to chloroplasts (Hedtke *et al.*, 1997, 2000). Consistent with this, a second class of NEP plastid promoters has also been identified (Allison *et al.*, 1996; Kapoor *et al.*, 1997). Both PEP and NEP transcribe different groups of plastid genes (Hajdukiewicz *et al.*, 1997). NEP mainly transcribes housekeeping genes during early chloroplast development and its activity is repressed in the subsequent light-dependent chloroplast maturation that is accompanied by the activation of PEP, and this switch in RNA polymerase is mediated by glutamyl-tRNA (Hanaoka *et al.*, 2005). Besides promoter elements, there are additional downstream sequences from the transcription start possibly acting as enhancer or repressor sequences that influence the transcription activation of plastid genes mostly in a co-regulation manner (Mayfield *et al.*, 1995; MacLean, 2005).

Post-transcriptional control may be a more important regulation of plastid gene expression than transcription activity since some plastid transcripts, like *psbA* transcripts, accumulate even though transcription activity has declined (Gruissem, 1989). Transcript stability is an important determinant of plastid transcript abundance that affects plastid gene expression, and measurement of mRNA stability has demonstrated different half-lives of plastid transcripts depending on the plastid developmental stage, plastid type and growth condition (Klaff and Gruissem, 1991; Mullet, 1993; Mayfield *et al.*, 1995). Transcript stability is regulated by 3'- and 5'-untranslated regions (UTRs), mRNA-ribosome interaction, and some nuclear-encoded factors (Mayfield *et al.*, 1995). Transcript processing also plays a regulatory role in plastid gene expression although a limited one (Mayfield *et al.*, 1995).

It has been proposed that translational and post-translational regulation is the dominant regulatory mechanism for plastid gene expression because of the lack of correspondence between plastid mRNA and protein levels in different growth conditions (Gruissem, 1989). For instance, most of the chloroplast-encoded proteins for thylakoid membrane complexes are not detectable in dark-grown plants despite the high transcript abundance from some of the genes. Existing evidence has supported a model in which nuclear-encoded translational activator proteins may bind to the 5'-UTR of the targeted mRNA, which could result in alteration of the secondary structure around the ribosome-binding site to facilitate ribosome binding, or they could act as mRNA-

specific initiation factors required for downstream events in translational initiation (Mayfield *et al.*, 1995). Post-translational processing involving proteolytic activity and phosphorylation appear to influence protein levels and complex assembly in plastids (Gruissem and Tonkyn, 1993). A number of photosynthetic proteins require non-covalent attachment of prosthetic groups such as chlorophylls, carotenoids, quinones, haem and ions in order to stabilise and function (Gruissem and Tonkyn, 1993). For instance, polycistronic transcripts of *psaA* and *psaB*, which encode two apoproteins of the P700-chlorophyll *a* complex of PSI, are found in both light- and dark-grown rice (*Oryza sativa*) and barley (*Hordeum vulgare*) seedlings, but the P700-chlorophyll *a* complex can be detected only in light-grown seedlings, suggesting that light regulation takes place at a translational or post-translational level (Chen *et al.*, 1992; Kim *et al.*, 1994). Chlorophyll, which is produced by a light-induced biosynthesis, promotes stability and accumulation of P700 and D1 chlorophyll proteins, suggesting a post-translational regulation via complex assembly (Kim *et al.*, 1994).

1.5 Nuclear control of plastid gene expression

Since gene transfer has occurred from plastid to nucleus, plastids are genetically dependent on the host nucleus for their functions. Hence anterograde (from the nucleus to the plastid) regulatory mechanisms for this set of genes has received much emphasis (Goldschmidt-Clermont, 1998; Leon *et al.*, 1998). The "cytoplasmic control principle" that proposes complete control from nucleus to plastid has previously been suggested (Ellis, 1977). Nuclear-encoded proteins are involved in plastid gene expression at transcriptional, post-transcriptional, translational and post-translational levels (Mayfield *et al.*, 1995; Leon *et al.*, 1998). Plastid growth and development also depends on the expression of nuclear genes that are responsible for providing the plastids' nutrients, proteins and regulatory factors (Bogorad, 1991; Leon *et al.*, 1998).

In the mechanisms for regulating the production of proteins in two compartments, cytosol and plastid, destined to be parts of the same complex in the plastid, it had been hypothesised that the nuclear gene product itself, or some signal that reports the level of that protein, moderates the transcription or translation of the plastid gene (Bogorad, 1991). A good example of this regulatory system is Rubisco, a holoenzyme consisting of eight nuclear-encoded small subunits (RbcS) and eight

plastid-encoded large subunits (*RbcL*). Analyses involving inhibitors, mutants and transgenic plants have shown that inhibiting the expression of one subunit does not directly affect the expression of the other (Radetzky and Zetsche, 1987; Rodermel *et al.*, 1988; Khrebtukova and Spreitzer, 1996). Therefore the notion that the expression of *RbcS* and *rbcL* is not tightly co-ordinated is widely accepted nowadays. However, positive and negative regulation of *RbcS* and *rbcL* expression has also been proposed (Rodermel, 1999). There is now a considerable body of evidence showing that retrograde signals from chloroplasts regulate the expression of nuclear genes encoding photosynthesis-related proteins.

1.6 Carotenoid-deficient plants and norflurazon

Carotenoid-deficient mutants of maize (*Zea mays*) contain mutations in the genes encoding enzymes of carotenoid biosynthesis. Carotenoids protect chlorophyll from photooxidation and carotenoid-deficient plant lines produce bleached or white seedlings in the light (Anderson and Robertson, 1960). Carotenoid-deficient maize lines accumulate low amounts of proteins and transcripts of nuclear photosynthesis genes encoding light-harvesting chlorophyll *a/b*-binding proteins (*Lhc*) (Harpster *et al.*, 1984; Mayfield and Taylor, 1984). The repression of *Lhc* transcripts has also been reported in carotenoid-deficient *albina* mutants of barley (Batschauer *et al.*, 1986). The tomato (*Lycopersicon esculentum*) *ghost* mutant has a variegated green/white phenotype due to a block in carotenoid biosynthesis caused by an impaired gene that encodes plastid terminal oxidase (PTOX) associated with carotenoid desaturation (Rick *et al.*, 1959; Scolnik *et al.*, 1987; Josse *et al.*, 2000). Carotenoids are not synthesised in the white leaves resulting in chlorophyll destruction by photooxidation and little development of internal membrane structures in plastids (Scolnik *et al.*, 1987). Transcript levels for two nuclear photosynthesis genes, namely *RbcS* and *Lhc* genes, are low in white leaves but are similar to wild type in green leaves (Giuliano and Scolnik, 1988).

Carotenoid-deficient plants can also be produced by applying norflurazon, a carotenoid biosynthesis inhibitor interacting with phytoene desaturase (Reiß *et al.*, 1983; Sandmann *et al.*, 1989). Norflurazon treatment has been widely used to study plastid control of nuclear gene expression as a simple treatment that can lead to photooxidation of the internal compartment of chloroplasts, without affecting the

envelope membranes (Reiß *et al.*, 1983). Studies on norflurazon-treated barley, maize, mustard (*Sinapis alba*) and pea (*Pisum sativum*) have demonstrated reduced transcript levels from genes encoding components of light-harvesting complexes, photosynthetic electron-transfer chain, PSII oxygen-evolving complex and reductive pentose phosphate pathway by RNA blot and *in-vitro* translation (Mayfield and Taylor, 1984; Batschauer *et al.*, 1986; Oelmüller and Mohr, 1986; Oelmüller *et al.*, 1986; Burgess and Taylor, 1987, 1988; Sagar *et al.*, 1988).

Nuclear run-on assays and reporter gene expression from promoters in transgenic plants have shown that the reduced transcript abundance of these photosynthesis-related genes in photobleached carotenoid-deficient plants is caused by decreased rates of transcription. Decreased transcription of *Lhc* and *RbcS* genes has been observed in nuclei isolated from norflurazon-treated seedlings of barley, maize, pea and rye (*Secale cereale*) (Batschauer *et al.*, 1986; Burgess and Taylor, 1988; Ernst and Schefbeck, 1988; Sagar *et al.*, 1988), and white leaves of the tomato *ghost* mutant (Giuliano and Scolnik, 1988). Studies done with transgenic plants containing fusions of various photosynthesis-related gene promoters to reporter genes encoding neomycin phosphotransferase II (NPTII) and β -glucuronidase (GUS) have shown that the expression of the reporter genes is decreased in the presence of norflurazon suggesting that chloroplasts play an important role in the expression of nuclear photosynthesis genes (Simpson *et al.*, 1986; Stockhaus *et al.*, 1987, 1989; Susek *et al.*, 1993; Bolle *et al.*, 1994; Gray *et al.*, 1995; McCormac *et al.*, 2001).

Deletion analysis of promoters of photosynthesis-related genes has been attempted to identify a minimum region that is able to direct expression patterns in norflurazon-treated seedlings. However, the regulatory elements identified that respond to norflurazon treatment can not be separated from light-regulatory elements suggesting that light and plastid signalling pathways share the same *cis*-acting elements (Bolle *et al.*, 1994; Lübberstedt *et al.*, 1994; Gray *et al.*, 1995; Kusnetsov *et al.*, 1996; Hahn and Kück, 1999). Light is involved in the modulation of nuclear photosynthesis genes since a number of *cis*-acting light-responsive elements (LREs) have been found in the promoter regions of *Lhc* and *RbcS* (Terzaghi and Cashmore, 1995; Argüello-Astorga and Herrera-Estrella, 1998). Transgenic seedlings containing the GUS reporter gene

under the control of chimeric promoters with multiple copies of known light-regulatory elements also show decreased expression in the presence of norflurazon (Puente *et al.*, 1996; Martínez-Hernández *et al.*, 2002).

Nevertheless, studies on *Arabidopsis cue* (chlorophyll *a/b*-binding [CAB] protein-underexpressed) mutants (López-Juez *et al.*, 1998), pea *lip1* (light-independent photomorphogenesis), *Arabidopsis cop1* (constitutively photomorphogenic) mutants (Sullivan and Gray, 1999) and rice (Yoshida *et al.*, 1998) have supported a model in which light and plastid signalling pathways are distinct although they are closely related. The close association between these pathways is further demonstrated by the reports that *gun1* is a deetiolation mutant (Susek *et al.*, 1993; Mochizuki *et al.*, 1996).

1.7 Plastid-ribosome-deficient plants and inhibitors of plastid gene expression

Early work on barley *albostrians* and *Saskatoon*, two mutant lines with white leaves that lack plastid ribosomes and plastid protein synthesis, demonstrated that the activities of two nuclear genes encoding cytoplasmically synthesised Calvin cycle enzymes, phosphoribulokinase and NADPH-glyceraldehyde-3-phosphate dehydrogenase, were considerably lower in the white leaves of the lines compared to the pigmented leaves (Bradbeer and Börner, 1978; Bradbeer *et al.*, 1979). Bradbeer *et al.* (1979) proposed that plastid products can control the synthesis of plastid proteins on cytoplasmic ribosomes. Transcript levels of genes encoding Lhc, RbcS and a number of chloroplast enzymes involved in the Calvin cycle were strongly reduced as well (Hess *et al.*, 1991, 1994). It has recently been reported that ribosome-deficient plastids in barley *albostrians* affect the transcript abundance of NEP and its target genes suggesting that plastid translation affects a nuclear gene that is responsible for plastid transcription (Emanuel *et al.*, 2004).

Studies have demonstrated that inhibitors of plastid transcription, such as tagetitoxin (Rapp and Mullet, 1991) and nalidixic acid (Gray *et al.*, 1995), and plastid translation, such as chloramphenicol (Oelmüller *et al.*, 1986), streptomycin (Yoshida *et al.*, 1998), lincomycin and erythromycin (Sullivan and Gray, 1999), repress expression of nuclear photosynthesis-related genes. Tagetitoxin is an inhibitor of the plastid-encoded RNA polymerase (Mathews and Durbin, 1990). Reduced transcripts of *RbcS*

and *Lhc* genes have been reported in tagetitoxin-treated seedlings of barley, but plastid DNA replication was not affected (Rapp and Mullet, 1991). Nalidixic acid, a prokaryotic DNA gyrase inhibitor that affects plastid DNA replication and transcription (Sugino *et al.*, 1977), decreased expression of the GUS reporter gene under the control of *RbcS* and *PetH* (ferredoxin-NADP⁺ oxidoreductase) promoters in transgenic tobacco (*Nicotiana tabacum*) seedlings when applied during the first 2-3 days of seedling development (Gray *et al.*, 1995).

Streptomycin inhibits prokaryotic protein synthesis by interacting with the 30S ribosomal subunit (Biswas and Gorini, 1972), whereas chloramphenicol, erythromycin and lincomycin interact with the 23S rRNA/50S subunit of the elongating ribosomal complex (Douthwaite, 1992a, b). Lincomycin and erythromycin specifically affect plastid translation but not mitochondrial protein synthesis (Ellis and Hartley, 1971; Pope, 1976; Tassi *et al.*, 1983). Lincomycin treatment affects nuclear photosynthesis genes (*Lhcb1* and *RbcS*) but not nuclear genes for mitochondrial (*Atp2*) or cytosolic (*Act*) proteins (Gray *et al.*, 2003). As with nalidixic acid, the inhibitors of plastid translation repress nuclear gene expression when applied early in development of the seedlings (Oelmüller *et al.*, 1986; Bajracharya *et al.*, 1987; Gray *et al.*, 1995). This phenomenon suggests that chloroplast translation is required in early seedling development for the expression of nuclear genes encoding photosynthesis proteins. The plastid signalling pathway responding to the state of plastid translation has been shown to be separate from photosynthetic signalling by treating pea *lip1* and *Arabidopsis cop1* mutants, which express nuclear photosynthesis genes in the dark, with lincomycin or erythromycin (Sullivan and Gray, 1999). Lincomycin or erythromycin repressed the transcript abundance of nuclear photosynthesis genes in dark-grown shoots of pea *lip1* and *Arabidopsis cop1* mutants (Sullivan and Gray, 1999).

After reviewing the findings from carotenoid- or plastid-ribosome-deficient plants, Oelmüller (1989) and Taylor (1989) have proposed the existence of a plastid signal regulating the expression of nuclear genes especially those encoding photosynthesis-related proteins. This signal seemed to provide the nuclear genome with information about the developmental stages of the plastid such as early development, differentiation and senescence (Oelmüller, 1989; Taylor, 1989). However, it was suggested that the plastid signal rather provides quantitative modulation of the

expression of the nuclear photosynthesis genes than controls the developmental programme of nuclear genes (Taylor, 1989).

1.8 Tetrapyrrole intermediates

Accumulation of *Lhc* transcripts in *Chlamydomonas* has been reported to be repressed by chlorophyll-synthesis inhibitors that block late steps in the chlorophyll biosynthetic pathway, such as dipyrindyl and cycloheximide resulting in the accumulation of the porphyrin compounds, but not by chlorophyll-synthesis inhibitors that block chlorophyll synthesis prior to porphyrin formation, such as hemin, levulinic acid and dioxoheptanoic acid (Johanningmeier and Howell, 1984; Johanningmeier, 1988). Nuclear run-on assays showed that dipyrindyl treatment leads to decreased transcription of *Lhc* in *Chlamydomonas* (Jasper *et al.*, 1991). Decreased transcript amounts and transcription of *Lhc* were also observed in dipyrindyl-treated cress (*Lepidium sativum*) seedlings by RNA-gel-blot and nuclear run-on analysis (Kittsteiner *et al.*, 1991). Treatment with β -thujaplicin, which leads to inhibition of protochlorophyllide (Pchl_{id}) synthesis and accumulation of Mg-protoporphyrin monomethyl ester (MgPPMe), on cress seedlings showed that the light-induction of *Lhc* transcripts was inhibited by about 50 % (Oster *et al.*, 1996). Barley seedlings treated with amitrole, known as an inhibitor of carotenoid biosynthesis, accumulated high levels of Mg-protoporphyrin and its monomethyl ester and showed reduced induction of *Lhc* and *RbcS* transcripts in light suggesting that the tetrapyrrole intermediates may serve as negative signalling components for the regulation of photosynthesis-related genes (La Rocca *et al.*, 2001). Strand *et al.* (2003) has demonstrated that accumulation of Mg-protoporphyrin IX (Mg-ProtoIX), a tetrapyrrole intermediate, is accompanied by reduction of *LHCB* expression, suggesting that the intermediate acts as a plastid signal. Norflurazon-treated *Arabidopsis* seedlings accumulate more Mg-ProtoIX and Mg-ProtoIX-supplemented protoplasts showed repression of *LHCB* expression (Strand *et al.*, 2003).

However, the proposed negative regulatory role of Mg-protoporphyrin and its methyl ester does not appear to be incontestable. Reduced light induction of *Lhc* transcripts has been shown in the *Chlamydomonas brs-1* mutant that contains a frameshift mutation in the *ChlH* gene encoding the H subunit of Mg-chelatase

(Chekounova *et al.*, 2001) and accumulates protoporphyrin IX, which can not be converted to Mg-protoporphyrin (Johanningmeier and Howell, 1984). The expression of the nuclear heat-shock genes, *Hsp70A* and *Hsp70B* encoding cytosolic and plastid-localised heat-shock proteins in *Chlamydomonas*, has been reported to be induced by Mg-protoporphyrin and Mg-protoporphyrin dimethyl ester (Kropat *et al.*, 1997, 2000). The promoter region that directs the induction of the *Hsp70A* gene by Mg-protoporphyrin is also involved in light regulation (Kropat *et al.*, 1997). *Hsp70A* and *Hsp70B* genes in the *brs-1* mutant are not inducible by light (Kropat *et al.*, 1997), suggesting that Mg-protoporphyrin or its methyl ester is a positive regulator for light-regulated expression of *Hsp70* genes. Further complexity has been added to the issue when the light-inducible expression of an *Arabidopsis HSP70* gene was found to be delayed in a mutant affecting the CHLI subunit of Mg-chelatase, but not in a mutant affecting the H-subunit (Brusslan and Peterson, 2002).

1.9 Photosynthesis signals

Photosynthesis itself provides signals to modulate nuclear photosynthesis gene expression. Redox signals play roles in the regulation of nuclear photosynthesis gene expression (Escoubas *et al.*, 1995; Maxwell *et al.*, 1995; Durnford and Falkowski, 1997; Petracek *et al.*, 1997, 1998; Pfannschmidt *et al.*, 2001a, b; Pursiheimo *et al.*, 2001). Inhibitors known to block photosynthetic electron transport such as 3-(3',4'-dichlorophenyl)-1,1'-dimethylurea (DCMU) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DMBIB) have been demonstrated to affect mRNA accumulation (Escoubas *et al.*, 1995; Durnford and Falkowski, 1997), transcription (Escoubas *et al.*, 1995), post-transcriptional mRNA stabilisation (Petracek *et al.*, 1998; Sullivan and Gray, 2002), and polyribosome association (Petracek *et al.*, 1997) of nuclear photosynthesis genes. Mutations of two nuclear genes, *PetC* and *AtpD*, encoding the Rieske protein of the cytochrome *b₆f* complex and the δ -subunit of the chloroplast ATP synthase, showed lower transcript abundance of nuclear photosynthesis genes (Maiwald *et al.*, 2003). Light or temperature treatments, which are known to affect the plastid redox state, change nuclear photosynthesis gene expression as well (LaRoche *et al.*, 1991; Maxwell *et al.*, 1995; Pfannschmidt *et al.*, 2001a, b; Pursiheimo *et al.*, 2001). Among the photosynthetic electron transfer components, the redox state of the plastoquinone pool is most likely to be the source of retrograde signal(s) (Escoubas *et*

al., 1995; Pfannschmidt *et al.*, 2001a, b; Masuda *et al.*, 2003; Chen *et al.*, 2004). A phosphorylatable protein messenger (Escoubas *et al.*, 1995; Durnford and Falkowski, 1997; Pursiheimo *et al.*, 2001) may be involved in the pathway. DCMU affects the expression of nuclear photosynthesis genes in 4-week-old tobacco seedlings but not in 7-day-old seedlings, suggesting that photosynthetic signals work only in plants with an assembled photosynthetic apparatus (Gray *et al.*, 1995; Sullivan and Gray, 2002).

Carbon metabolites such as acetate, sucrose and its hexose components (glucose and fructose) repress expression of nuclear photosynthesis genes in unicellular algae (Steinmüller and Zetsche, 1984; Steinbiß and Zetsche, 1986; Kindle, 1987; Kishore and Schwartzbach, 1992) and higher plants (Sheen, 1990; Cheng *et al.*, 1992; Criqui *et al.*, 1992; Harter *et al.*, 1993; Krapp *et al.*, 1993; Van Oosten and Besford, 1994). The recessive maize mutant *sucrose export defective 1 (sxd1)*, which fails to efficiently export sucrose due to a compromised plasmodesmatal structure and/or function during leaf development, showed sugar accumulation that did not affect nuclear photosynthesis genes (Provencher *et al.*, 2001). Hexokinase appears to be a sensor for the sugar response in higher plants (Jang *et al.*, 1997). The *Arabidopsis CUE1* gene encoding a plastid phosphoenolpyruvate/phosphate translocator may play a role in metabolite-mediated regulation of nuclear genes (Streatfield *et al.*, 1999). Interestingly, the *Arabidopsis gun5* mutant was found to show a strong *gun* phenotype only in the presence of sucrose (McCormac and Terry, 2004). In addition, the redox and sugar signalling pathways have been shown to interact with each other in *Arabidopsis* (Oswald *et al.*, 2001).

1.10 Reactive oxygen species (ROS)

In the absence of protective carotenoids, as in carotenoid-deficient mutants or norflurazon-treated plants, chlorophylls can be photooxidised resulting in the production of ROS. Also, ROS can be generated as by-products that are rapidly converted into less toxic products, in chloroplasts by direct transfer of excitation energy from chlorophyll to produce singlet oxygen, or by univalent oxygen reduction at PSI, in the Mehler reaction (Foyer *et al.*, 1994; Allen, 1995). ROS production is likely to be increased at high light intensities and has been proposed to provide possible signalling intermediates (Pfannschmidt *et al.*, 2001a). ROS have been shown to alter nuclear gene

expression (Mullineaux and Karpinski, 2002). External H₂O₂ and high light can induce expression of nuclear genes related to stress responses such as the genes encoding cytosolic ascorbate peroxidases (cAPXs) that are responsible for H₂O₂ reduction to H₂O (Karpinski *et al.*, 1999; Kimura *et al.*, 2001). Transgenic tobacco expressing thylakoid-membrane-bound APX has been shown to have an enhanced H₂O₂-scavenging ability (Yabuta *et al.*, 2004). Similarly, the *fluorescent (flu)* mutant of *Arabidopsis* generates singlet oxygen, a non-radical ROS, after a dark-to-light shift and changes the transcript abundance of a number of nuclear genes (op den Camp *et al.*, 2003). *Arabidopsis* EXECUTER 1 (EX1) protein has been identified as a component in the singlet oxygen-mediated signalling pathway (Wagner *et al.*, 2004). ROS, as the physiological by-products of the mitochondrial electron transport respiratory chain, are known to play a role in the plant mitochondrion-to-nucleus signalling pathway (Maxwell *et al.*, 2002).

1.11 Synergy with mitochondria

Some mutations of mitochondrial genes in maize showing striped sectors of pale/yellow-green tissue on the leaves have been demonstrated to affect the development of chloroplasts (Roussell *et al.*, 1991; Gu *et al.*, 1993). The *non-chromosomal stripe 2 (ncs2)* mutant of maize has a DNA rearrangement in the mitochondrial genome that causes abnormal or prematurely arrested chloroplast development (Roussell *et al.*, 1991). Similarly, the *ncs6* mutant of maize has a partial deletion of the mitochondrial cytochrome oxidase subunit 2 gene (*cox2*) that leads to the alteration of the structure and function of chloroplasts (Gu *et al.*, 1993). Nuclear recessive mutations at the *CHLOROPLAST MUTATOR (CHM)* locus of *Arabidopsis* can cause rearrangement of the mitochondrial genome and produce a variegated phenotype (Martínez-Zapater *et al.*, 1992; Sakamoto *et al.*, 1996). Both chloroplasts and mitochondria are involved in the synthesis of sucrose and amino acids, and in the provision of redox equivalents for the reduction of hydroxypyruvate in the peroxisomal matrix (Krömer, 1995). It appears that some form of interorganellar communication may be needed between mitochondria and plastids to coordinate the development of photosynthetically competent chloroplasts.

Treatments of tobacco seedlings with lincomycin and erythromycin, which specifically affect plastid translation but not mitochondrial protein synthesis (Ellis and

Hartley, 1971; Pope, 1976; Tassi *et al.*, 1983), reduce nuclear photosynthesis gene expression, suggesting that mitochondrial protein synthesis is not involved in the generation of the plastid signal. However, mutations of a nuclear gene *PROLYL-tRNA SYNTHETASE1 (PRORS1)* in *Arabidopsis* reduce protein synthesis in both plastids and mitochondria, leading to altered gene expression of nuclear genes for photosynthetic light reactions and chloroplast proteins (Pesaresi *et al.*, 2006). On the other hand, the double mutant of *mrpl11* and *prpl11*, which are impaired in the mitochondrial and plastid ribosomal L11 proteins respectively, but neither of the single mutants, resulted in strong down-regulation of nuclear photosynthetic genes. This implies that signals from both plastids and mitochondria act synergistically in the regulation of nuclear photosynthetic gene expression (Pesaresi *et al.*, 2006).

1.12 genomes uncoupled (*gun*) mutants

The first attempt to identify mutants in chloroplast signalling has been made using mutagenised transgenic *Arabidopsis* plants containing a construct of *LHCBI.2* promoter-*uidA* (GUS) (Susek *et al.*, 1993). Five *Arabidopsis* genomes uncoupled (*gun*) mutants (*gun1-gun5*) showing GUS activity in the presence of norflurazon were isolated (Susek *et al.*, 1993; Mochizuki *et al.*, 2001). Since *LHCBI* transcription is normally repressed in norflurazon-treated plants but the *gun* mutants express the *uidA* gene under the control of the *LHCBI* promoter in the presence of norflurazon, the mutants have uncoupled transcription of *LHCBI* from its normal dependence on the functional plastids.

Genetic and molecular characterisations of the *gun* mutants have been carried out and some of the *gun* mutations have been identified to be in the genes encoding enzymes of the tetrapyrrole biosynthesis pathway. *GUN5* encodes the H subunit of Mg-chelatase, whereas *GUN2* and *GUN3* are allelic to *LONG HYPOCOTYL 1 (HY1)* and *HY2* (Mochizuki *et al.*, 2001) encoding respectively haem oxygenase (Davis *et al.*, 1999; Muramoto *et al.*, 1999) and phytychromobilin synthase (Kohchi *et al.*, 2001), enzymes of phytychromobilin synthesis. *GUN4* binds the product and substrate of Mg-chelatase and activates Mg-chelatase (Larkin *et al.*, 2003) through a cleft in the protein structure (Davison *et al.*, 2005; Verdecia *et al.*, 2005).

Strand *et al.* (2003) provided evidence to support the previous findings that Mg-ProtoIX, a tetrapyrrole intermediate, is one of the signals between the chloroplast and nucleus with results from various feeding experiments, inhibitor treatments and analyses on tetrapyrrole biosynthetic mutants. In the study, accumulation of Mg-ProtoIX was reported to be accompanied by reduction of *LHCB* expression. Mg-ProtoIX-supplemented *Arabidopsis* protoplasts showed repression of *LHCB* expression and treatment with dipyriddy, which is a Fe-chelator known to increase Mg-ProtoIX and its methyl esters, reverses phenotype of *gun* mutants (Strand *et al.*, 2003). A number of mutants with different lesions in the tetrapyrrole biosynthesis pathway, such as mutants for porphobilinogen deaminase, the D-subunit of Mg-chelatase and coproporphyrinogen oxidase, all of which are predicted to have decreased Mg-ProtoIX, also show a *gun* phenotype (Strand *et al.*, 2003).

Gray (2003) proposed several key questions concerning the role of Mg-ProtoIX in chloroplast-to-nucleus signalling since it is still unclear whether Mg-ProtoIX is the signal that leaves the chloroplast or is a chloroplast-located constituent of a multi-component signal transduction pathway, and whether Mg-ProtoIX is involved in regulating nuclear gene expression during normal plant development or only under extreme conditions, such as herbicide treatment. The barley *xantha-1* mutant, which is defective in a gene encoding MgPPMe cyclase (Rzeznicka *et al.*, 2005), accumulates high level of MgPPMe (Gadjieva *et al.*, 2005). *xantha-1* seedlings contain reduced levels of *Lhc* transcripts in the presence of norflurazon but accumulate *Lhc* transcripts in the absence of norflurazon despite the high level of MgPPMe (Gadjieva *et al.*, 2005). Since the plastid membrane system in the mutant is more developed without norflurazon treatment, it has been speculated that tetrapyrrole intermediates may only function as signal molecules at an early developmental stage of chloroplast development (Gadjieva *et al.*, 2005).

The *Arabidopsis gun1* mutant appears to be distinct from the other *gun* mutants (*gun2-gun5*) and the mutation has yet to be identified. Studies on double mutants have concluded that *GUN1* does not work in the same pathway as the other *GUN* genes (Vinti *et al.*, 2000; Mochizuki *et al.*, 2001). From microarray analysis, expression profiles of 152 norflurazon-responsive genes were similar and clustered together in *gun2* and *gun5*, whereas *gun1* showed a different profile (Strand *et al.*, 2003). Although

the GUN1 pathway is distinct from the tetrapyrrole-mediated plastid-to-nucleus signalling pathway (Vinti *et al.*, 2000; Mochizuki *et al.*, 2001; Strand *et al.*, 2003), these two pathways appear to interact to give rise to the *gun* phenotype. Double mutants of *gun1gun4* and *gun1gun5* showed a stronger *gun* phenotype indicating that the pathways are distinct but interacting synergistically (Mochizuki *et al.*, 2001; McCormac and Terry, 2004). Analysis of many mutants and conditions using a gene-sequence-tag array of approximately 3000 nuclear sequences encoding chloroplast proteins suggests that a “master switch” controls the expression of many of these genes and *gun1* and *gun5* mutants are clustered into the same response class (Richly *et al.*, 2003).

gun1 showed higher expression of nuclear photosynthesis genes in the presence of chloramphenicol than the wild type (Susek *et al.*, 1993). Further analysis with lincomycin has revealed a basis to distinguish the uncharacterised *gun1* from other *gun* mutants. *gun* mutants express nuclear photosynthesis genes in the presence of norflurazon, whereas only *gun1* expresses the genes in the presence of lincomycin (Gray *et al.*, 2003; JH Wang, JA Sullivan and JC Gray, unpublished). This implies that GUN1 is part of an uncharacterised signalling pathway involving plastid protein synthesis. Recently, the *Arabidopsis abscisic acid-insensitive 4 (abi4)* mutant has been reported to display a weak *gun* phenotype and may be part of the plastid-to-nucleus signalling pathway responding to the state of plastid protein synthesis (Nott *et al.*, 2006). Promoter elements for retrograde signalling appear to be closely related to G-box (Strand *et al.*, 2003) and perhaps binding of ABI4 to elements close to the G-box prevents light-dependent activation of nuclear gene (Nott *et al.*, 2006).

After all these studies and mutant characterisations, it is clear that plastid-to-nucleus signalling exists to regulate the expression of nuclear genes for photosynthesis components. However, it is also clear that the originally proposed model of a single ‘plastid signal’ or ‘plastid factor’ involved in the plastid-to-nucleus signalling (Oelmüller and Mohr, 1986; Oelmüller *et al.*, 1986; Taylor, 1989) is too simple. There are multiple signals produced by plastids (Sullivan and Gray, 2002; Surpin *et al.*, 2002) and Mg-ProtoIX may be one of the signalling molecules (Strand *et al.*, 2003).

1.13 Seedling development and abscisic acid (ABA)

Dark-grown *Arabidopsis gun1* seedlings are defective in greening on illumination compared to the wild type (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). This suggests that the plastid-to-nucleus signalling pathway responding to the state of plastid protein synthesis plays a role in seedling development especially in the regulation of transition from heterotrophic to photoautotrophic growth. ABI4, a possible component of the plastid-to-nucleus signalling pathway (Nott *et al.*, 2006), mediates sugar and ABA responsiveness by direct binding to a light-responsive element (Acevedo-Hernández *et al.*, 2005) and regulates lipid mobilisation that is required to fuel seedling establishment (Penfield *et al.*, 2006a). GUN5 (Mg-chelatase H subunit), which interacts with GUN1 (Mochizuki *et al.*, 2001; McCormac and Terry, 2004), has been reported to be an ABA receptor (Shen *et al.*, 2006).

Higher plants assume different growth patterns under dark or light conditions during early development (McNellis and Deng, 1995). Before emerging from the soil, dicotyledonous seedlings follow a skotomorphogenic (etiolated) growth pattern with elongated hypocotyls, small folded cotyledons and apical hooks (Staub and Deng, 1996). The etiolated seedlings show little or no expression of photosynthesis-related genes and do not contain developed chloroplasts (Chory *et al.*, 1996; Staub and Deng, 1996). When the seedlings emerge into the light, the growth is switched to photomorphogenic development that is accompanied by inhibition of hypocotyl elongation, open and expanded cotyledons, lack of an apical hook, developed chloroplasts and photosynthetic gene expression (Chory *et al.*, 1996; Staub and Deng, 1996).

Different spectral regions of light are able to elicit photomorphogenic development (Beggs *et al.*, 1980) suggesting that photomorphogenesis is facilitated through several photoreceptors recognising different regions of the light spectrum. These photoreceptors include phytochromes (red/far-red photoreceptors) (Quail *et al.*, 1995), cryptochromes (Cashmore *et al.*, 1999) and phototropins (Briggs and Christie, 2002) (blue/UV-A receptors), and unknown photoreceptor(s) for UV-B (Broshé and Strid, 2003). When light is perceived by the photoreceptors, it is transduced to a group of components acting as negative regulators essential for repressing photomorphogenic

development in the dark such as products encoded by five *COP* genes (*COP1*, 8, 9, 10 and 11), *DE-ETIOLATED1* (*DET1*) and four *FUSCA* (*FUS*) genes (*FUS4*, 5, 11 and 12) (Wei and Deng, 1996).

Phytohormones appear to play a role in light-dependent seedling development especially, in some cases, as downstream effectors of the phytochrome transduction pathway (Wei and Deng, 1996; Nemhauser and Chory, 2002). All major phytohormones appear to interact with the components of light signalling. For examples, auxin/indole-3-acetic acid (Aux/IAA) proteins, responsible for the auxin response, are phosphorylated by phytochrome A (Colón-Carmona *et al.*, 2000); exogenous application of cytokinins promotes de-etiolation (Chory *et al.*, 1994) and the cytokinin-insensitive mutant, *cin4*, was found to be allelic to *cop10* (Vogel *et al.*, 1998); double mutants of gibberellin-deficient *gal* and phytochrome B-deficient *phyB* have been shown to have increased responsiveness to gibberellins (Reed *et al.*, 1996); *DET2* encodes a steroid reductase for the brassinosteroid biosynthesis pathway (Li *et al.*, 1996); and ethylene regulates cell expansion in a light-dependent manner (Smalle *et al.*, 1997; Raz and Ecker, 1999). These phytohormones have been implicated in photomorphogenesis, with cytokinin promoting photomorphogenesis, and auxin, brassinosteroids and gibberellins showing an opposite action (Chory *et al.*, 1994; Nemhauser and Chory, 2002). Although ABA acts in opposition to brassinosteroids and gibberellins in some contexts, an ABA response appears to be negatively regulated by phytochrome action (Weatherwax *et al.*, 1996; Riemann *et al.*, 2003) and required for etiolated development (Rohde *et al.*, 2000; Nemhauser and Chory, 2002).

Developing seedlings devote their nutritional reserves accumulated during mid- to late-stages of embryogenesis almost exclusively to hypocotyl extension and development of the photosynthetic machinery after reaching the light (McNellis and Deng, 1995; Fujiwara *et al.*, 2002). Seed storage proteins are degraded during germination to supply amino acids for development (Fujiwara *et al.*, 2002) and storage lipids are mobilised to provide an energy source for the seedlings (Penfield *et al.*, 2006b). *ABI3*, *FUS3* and *LEAFY COTYLEDON 1* (*LEC1*) appear to act as important regulatory components of mid- to late-stages of embryogenesis as accumulation of seed storage and late embryogenesis abundant (LEA) proteins is severely reduced in mutants of these components (Nambara *et al.*, 1992; Vicent *et al.*, 2000; Fujiwara *et al.*, 2002).

Expression of seed storage protein or LEA protein genes is down-regulated in plant mutants deficient in ABA biosynthesis, although not to the same extent as in ABA-insensitive mutants (Koornneef *et al.*, 1989; Kriz *et al.*, 1990; Meurs *et al.*, 1992; Finkelstein, 1993; Paiva and Kriz, 1994; Parcy *et al.*, 1994). Genes encoding seed storage and LEA proteins have also been shown to be positively regulated by ABA (for review, Chandler and Robertson, 1994). In contrast, ABA negatively regulates photosynthesis genes during embryogenesis (Medford and Sussex, 1989; Chang and Walling, 1991). Therefore ABA plays an important role in mid and late embryogenesis.

ABA is required for seed dormancy since mutants deficient in ABA content, such as *Arabidopsis aba-deficient (aba)* mutants, or sensitivity, such as *Arabidopsis abi* mutants, produce viviparous or precociously germinating seeds (Bewley, 1997). Reciprocal crosses between *aba* mutants and the wild type showed that there are maternal and embryonic sources of ABA in developing seeds (Karssen *et al.*, 1983). However, the initiation of seed dormancy correlates well with the presence of the embryonic ABA but not with the maternal ABA (Karssen *et al.*, 1983). Evidence from maize also suggest that maternal ABA is not sufficient to suppress germination since viviparous seeds segregate on self-pollinated ears of heterozygous plants of various ABA-deficient mutants, and genetically normal endosperm does not complement the viviparous embryos of ABA-deficient mutants (McCarty, 1995). Seed germination can also be prevented by exogenous ABA at the stage of radicle extension (Bewley, 1997). During radicle extension, the cell wall loosening but not the osmotic potential and water up-take is prevented by the presence of ABA (Schopfer and Plachy, 1985).

Gibberellins also play an important role in seed germination (Bentsink and Koornneef, 2002). Mutants deficient in gibberellins require exogenous gibberellins to germinate and inhibitors of gibberellin biosynthesis, such as paclobutrazol and tetcyclacis, prevent germination (Koornneef and van der Veen, 1980; Debeaujon and Koornneef, 2000). Double mutants of gibberellin and ABA biosynthesis pathways showed higher ability to germinate compared to monogenic mutants that are deficient in gibberellins (Koornneef *et al.*, 1982; Debeaujon and Koornneef, 2000). This suggests that gibberellins overcome the ABA-induced dormancy for germination since reduced dormancy of *aba* mutants is accompanied by a reduced requirement for gibberellins to achieve germination (Koornneef *et al.*, 1982; Debeaujon and Koornneef, 2000).

Gibberellin biosynthesis in germinating seeds is induced by light as the expression of gibberellin 3 β -hydroxylase genes is regulated by phytochromes (Yamaguchi *et al.*, 1998). Light also decreases ABA content of germinating seeds prior to radicle protrusion (Tillberg, 1992).

Besides seed development and germination, ABA shows some regulatory effects during seedling development. Increases in the root/shoot growth ratio can be induced by water deficit, which results in high levels of endogenous ABA, and exogenous ABA (Watts *et al.*, 1981; Biddington and Dearman, 1982). Expression of *RbcS* and *Lhc* is negatively regulated by the high level of endogenous ABA related to water deficit in tomato leaves (Bartholomew *et al.*, 1991). Seedlings accumulate ABA in the dark and the ABA content decreases on illumination (Williams *et al.*, 1994; Weatherwax *et al.*, 1996; Riemann *et al.*, 2003). The dark-induced ABA has been shown to suppress expression of *RbcS* and *Lhcb* (Weatherwax *et al.*, 1996). ABA-INSENSITIVE 3 (ABI3) plays a role in plastid and leaf development in dark-grown *Arabidopsis* seedlings (Rohde *et al.*, 2000). ABI4 has also been demonstrated to mediate sugar and ABA responsiveness by direct binding to light-responsive element (Acevedo-Hernández *et al.*, 2005) and regulates lipid mobilisation for seedling development (Penfield *et al.*, 2006a).

1.14 Project aim

The overall aim of this research project is to understand the role of plastid protein synthesis in plastid-to-nucleus signalling that regulates the expression of nuclear genes for photosynthesis components. The main approach for this study is to characterise *Arabidopsis gun1*-like mutants using genetic and molecular means in the hope that components of the plastid-protein-synthesis-related plastid signalling pathway may be identified. The proposed experiments built directly on previous work with pea and tobacco (Gray *et al.*, 1995; Sullivan and Gray, 1999; Sullivan and Gray, 2002) and with the collection of putative *Arabidopsis gun1*-like mutants (C Meade, JA Sullivan and JC Gray, unpublished).

Since the plastid-to-nucleus signalling pathway responding to the state of plastid protein synthesis is largely uncharacterised, it will be of interest to elucidate the pathway by isolating more mutants for the components constituting the pathway. A collection of *Arabidopsis gun* mutants has been produced following ethyl methanesulphonate (EMS) or gamma radiation treatment of a transgenic line containing a tobacco *RbcS* promoter-green fluorescent protein (GFP) construct (Sullivan, 1999). Lines that showed GFP expression in the presence of norflurazon and lincomycin have been isolated as putative *gun1*-like mutants.

The first objective of the work was to select putative *Arabidopsis gun1*-like mutants that show strong phenotypes such as altered transcript abundance of nuclear photosynthesis genes in the presence of norflurazon or lincomycin and altered greening pattern, which are known phenotypes of the previously isolated *gun1-1* (Susek *et al.*, 1993; Mochizuki *et al.*, 1996; JH Wang, JA Sullivan and JC Gray, unpublished). The second objective was to characterise the phenotypes of the selected mutant lines with molecular or biochemical techniques including the transcript profiles using RNA-gel-blot and microarray analyses. The third objective was to characterise the selected mutant lines genetically. The nature of the mutations in the mutant lines were to be examined and their relationship to *gun1* were to be established. Genetic mapping was to be conducted to map the precise locations of the mutations in the *Arabidopsis* genome and segregation analysis to investigate if the mutant phenotypes are genetically linked.

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Chapter 2
Materials and methods

2. Materials and methods

2.1 Plant materials

Seeds of wild-type *Arabidopsis thaliana* ecotypes, Columbia (Col-0), Wassilewskaja (Ws) and Landsberg *erecta* (Ler) were obtained from the laboratory stock of the Molecular Biology Group, Department of Plant Sciences, University of Cambridge, UK. PR28.1N, PR32.2N, PR32.4N, PR48.2N and PR107.11N were putative *gun1*-like mutants in a Ws background and isolated in the Molecular Biology Group, Department of Plant Sciences, University of Cambridge, UK (section 3.1 for more description). 3R12 was the parental line for the putative *gun1*-like mutants (Sullivan, 1999) and *gun1-100* was a *gun1*-like mutant selected from the same experiment and later found to be allelic to *gun1-1* (JH Wang, JA Sullivan and JC Gray, unpublished). Seeds of *gun1-1* (Susek *et al.*, 1993; Mochizuki *et al.*, 1996) in the Col background were obtained from J. Chory (Plant Biology Laboratory and Howard Hughes Medical Institute, The Salk Institute, La Jolla, CA 92037, USA).

2.2 Plant growth procedures

2.2.1 Seed sterilisation

Seeds were surface-sterilised by immersion in 70 % (v/v) ethanol, prepared with sterile deionised water, for 2 min. The ethanol was replaced with 10 % (v/v) sodium hypochlorite (Fisher Scientific, Loughborough, UK) and 0.5 % (v/v) Tween 20 (Sigma, Poole, UK), prepared with sterile deionised water, and the seeds were soaked in the solution for 15 min with gentle agitation. The hypochlorite solution was then removed using a pipettor and the seeds were washed 4 times with sterile deionised water.

2.2.2 Growth of *Arabidopsis thaliana*

For soil-grown seedlings, seeds were sown on a 3:2 (v/v) mixture of Levington M3 (medium structure and high nutrient) compost (Scotts UK Professional, Bramford, UK) and fine vermiculite (William Sinclair Horticulture, Lincoln, UK). The soil

mixture was watered with 0.2 g/l Intercept 70WG (Scotts UK Professional), a systemic and curative insecticide that gives protection and control of some common pests. The seeds were stratified at 4 °C for 3 days and removed to a 22 °C growth room with alternating cool white (Osram, Munich, Germany) and GRO-LUX (Sylvania, Erlangen, Germany) fluorescent lights (average light intensity 40 $\mu\text{mole m}^{-2}\text{s}^{-1}$; 16-h diurnal photoperiod).

For plate-grown seedlings, seeds were surface-sterilised as described in section 2.2.1 and spread on 0.7 % (w/v) micro agar (Duchefa Biochemie, Haarlem, The Netherlands) containing half-strength MS medium (Murashige and Skoog, 1962) (Duchefa Biochemie) in a 9-cm petri dish. Norflurazon (5 μM) (Sandoz Agro, Des Plaines, IL, USA) and 0.5 mM lincomycin hydrochloride (Duchefa Biochemie) were added to the medium whenever specified. When seedlings were to be used for RNA-gel-blot analysis, the seeds were sown onto a sterilised 20- μm nylon mesh circle 8.5 cm in diameter (Normesh, Oldham, UK) overlaid onto the medium. The seeds were stratified at 4 °C in the dark (petri dish wrapped in two layers of aluminium foil) overnight and then grown at 22 °C under a mixture of cool white and GRO-LUX fluorescent lights in 3:2 ratio (average light intensity 60 $\mu\text{mole m}^{-2}\text{s}^{-1}$; 16-h diurnal photoperiod) for the indicated period of time. For dark treatment, plates were wrapped in two layers of aluminium foil for the indicated periods after being stratified overnight at 4 °C and light-treated for 24 h in a 22 °C growth room with a mixture of cool white and GRO-LUX fluorescent lights in 3:2 ratio (average light intensity 60 $\mu\text{mole m}^{-2}\text{s}^{-1}$; 16-h diurnal photoperiod).

2.2.3 Silique collection

Plants were grown in soil as described in section 2.2.2. When the plants had reached maturity, flowers were marked with threads and siliques were harvested in pools corresponding to four developmental stages: early embryogenesis (1-5 days after flowering, DAF), maturation (8-11 DAF), late embryogenesis (17-21 DAF) and dry seeds (>21 DAF) (Baud *et al.*, 2002). The siliques or seeds were subjected to RNA extraction as described in section 2.6.2.

2.3 Emasculation and plant crossing

Arabidopsis plants were soil-grown (section 2.2.2) to the flowering stage. Flower buds that were not too young (small and soft flower buds) or too old (opening flower buds with emerging white petals; tend to have started self-fertilisation) were chosen for emasculation. All other plant parts in the immediate vicinity including flower buds that are too old or too young for emasculation were removed. In the chosen flower buds, all flower parts except the pistils were removed with a pair of fine forceps, which were cleaned by dipping in 70 % (v/v) ethanol between flower buds.

Stigmas of the prepared recipient flowers were brushed with the stamens of fully open donor flowers. The brushing was performed at least twice to make sure that there were plenty of pollen grains at the stigmas. The recipient flowers were then labelled with threads of different colours. The plants were grown at 22 °C with alternating cool white and GRO-LUX fluorescent lights (average light intensity 40 $\mu\text{mole m}^{-2}\text{s}^{-1}$; 16-h diurnal photoperiod). Other plant parts in the vicinity were regularly pruned until the siliques were formed and became mature.

Mature siliques were collected in 1.5 ml microcentrifuge tubes. A hole was made in the lid of each tube to allow the release of water vapour. All the tubes were incubated at 30 °C for a couple of weeks to dry.

2.4 Growth and storage of bacterial strains

Plasmid-containing bacterial cells were streaked on 1.5 % (w/v) agar plates containing LB medium (1 % [w/v] bacto-tryptone [Difco, East Molesey, UK], 0.5 % [w/v] bacto-yeast extract [Difco], 0.5 % [w/v] sodium chloride, adjusted to pH 7.2 with 0.1 M sodium hydroxide and autoclaved to sterilise) and 50 $\mu\text{g/ml}$ kanamycin (Melford Laboratories, Ipswich, UK), grown overnight at 37 °C and single colonies were used to inoculate 5 ml of liquid LB medium with 50 $\mu\text{g/ml}$ kanamycin (Melford Laboratories). Cultures were grown for 14-18 h at 37 °C with shaking (180 rpm). Glycerol stocks for the bacterial strain contained 0.6 ml of the liquid bacterial culture and 0.4 ml 50 % (v/v) glycerol. The glycerol stocks were stored at -80 °C.

2.5 DNA procedures

2.5.1 Plant genomic DNA extraction

Mature leaves (3-4) or 30-50 seedlings of *Arabidopsis thaliana* were put in a 1.5 ml microcentrifuge tube, frozen with liquid nitrogen, and ground to fine powder with a pestle. Extraction buffer (350 μ l of 0.2 M Tris-HCl pH 8.0, 0.25 M NaCl, 25 mM EDTA and 0.1 % [w/v] SDS) was added. The mixture was further ground, vortexed, and centrifuged at 10000 x g for 20 min. Approximately 350 μ l supernatant was recovered and transferred to a fresh tube. Tris-buffered phenol/chloroform (1:1 [v/v]) (350 μ l) was added, mixed by inversion, and centrifuged at 10000 x g for 15 min. Upper aqueous phase (approximately 350 μ l) was transferred to a fresh tube. Sodium acetate (35 μ l of 3 M made up with 11.5 ml of glacial acetic acid, 60 ml of 5 M sodium acetate and sterile deionised water to 100 ml) and 700 μ l 100 % (v/v) ethanol were added. The mixture was incubated at -20 °C for 2 h and centrifuged at 10000 x g for 30 min. The supernatant was decanted carefully and the pellet was washed by addition of 200 μ l 70 % (v/v) ethanol and centrifugation for 5 min at 10000 x g. The ethanol was discarded and the pellet was air dried before being resuspended in 150 μ l sterile deionised water. The sample was stored at -20 °C immediately.

The concentration and purity of the DNA sample was determined by measuring the absorbance at 260 and 280 nm of a known dilution of the sample. A 50 μ g/ml solution of DNA has an absorbance of 1.0 at 260 nm (Sambrook *et al.*, 1989). An A_{260}/A_{280} ratio between 1.8 and 2.0 indicates that protein contamination in the DNA solution is minimal.

2.5.2 PCR

A PCR reaction (100 μ l) contained 200 ng DNA or 2-6 μ l of complementary DNA (cDNA) (section 2.6.8 for cDNA preparation) depending of the transcript abundance, 1 x NH_4 reaction buffer (Bioline, London, UK), 1.5 mM MgCl_2 (Bioline), 1.25 mM dNTPs, 100 pmoles of each primer (Invitrogen, Paisley, UK), 6.25 units BioTaq DNA polymerase (Bioline), and sterile deionised water to 100 μ l.

PCR amplifications were carried out in a Flexigene or Techgene programmable thermal controller (Techne, Stone, UK). The cycling conditions were 94 °C for 5 min, followed by 30 cycles of 94 °C for 45 s, 52 °C for 30 s and 72 °C for 1 min 30 s, and finally 72 °C for 10 min before cooling to 4 or 10 °C.

2.5.3 Digestion with restriction enzymes

A typical restriction enzyme digestion was performed on 1-5 µg DNA in a total volume of 20 µl. It consisted of 5 units of each of the restriction enzymes used (Roche, Lewes, UK or New England BioLabs, Hitchin, UK), 1 x enzyme buffer (supplied with the enzymes), 100 µg/ml bovine serum albumin (BSA) (New England BioLabs), and sterile deionised water to 20 µl. The reaction was carried out overnight at a temperature appropriate for the enzymes (typically 37 °C).

2.5.4 Agarose gel electrophoresis of DNA

A 1-2 % (w/v) agarose gel with 1 x TBE buffer (90 mM Tris-borate pH 8.0 and 2 mM EDTA) and 0.02-0.1 mg/ml ethidium bromide was set in an electrophoresis tray at room temperature for approximately 1 h. Once the gel was set, it was placed into an electrophoresis tank containing 1 x TBE buffer. DNA samples (10-15 µl) containing 1-2 µl of bromophenol blue loading buffer (0.25 % [w/v] bromophenol blue, 30 % [v/v] glycerol in water) and marker DNA, such as HyperLadder I (Bioline), were loaded into the wells and electrophoresis was carried out for 1 h at 70 V. The DNA was visualised using an UV transilluminator and a photographic image taken using a Polaroid MP4⁺ Instant Camera System or an AlphaImager 1200 controlled by AlphaEase v3.3b software (Alpha Innotech, San Leandro, CA, USA).

2.5.5 DNA sequencing

Sequencing of DNA fragments from PCR reactions was carried out at the DNA Sequencing Facility, Department of Genetics, University of Cambridge, UK. PCR products were electrophoresed and the desired bands were cut out with a scalpel. DNA was extracted using QIAquick Gel Extraction Kit (QIAGEN, Crawley, UK) and

quantified by comparison with size and concentration markers of HyperLadder I in an agarose gel (section 2.5.4). PCR products (10 µl of 15 ng/ul) and appropriate primers (10 µl of 1 pmol/ul) were supplied to the Facility. Sequencing reactions were performed using Big Dye Terminator Cycle Sequencing Reaction Mix (ABI, Warrington, UK) and labelled fragments were analysed using an ABI 3100 Capillary Sequencing Machine.

2.5.6 Genetic mapping

2.5.6.1 Cleaved amplified polymorphic sequences (CAPS)

The CAPS procedure for mapping *Arabidopsis* mutations was described in Konieczny and Ausubel (1993). The information on the CAPS markers, such as their map positions, primer sequences, product sizes and restriction enzymes as listed in Appendix II, was obtained from the website of The Arabidopsis Information Resource (TAIR; <http://www.arabidopsis.org/>), the website of the Laboratory of Fred Ausubel in the Department of Molecular Biology at The Massachusetts General Hospital, Boston, MA 02114, USA (<http://ausubellab.mgh.harvard.edu/>), and Baumbusch *et al.* (2001). Some primers were modified for better band resolution in agarose gel (Appendix II for details).

F1 or F2 mapping individuals, produced as described in sections 4.2 and 4.3, were allowed to grow to maturity (soil-growing procedure described in section 2.2.2). DNA from each individual was extracted as described in section 2.5.1. PCR (50-µl reaction) with the CAPS primers was then performed as described in section 2.5.2. The PCR product was precipitated by addition of 0.1 vol of 3 M potassium acetate and 2 vol of chilled 100 % (v/v) ethanol. The mixture was incubated at -20 °C for 2 h and centrifuged at 10000 x g for 30 min. The supernatant was decanted carefully and the pellet was washed by addition of 200 µl 70 % (v/v) ethanol and centrifugation for 5 min at 10000 x g. The ethanol was discarded and the pellet was air dried before being resuspended in 150 µl sterile deionised water. The cleaned PCR product was digested (section 2.5.3) and electrophoresed on a 1-2 % (w/v) agarose gel depending on the band sizes (section 2.5.4).

2.5.6.2 Simple sequence length polymorphism (SSLP)

SSLP markers for genetic mapping in *Arabidopsis* were initially reported in Bell and Ecker (1994). The information on the SSLP markers, such as their map positions, primer sequences and product sizes, was obtained from the TAIR website, as listed in Appendix II. F1 or F2 mapping individuals as described in sections 4.2 and 4.3 were allowed to grow to maturity (soil-growing procedure described in section 2.2.2). DNA from each individual was extracted as described in section 2.5.1. PCR (50- μ l reaction) with the SSLP primers was then performed as described in section 2.5.2. The PCR products were electrophoresed on a 1-2 % (w/v) agarose gel depending on the band sizes (section 2.5.4).

2.5.6.3 Single nucleotide polymorphism (SNP)

A PCR-based SNP mapping procedure for *Arabidopsis* was described in Drenkard *et al.* (2000). Information of the SNP markers was obtained from the TAIR website and the Monsanto *Arabidopsis* SNP and *Ler* Sequence Collection (Jander *et al.*, 2002). The information on the SNP markers, such as their map positions, primer sequences and product sizes, is listed in Appendix II. F2 mapping individuals selected as described in section 4.2 were allowed to grow to maturity (soil-growing procedure described in section 2.2.2). DNA from each individual was extracted as described in section 2.5.1. PCR (25- μ l reaction) with the SNP primers was then performed as described in section 2.5.2. For each line, two PCR reactions were prepared. One of them was performed with the primer pair specific to the *Ws* allele and the other one was specific for the *Ler* allele. The CAPS AP3-LINKED (uncut product size, 0.72 kbp) primer pair was added (12.5 pmoles in 25- μ l PCR reaction) to the PCR reactions as the internal control. The PCR products were electrophoresed on a 1-2 % (w/v) agarose gel depending on the band sizes (section 2.5.4).

2.6 RNA procedures

2.6.1 Preparation of RNase-free solutions and equipment

All solutions were treated with diethyl pyrocarbonate (DEPC) or made using DEPC-treated water to eliminate RNase contamination. DEPC was added to solutions (1 ml/l) which were then stirred, using a magnetic stirrer, overnight. Solutions were then autoclaved (100 kNm^{-2} for 15 min at $121 \text{ }^\circ\text{C}$) to hydrolyse any remaining DEPC before use. Glassware was baked overnight at $161 \text{ }^\circ\text{C}$ before use and, whenever possible, sterile, RNase-free and disposable plasticware was used. Gloves were worn at all times to avoid RNase contamination.

2.6.2 Total RNA extraction

RNA was prepared from plant materials with Concert Plant RNA Reagent (Invitrogen) using the manufacturer's large-scale isolation protocol or TriPure Isolation Reagent (Roche) using the manufacturer's isolation protocol with the modifications described below.

For extraction with Concert Plant RNA Reagent, plant tissue was ground to a fine powder in a mortar with a pestle in liquid nitrogen. For approximately 1 g of fresh tissue, 5 ml Concert Plant RNA Reagent was added to the mortar and the contents were mixed well. The mixture was then passed through a $70\text{-}\mu\text{m}$ cell strainer (BD Falcon, Cowley, UK) into a 50 ml tube. For each 5 ml of the mixture, 1 ml of 5 M sodium chloride and 3 ml chloroform were added. The mixture was vortexed for 30 s and centrifuged at $3500 \times g$ for 30 min at $4 \text{ }^\circ\text{C}$. The colourless aqueous upper layer was transferred to a 15 ml tube and 1 vol of water-saturated phenol/chloroform/isoamyl alcohol (PCI; 25:24:1 [v/v/v]) was added and vortexed. The mixture was centrifuged at $3500 \times g$ for 15 min at $4 \text{ }^\circ\text{C}$. The supernatant was transferred to a fresh tube and the PCI extraction steps were repeated for another two rounds. After the last round of PCI extraction, 0.9 vol of isopropanol was added to the supernatant to precipitate RNA. The mixture was mixed by inversion and centrifuged at $2600 \times g$ for 30 min at $4 \text{ }^\circ\text{C}$. The supernatant was decanted carefully and the pellet was washed by addition of 5-10 ml of

75 % (v/v) ethanol prepared with DEPC-treated sterile deionised water and centrifugation for 5 min at 2600 x g. The ethanol was discarded and the pellet was resuspended in 20-100 μ l of DEPC-treated sterile deionised water, depending on the pellet size. The solution was incubated at 65 °C for 3 min, vortexed, centrifuged at 2600 x g for 1 min at 4 °C, and transferred to a fresh RNase-free microcentrifuge tube. The RNA sample was stored at -70 °C immediately.

For extraction with TriPure Isolation Reagent, plant tissue (approximately 200-400 mg) was ground to a fine powder in a mortar with a pestle in liquid nitrogen. TriPure Isolation Reagent (1 ml) was added to the mortar and mixed well with the ground tissue. The mixture was then transferred to an RNase-free microcentrifuge tube and incubated for 5 min at room temperature. Chloroform (200 μ l) was added, vortexed for 30 s, and centrifuged at 10000 x g for 15 min at 4 °C. The colourless aqueous upper layer was transferred to a fresh RNase-free microcentrifuge tube and 500 μ l of isopropanol was added to precipitate RNA. The contents of the tube were mixed by inversion and centrifuged at 10000 x g for 10 min at 4 °C. The supernatant was discarded and the RNA pellet was washed using 1 ml of 75 % (v/v) ethanol followed by centrifugation at 5000 x g for 5 min at 4 °C. The pellet was dried at room temperature and dissolved in 15-25 μ l of DEPC-treated sterile deionised water, depending on the pellet size. The solution was incubated at 65 °C for 10 min, vortexed, centrifuged at 5000 x g for 1 min at 4 °C, and transferred to a fresh RNase-free microcentrifuge tube. The RNA sample was stored at -70 °C immediately.

The concentration and purity of the RNA sample was determined by measuring the absorbance at 260 and 280 nm of a known dilution of the sample. A 40 μ g/ml solution of RNA has an absorbance of 1.0 at 260 nm (Sambrook *et al.*, 1989). An A_{260}/A_{280} ratio between 1.8 and 2.0 indicates that protein contamination in the RNA solution is minimal.

2.6.3 Agarose-gel electrophoresis of RNA

An agarose gel (1.2 % [w/v]) with 1 x running buffer (20 mM MOPS pH 7.0, 8 mM sodium acetate, 1 mM EDTA), 2 % (v/v) formaldehyde and 0.02-0.1 mg/ml

ethidium bromide was set in an RNase-free gel casting tray at room temperature for about 2 h. RNA samples were prepared by adding equal quantities of RNA (7-15 μg as indicated in the text) to 5 μl of sample buffer (48 % [v/v] deionised formamide, 1 x running buffer, 6 % [v/v] formaldehyde, 5 % [v/v] glycerol and 0.02 % [w/v] bromophenol blue) and incubating at 65 °C for 15 min. The samples were loaded on the gel and electrophoresed in 1 x running buffer at 70 V until the loading dye had migrated 7-8 cm.

2.6.4 RNA-gel blotting

RNA-gel-blot analysis was initially described in Alwine *et al.* (1977). When electrophoresis was complete the gel was removed from the tank. The rRNA was visualised on a UV transilluminator and a picture was taken using a Polaroid MP4⁺ Instant Camera System or an AlphaImager 1200 controlled by AlphaEase v3.3b software. The gel was then soaked in DEPC-treated water and shaken for 20 min to remove formaldehyde. The gel was cut to a suitable size with an orientation mark and placed on a piece of Whatman 3MM chromatography paper which had been wetted with 10 x SSC (1.5 M sodium chloride, 0.15 M sodium citrate). The edges of the paper were dipped in a reservoir of 10 x SSC. A piece of GeneScreen Plus hybridization transfer membrane (PerkinElmer, Beaconsfield, UK) was cut to the size of the gel, soaked in 10 x SSC and placed on top of the gel. Pieces of Whatman 3MM chromatography paper (approximately 30) and a 3-cm thickness of paper towels cut to the size of the gel were placed on top of the membrane. A glass plate and a 500 g weight were placed on top and transfer was carried out overnight. After the transfer, the membrane was washed with 2 x SSC to remove any residual agarose gel. The RNA was fixed to the membrane by air drying for 2 h at room temperature or 1 h at 37 °C.

2.6.5 Preparation of random primer-labelled DNA probe

Radiolabelled probes were prepared by the random-primer method (Feinberg and Vogelstein, 1983). Probe DNA (40 ng in 40 μl of water) (section 2.6.9 for probe preparation) was heated in an 1.5 ml microcentrifuge tube at 95 °C for 5 min and 12 μl oligo-labelling buffer (0.25 M Tris-HCl pH8.0, 25 mM MgCl_2 , 50 mM 2-

mercaptoethanol, 0.1 mM dCTP, 0.1 mM dTTP, 0.1 mM dGTP, 1 M HEPES-NaOH pH 6.6, 0.54 mg/ml random hexanucleotides [Amersham Biosciences, Little Chalfont, UK], 24 µg BSA, 4 µl (40 µCi/1.48 MBq) [α - 32 P]-dATP (3000 Ci/mmol) (Amersham Biosciences), and 4.8 units Klenow fragment of *Escherichia coli* DNA polymerase I (Roche) were added and incubated for 3-6 h at room temperature. The unincorporated nucleotides were separated from the probe using a MicroSpin S-200 HR column according to the manufacturer's protocol (Amersham Biosciences). The probe was collected in a screw-cap 1.5 ml microcentrifuge tube with 200 µl of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) by centrifugation.

2.6.6 Hybridisation of probe to RNA-gel blot

The GeneScreen Plus membrane from section 2.6.4 was wetted with 2 x SSC and incubated for 2-4 h in 10 ml pre-hybridisation buffer (50 % [v/v] deionised formamide, 2 x SSC, 1 % [w/v] SDS, 0.15 % [w/v] polyvinyl pyrrolidone MW 40,000 [Sigma], 0.15 % [w/v] BSA, 0.15% [w/v] Ficoll 400 [Sigma] and 0.5 mg/ml fish-milt DNA [sodium salt] [ICN Biomedicals, Thame, UK]) at 42 °C. The pre-hybridisation buffer was changed to 10 ml hybridisation buffer (pre-hybridisation buffer containing 10 % [w/v] dextran sulphate [Duchefa Biochemie]), the probe was added and hybridisation was carried out overnight at 42 °C in a Hybaid hybridisation oven. After hybridisation, the membrane was washed in 2 x SSC for 15 min at room temperature, 2 x SSC containing 1 % (w/v) SDS for 15 min at 65 °C, and 0.1 x SSC for 15 min at 65 °C. The membrane was wrapped in Saran film and put in a storage phosphor screen cassette (Molecular Dynamics; Amersham Biosciences) (section 2.6.7). Probe was removed from the membrane by washing with a boiling solution of 0.1 x SSC containing 1 % (w/v) SDS for 15 min and rinsing twice with 2 x SSC for 15 min. The membrane was then either re-probed or wrapped in Saran film for storage.

2.6.7 Visualisation and quantification

The probed membrane, wrapped in Saran film, was exposed to a storage phosphor screen (Molecular Dynamics; Amersham Biosciences) for 24 h. The screen was then visualised by a Typhoon 8600 Variable Mode Imager controlled by Typhoon

Scanner Control software version 1.0 (Molecular Dynamics; Amersham Biosciences) and quantified with ImageQuant software version 5.1 (Molecular Dynamics; Amersham Biosciences). Depending on the strength of the signal, the membrane was then returned to the cassette for 1 h - 3 days. The signals from the test gene probes, which were given as intensity units by the imaging system, were standardised to the signal from a constitutive gene probe generated from *Arabidopsis ACTIN-7 (ACT7)* gene as a loading control. Ethidium bromide-stained rRNA bands were also shown as a loading control especially when the suitability of *ACT7* to be a loading control was uncertain. When replicates were indicated in the text, three replicates for each line or treatment were performed. Averages and standard errors were calculated from the replicates.

2.6.8 cDNA preparation

RNA (10 µg) extracted from mature leaves of the *Ws* ecotype (section 2.6.2) was mixed with 2 µg of oligo (dT) primer (Roche) and made up to 10 µl with DEPC-treated sterile deionised water before being incubated at 70 °C for 10 min and put on ice for 1 min. The mixture was then added to 800 units of M-MLV reverse transcriptase (Promega, Southampton, UK), 1 x M-MLV RT buffer (Promega), 2 mM dNTPs (Bioline), and DEPC-treated sterile deionised water to 30 µl. The mixture was incubated at 42 °C for 2 h followed by 94 °C for 1 min and cooled to 4 °C. The cDNA was stored at -20 °C.

2.6.9 Probes for RNA-gel-blot analysis

Probes for RNA blot analysis except *ACT7* were generated from cDNA (section 2.6.8) using PCR (section 2.5.2) with different annealing temperatures. Sequences of the primers, product sizes, annealing temperatures and target transcript sizes are given in Appendix I. PCR products were electrophoresed on a 1 % (w/v) agarose gel (section 2.5.4) to check the size and were quantified by reference to the HyperLadder I. The desired bands were cut out with a scalpel and DNA was extracted using QIAquick Gel Extraction Kit (QIAGEN), and confirmed by sequencing (section 2.5.5).

The 509-bp probe for *ACT7* (generated from At5g09810; transcript size is approximately 1700 nt) was carried in a plasmid, pCR-Blunt-ArabACTIN, produced by inserting the probe fragment into a pCR-Blunt vector (Invitrogen), obtained from N.J. Brown (Department of Plant Sciences, University of Cambridge, Downing Street, Cambridge, CB2 3EA, UK). The plasmid was in turn carried by *E. coli* DH5 α cell (Hanahan, 1985) (section 2.4 for growth and storage of bacterial strain). A large amount of the plasmid was prepared using a HiSpeed Plasmid Midi Kit (QIAGEN). The probe fragment was cut out with restriction enzyme *EcoR* I (section 2.5.3), electrophoresed on a 1 % (w/v) agarose gel (section 2.5.4), confirmed for the size and quantified using HyperLadder I. The desired band was cut out with a scalpel and DNA was extracted using QIAquick Gel Extraction Kit (QIAGEN).

2.6.10 Microarray analysis

2.6.10.1 RNA clean-up

Prior to delivery for microarray analysis, total RNA extracted using Concert Plant RNA Reagent, as described in section 2.6.2, was made up to a volume of 200 μ l using DEPC-treated sterile deionised water and applied to a MicroCon YM-30 column (Millipore, Watford, UK) inserted in a vial. The assembly was centrifuged at 12000 \times g for 10 min at 4 °C to concentrate the solution. The column was then inverted in a fresh vial and centrifuged for 3 min at 3000 \times g to collect the concentrated RNA solution of approximately 5-8 μ l. The volume of the RNA solution was increased to 10 μ l by addition of DEPC-treated sterile deionised water.

2.6.10.2 Affymetrix GeneChip expression analysis

The entire process of biotin labelled-complementary RNA (cRNA) preparation, hybridisation to Affymetrix ATH-121501 microarrays, array scanning, and data normalisation was carried out by the Medical Research Council (MRC) GeneService at Hinxton Hall, Cambridge, UK, according to standard Affymetrix protocols. Data were returned as text files and analysed using GeneSpring 7 (Silicon Genetics, San Carlos, CA, USA). Three replicates for each test line were performed and average values of

transcript abundance were used. Correspondence between the Affymetrix probe set ID, Arabidopsis Genome Initiative (AGI) codes, and the National Center for Biotechnology Information (NCBI) Reference Sequence (RefSeq) mRNA ID was determined using Affymetrix NetAffx Analysis Center (<http://www.affymetrix.com/>).

Gene Ontology (GO) categorisations of cellular location, biological process and molecular function on GO-annotated *Arabidopsis* genes were performed using the TAIR GO Annotations tool (<http://www.arabidopsis.org/>). In order to search for common *cis*-regulatory sequences in upstream regions of highly affected genes, the "Find Potential Regulatory Sequences" tool in GeneSpring was used based on the following criteria: 6-8 nt without any point discrepancies within 10-1000 nucleotides upstream of the translation start site of each gene. A cut-off P-value of 1×10^{-10} was used in all statistical analysis relative to upstream regions of other (unselected) genes. Sequences of the *Arabidopsis thaliana* genome were downloaded from the NCBI Genome database (ftp://ftp.ncbi.nih.gov/genomes/Arabidopsis_thaliana/).

2.7 Abscisic acid (ABA) procedures

2.7.1 Preparation of ABA solution

(±) *cis-trans* ABA (A1049; Sigma) was dissolved in 1 N NaOH and diluted in sterile deionised water to a final stock concentration of 25 mM.

2.7.2 Germination assay

For the seed germination assay on ABA, 25-150 seeds of each test line were sterilised and plated on 0.7 % (w/v) agar with half-strength MS and ABA of different concentrations as indicated in the text. The plates were stratified and transferred to the light as described in sections 2.2.1 and 2.2.2. Controls without ABA in the medium were prepared in a separate room to avoid possible contamination. After 10 days, the number of seeds showing radicle emergence observable to the naked eye was determined. When replicates are indicated in the text, three replicates for each line or

treatment were performed. Averages and standard errors were calculated from the replicates.

2.7.3 Measurement of endogenous ABA

The concentration of ABA in seedling tissue was determined by radioimmunoassay as initially described in Quarrie *et al.* (1988) and performed entirely by J.C. Theobald (Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK) without knowing the identity of the samples during the process. 7-day-old seedlings (500-1000) were ground to a fine powder in a mortar with a pestle in liquid nitrogen. The powder was freeze-dried using an Edwards Super Modulyo Freeze-dryer and sent to Lancaster. The powder was extracted with distilled water at a ratio of 20:1 (water volume : leaf dry weight) on a rotary shaker at 5-10 °C overnight. Extracts were clarified by centrifugation (10000 x g for 5 min) and duplicate aliquots of the supernatant were taken for assay.

In 2 ml microtubes (Sarstedt, Beaumont Leys, UK), 50 µl of sample or one of the six standards that ranged in 2-fold dilutions from 4000 to 125 pg of (\pm) *cis-trans* ABA, was added to 200 µl of PBS buffer (22 mM NaH₂PO₂, 30 mM Na₂HPO₄, 50 mM NaCl, pH 6.0), followed by 100 µl of DL-*cis,trans*-[G-³H]ABA (Amersham Biosciences) and 100 µl of MAC 252 monoclonal antibody against (S)-*cis,trans*-ABA (obtained from G.W. Butcher, Babraham Institute, Cambridge, CB2 4AT, UK). After vortexing, samples were incubated in the dark at 4 °C for 45 min. To remove any unbound label, 500 µl of saturated ammonium sulphate was added, the sample mixed, left for 30 min at room temperature, centrifuged at 10000 x g for 4 min, and the supernatant discarded. This was repeated with 1 ml of 50 % (w/v) saturated ammonium sulphate to further wash the pellet, and the precipitate was resuspended in 100 µl of water before adding 1.5 ml of Ecoscint-H (National Diagnostics, Hull, UK) for liquid scintillation counting on a Tri-Carb 1600TR (Packard Instrument Company, Meriden, CT, USA).

ABA amounts were calculated from the radioactivity (cpm) present in the pellets. The curve obtained from the standards was linearised by plotting the logit-

transformation of the data against the natural logarithm of the amount of unlabelled ABA added. ABA concentrations of unknowns were calculated by interpolation. By using spike dilution tests (Rosher *et al.*, 1985), it had previously been determined that there was no significant cross reactivity of MAC 252 in leaf extracts of *Arabidopsis thaliana* (JC Theobald, unpublished). Three replicates were prepared for each line and 2-4 measurements were performed for each replicate. All these measurements for a replicate were averaged. All measurements of ABA concentration were normalised to the dry weight of seedling tissue (μg of ABA/g of seedling dry weight).

2.8 Pigment analysis

Chlorophyll was extracted using a protocol modified from that described in Moran and Porath (1980). Approximately 200 mg of seedlings was immersed in 1.5 ml *N,N*-dimethylformamide (DMF) for 24 h at 4 °C in complete darkness (wrapped with aluminium foil). The extract was subjected to spectrophotometric measurements at 664, 647, 625 and 603 nm using a UV/VIS Spectrometer Lambda 9 (PerkinElmer) and the absorbance spectrum between 600 and 700 nm was drawn when indicated in the text. Total chlorophyll was sum of chlorophyll *a* and *b* calculated using equations 14 and 15 described by Moran (1982) and standardised to the fresh weight of seedling tissue (μg of chlorophyll/g of seedling fresh weight).

$$\text{Chlorophyll } a \text{ } (\mu\text{g/ml}) = 12.81 A_{664} - 2.16 A_{647} + 1.44 A_{625} - 4.91 A_{603}$$

$$\text{Chlorophyll } b \text{ } (\mu\text{g/ml}) = -4.93 A_{664} + 26.01 A_{647} + 3.74 A_{625} - 15.55 A_{603}$$

Three replicates for each line or treatment were performed. Averages and standard errors were calculated from the replicates.

2.9 Microscopy and photography

2.9.1 Photography

Photographic images were taken using a Canon EOS 300D digital camera. For close-up images of seedlings, a Leica MZFLIII dissecting microscope was used to view

the specimens. Images were then taken using a Nikon Coolpix 950 digital camera attached to the dissecting microscope.

2.9.2 Fluorescence microscopy

Specimens were mounted with water on a slide covered with a cover slip and viewed with a Nikon Optiphot 2 epifluorescence microscope. In order to examine GFP fluorescence, an Omega Optical filter set (XF76 NM574; 405DF40, excitation 405 ± 20 ; dichroic 450DRLP02; 520DF40, emission 520 ± 20) (Glen Spectra, Stanmore, UK) suitable for the main 395 nm excitation and 507 nm emission peaks of GFP5 (Siemering *et al.*, 1996) was used. For chlorophyll that is excited optimally at 460 nm and emits at 685 nm, another filter set (B-2A, excitation 470/40; dichroic DM510; BA520, emission LP520) (Nikon, Kingston upon Thames, UK) was used. Microphotographic images were taken using a Nikon Coolpix 950 or 8700 digital camera attached to the microscope.

2.9.3 GFP quantification and frequency histogram

GFP was quantified in terms of the average green pixel intensity from the microphotographic images of cotyledons, taken as described in section 2.9.2, using MetaMorph software version 4.01 (Universal Imaging, Marlow, UK). Frequency histograms to show the distribution of GFP intensities of F2 individuals for genetic analysis were drawn using Microsoft Excel 2002.

2.9.4 Transmission electron microscopy (TEM)

The entire process of fixation, sectioning, staining and viewing was performed at the Multi-Imaging Centre, Department of Anatomy, University of Cambridge, UK. The process was performed as described in Peracchia and Mittler (1972) with modifications. Seedlings were fixed by immersion in 0.1 M PIPES buffer (pH 7.4) containing 4 % (v/v) glutaraldehyde, 0.3 % (v/v) hydrogen peroxide and 2 mM CaCl_2 . Tissues were fixed for 4-6 h at 4 °C, washed twice in buffer (0.1 M PIPES, pH 7.4), and stored at 4 °C. After buffer washes, they were post-fixed in 1 % (w/v) osmium ferricyanide for 1 h,

rinsed 3 times in deionised water, and stained in 2 % (w/v) uranyl acetate for 1 h. They were rinsed in deionised water and dehydrated in an ascending series of ethanol solutions to absolute ethanol (3 x 70 % [v/v], 3 x 95 % [v/v] and 3 x 100 % [v/v]), rinsed twice in acetonitrile, and embedded in Spurr's epoxy resin. Sections (50 nm) were cut on a Leica Ultracut UCT, stained with saturated uranyl acetate in 50 % (v/v) ethanol and lead citrate (Reynolds, 1963), and viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV.

2.10 Statistical analysis

A two-tailed chi-square test was performed to test the observed distributions of phenotypes against the expected distributions using QuickCalcs, online calculators for scientists (<http://www.graphpad.com/quickcalcs/>) (GraphPad Software, San Diego, CA, USA). The cut-off P-value was set at 5 %. P-values higher than 5 % were considered not significant (^{ns}) and P-values lower than 5 % were considered significant (*). P-values lower than 1 % were considered very significant (**).

Chapter 3
Characterisation of
putative *gun1*-like mutants

3. Characterisation of putative *gun1*-like mutants

3.1 Introduction

Plastid translation or protein synthesis is required for the expression of nuclear photosynthesis genes, because inhibition of plastid protein synthesis results in decreased nuclear photosynthesis gene expression (more discussion in section 1.7). Experiments with two inhibitors, norflurazon and lincomycin, have demonstrated the basis for distinguishing *gun1* from other *gun* mutants. All *gun* mutants express nuclear photosynthesis genes in the presence of norflurazon, but only *gun1* expresses the genes in the presence of lincomycin (Gray *et al.*, 2003; JH Wang, JA Sullivan and JC Gray, unpublished). This implies that *GUN1* is part of an uncharacterised plastid-to-nucleus signalling pathway involving plastid protein synthesis.

In order to understand more about the plastid-protein-synthesis-related plastid-to-nucleus signalling, an attempt to isolate more mutants in components of the pathway has been made. A collection of *Arabidopsis gun* mutants was produced following ethyl methanesulphonate (EMS) treatment of a transgenic line containing a reporter gene encoding endoplasmic-reticulum-targeted green fluorescent protein (GFP) under the control of a tobacco *RbcS* promoter (Sullivan, 1999; Gray *et al.*, 2003). Screening of the mutagenised seed pools for *gun* mutants that express GFP in the presence of norflurazon and lincomycin (a *gun1*-like phenotype) does not require the extended histochemical staining of detached cotyledons necessary for GUS detection in the previous screen for *gun* mutants (Susek *et al.*, 1993).

From approximately 15000 seeds of the parental line mutagenised with 50 mM or 100 mM EMS, a number of putative *gun* mutants were identified in the initial screening with norflurazon, although the majority of them are so far uncharacterised. Through genetic analysis, at least one of the putative mutant lines was concluded to be allelic to the previously isolated *gun1-1* and was designated *gun1-100* (JH Wang, JA Sullivan and JC Gray, unpublished). Two approaches were used in screening for *gun1*-like mutants in the hope that mutants showing *gun1* phenotypes, but not allelic to *gun1-1*, would represent other components of the same pathway (C Meade, JA Sullivan and

JC Gray, unpublished). In the first approach, mutagenised seeds were screened on norflurazon and, for those expressing GFP, secondary screening on lincomycin was conducted. Approximately ten lines that were expressing GFP in both norflurazon and lincomycin treatments were identified as putative *gun1*-like mutants. In the second approach, mutagenised seeds were screened on lincomycin and, for those expressing GFP, secondary screening with norflurazon was conducted. This approach was an attempt to isolate lines that express GFP on lincomycin, but not on norflurazon. However, no line with this phenotype was isolated. This approach produced approximately 25 lines that were expressing GFP in both lincomycin and norflurazon treatments (C Meade, JA Sullivan and JC Gray, unpublished).

Five putative *gun1*-like mutants, PR28.1N, PR32.2N, PR32.4N, PR48.2N and PR107.11N, were chosen for further examination. The names of the lines indicate their origin; for example, PR28.1N was the first putative mutant selected from the norflurazon screen of M2 pool 28. These lines were subjected to RNA-gel-blot analysis to investigate the transcript abundance of endogenous nuclear photosynthesis genes, such as *RBCS* and *LHCBI*, and by pigment analysis of the illuminated dark-grown seedlings in this study.

3.2 GFP expression in putative *gun1*-like mutants

PR28.1N, PR32.2N, PR32.4N, PR48.2N and PR107.11N in the Ws background were selected as high GFP-expressers on both norflurazon and lincomycin treatments. Seeds from the parental line (3R12), *gun1-100* and the mutant lines were sterilised, plated, stratified and light-treated, as described in sections 2.2.1 and 2.2.2. The seeds were germinated on 0.7 % agar with half-strength MS either with water, 5 μ M norflurazon or 0.5 mM lincomycin and grown for 5 days in the light after stratification. The chlorophyll and GFP fluorescence of the cotyledons of the seedlings were examined using a Nikon Optiphot 2 epifluorescence microscope (section 2.9.2) and microphotographic images were taken using a Nikon Coolpix 950 digital camera attached to the microscope.

Contrary to the previous report, the parental line, 3R12, did not show GFP expression in the untreated control condition (Figure 3.1) (Sullivan, 1999). One possible explanation was that the transgene had been silenced after generations (Stam *et al.*, 1997). All putative *gun1*-like mutants showed GFP expression in the presence or absence of norflurazon or lincomycin, like *gun1-100*. Therefore, the previous observation that these putative *gun1*-like mutants showed GFP expression in the presence of norflurazon and lincomycin was confirmed (C Meade, JA Sullivan and JC Gray, unpublished). However the intensities of GFP fluorescence were different among the lines. Some lines showed brighter GFP fluorescence on different treatments, such as PR28.1N on water and norflurazon, *gun1-100* on norflurazon, and PR48.2N on lincomycin. Except *gun1-100*, the brighter fluorescence of PR28.1N and PR48.2N seedlings was observed to be due to the variation of fluorescence intensity.

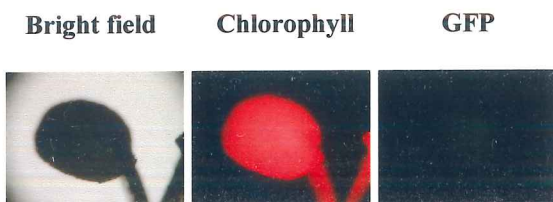
The red chlorophyll fluorescence was not detected in the norflurazon-treated seedlings. Norflurazon inhibits carotenoid biosynthesis, resulting in chlorophyll photooxidation (Frosch *et al.*, 1979; Reiß *et al.*, 1983). The red chlorophyll fluorescence was also low in the lincomycin-treated seedlings supporting the previous report on inhibitory effect of lincomycin on chlorophyll accumulation (Hiller *et al.*, 1977). However, lincomycin-treated PR32.2N, PR32.4N and PR107.11N seedlings showed red chlorophyll fluorescence higher than the norflurazon-treated seedlings but lower than the untreated seedlings.

3.3 Transcript abundance of nuclear photosynthesis genes in putative *gun1*-like mutants

Putative *gun1*-like mutants PR28.1N, PR32.2N, PR32.4N, PR48.2N and PR107.11N were subjected to a preliminary round of RNA-gel-blot analysis to investigate the transcript abundance of the *RBCS* nuclear photosynthesis gene family in the mutant seedlings treated with the norflurazon or lincomycin to confirm their *gun1*-like phenotype. The *RBCS* genes are well-studied plastid-regulated nuclear photosynthesis genes with high transcript abundance in plants (Oelmüller, 1989; Taylor, 1989; Rodermel, 1999).

3R12
(parental
line)

Water



Norflurazon



Lincomycin



gun1-100

Water



Norflurazon



Lincomycin



PR28.1N

Water



Norflurazon



Lincomycin



PR32.2N

Water



Norflurazon



Lincomycin



Continued

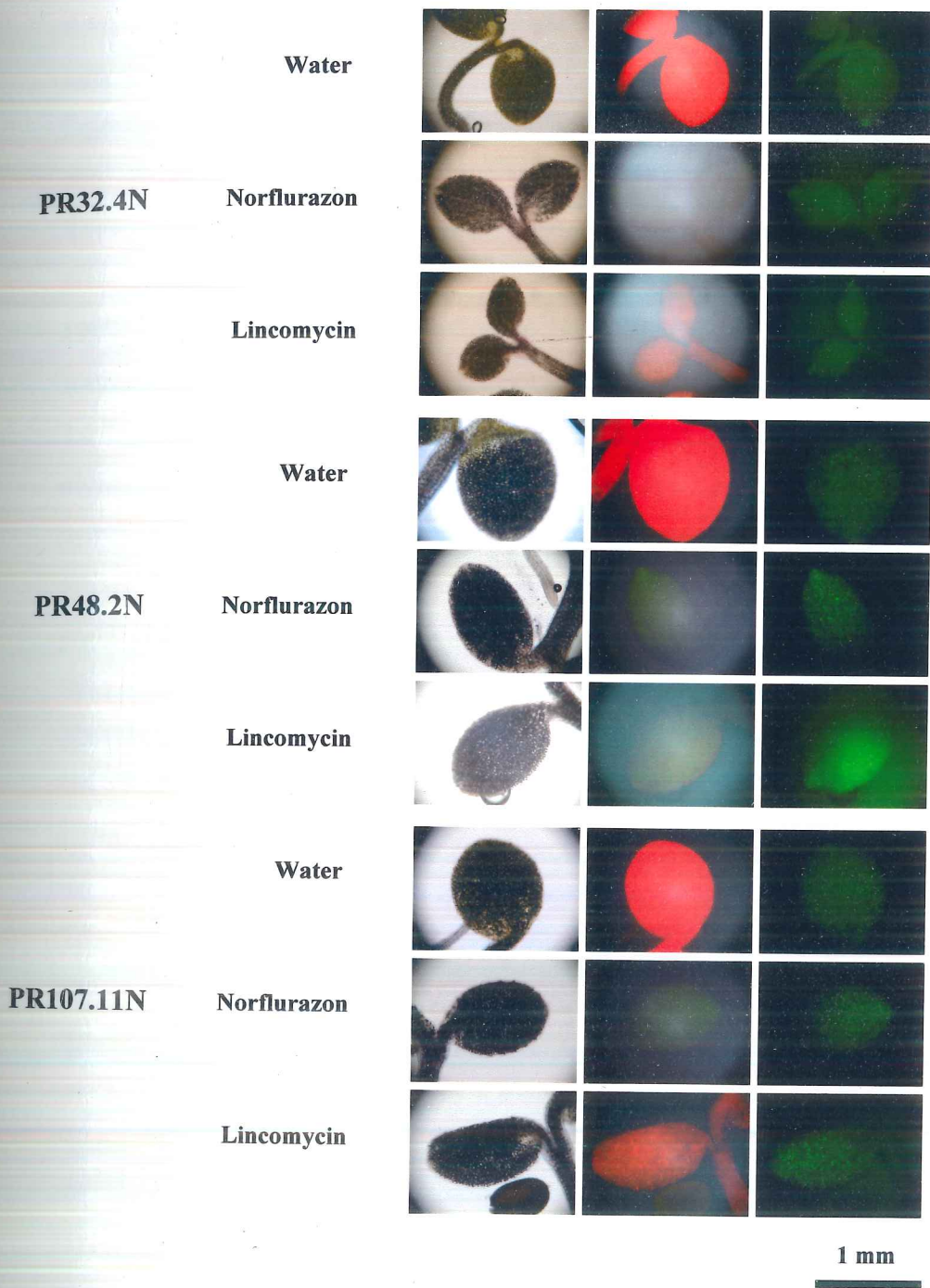


Figure 3.1 Effect of norflurazon and lincomycin on green fluorescent protein (GFP) fluorescence in seedlings of putative *gun1*-like mutants.

3R12 (parental line), *gun1-100*, PR28.1N, PR32.2N, PR32.4N, PR39.2N, PR48.2N and PR107.11N seeds were germinated on 0.7 % agar with half-strength MS either with water, 5 μ M norflurazon or 0.5 mM lincomycin and grown for 5 days in the light. Cotyledons of the seedlings were viewed for chlorophyll and green fluorescent protein (GFP) fluorescence using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 950 digital camera.

Seeds from *Arabidopsis thaliana* ecotype Ws (wild type), *gun1-1* and the mutant lines were prepared as described in section 3.2. The seeds were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the dark followed by 2 days in the light either with water (LW), 5 μ M norflurazon (LN) or 0.5 mM lincomycin (LL), or in continuous darkness for 7 days with water (DW) after stratification and light treatment. These growth conditions have been used previously in the laboratory for the study of plastid signalling in tobacco, pea and *Arabidopsis* (Gray *et al.*, 1995; Sullivan and Gray, 1999; Sullivan and Gray, 2002; Gray *et al.*, 2003; Brown *et al.*, 2005). After 7 days, the seedlings were harvested for RNA extraction using TriPure Isolation Reagent (section 2.6.2). Total RNA (9-15 μ g) extracted from each line and treatment was separated by electrophoresis on a 1.2 % agarose gel and blotted on a piece of GeneScreen Plus hybridization transfer membrane before being subjected to probing with 32 P-labelled probes (sections 2.6.3-2.6.6) generated from *RBCS-1A* and *ACT7* (section 2.6.9). The probe for *RBCS-1A* was predicted to hybridise to other members of the *RBCS* gene family, such as *RBCS-1B*, *RBCS-2B* and *RBCS-3B*, due to close sequence similarity (Krebbers *et al.*, 1988). Similarly, the probe for *ACT7* was expected to hybridise to a family of *ACT* genes, such as *ACT1*, *ACT2*, *ACT3*, *ACT4* and *ACT8*. The similarity among the *ACT* sequences corresponding to the probe sequence was observed to be more than 80 % by a NCBI Basic Local Alignment Search Tool (BLAST) search (<http://www.ncbi.nlm.nih.gov/>). Hybridisation signals from the RNA-gel blots shown in Figure 3.2 were quantified using a Typhoon 8600 Variable Mode Imager and standardised to *ACT7* signal to account for differences in the loading of total RNA (section 2.6.7). Ethidium bromide-stained rRNAs are shown in Figure 3.2 as a loading control. Each of the mutant lines was analysed independently and the transcript abundance in the seedlings grown on norflurazon or lincomycin, and in darkness were expressed relative to the transcript abundance in the seedlings of the same mutant grown on water for 5 days in the dark and 2 days in the light, which was given an arbitrary value of 100 (Figure 3.3).

Wild-type Ws seedlings showed lower transcript abundance of *RBCS* with treatments of norflurazon and lincomycin, and in darkness (Figures 3.2 and 3.3). *RBCS* genes are known to be regulated by light (Dedonder *et al.*, 1993; for review, Thompson and White, 1991) and plastid signals (for reviews, Gray *et al.*, 2003; Nott *et al.*, 2006). *gun1-1* displayed higher transcript abundance of *RBCS* in the presence of norflurazon or

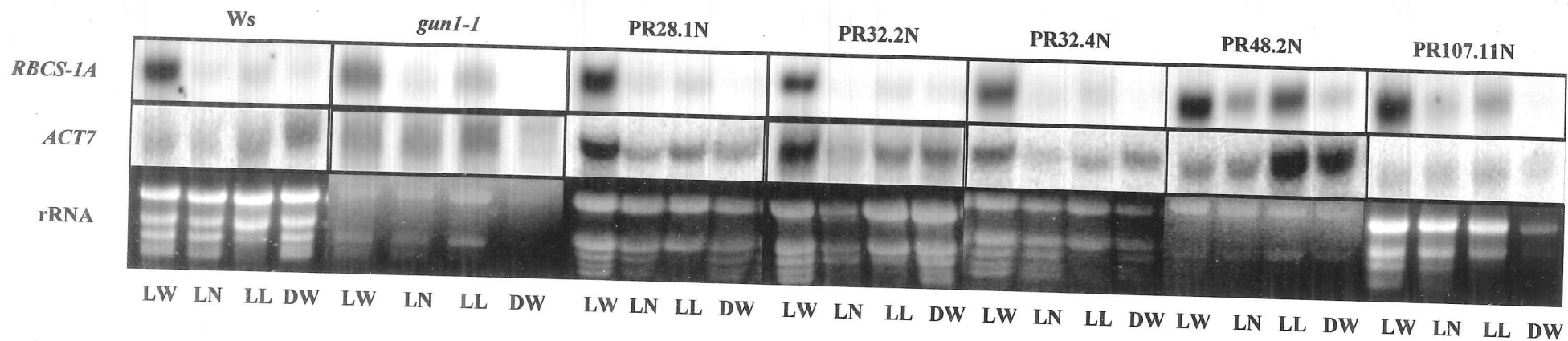


Figure 3.2 Effect of light, norflurazon and lincomycin on *RBCS* transcript abundance in seedlings of putative *gun1*-like mutants.

Wild-type Ws, *gun1-1*, PR28.1N, PR32.2N, PR32.4N, PR39.2N, PR48.2N, and PR107.11N seeds were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the dark followed by 2 days in the light, either with water (LW), 5 μM norflurazon (LN) or 0.5 mM lincomycin (LL) or in continuous darkness for 7 days with water (DW). Total RNA was extracted from the seedlings using TriPure Isolation Reagent and 9-15 μg of the total RNA was subjected to RNA-gel-blot analysis using a ³²P-labelled probe from a nuclear photosynthesis gene, *RBCS-1A*. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

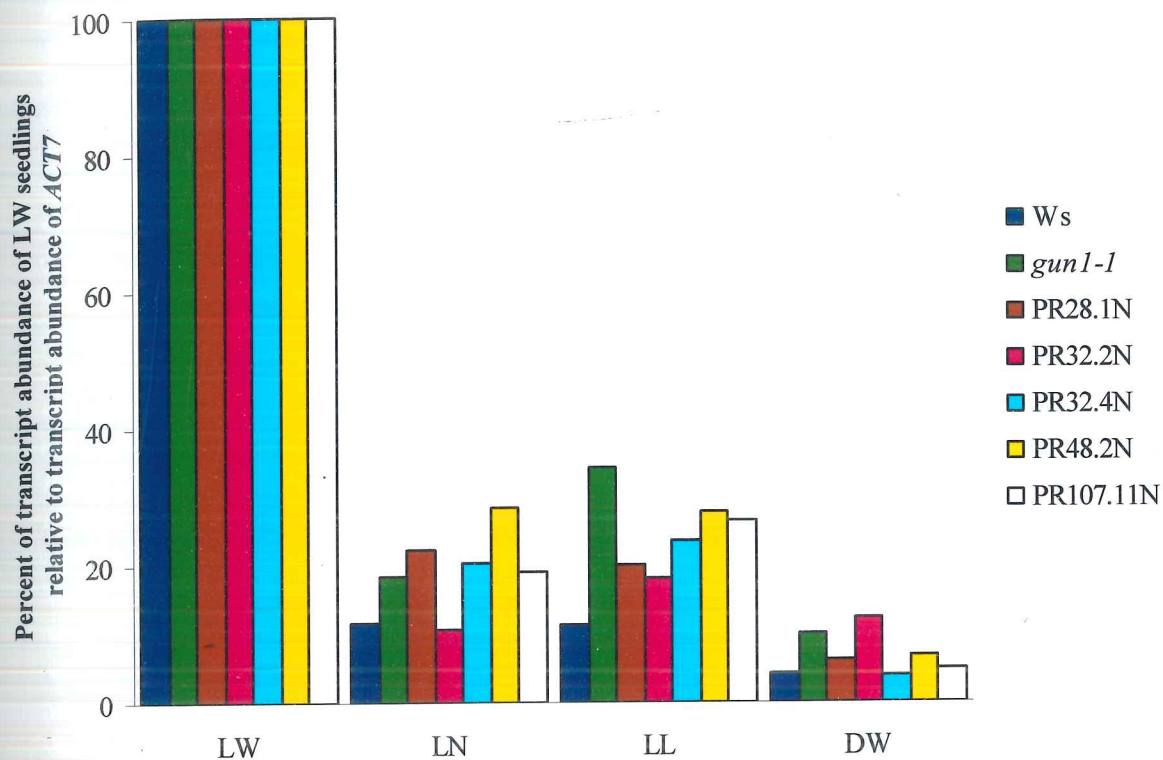


Figure 3.3 Quantification of the effect of light, norflurazon and lincomycin on transcript abundance of *RBCS* in seedlings of putative *gun1*-like mutants.

Hybridisation signals from the RNA-gel blots shown in Figure 3.2 were quantified using a Typhoon 8600 Variable Mode Imager and standardised to *ACT7* signal to account for differences in the loading of total RNA. For each line, the amounts of transcripts were expressed relative to the seedlings grown on water for 5 days in the dark and 2 days in the light, which was given an arbitrary value of 100. Abbreviations are as given in the legend of Figure 3.2.

lincomycin compared to the wild-type seedlings. The results also showed that most of the putative *gun1*-like mutants, like *gun1-1*, had higher transcript abundance of *RBCS* in the presence of norflurazon or lincomycin compared to the transcript abundance in wild-type Ws seedlings. Among the putative *gun1*-like mutants, PR48.2N seedlings showed the biggest differences of *RBCS* transcript abundance compared to the wild type, namely 2.5- and 2.4-fold higher with norflurazon and lincomycin treatments, respectively. PR28.1N seedlings showed *RBCS* transcripts 1.9- and 1.7-fold higher than the wild-type seedlings with norflurazon and lincomycin treatments, respectively. Seedlings of PR32.2N had wild-type level of transcript abundance in norflurazon and just slightly higher (1.6-fold) in lincomycin and, hence were unlikely to be genuine *gun1*-like mutants. PR32.4N seedlings showed 1.8- and 2.1-fold more transcripts with norflurazon and lincomycin treatments, respectively. PR107.11N seedlings showed 1.7- and 2.3-fold higher transcript abundance with norflurazon and lincomycin treatments, respectively.

3.4 Greening abilities of putative *gun1*-like mutants

gun1-1 is deficient in greening after illumination of the dark-grown seedlings compared to the wild type (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). The putative *gun1*-like mutants were examined for their greening phenotype on illumination of dark-grown seedlings. Seeds from wild types, Col and Ws, *gun1-1*, *gun1-100*, and the putative *gun1*-like mutants, PR28.1N, PR32.2N, PR32.4N, PR48.2N and PR107.11N were prepared as described in section 3.2. The seeds were germinated on 0.7 % agar with half-strength MS and grown for 6 or 10 days in the dark followed by 1 day in the light to give 6D1L and 10D1L samples. The seedlings were harvested for chlorophyll extraction with *N,N*-dimethylformamide (DMF) using a protocol modified from that described in Moran and Porath (1980) (section 2.8). The seedling extracts were subjected to spectrophotometric measurements and chlorophyll amounts were calculated using equations described by Moran (1982) (section 2.8). Total chlorophyll was standardised to the fresh weight of seedling tissue (μg of chlorophyll/g of seedling fresh weight). Three replicates for each measurement were performed to obtain the means and SEM.

For seedlings grown for 6 days in the dark followed by 1 day in the light, *gun1-1* accumulated much less total chlorophyll (0.3 $\mu\text{g/g}$) than its wild type, Col (13.9 $\mu\text{g/g}$) (Figure 3.4). This was consistent with the previous observation that *gun1-1* is defective in greening after transition from dark- to light-growth (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). Curiously *gun1-100*, another allele of *gun1-1*, showed a chlorophyll content only 50 % lower than its wild type, Ws. This could be due to the effect of different alleles or different genetic backgrounds. All the putative *gun1*-like mutants, except PR48.2N, contained similar amounts of chlorophyll to the wild-type Ws. PR48.2N accumulated much higher total chlorophyll (62.2 $\mu\text{g/g}$) than its wild type, Ws (16.1 $\mu\text{g/g}$).

For seedlings grown for 10 days in the dark followed by 1 day in the light, all the lines accumulated low levels of total chlorophyll. *gun1-1* and all putative *gun1*-like mutants, except PR48.2N, contained similar amounts of chlorophyll to their wild types after 10 days in the dark followed by 1 day in the light. PR48.2N accumulated the highest level of total chlorophyll (6.4 $\mu\text{g/g}$) compared to other tested lines, including the wild-type Ws (1.4 $\mu\text{g/g}$), after the treatment.

This experiment demonstrated that PR48.2N had a distinct mutant phenotype from the rest of the putative *gun1*-like mutants tested. PR48.2N was therefore selected for further characterisation of its mutant phenotypes. The defective greening phenotype of PR48.2N was different from *gun1-1*; *gun1-1* is a loss-of-greening-ability mutant (Susek *et al.*, 1993; Mochizuki *et al.*, 1996), whereas PR48.2N was a gain-of-greening-ability mutant.

3.5 Transcript abundance of nuclear photosynthesis genes in PR48.2N

PR48.2N showed the strongest *gun1*-like phenotype with respect to transcript abundance among the tested mutant lines in section 3.2 and was subjected to further analysis. The transcript abundance of two classes of nuclear photosynthesis genes, *RBCS* and *LHCBI*, in PR48.2N seedlings treated with norflurazon or lincomycin was investigated together with the wild-type Ws. Transcript abundances of the nuclear photosynthesis genes in Ws and PR48.2N seedlings were compared directly on the same RNA-gel blot.

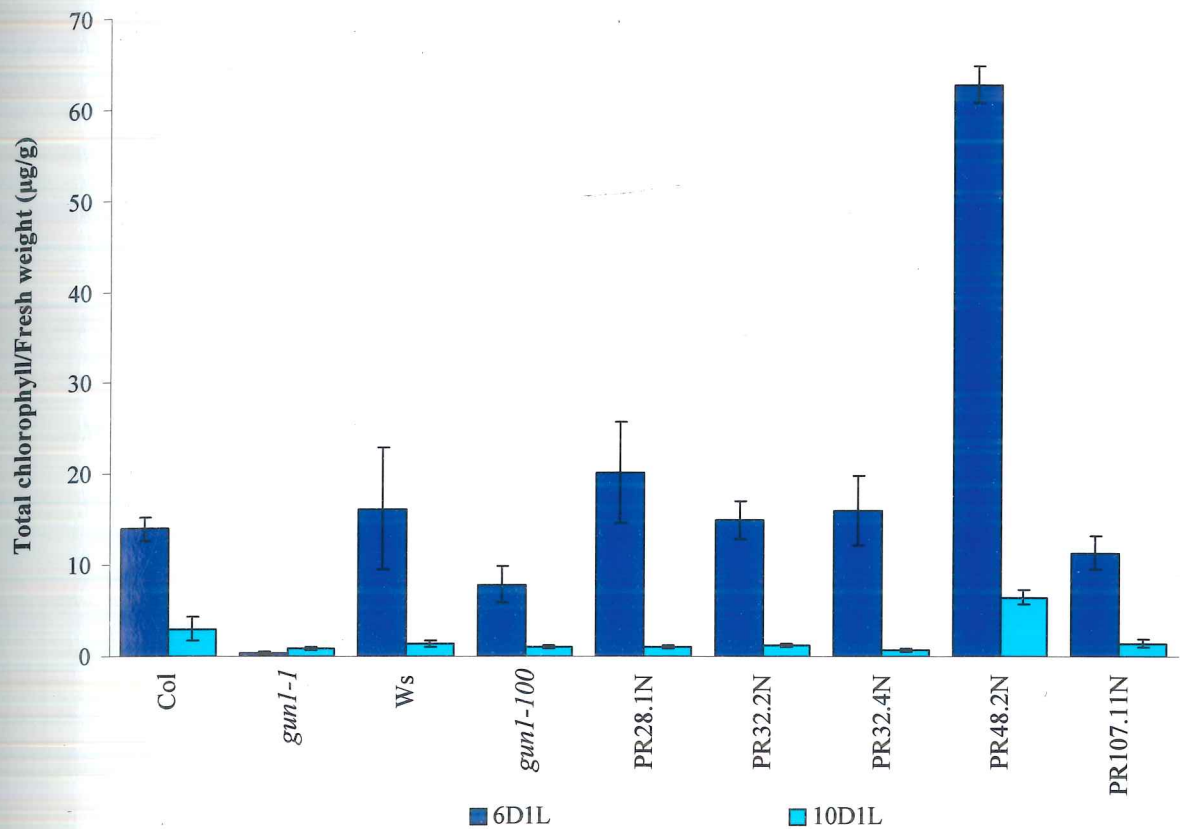


Figure 3.4 Chlorophyll content of seedlings of putative *gun1*-like mutants after 6 or 10 days in the dark followed by 1 day in the light.

Wild-type Col and Ws, and mutant, *gun1-1*, *gun1-100*, PR28.1N, PR32.2N, PR32.4N, PR39.2N, PR48.2N and PR107.11N, seedlings were grown on 0.7 % agar with half-strength MS for 6 (6D1L) or 10 (10D1L) days in the dark followed by 1 day in the light. Chlorophylls were extracted using *N,N*-dimethylformamide (DMF) and measured with a UV/VIS Spectrometer Lambda 9. Total chlorophyll was calculated using an equation described by Moran (1982) on a basis of seedling fresh weight. Results are means \pm SEM for 3 replicates.

Wild-type Ws and PR48.2N seedlings were grown as previously (section 3.3) but RNA extraction was performed with Concert Plant RNA Reagent using the manufacturer's large-scale isolation protocol with modifications (section 2.6.2). Total RNA (7 µg) extracted from each line and treatment was subjected to RNA-gel-blot analysis (section 3.3) using ³²P-labelled probes generated from nuclear photosynthesis genes *RBCS-1A* and *LHCBI.2* (section 2.6.9). The probe for *RBCS-1A* was described in section 3.3 and the probe for *LHCBI.2* was expected to hybridise to other members of the *LHCBI* gene family, such as *LHCBI.1*, *LHCBI.3*, *LHCBI.4* and *LHCBI.5*, due to close sequence homology (Jansson, 1999). Other *LHCB* members showed low similarity ($\leq 30\%$) to this probe sequence by a NCBI BLAST search. The amounts of transcripts were expressed relative to wild-type Ws grown on water for 5 days in the dark and 2 days in the light, which was given an arbitrary value of 100. The experiment was repeated independently three times to obtain the means and SEM.

PR48.2N seedlings showed 2-fold higher transcript abundance of the nuclear photosynthesis genes after treatments of norflurazon or lincomycin compared to the wild type (Figures 3.5 and 3.6). However, PR48.2N also showed a 2-fold higher transcript abundance of the nuclear photosynthesis genes in the light in the absence of inhibitors. Therefore, it could be argued that PR48.2N was an overexpresser of the nuclear photosynthesis genes with a higher baseline of the transcript levels. So when chloroplast functions were perturbed and the nuclear photosynthesis genes were down-regulated, PR48.2N had higher levels of the transcripts compared to the wild type. Wild-type Ws showed lower transcript abundance of *RBCS* and *LHCBI* after treatments of norflurazon, lincomycin and darkness. Like *RBCS*, as discussed in section 3.3, *LHCBI* genes are also regulated by light (Karlin-Neumann *et al.*, 1988; for review, Thompson and White, 1991) and plastid signals (for reviews, Gray *et al.*, 2003; Nott *et al.*, 2006).

Since PR48.2N showed higher transcript abundance of nuclear photosynthesis genes with or without treatments of norflurazon or lincomycin, it was considered to be distinct from *gun1-1*, but was an interesting mutant to pursue further. No *Arabidopsis* mutants with higher transcript levels of nuclear photosynthesis genes under normal growth conditions had previously been reported, although *Arabidopsis* mutants showing

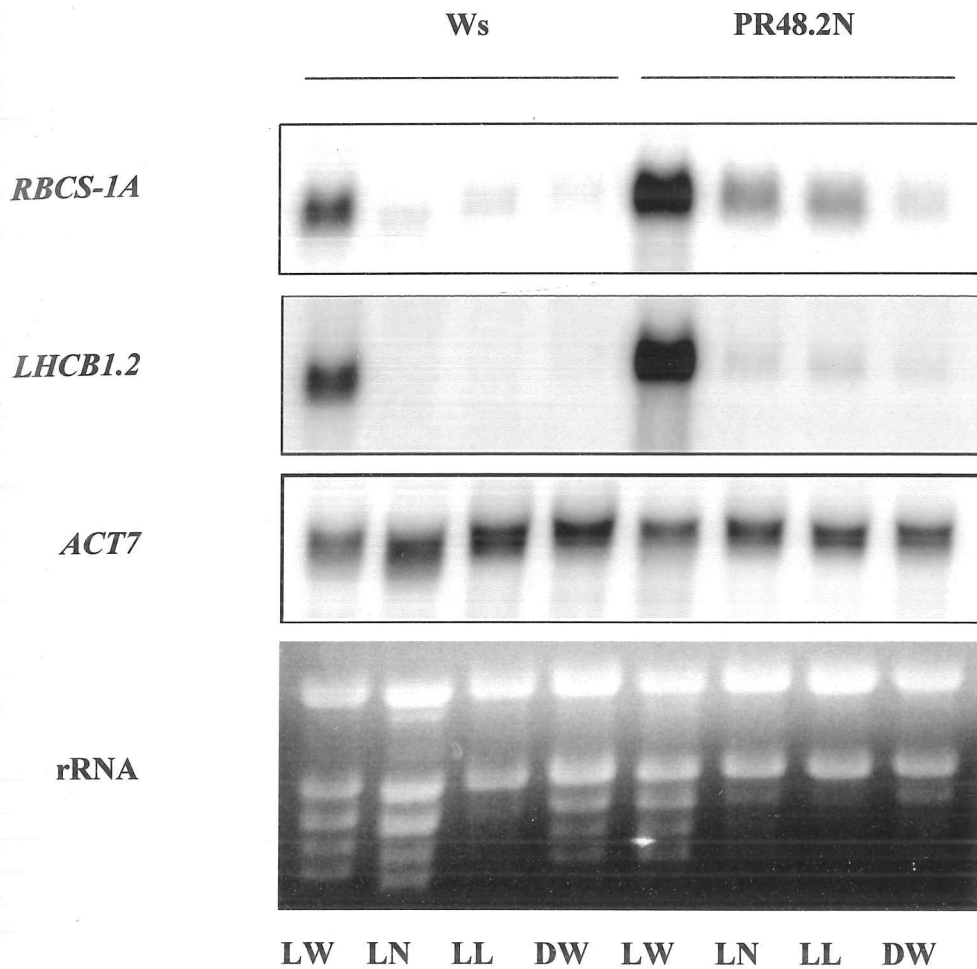


Figure 3.5 Effect of light, norflurazon and lincomycin on transcript abundance of nuclear photosynthesis genes in wild-type Ws and PR48.2N seedlings.

Wild-type Ws and PR48.2N seeds were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the dark followed by 2 days in the light either with water (LW), 5 μM norflurazon (LN) or 0.5 mM lincomycin (LL) or in continuous darkness for 7 days with water (DW). Total RNA was extracted from the seedlings using Concert Plant RNA Reagent and 7 μg of the total RNA was subjected to RNA-gel-blot analysis using ³²P-labelled probes from nuclear photosynthesis genes, *RBCS-1A* and *LHCB1.2*. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

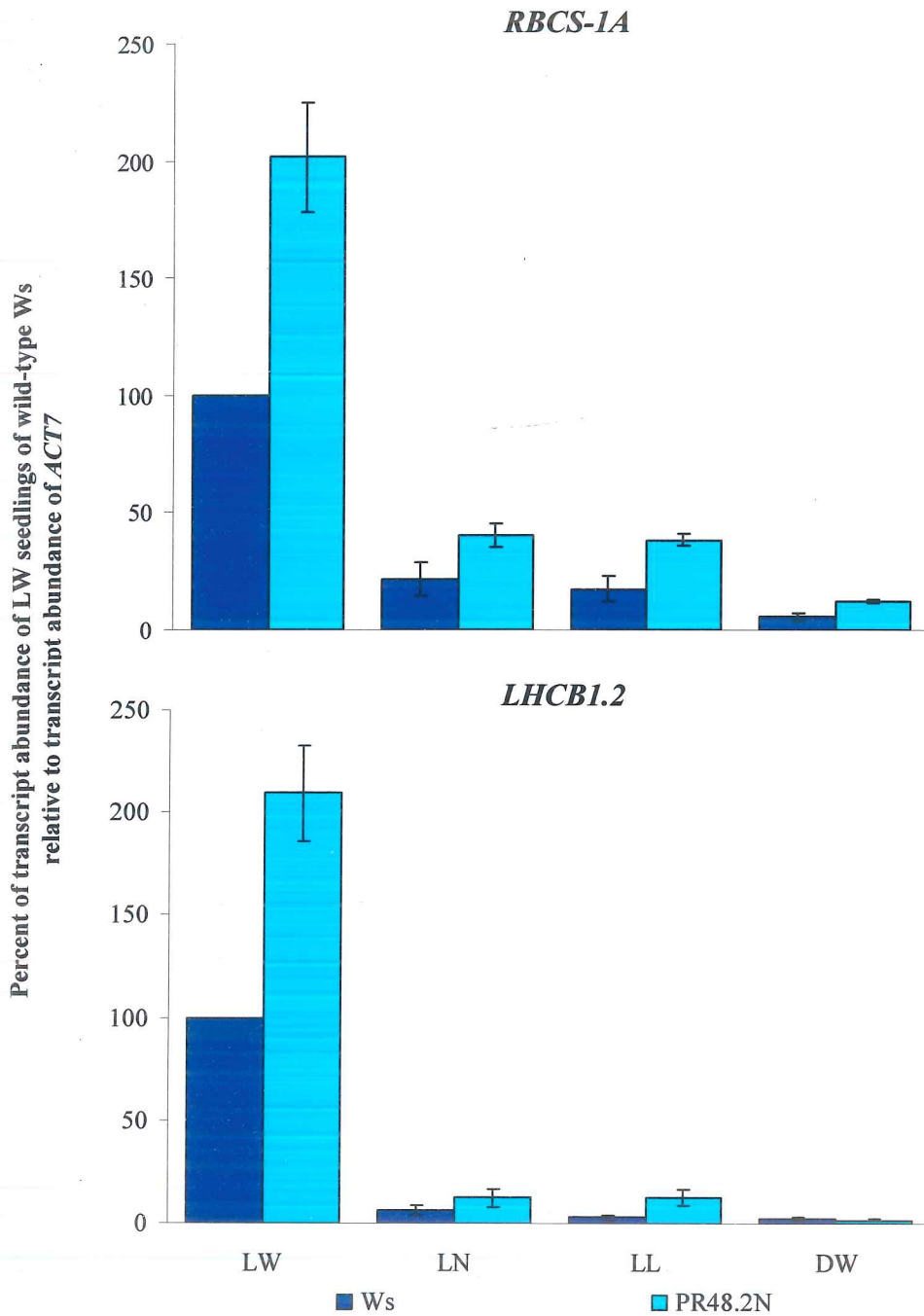


Figure 3.6 Quantification of the effect of light, norflurazon and lincomycin on transcript abundance of nuclear photosynthesis genes in wild-type Ws and PR48.2N seedlings.

Hybridisation signals from the RNA-gel blots shown in Figure 3.5 were quantified using a Typhoon 8600 Variable Mode Imager and standardised to *ACT7* signal to account for differences in the loading of total RNA. For each probe, the amounts of transcripts were expressed relative to wild-type Ws grown on water for 5 days in the dark and 2 days in the light, which was given an arbitrary value of 100. Abbreviations are as given in the legend of Figure 3.5. Results are means \pm SEM for 3 independent experiments.

lower levels of nuclear photosynthesis gene transcripts, such as *cue* (*cab-underexpressed*) mutants, had been previously isolated (Li *et al.*, 1995; López-Juez *et al.*, 1998). The higher abundance of nuclear photosynthesis gene transcripts in PR48.2N seedlings in the presence of norflurazon or lincomycin appeared to be an effect of high transcript levels of the genes rather than altered plastid-to-nucleus signalling.

3.6 Greening ability of PR48.2N

As demonstrated in section 3.4, PR48.2N was a mutant with altered chlorophyll accumulation during the transition from dark- to light-growth. In order to examine chlorophyll accumulation in PR48.2N and the wild-type Ws after various lengths of dark treatment, a time-course experiment was conducted with PR48.2N and wild-type Ws seedlings illuminated for a 16-h diurnal photoperiod after being grown for 2-10 days in the dark. Seedlings of wild-type Ws and PR48.2N were grown and chlorophyll was extracted and measured as described in section 3.4. The seeds were germinated on 0.7 % agar with half-strength MS and grown for 2, 3, 4, 5, 6, 7, 8, 9 or 10 days in the dark followed by 1 day in the light (abbreviated as 2D1L, 3D1L, 4D1L, 5D1L, 6D1L, 7D1L, 8D1L, 9D1L and 10D1L). The seedling extracts were subjected to spectrophotometric measurements and absorption spectra from 600 to 700 nm were drawn. There were three replicates for each measurement to obtain the means and SEM (section 2.8).

PR48.2N seedlings were paler than the wild-type seedlings at 2D1L (Figure 3.7). However, at 6D1L or 10DL, PR48.2N seedlings were greener than the wild-type seedlings. Absorbance spectra of extracts from 2D1L seedlings showed that the absorption maximum for chlorophyll *a* at 664 nm was higher in wild-type Ws than PR48.2N (Figure 3.8). For extracts from 6D1L seedlings, the 664-nm absorbance maximum of PR48.2N was higher than the wild-type Ws. Absorbance spectra of extracts from the wild-type Ws seedlings at 10D1L showed hardly any absorbance at 664 nm, whereas the 664-nm absorbance maximum of the PR48.2N seedling extract was higher than the wild type. Total chlorophyll of wild-type Ws and PR48.2N seedlings decreased from 2D1L to 10D1L. PR48.2N seedlings accumulated less chlorophyll compared to the wild type at 4D1L and before, whereas at 5D1L and after PR48.2N seedlings accumulated more chlorophyll than the wild type (Figure 3.9). Total chlorophyll of wild-type Ws seedlings decreased from 2D1L (189.6 µg/g) to 10D1L

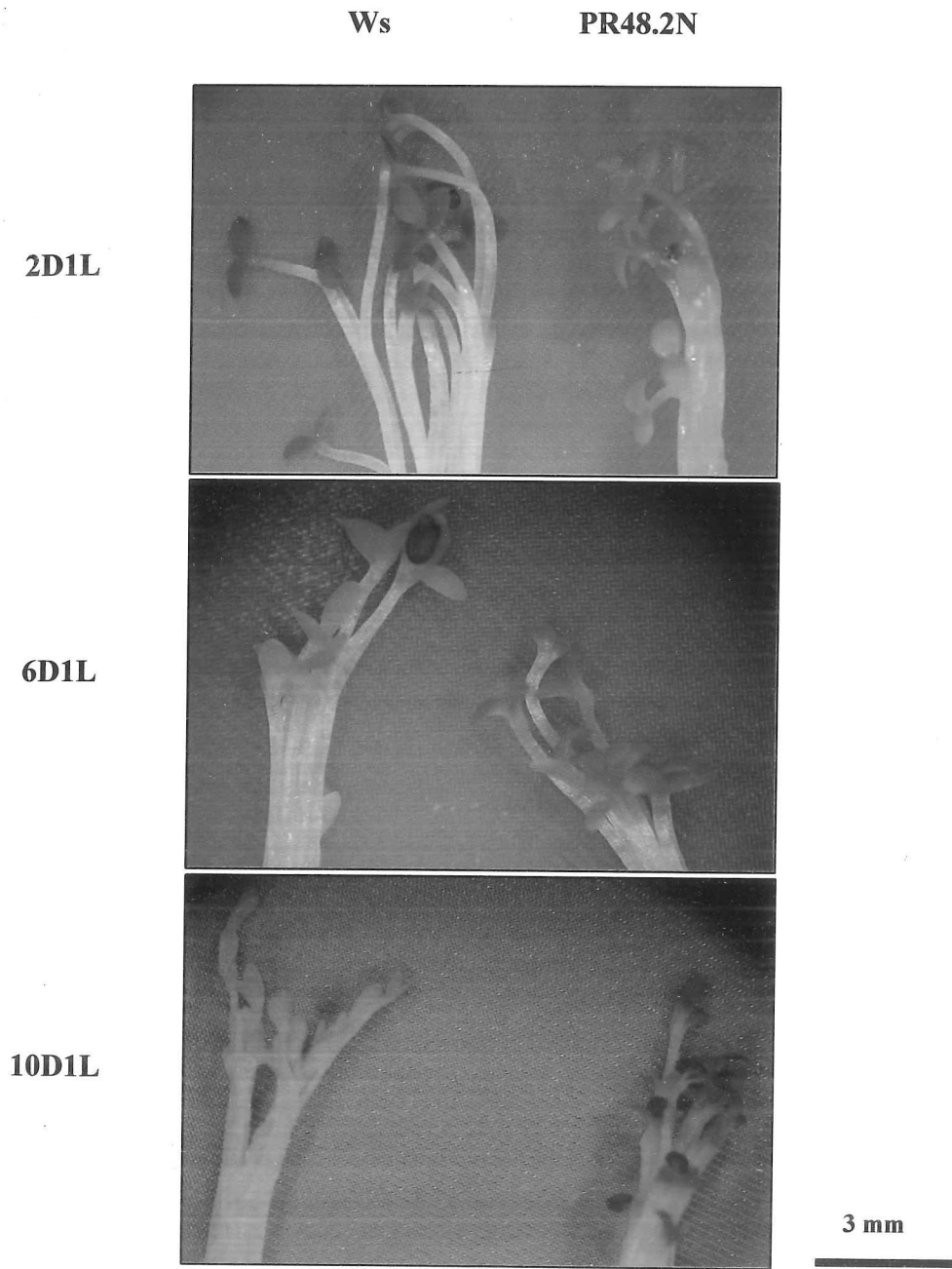


Figure 3.7 Wild-type Ws and PR48.2N seedlings after 2, 6 or 10 days in the dark followed by 1 day in the light.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D1L), 6 (6D1L) or 10 (10D1L) days in the dark followed by 1 day in the light after stratification and light treatment. Seedlings were viewed using a Leica MZFLIII dissecting microscope and images were taken using a Nikon Coolpix 950 digital camera attached to the dissecting microscope.

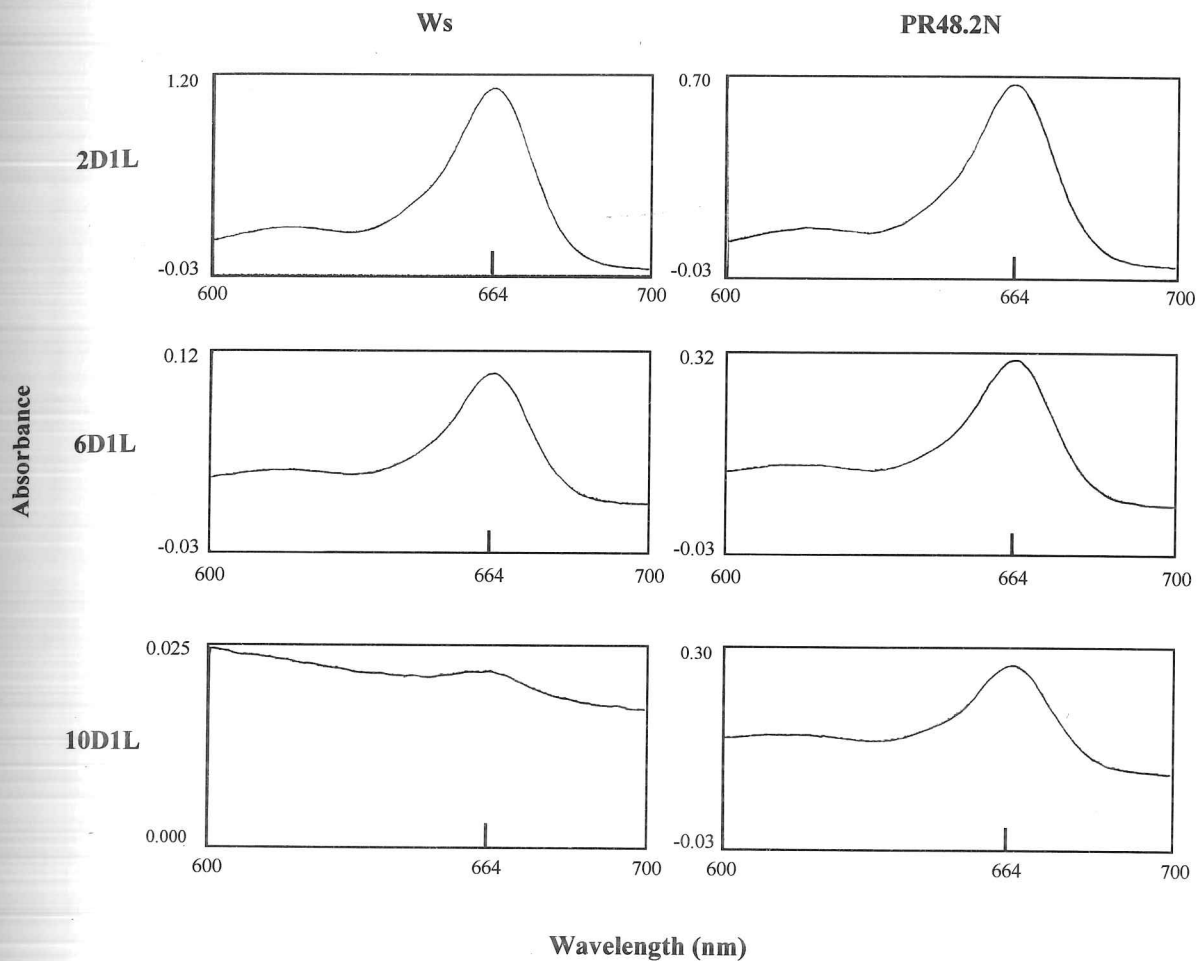


Figure 3.8 Absorbance spectra of seedlings extracts of wild-type Ws and PR48.2N seedlings after 2, 6 or 10 days in the dark followed by 1 day in the light.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D1L), 6 (6D1L) or 10 (10D1L) days in the dark followed by 1 day in the light. Chlorophylls were extracted from approximately 200 mg of fresh seedlings using *N,N*-dimethylformamide (DMF) and absorbance spectra of the extracts between wavelengths of 600 and 700 nm were drawn using a UV/VIS Spectrometer Lambda 9 (PerkinElmer). Absorbance maxima were at approximately 664 nm.

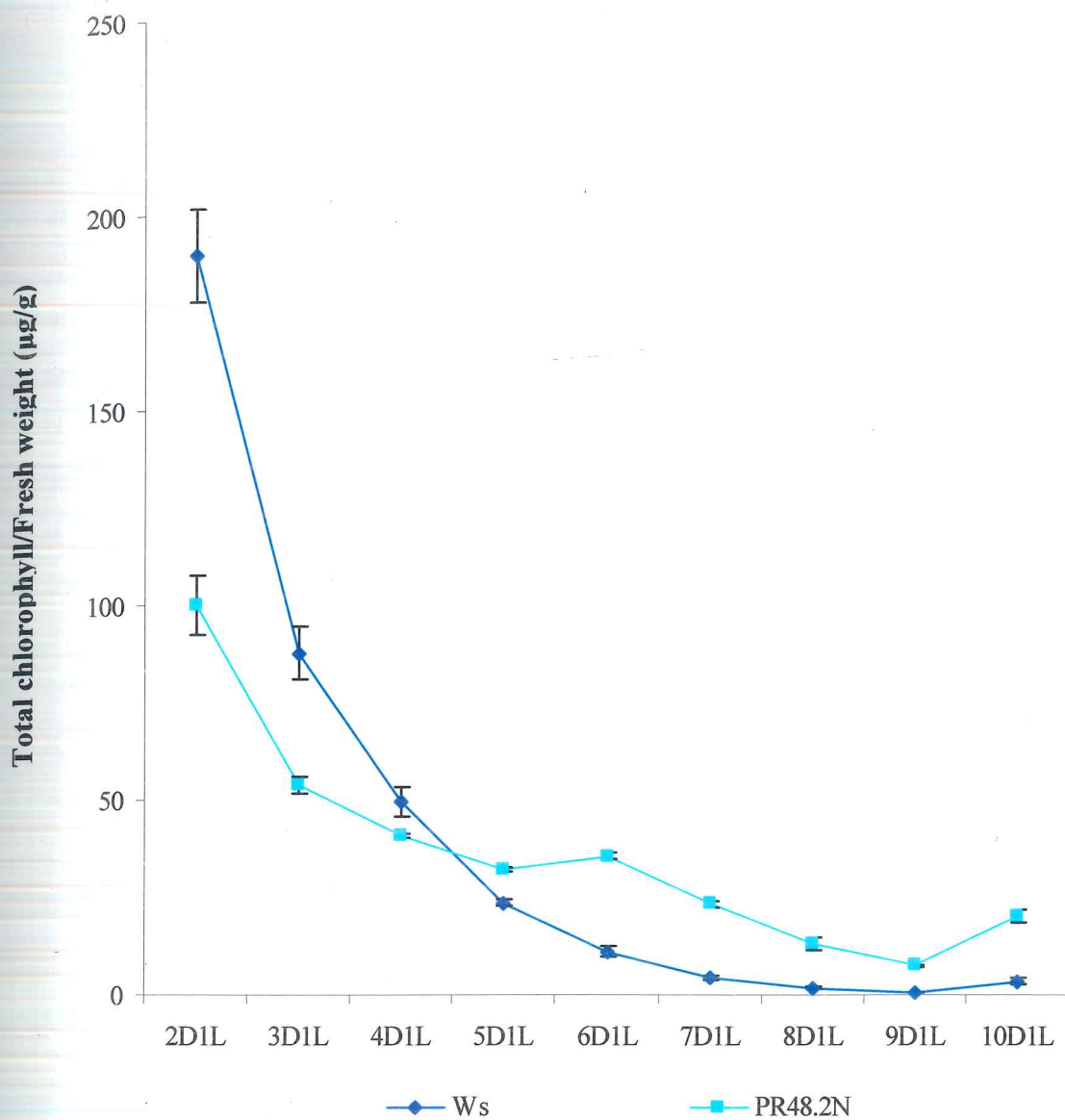


Figure 3.9 Chlorophyll content of wild-type Ws and PR48.2N seedlings after 2, 3, 4, 5, 6, 7, 8, 9 or 10 days in the dark followed by 1 day in the light.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D1L), 3 (3D1L), 4 (4D1L), 5 (5D1L), 6 (6D1L), 7 (7D1L), 8 (8D1L), 9 (9D1L) or 10 (10D1L) days in the dark followed by 1 day in the light. Chlorophylls were extracted using *N,N*-dimethylformamide (DMF) and measured with a UV/VIS Spectrometer Lambda 9 (PerkinElmer). Total chlorophyll was calculated using an equation described by Moran (1982) on a basis of seedling fresh weight. Results are means \pm SEM for 3 replicates.

(3.4 $\mu\text{g/g}$), whereas the total chlorophyll of PR48.2N seedlings decreased from 2D1L (99.9 $\mu\text{g/g}$) to 10D1L (20.1 $\mu\text{g/g}$).

PR48.2N appeared to be a mutant with a lowered greening ability after shorter periods of darkness, such as 2 to 4 days in the dark, but an enhanced greening ability after prolonged dark treatments, such as 5 to 10 days in the dark. This phenotype was not observed in *gun1-1*, as the proportion of *gun1-1* seedlings that turn green after a short period of darkness, such as 3 days in the dark, is similar to the proportion of wild-type Col seedlings that turn green after the same treatment, but the ability of *gun1-1* seedlings to green drops after longer periods of darkness (6 days or more) (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). Greening after a short period of darkness may be regarded as a normal plant process since most seeds have to experience some darkness, for instance in the soil, before reaching the light. However, greening after prolonged darkness may be considered a rare process for plants since this condition is not common in nature and the seedlings have to devote most of their limited stored energy reserves to hypocotyl extension (McNellis and Deng, 1995). The fact that PR48.2N seedlings showed different phenotypes after short and long periods of darkness suggests that greening processes after a short period of darkness and a prolonged one are differentially regulated.

3.7 Ultrastructure of plastids during the greening process

Since a PR48.2N had an altered pattern of chlorophyll accumulation following illumination of dark-grown seedlings, the ultrastructure of plastids of wild-type Ws and PR48.2N during the greening process was investigated using TEM. The entire process of fixation, sectioning, staining and viewing was performed at the Multi-Imaging Centre, Department of Anatomy, University of Cambridge, UK. Wild-type Ws and PR48.2N seedlings were grown for 2, 6 or 10 days in the dark after stratification and light treatment (abbreviated as 2D, 6D and 10D) and 2D1L, 6D1L or 10D1L as previously described (sections 3.4 and 3.6). Seedlings were fixed in glutaraldehyde and hydrogen peroxide, embedded in Spurr's epoxy resin, and stained with uranyl acetate and lead citrate (Peracchia and Mittler, 1972), before being viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV (section 2.9.4).

For 2D seedlings, prolamellar bodies were observed in the etioplasts present in the dark-grown cotyledons of both wild-type Ws and PR48.2N seedlings (Figures 3.10A and D). However, long primary thylakoids radiating out from the prolamellar bodies had formed in the etioplasts of wild-type Ws cotyledons at 2D and prolamellar bodies in 2D PR48.2N seedlings appeared to be smaller (Figure 3.10A). For 6D seedlings, both wild-type Ws and PR48.2N cotyledons contained similar etioplasts with typical prolamellar bodies at the centre (Figures 3.10B and E). For seedlings grown for 10D, etioplasts of PR48.2N cotyledons (Figure 3.10F) were similar to the wild-type etioplasts after 2D (Figure 3.10A), which had formed long primary thylakoids from the prolamellar bodies, but smaller. Etioplasts of wild-type Ws cotyledons at 10D were different from those in PR48.2N. The etioplasts contained some rudimentary membrane and lacked signs of differentiation (Figure 3.10C). They were similar to wild-type *Arabidopsis* etioplasts observed in plants dark-grown for 21 days (Rohde *et al.*, 2000).

For illuminated seedlings after 2D, normal chloroplasts with fully developed thylakoid membranes and starch grains were observed in wild-type Ws cotyledons (Figure 3.11A), whereas in PR48.2N cotyledons, chloroplasts with fewer thylakoid membranes and almost devoid of starch grains were observed (Figure 3.11D). This observation coincided with less chlorophyll accumulation of PR48.2N seedlings compared to the wild-type Ws seedlings after 2D1L, as described in sections 3.4 and 3.6. The wild-type plastids after 2D appeared to be more able to differentiate than the PR48.2N plastids, since the wild-type Ws etioplasts had formed primary thylakoids after 2D and differentiated into chloroplasts with more thylakoid membranes and starch grains when illuminated.

For illuminated seedlings after 6D, both wild-type Ws and PR48.2N chloroplasts in the cotyledons showed similar structures. The chloroplasts of both wild-type Ws and PR48.2N cotyledons at 6D1L contained fewer thylakoid membranes compared to the wild-type chloroplasts at 2D1L and were almost devoid of starch grains (Figures 3.11B and E). This corresponds to the much lower chlorophyll content of both wild-type Ws and PR48.2N seedlings after 6D1L compared to the wild-type Ws seedlings after 2D1L (sections 3.4 and 3.6). Although PR48.2N seedlings accumulated more chlorophyll at 6D1L compared to the wild-type Ws seedlings, etioplasts of wild-type Ws and PR48.2N cotyledons at 6D and chloroplasts at 6D1L were similar.

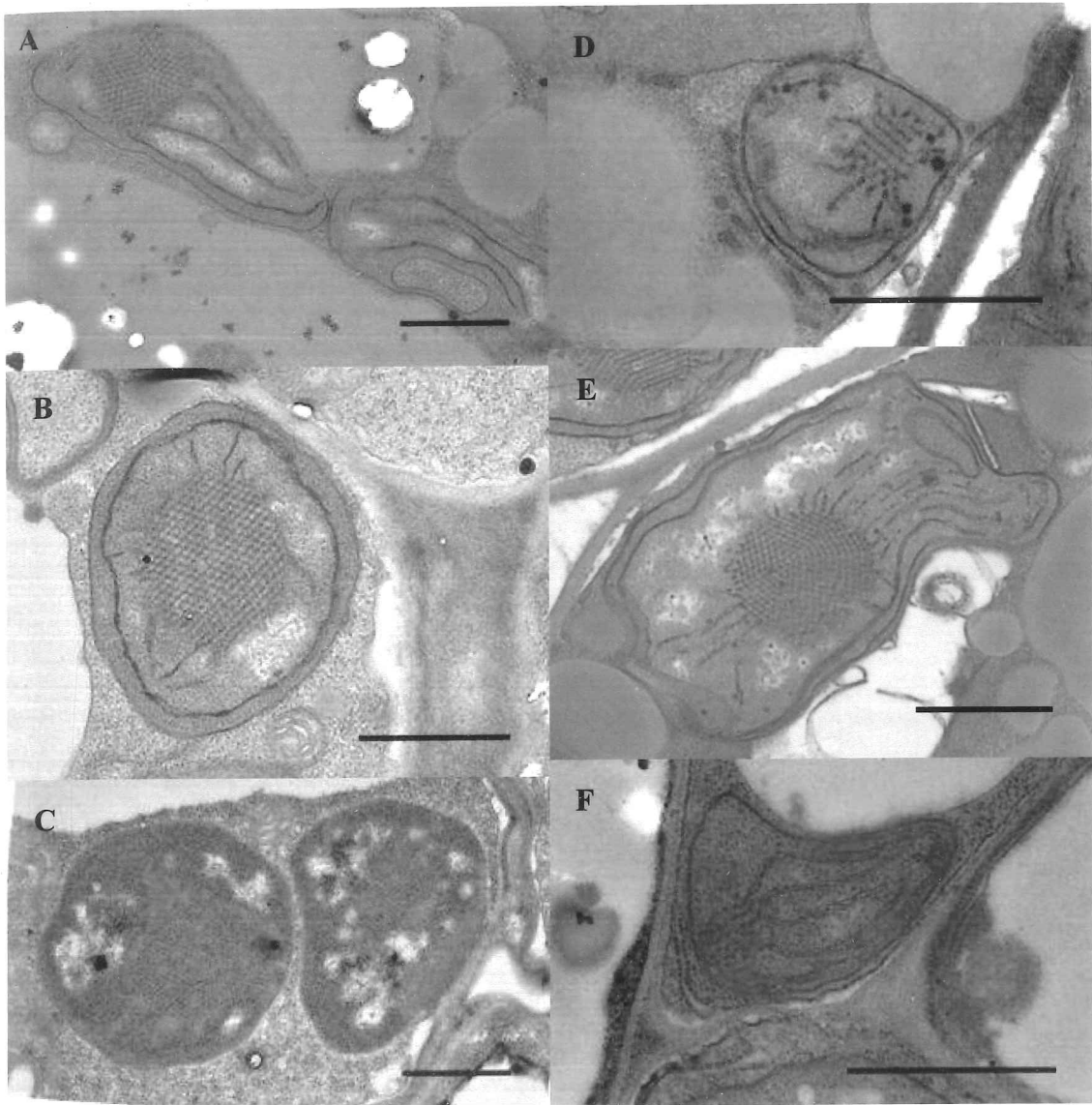


Figure 3.10 Transmission electron microscopy of plastids from cotyledons of wild-type Ws and PR48.2N seedlings after 2, 6 or 10 days in the dark.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D), 6 (6D) or 10 (10D) days in the dark. Seedlings were fixed in glutaraldehyde and hydrogen peroxide, embedded in Spurr's epoxy resin, and stained with uranyl acetate and lead citrate before being viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV. Bars = 1 μ m.

(A), (B) and (C) Plastids from cotyledons of wild-type Ws seedlings grown for (A) 2D, (B) 6D and (C) 10D.

(D), (E) and (F) Plastids from cotyledons of PR48.2N seedlings grown for (D) 2D, (E) 6D and (F) 10D.

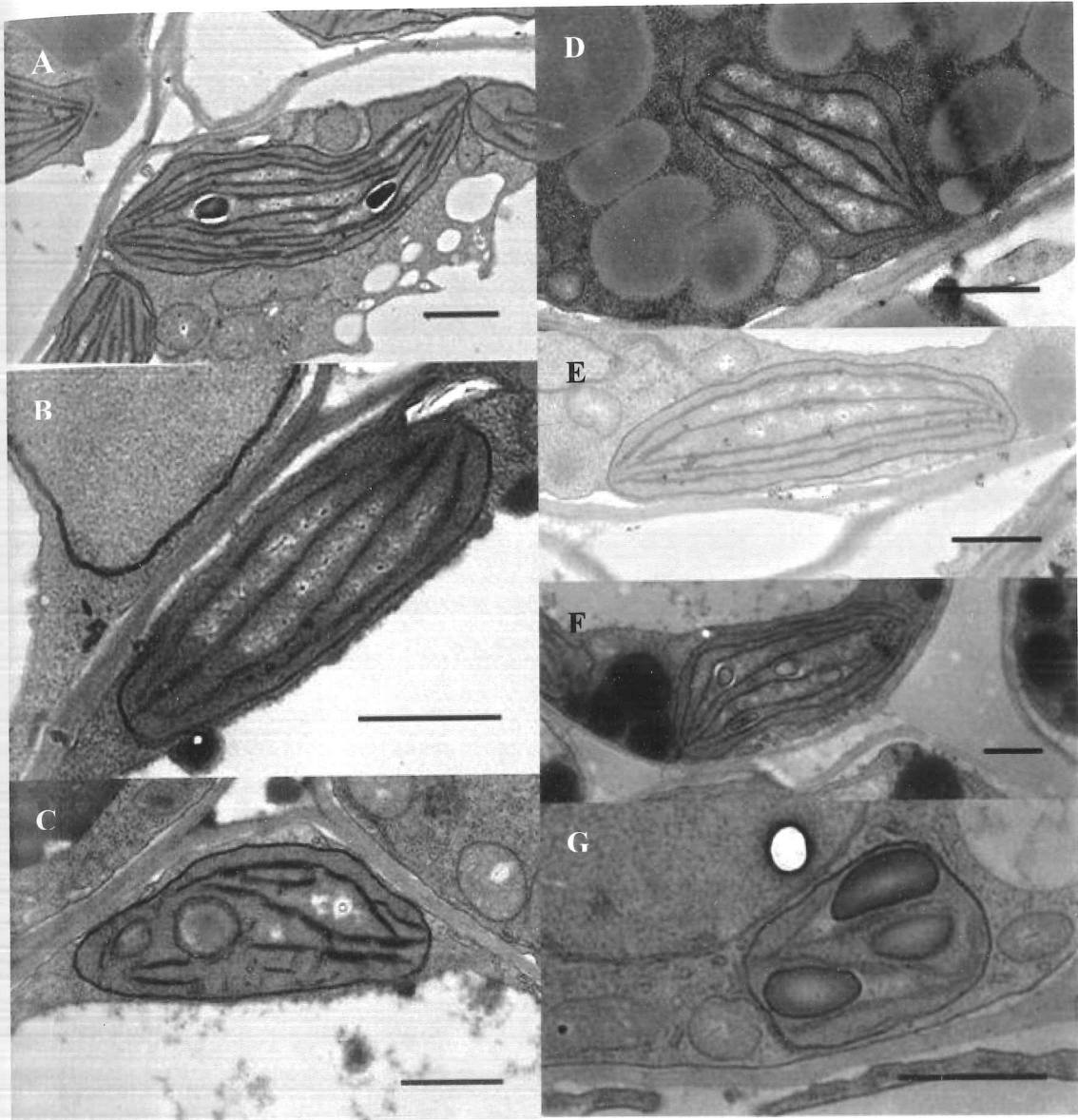


Figure 3.11 Transmission electron microscopy of plastids from cotyledons of wild-type Ws and PR48.2N seedlings after 2, 6 or 10 days in the dark followed by 1 day in the light.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D1L), 6 (6D1L) or 10 (10D1L) days in the dark followed by 1 day in the light. Seedlings were fixed in glutaraldehyde and hydrogen peroxide, embedded in Spurr's epoxy resin, and stained with uranyl acetate and lead citrate before being viewed in a FEI Philips CM100 transmission electron microscope operated at 80 kV. Bars = 1 μ m.

(A), (B) and (C) Plastids from cotyledons of wild-type Ws seedlings grown for (A) 2D1L, (B) 6D1L and (C) 10D1L.

(D), (E), (F) and (G) Plastids from cotyledons of PR48.2N seedlings grown for (D) 2D1L, (E) 6D1L and (F and G) 10D1L.

For illuminated seedlings after 10D, chloroplasts with thylakoid membranes and starch grains were observed in PR48.2N cotyledons but not in wild-type Ws seedlings (Figures 3.11C and F). However, the number of thylakoid membranes was lower than that found in wild-type chloroplasts after 2D1L. Interestingly, PR48.2N chloroplasts were almost devoid of starch grains at 2D1L and 6D1L, but starch grains were observed at 10D1L. An exceptional plastid that resembled an amyloplast was observed in a PR48.2N cotyledon after 10D1L (Figure 3.11G). In wild-type Ws chloroplasts at 10D1L, fewer thylakoid membranes were formed and the whole internal structure was not well integrated (Figure 3.11C). This agrees with the findings in sections 3.4 and 3.6 that PR48.2N seedlings accumulated more total chlorophyll than the wild-type Ws seedlings after 10D1L. The etioplasts of wild-type Ws cotyledons after 10D may be in an arrested state after being too long in the dark because they did not differentiate into proper chloroplasts after 10D1L and wild-type seedlings did not green properly after 10D1L.

In conclusion, the mutation in PR48.2N affected the ultrastructure of etioplasts in dark-grown seedlings and of chloroplasts during the process of greening.

3.8 Genome-wide comparison of transcript abundance between wild-type Ws and PR48.2N

From the analyses of transcript abundance of nuclear photosynthesis genes and of chlorophyll accumulation after various periods of darkness, PR48.2N was shown to be a different mutant from *gun1-1*. One of the differences between PR48.2N and *gun1-1*, namely increased transcript abundance of nuclear photosynthesis genes in the absence of treatments affecting plastid function, was considered worth pursuing further, as discussed in section 3.5. However, RNA-gel-blot analysis is rather limiting in the number of genes that can be examined in parallel. Microarray analysis, which is able to make genome-wide comparisons of transcript abundance between samples, was conducted on PR48.2N and wild-type Ws seedlings in the absence of inhibitors.

Microarray analysis was conducted using Affymetrix ATH-121501 arrays that carry approximately 22750 probe sets representing approximately 23750 genes, a large majority of the genome of *Arabidopsis thaliana* (Redman *et al.*, 2004). Total RNA was

extracted from wild-type Ws and PR48.2N seedlings grown for 5 days in the dark followed by 2 days in the light (untreated light-grown seedlings [LW] in sections 3.3 and 3.5), using Concert Plant RNA Reagent as previously described (section 3.5). The total RNA was processed with an additional cleaning-up procedure using a MicroCon YM-30 column to avoid the contamination of any inhibitors for the later steps (section 2.6.10.1) and shipped to the MRC GeneService at Hinxton Hall, Cambridge, UK where biotin-labelled cRNA was prepared according to standard operating procedures and hybridised to the surface of an Affymetrix ATH-121501 array. Three independent extractions and hybridisations were carried out for each line. After hybridisation, arrays were washed and scanned, and normalised data were returned. The data were then loaded into GeneSpring software and the replicate values were averaged. Data analysis was then performed using GeneSpring and the TAIR website (section 2.6.10.2).

Affymetrix values representing the transcript abundance of genes between the lines tested were compared. Differences in transcript levels are likely to be inaccurately estimated by microarrays when the transcript abundance of genes being compared is at low levels (Holland, 2002; Czechowski *et al.*, 2004). Therefore it has been proposed that genes with low transcript abundance are excluded from comparison in microarray analyses (Finkelstein *et al.*, 2002; Simon *et al.*, 2003). In this study, no comparison was made between transcript abundance values lower than 10. When a cut-off of 2-fold change in the transcript abundance values between PR48.2N and wild-type Ws was used to define differential transcript levels of genes in PR48.2N seedlings relative to wild-type Ws seedlings in this microarray study, 533 genes showed higher transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings, whereas 2024 genes showed lower transcript abundance. When a cut-off of 3-fold change was used, more manageable gene lists with fewer genes were produced (Schena *et al.*, 1995, 1996).

Figure 3.12 shows the average, normalised Affymetrix values of transcript abundance of genes represented on the ATH-121501 array of PR48.2N from three independent hybridisations plotted on a log scale against wild-type Ws. After the removal of genes showing less than 3-fold change and comparisons between abundance values lower than 10, 116 genes showed higher transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings. This was about 0.5 % of the genes

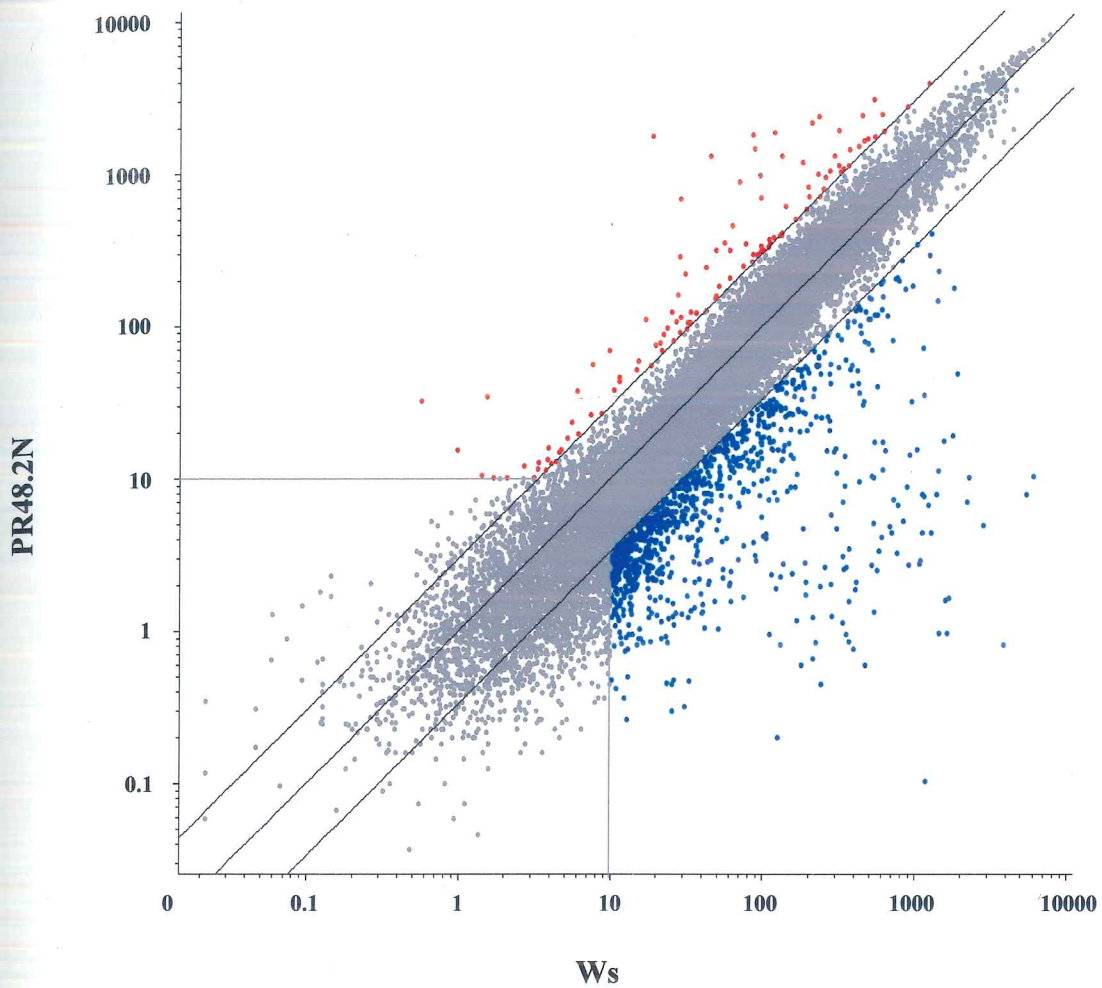


Figure 3.12 Genome-wide comparison of transcript levels between wild-type Ws and PR48.2N seedlings using Affymetrix microarray analysis.

Labelled-complementary RNA (cRNA), prepared from total RNA extracted from wild-type Ws and PR48.2N seedlings grown on 0.7 % agar with half-strength MS for 5 days in the dark followed by 2 days in the light, was hybridised to Affymetrix ATH-121501 microarrays. The figure shows the average normalised transcript abundance values of PR48.2N from three independent hybridisations plotted on a log scale against wild-type Ws. The regression line (middle, diagonal grey line) and the 3-fold lines (diagonal grey lines above and below the regression line) are shown. A cut-off of 3-fold change in abundance values was used to define differential transcript abundance. No comparison was made between abundance values lower than 10 (below and before the horizontal and vertical grey lines). Genes with higher abundance values in PR48.2N were coloured red (●) and those with lower abundance values were coloured blue (●).

represented on the Affymetrix chip. The 50 most affected genes are listed in Table 3.1. A germination protein-related gene (At3g29970) with no clear function in *Arabidopsis*, but similar to a barley gene encoding HvB12D present in the aleurone layer and embryos of developing and germinating seed (Aalen *et al.*, 1994), was top of the list showing a 94.5-fold increase in transcript abundance in PR48.2N seedlings compared to wild-type Ws. The genes that showed higher transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings were subjected to Gene Ontology (GO) categorisations of the cellular location, biological process and molecular function of the expressed proteins using the TAIR GO Annotations tool. The results showed that the functions and localisations of the products of these genes were largely unknown, although a small portion of them were predicted to be targeted to organelles such as chloroplasts (15.1%) and mitochondria (11.9%) (Figure 3.13). That the functions and Gene Ontologies of many of the genes with higher transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings had not been identified indicates that the gene products might represent some uncharacterised processes or unidentified components of known processes.

Conversely, 975 genes showed lower transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings (Figure 3.12). This was about 4.1 % of the genes represented on the Affymetrix chip. The 50 most affected genes are listed in Table 3.2. They were mainly genes involved in late embryogenesis and seed maturation, including genes encoding seed storage proteins, LEA proteins and oleosins (for review of transcripts present in immature and dry seeds, Delseny *et al.*, 2001). Two genes encoding seed storage proteins, namely 2S seed storage protein 2 (At4g27150) encoding a 2S albumin and cruciferin A, *CR1* (At5g44120) encoding a 12S globulin, were top of the list showing approximately 11000- and 4700-fold lower transcript abundance in PR48.2N seedlings compared to wild-type Ws, respectively. There were three more genes encoding seed storage proteins, namely cruciferin 3, *CR3* (At4g28520) encoding a 12S globulin, 2S seed storage protein 3 (At4g27160) and 2S seed storage protein 1 (At4g27140) encoding 2S albumins, on the list. Two genes encoding LEA proteins showed lower transcript abundance in PR48.2N seedlings: one similar to *Brassica napus* LEA76 (At3g15670) showed approximately 1700-fold lower transcript abundance in PR48.2N seedlings compared to wild-type Ws, whereas the other one, *Arabidopsis* LEA M17 (At2g41260) showed approximately 1500-fold lower transcript

Table 3.1 List of 50 *Arabidopsis* genes with the highest ratios of transcript levels in PR48.2N seedlings relative to wild-type Ws seedlings.

Labelled-complementary RNA (cRNA), prepared from total RNA extracted from wild-type Ws and PR48.2N seedlings grown on 0.7 % agar with half-strength MS for 5 days in the dark followed by 2 days in the light, was hybridised to Affymetrix ATH-121501 microarrays. The table shows 50 *Arabidopsis* genes with the highest ratios of transcript levels in PR48.2N seedlings relative to wild-type Ws seedlings. Transcript abundance values were average, normalised Affymetrix values from three independent hybridisations. Ratios are given as the ratios of transcript abundance values between PR48.2N and wild-type Ws seedlings.

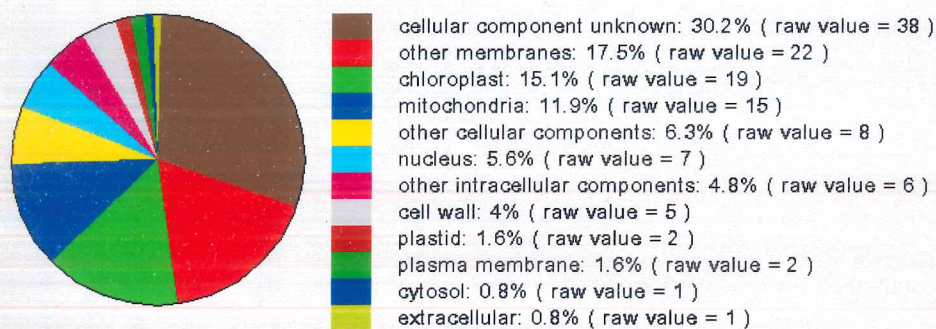
AGI code	Gene title (Affymetrix)	Abundance value (Ws)	Abundance value (PR48.2N)	Ratio (PR48.2N/Ws)
At3g29970	germination protein-related	18.7	1764.8	94.5
At2g02730	expressed protein	0.6	32.5	57.5
At2g17850	senescence-associated family protein	45.0	1282.8	28.5
At1g76650	calcium-binding EF hand family protein	28.5	673.2	23.6
At3g21860	E3 ubiquitin ligase SCF complex subunit SKP1/ASK1 (At10), putative	1.5	34.6	22.6
At1g77120	alcohol dehydrogenase (ADH)	85.0	1771.5	20.8
At3g02550	LOB domain protein 41/lateral organ boundaries domain protein 41 (LBD41)	87.2	1449.5	16.6
At2g15020	expressed protein	1.0	15.4	15.9
At4g39675	expressed protein	117.3	1835.8	15.6
At1g43800	acyl-(acyl-carrier-protein) desaturase, putative/stearoyl-ACP desaturase, putative	69.6	882.3	12.7
At1g33055	expressed protein	207.9	2141.1	10.3
At4g10270	wound-responsive family protein	95.0	962.9	10.1
At3g03270	universal stress protein (USP) family protein/early nodulin ENOD18 family protein	231.2	2325.2	10.1
At4g33560	expressed protein	28.3	282.6	10.0
At1g35140	phosphate-responsive protein, putative	132.4	1292.0	9.8
At2g14210	MADS-box protein (ANR1)	7.5	56.4	7.5
At4g36450	mitogen-activated protein kinase, putative/MAPK, putative (MPK14)	1.4	10.5	7.5
At4g30140	GDSL-motif lipase/hydrolase family protein	62.3	450.9	7.2
At5g10040	expressed protein	9.7	69.3	7.2
At5g39890	expressed protein	96.0	684.4	7.1
At3g27220	kelch repeat-containing protein	30.8	218.2	7.1
At3g10040	expressed protein	16.8	109.7	6.5
At5g66985	expressed protein	48.6	313.6	6.5
At5g15120	expressed protein	181.4	1167.7	6.4
At5g42200	zinc finger (C3HC4-type RING finger) family protein	54.7	348.3	6.4
At2g25780	hypothetical protein	6.0	37.2	6.2
At2g16060	non-symbiotic haemoglobin 1 (HB1) (GLB1)	310.2	1914.7	6.2

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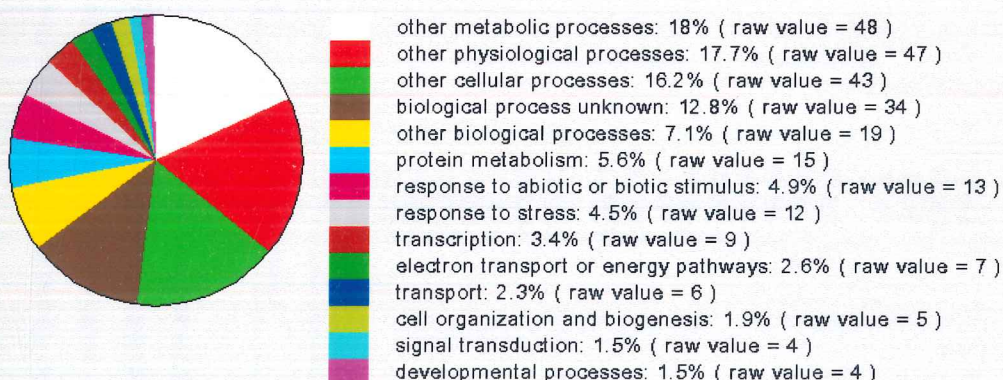
At2g18260	syntaxin-related protein, putative (SYP112)	1.7	10.1	6.0
At5g23350	GRAM domain-containing protein/ABA-responsive protein-related GRAM	27.3	158.7	5.8
(At5g23360*)	domain-containing protein/ABA-responsive protein-related			
At4g33070	pyruvate decarboxylase, putative	41.8	241.7	5.8
At5g57560	xyloglucan:xyloglucosyl transferase/xyloglucan endotransglycosylase/endo-xyloglucan transferase (TCH4)	531.1	3039.4	5.7
At4g08950	phosphate-responsive protein, putative (EXO)	440.8	2375.0	5.4
At2g41850	endo-polygalacturonase, putative	59.6	312.1	5.2
At5g10530	lectin protein kinase, putative	2.0	10.1	5.0
At1g69570	Dof-type zinc finger domain-containing protein	24.9	122.4	4.9
At5g20830	sucrose synthase/sucrose-UDP glucosyltransferase (SUS1)	76.5	347.1	4.5
At5g39820	no apical meristem (NAM) family protein	2.7	12.0	4.5
At1g72430	auxin-responsive protein-related	290.0	1296.7	4.5
At5g60850	Dof-type zinc finger domain-containing protein	140.0	606.7	4.3
At5g41080	glycerophosphoryl diester phosphodiesterase family protein	228.3	979.1	4.3
At4g26260	expressed protein	5.5	23.4	4.3
At1g69190	dihydropterin pyrophosphokinase, putative/dihydropteroate synthase, putative/DHPS, putative	3.8	15.9	4.2
At5g10210	expressed protein	23.2	96.4	4.1
At1g29090	peptidase C1A papain family protein	196.5	805.4	4.1
At4g10260	pfkB-type carbohydrate kinase family protein	11.3	46.4	4.1
At1g48100	glycoside hydrolase family 28 protein/polygalacturonase (pectinase) family protein	26.6	107.3	4.0
At3g47340	asparagine synthetase 1 (glutamine-hydrolyzing)/glutamine-dependent asparagine synthetase 1 (ASN1)	601.9	2428.3	4.0
At3g14280	expressed protein	28.4	114.2	4.0
At3g43190	sucrose synthase, putative/sucrose-UDP glucosyltransferase, putative	21.9	87.9	4.0
At1g03445	kelch repeat-containing protein/ serine/threonine phosphoesterase family protein	15.1	59.0	3.9

* Cross hybridisation.

Functional Categorization by Genes for : GO Cellular Component



Functional Categorization by Genes for : GO Biological Process



Functional Categorization by Genes for : GO Molecular Function

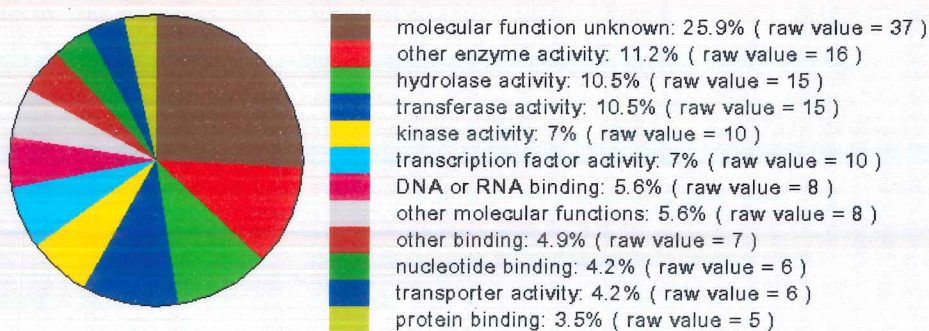


Figure 3.13 Gene Ontology (GO) categorisations of function and location of products of *Arabidopsis* nuclear genes with transcript levels 3-fold higher in PR48.2N seedlings relative to wild-type Ws.

Products of *Arabidopsis* nuclear genes with transcript levels 3-fold higher in PR48.2N seedlings relative to wild-type Ws seedlings were categorised into their cellular locations, biological processes and molecular functions using The Arabidopsis Information Resource (TAIR) Gene Ontology (GO) Annotations tool (<http://www.arabidopsis.org/>). Raw value = number of gene(s) in the category.

Table 3.2 List of 50 *Arabidopsis* genes with the lowest ratios of transcript levels in PR48.2N seedlings relative to wild-type Ws seedlings.

Labelled-complementary RNA (cRNA), prepared from total RNA extracted from wild-type Ws and PR48.2N seedlings grown on 0.7 % agar with half-strength MS for 5 days in the dark followed by 2 days in the light, was hybridised to Affymetrix ATH-121501 microarrays. The table shows the 50 *Arabidopsis* genes with the lowest ratios of transcript levels in PR48.2N seedlings relative to wild-type Ws seedlings. Transcript abundance values were average, normalised Affymetrix values from three independent hybridisations. Ratios are given as the ratios of transcript abundance values between PR48.2N and wild-type Ws seedlings.

AGI code	Gene title (Affymetrix)	Abundance value (Ws)	Abundance value (PR48.2N)	Ratio (PR48.2N/Ws)
At4g27150	2S seed storage protein 2/2S albumin storage protein/NWMU2-2S albumin 2	1168.5	0.1	8.83×10^{-5}
At5g44120	12S seed storage protein (CRA1)	3831.9	0.8	2.13×10^{-4}
At3g15670	late embryogenesis abundant protein, putative/LEA protein, putative	1625.0	1.0	5.96×10^{-4}
At2g41260	glycine-rich protein/late embryogenesis abundant protein (M17)	1439.0	1.0	6.75×10^{-4}
At5g40420	glycine-rich protein/oleosin	1656.8	1.6	9.93×10^{-4}
At1g48130	peroxiredoxin (PER1)/rehydrin, putative	1587.1	1.6	1.01×10^{-3}
At3g51810	Em-like protein GEA1 (EM1)	470.0	0.6	1.28×10^{-3}
At4g28520	12S seed storage protein, putative/cruciferin, putative	5372.8	7.9	1.47×10^{-3}
At4g21020	late embryogenesis abundant domain-containing protein/LEA domain-containing protein	123.9	0.2	1.61×10^{-3}
At5g54740	protease inhibitor/seed storage/lipid transfer protein (LTP) family protein	6004.7	10.3	1.72×10^{-3}
At1g75830	plant defensin-fusion protein, putative (PDF1.1)	2813.8	4.9	1.76×10^{-3}
At4g09610	gibberellin-regulated protein 2 (GASA2)/gibberellin-responsive protein 2	241.3	0.4	1.86×10^{-3}
At3g12960	expressed protein	393.0	0.8	1.92×10^{-3}
At4g26740	embryo-specific protein 1 (ATS1)	365.2	0.9	2.35×10^{-3}
At4g27160	2S seed storage protein 3/2S albumin storage protein/NWMU2-2S albumin 3	537.8	1.3	2.50×10^{-3}
At3g27660	glycine-rich protein/oleosin	1069.9	2.8	2.58×10^{-3}
At3g22500	late embryogenesis abundant protein (ECP31)/LEA protein	354.2	1.0	2.69×10^{-3}
At2g35300	late embryogenesis abundant group 1 domain-containing protein/LEA group 1 domain-containing protein	1075.5	2.9	2.71×10^{-3}
At2g21490	dehydrin family protein	897.9	2.7	3.01×10^{-3}
At1g27470	transducin-related/WD-40 repeat protein-related	211.2	0.7	3.10×10^{-3}
At3g22640	cupin family protein	2203.3	7.0	3.19×10^{-3}
At5g44310	late embryogenesis abundant domain-containing protein/LEA domain-containing protein	178.1	0.6	3.37×10^{-3}
At3g19920	expressed protein	331.0	1.1	3.44×10^{-3}

Continued

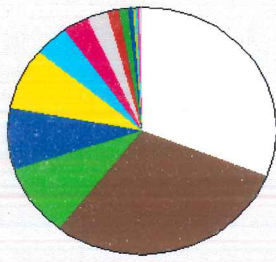
At4g25140	glycine-rich protein/oleosin	1262.5	4.4	3.48×10^{-3}
At2g23240	plant EC metallothionein-like family 15 protein	221.2	0.8	3.81×10^{-3}
At3g54940	cysteine proteinase, putative	1105.2	4.4	3.96×10^{-3}
At1g32560	late embryogenesis abundant group 1 domain-containing protein/LEA group 1 domain-containing protein	759.8	3.2	4.26×10^{-3}
At3g58450	universal stress protein (USP) family protein	364.4	1.6	4.27×10^{-3}
At2g36640	late embryogenesis abundant protein (ECP63)/LEA protein	283.5	1.3	4.44×10^{-3}
At4g16160	mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein	562.4	2.5	4.47×10^{-3}
At1g54870	short-chain dehydrogenase/reductase (SDR) family protein	2248.2	10.2	4.55×10^{-3}
At1g54860	expressed protein	405.8	1.9	4.59×10^{-3}
At4g09600	gibberellin-regulated protein 3 (GASA3)/gibberellin-responsive protein 3	411.3	2.1	5.13×10^{-3}
At2g40170	Em-like protein GEA6 (EM6)	517.8	2.7	5.26×10^{-3}
At1g73190	tonoplast intrinsic protein, alpha/alpha-TIP (TIP3.1)	1226.3	6.7	5.46×10^{-3}
At2g41280	late embryogenesis abundant protein (M10)/LEA protein M10	213.3	1.2	5.77×10^{-3}
At5g50600	short-chain dehydrogenase/reductase (SDR) family protein/reductase (SDR) family protein short-chain dehydrogenase	1368.3	7.9	5.77×10^{-3}
At3g53040	late embryogenesis abundant protein, putative/LEA protein, putative	574.9	3.4	5.85×10^{-3}
At3g22490	late embryogenesis abundant protein, putative/LEA protein, putative	129.7	0.8	6.28×10^{-3}
At1g05510	expressed protein	829.3	5.2	6.30×10^{-3}
At3g01570	glycine-rich protein/oleosin	692.8	4.4	6.34×10^{-3}
At2g28490	cupin family protein	1141.4	7.6	6.68×10^{-3}
At4g27140	2S seed storage protein 1/2S albumin storage protein/NWMU1-2S albumin 1	599.9	4.1	6.83×10^{-3}
At4g10020	short-chain dehydrogenase/reductase (SDR) family protein	167.1	1.2	6.98×10^{-3}
At3g21370	glycosyl hydrolase family 1 protein	679.9	4.8	7.07×10^{-3}
At5g66780	expressed protein	1103.3	7.8	7.08×10^{-3}
At2g25890	glycine-rich protein/oleosin	277.4	2.0	7.28×10^{-3}
At2g02120	plant defensin-fusion protein, putative (PDF2.1)	352.2	2.6	7.46×10^{-3}
At3g21380	jacalin lectin family protein	336.7	2.5	7.55×10^{-3}
At3g02480	ABA-responsive protein-related	1424.1	11.1	7.80×10^{-3}

abundance. There were 11 more LEA proteins, putative LEA proteins and LEA domain-containing proteins in the list. A gene (At5g40420) encoding an oleosin protein also showed 1000-fold lower transcript abundance in PR48.2N seedlings compared to wild-type Ws. There were four more genes encoding oleosins in the list. GO categorisations showed that the functions and localisations of the products of the majority of the genes showing low transcripts level in PR48.2N seedlings were not known as well (Figure 3.14). For those genes successfully categorised into known cellular locations, biological processes or molecular functions, no over-represented Gene Ontology was observed.

3.9 Transcript abundance of nuclear photosynthesis and seed protein genes

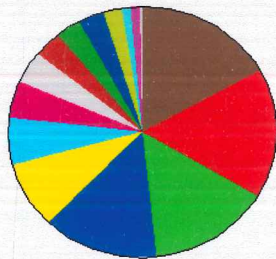
PR48.2N was initially shown to be a mutant with higher transcript abundance of nuclear photosynthesis genes, *RBCS* and *LHCBI*, by RNA-gel-blot analysis (section 3.5), whereas microarray analysis detected a lower transcript abundance of genes encoding some seed proteins (section 3.8). To compare the transcript abundance of nuclear photosynthesis and seed protein genes in the microarray analysis, the average, normalised abundance values of members of these two groups of genes were plotted on a log scale against the wild-type Ws (Figure 3.15). For nuclear photosynthesis genes, a gene probe representing four genes encoding RBCS (At1g67090, At5g38410, At5g38420 and At5g38430) and 16 gene probes representing 21 genes encoding LHC proteins (At1g29910, At1g29920, At1g29930, At1g15820, At1g19150, At1g45474, At1g61520, At2g05070, At2g05100, At2g34420, At2g34430, At2g40100, At3g08940, At3g27690, At3g47470, At3g54890, At3g61470, At4g10340, At5g01530, At5g28450 and At5g54270) were included in the scatter plot, whereas for seed protein genes, eight gene probes representing genes encoding eight seed storage proteins consisting of both 12S globulins and 2S albumins (At1g03880, At4g27140, At4g150, At4g27160, At4g27170, At4g28520, At5g44120 and At5g54740), 13 gene probes representing genes encoding 13 confirmed and putative LEA proteins (At1g01470, At1g52690, At2g36640, At2g40170, At2g41260, At2g41280, At2g46140, At3g15670, At3g22490, At3g22500, At3g50790, At3g51810 and At3g53040), and eight gene probes representing genes encoding eight oleosin proteins (At2g25890, At3g01570, At3g18570, At3g27660, At4g25140, At5g40420, At5g51210 and At5g56100) were included.

Functional Categorization by Genes for : GO Cellular Component



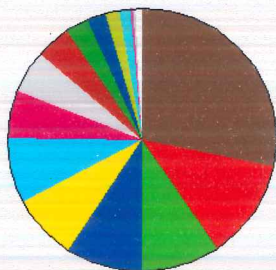
other membranes:	31% (raw value = 298)
cellular component unknown:	29.4% (raw value = 283)
mitochondria:	9.6% (raw value = 92)
nucleus:	8% (raw value = 77)
chloroplast:	8% (raw value = 77)
other cellular components:	4.2% (raw value = 40)
other intracellular components:	3% (raw value = 29)
other cytoplasmic components:	2.6% (raw value = 25)
cell wall:	1.4% (raw value = 13)
extracellular:	1% (raw value = 10)
cytosol:	0.6% (raw value = 6)
plasma membrane:	0.4% (raw value = 4)
ER:	0.2% (raw value = 2)
plastid:	0.2% (raw value = 2)
ribosome:	0.2% (raw value = 2)
Golgi apparatus:	0.1% (raw value = 1)

Functional Categorization by Genes for : GO Biological Process



biological process unknown:	16.9% (raw value = 312)
other metabolic processes:	16.7% (raw value = 309)
other physiological processes:	14.7% (raw value = 272)
other cellular processes:	13.9% (raw value = 257)
other biological processes:	8.5% (raw value = 158)
response to abiotic or biotic stimulus:	6.1% (raw value = 113)
transcription:	4.6% (raw value = 85)
protein metabolism:	4.1% (raw value = 75)
developmental processes:	3.5% (raw value = 64)
transport:	3.2% (raw value = 59)
response to stress:	3% (raw value = 56)
electron transport or energy pathways:	2.3% (raw value = 42)
cell organization and biogenesis:	1.2% (raw value = 22)
signal transduction:	1% (raw value = 18)
DNA or RNA metabolism:	0.4% (raw value = 7)

Functional Categorization by Genes for : GO Molecular Function



molecular function unknown:	28.2% (raw value = 298)
other enzyme activity:	12.3% (raw value = 130)
hydrolase activity:	9.6% (raw value = 101)
other binding:	9.3% (raw value = 98)
transferase activity:	8.2% (raw value = 86)
transcription factor activity:	8.2% (raw value = 86)
other molecular functions:	5.9% (raw value = 62)
DNA or RNA binding:	5.4% (raw value = 57)
transporter activity:	4.3% (raw value = 45)
protein binding:	2.8% (raw value = 30)
kinase activity:	2.2% (raw value = 23)
nucleotide binding:	1.7% (raw value = 18)
nucleic acid binding:	1.4% (raw value = 15)
receptor binding or activity:	0.3% (raw value = 3)
structural molecule activity:	0.3% (raw value = 3)

Figure 3.14 Gene Ontology (GO) categorisations of function and location of products of *Arabidopsis* nuclear genes with transcript levels 3-fold lower in PR48.2N seedlings relative to wild-type Ws.

Products of *Arabidopsis* nuclear genes with transcript levels 3-fold lower in PR48.2N seedlings relative to wild-type Ws seedlings were categorised into their cellular locations, biological processes and molecular functions using The Arabidopsis Information Resource (TAIR) Gene Ontology (GO) Annotations tool (<http://www.arabidopsis.org/>). Raw value = number of gene(s) in the category.

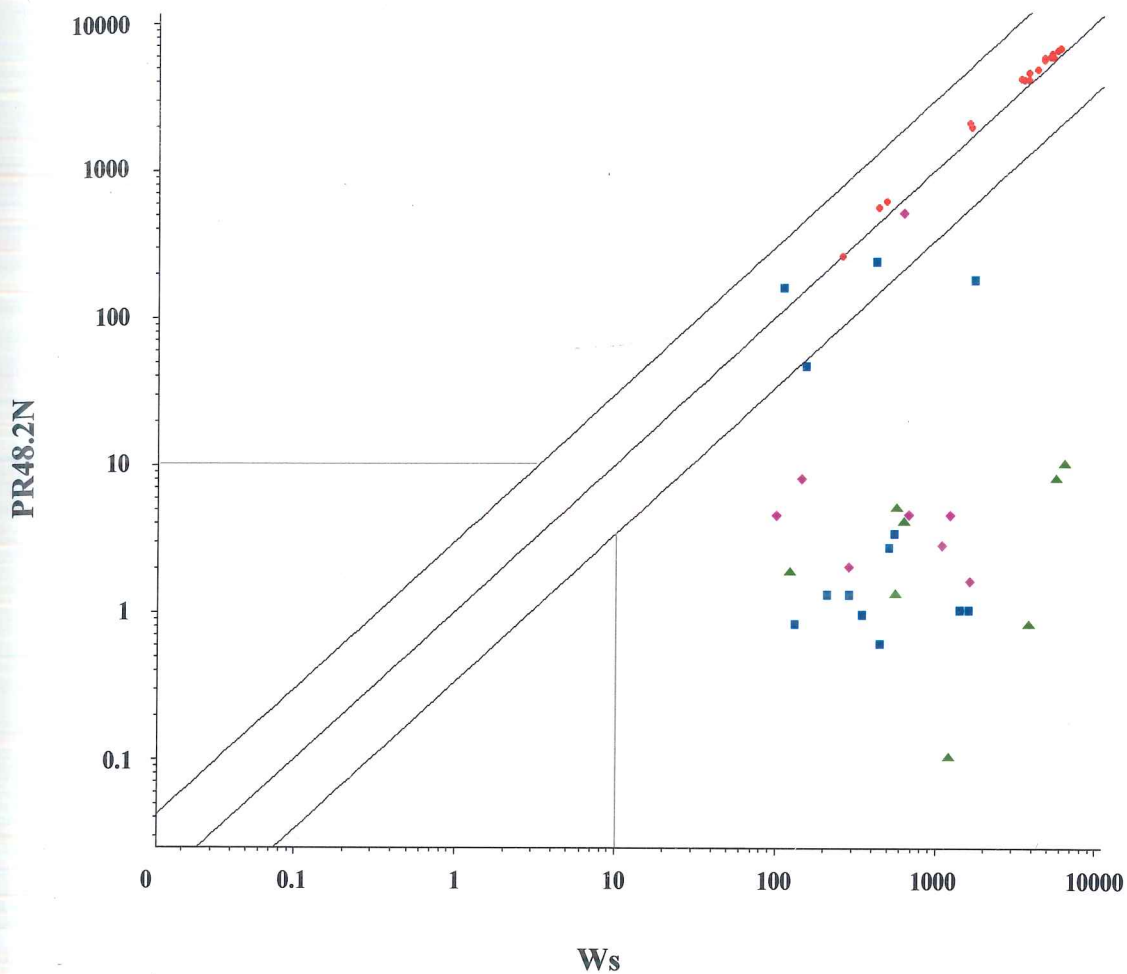


Figure 3.15 Comparison of transcript levels of nuclear genes encoding photosynthesis proteins, seed storage proteins, late embryogenesis abundant (LEA) proteins and oleosins between wild-type Ws and PR48.2N seedlings using Affymetrix microarray analysis.

Labelled complementary RNA (cRNA), prepared from total RNA extracted from wild-type Ws and PR48.2N seedlings grown on 0.7 % agar with half-strength MS for 5 days in the dark followed by 2 days in the light, was hybridised to Affymetrix ATH-121501 microarrays. The figure shows the average normalised abundance values of four groups of selected gene transcripts in PR48.2N plotted on a log scale against wild-type Ws. The regression line (middle, diagonal grey line) and the 3-fold lines (diagonal grey lines above and below the regression line) are shown. Transcript levels of genes encoding nuclear photosynthesis proteins (●), seed storage proteins (▲), late embryogenesis abundant (LEA) proteins (■) and oleosins (◆) were compared between wild-type Ws and PR48.2N.

The scatter plot shows that transcript abundance of most of the nuclear photosynthesis genes was slightly higher in PR48.2N seedlings than in wild-type seedlings (Figure 3.15). However, the differences of the transcript levels were mainly lower than 2-fold. Unlike the nuclear photosynthesis genes, the seed protein genes displayed lower transcript abundance in PR48.2N seedlings relative to wild-type Ws seedlings. All of the seed storage proteins included in the scatter plot showed very low transcript abundance in PR48.2N seedlings. Most of the LEA protein genes (11 out of 13) showed lower transcript levels in PR48.2N relative to wild-type Ws by at least 3-fold. All oleosin genes except one showed at least 3-fold lower transcript levels in PR48.2N relative to wild-type Ws.

Previous analysis by RNA-gel blot indicated that PR48.2N contained 2-fold more *RBCS* and *LHCBI* transcripts than wild type (section 3.5), but this phenotype was not observed by microarray analysis. This may be explained by different sensitivities of the techniques. Measurements of change of transcript abundance obtained from RNA-gel-blot and microarray analyses usually match in terms of the direction of change but not always the magnitude especially with different concentrations of the transcripts, since both techniques possess different sensitivities (Schena *et al.*, 1995; Kane *et al.*, 2000). The change in transcript abundance of nuclear photosynthesis genes in PR48.2N compared to wild type (2-fold) determined using RNA-gel-blot analysis may be too small to be resolved confidently using microarrays. From the comparison of differential transcript abundance of nuclear photosynthesis and seed protein genes, it was apparent that the transcript abundance of seed protein genes showed a much greater magnitude of change in PR48.2N seedlings relative to wild-type Ws seedlings. The lower transcript abundance of seed protein genes therefore appeared to be one of the major phenotypes of PR48.2N.

3.10 Transcript abundance of *CRA1*, a 12S seed storage protein gene

In order to verify the results from microarray analysis and to examine the transcript profile of the genes showing lower transcript abundance in PR48.2N, the effect of light, norflurazon and lincomycin on the transcript abundance of a 12S seed storage protein gene, *CRA1* (Pang *et al.*, 1988), in *gun1-1* and PR48.2N seedlings was studied with their wild types using RNA-gel-blot analysis. *CRA1* was chosen for this

diagnostic verification because the gene showed the second lowest transcript abundance in PR48.2N relative to the wild type after 2S seed storage protein 2 but higher transcript abundance compared to 2S seed storage protein 2, which facilitated its detection by RNA-gel-blot analysis (Table 2). Seedlings of Col, Ws, *gun1-1* and PR48.2N were grown, total RNA was extracted, and RNA-gel-blot analysis was performed as previously (section 3.5). A probe generated from *CRA1* was utilised for this experiment (section 2.6.9). There was no noteworthy cross-hybridisation predicted for this probe. The sequence of *CRA1* corresponding to the probe sequence was found to share only 30 % identity with the sequence of *CRUCIFERIN 2 (CRU2)*, which is the best match in a NCBI BLAST search.

CRA1, the 12S seed storage protein gene, showed lower transcript abundance in light-grown *Arabidopsis* seedlings compared to the dark-grown seedlings (Figures 3.16 and 3.17). In wild-type Ws, there were approximately 4-fold more transcripts of *CRA1* in dark-grown seedlings compared to the light-grown seedlings, whereas in wild-type Col, there were approximately 10-fold more transcripts of *CRA1* in dark-grown seedlings compared to the light-grown seedlings. Therefore *CRA1* appeared to be down-regulated by light. PR48.2N showed lower abundance of *CRA1* transcripts compared to the wild-type Ws in all treatments. For light-grown PR48.2N seedlings, there were approximately 3-fold fewer *CRA1* transcripts compared to the light-grown wild-type Ws seedlings, whereas for dark-grown PR48.2N seedlings, there were approximately 6-fold fewer *CRA1* transcripts compared to the dark-grown wild-type Ws seedlings. These observations were qualitatively similar to the microarray results in sections 3.8 and 3.9 that transcript abundance of *CRA1* in PR48.2N seedlings was much lower than wild-type Ws seedlings. However, the microarray analysis showed 4700-fold fewer transcripts, whereas only 3- to 6-fold differences were detected by RNA-gel-blot analysis. As discussed before, measurements of change of transcript abundance obtained from RNA-gel-blot and microarray analyses usually agree in terms of the direction of change but not always the magnitude especially with different abundance of the transcripts as both techniques possess different sensitivities (Schena *et al.*, 1995; Kane *et al.*, 2000).

There was no obvious difference in *CRA1* transcripts between the lincomycin-treated wild-type seedlings and the untreated controls. Therefore *CRA1* appeared not to

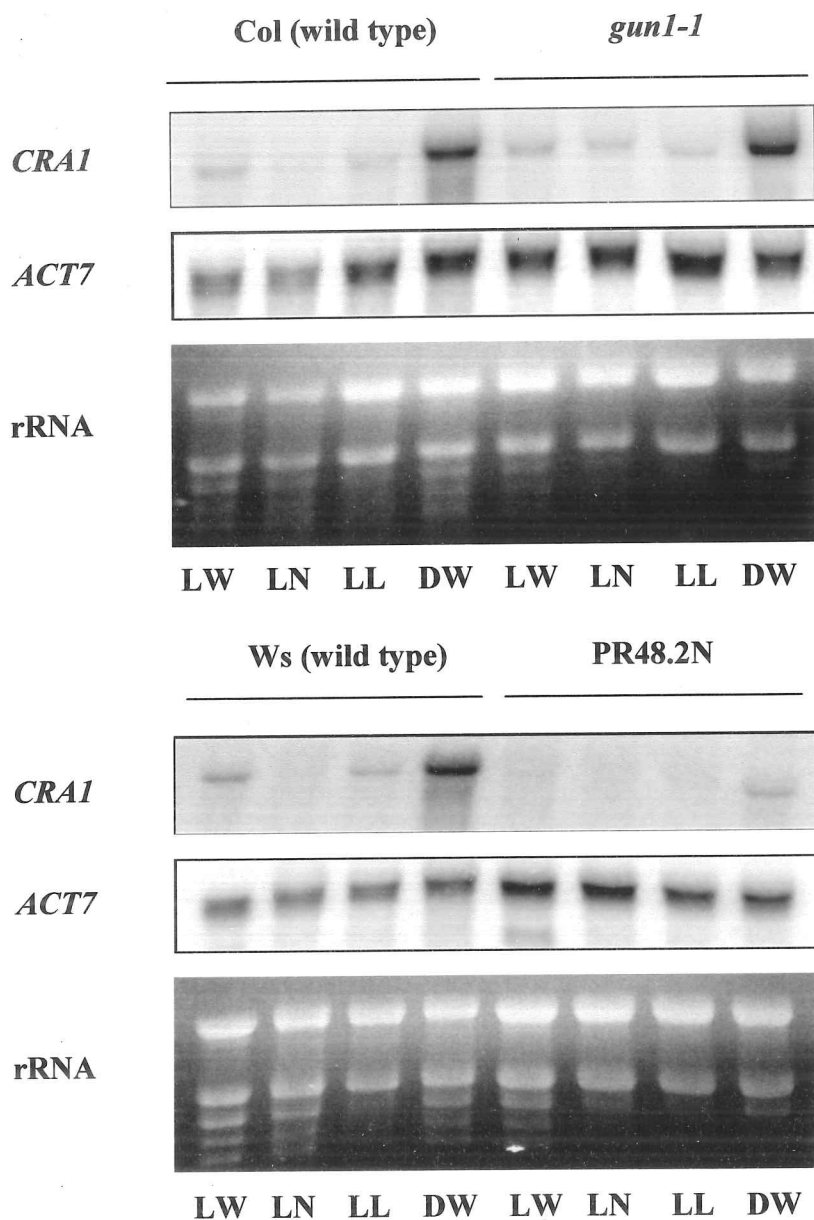


Figure 3.16 Effect of light, norflurazon and lincomycin on the transcript abundance of a 12S seed storage protein gene, *CRA1*, in *gun1-1* and PR48.2N seedlings with their wild types.

Seeds of wild types, Col and Ws, and mutants, *gun1-1* and PR48.2N, were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the dark followed by 2 days in the light either with water (LW), 5 μ M norflurazon (LN) or 0.5 mM lincomycin (LL) or in continuous darkness for 7 days with water (DW). Total RNA was extracted from the seedlings using Concert Plant RNA Reagent and 7 μ g of the total RNA was subjected to RNA-gel-blot analysis using a 32 P-labelled probe from a 12S seed storage protein gene, *CRA1*. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

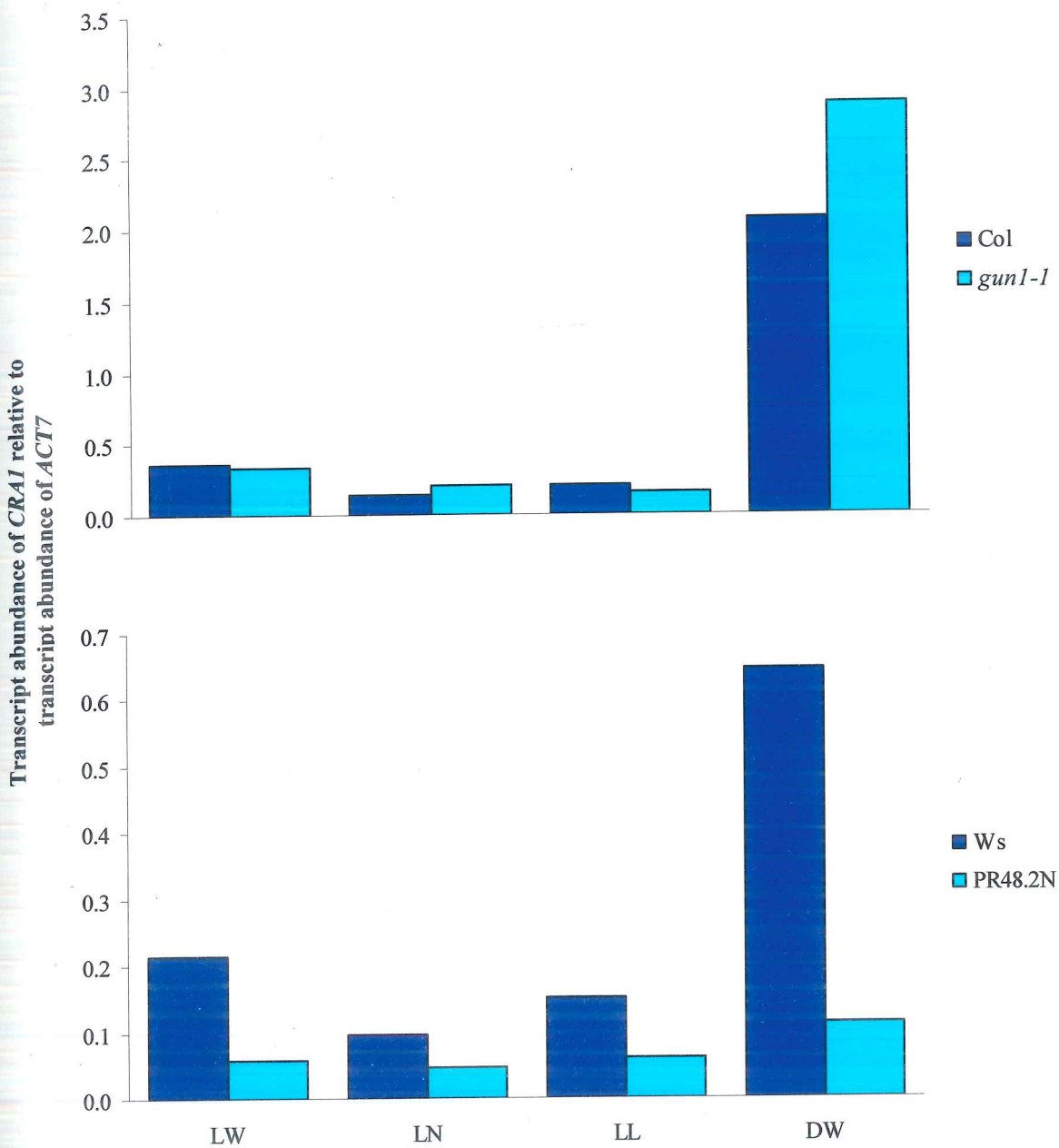


Figure 3.17 Quantification of the effect of light, norflurazon and lincomycin on transcript abundance of a 12S seed storage protein gene, *CRA1*, in *gun1-1* and PR48.2N seedlings with their wild types.

Hybridisation signals from the RNA-gel blots shown in Figure 3.16 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardised to *ACT7* signal to account for differences in the loading of total RNA. Abbreviations are as given in the legend of Figure 3.16.

be regulated by the plastid signal responding to the state of plastid protein synthesis. However, there were slightly fewer *CRA1* transcripts in norflurazon-treated wild-type seedlings compared to the untreated controls. This may be a consequence of the facts that norflurazon is an inhibitor of carotenoid biosynthesis, which provides the precursor for ABA biosynthesis (Debeaujon and Koornneef, 2000), and genes encoding seed storage proteins are positively regulated by ABA (for review, Chandler and Robertson, 1994). The inhibitory effect of norflurazon on ABA biosynthesis may result in lower *CRA1* transcript abundance in norflurazon-treated wild-type seedlings. However, transcript abundance of *CRA1* in norflurazon-treated seedlings of *gun1-1* was not lower than its untreated control. This could mean either *gun1-1* was a mutant deficient in one of the ABA-related pathways or *CRA1* was regulated by some plastid signals. Unlike PR48.2N seedlings, *gun1-1* seedlings did not show lower abundance of *CRA1* transcript relative to the wild type. Therefore, PR48.2N is likely to contain a mutation in a different gene to *gun1-1*.

3.11 Transcript abundance of seed protein genes during stages of seed development

The genes that showed lower transcript levels in PR48.2N seedlings relative to wild-type Ws were mainly genes involved in late embryogenesis and seed maturation (sections 3.8 and 3.9). However, previous experiments investigating the transcript abundance of these genes in PR48.2N were conducted in seedlings rather than developing seeds. The altered transcript abundance of seed protein genes in PR48.2N seedlings might be due to decreased expression in developing and mature seeds. The transcript levels of genes encoding seed storage proteins, LEA proteins and oleosins were investigated in various stages of developing seeds of wild-type Ws and PR48.2N.

Silques corresponding to four developmental stages: early embryogenesis (1-5 days after flowering, DAF), maturation (8-11 DAF), late embryogenesis (17-21 DAF) and dry seeds (>21 DAF) (Baud *et al.*, 2002) were harvested from mature wild-type Ws and PR48.2N plants, as described in section 2.2.3. The siliques or seeds collected were subjected to RNA extraction with Concert Plant RNA Reagent using the manufacturer's large-scale isolation protocol with modifications (section 2.6.2). Total RNA (15 µg) was subjected to RNA-gel-blot analysis using ³²P-labelled probes (sections 2.6.3-2.6.6) from

seed protein genes, namely *CRA1* encoding a 12S seed storage protein as described in section 3.10, *OLEO2* encoding a type 2 oleosin (Zou *et al.*, 1996) and *LEA76* encoding a late embryogenesis abundant protein 76 homologue (Harada *et al.*, 1989) (section 2.6.9). There was no noteworthy cross-hybridisation predicted for these probes by BLAST searches as previously described (section 3.3). Ethidium bromide-stained rRNAs were shown as a loading control (Figure 3.18).

Transcript abundance of the seed protein genes was not obviously different between developing or mature seeds of wild-type Ws and PR48.2N (Figure 3.18). At the stage of 1-5 DAF corresponding to early embryogenesis, no transcripts were detected for genes encoding *CRA1*, *OLEO2* or *LEA76* in either wild-type Ws or PR48.2N. Within the first 5 days of embryogenesis in *Arabidopsis*, transcripts encoding seed storage proteins, an oleosin and LEA proteins are undetectable (Parcy *et al.*, 1994).

At the stage of 8-11 DAF corresponding to seed maturation, transcripts encoding *CRA1* and *OLEO2*, but not *LEA76*, were detected in both wild-type Ws and PR48.2N. PR48.2N showed slightly lower transcript abundance of *CRA1* and *OLEO2*. During the stage of seed maturation, abundance of the transcripts encoding seed storage proteins and an oleosin increases (Parcy *et al.*, 1994). However, transcripts encoding LEA proteins are not yet detectable at this stage (Parcy *et al.*, 1994).

At the stage of 17-21 DAF corresponding to late embryogenesis, transcripts were detected for genes encoding *CRA1*, *OLEO2* and *LEA76* in both wild-type Ws and PR48.2N. No obvious difference in transcript abundance of *CRA1*, *OLEO2* and *LEA76* was observed between wild-type Ws and PR48.2N. During the stage of late embryogenesis, abundance of the transcripts encoding seed storage proteins, an oleosin, and LEA proteins achieves the highest level (Parcy *et al.*, 1994).

For dry mature seeds (>21 DAF), transcripts were detected in low quantity for genes encoding *CRA1*, *OLEO2* and *LEA76* in both wild-type Ws and PR48.2N. No obvious difference in transcript abundance of *CRA1*, *OLEO2* and *LEA76* was observed between wild-type Ws and PR48.2N at the stage of >21 DAF. The low abundance of transcripts encoding *CRA1*, *OLEO2* and *LEA76* at the stage of >21 DAF could be due to the degradation of total RNA indicated by the rRNAs in Figure 3.18. Isolating total

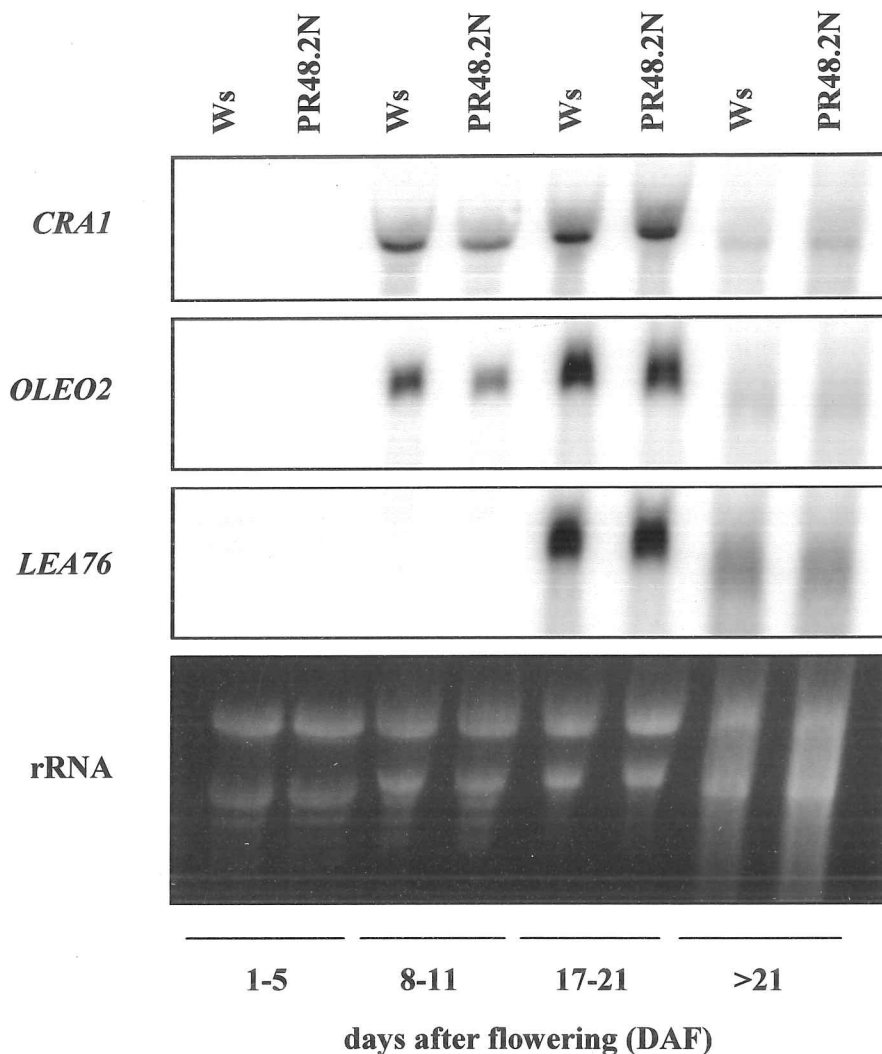


Figure 3.18 Transcript abundance of genes encoding a 12S seed storage protein (*CRA1*), an oleosin type 2 (*OLEO2*), and a late embryogenesis abundant 76 homologue protein (*LEA76*) in wild-type Ws and PR48.2N at different stages of seed development.

Total RNA was extracted from siliques of different stages, namely 1-5, 8-11 and 17-21 days after flowering (DAF) corresponding to early embryogenesis, maturation and late embryogenesis, and dry seeds (>21 DAF) of wild-type Ws and PR48.2N using Concert Plant RNA Reagent and 15 µg of the total RNA was subjected to RNA-gel-blot analysis using ³²P-labelled probes from genes encoding a 12S seed storage protein (*CRA1*), an oleosin type 2 (*OLEO2*), and a late embryogenesis abundant 76 homologue protein (*LEA76*). Ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as a loading control.

RNA of good quality from *Arabidopsis* dry mature seeds is a challenging task (Tai *et al.*, 2004). Despite the degradation, the intensity of ethidium bromide-stained rRNA in Figure 3.18 indicated that the loading of total RNA was roughly equal.

As reported by Parcy *et al.* (1994), the transcripts encoding seed storage proteins and an oleosin reach the highest amount during the late embryogenesis and then decrease. For transcripts encoding LEA proteins in *Arabidopsis*, the accumulation reaches a maximum during late embryogenesis, and some, but not all, decrease after the plateau (Parcy *et al.*, 1994). Another study on accumulation kinetics of transcripts encoding proteins involved in cotton (*Gossypium hirsutum*) seed development also reported the same observation that seed storage protein and oleosin transcripts reach the highest quantity during maturation and then decrease during the stages of post-abscission and desiccation (Hughes and Galau, 1989; Hughes *et al.*, 1993). All transcripts encoding cotton LEA-A class proteins, to which LEA76 belongs (Parcy *et al.*, 1994), reach a maximal level during maturation and show a decrease during the stages of post-abscission and desiccation (Hughes and Galau, 1989; Hughes *et al.*, 1993).

PR48.2N showed an altered phenotype in transcript abundance of a group of genes involved in late embryogenesis and seed maturation. However, this altered phenotype was observed only in PR48.2N seedlings (sections 3.8, 3.9 and 3.10). Although there was a slight decrease in accumulating transcripts of *CRA1* and *OLEO2* at the stage of 8-11 DAF corresponding to seed maturation, there was no other apparent difference observed in the transcript abundance of *CRA1*, *OLEO2* and *LEA76* during the stages of seed development. Hence, the seedling phenotype of PR48.2N was unlikely to be a consequence of decreased expression during embryogenesis and seed maturation.

3.12 Transcript abundance of nuclear photosynthesis and seed protein genes during the greening process

PR48.2N was initially identified as a mutant showing higher transcript abundance of nuclear photosynthesis genes in seedlings grown for 5 days in the dark followed by 2 days in the light compared to the wild type (sections 3.3 and 3.5). In addition it showed low transcript abundance of a group of genes involved in late

embryogenesis and seed maturation in the seedlings (sections 3.8, 3.9 and 3.10) rather than the developing seeds (section 3.11). PR48.2N also showed other mutant phenotypes, including lower chlorophyll accumulation after a short period of darkness, and higher chlorophyll accumulation after prolonged darkness (sections 3.4 and 3.6). In order to examine whether the transcripts of nuclear photosynthesis and seed protein genes changed during the greening process, RNA-gel-blot analysis was performed on greening seedlings of wild-type and PR48.2N after various lengths of dark treatment.

Dark-grown (2D, 3D, 4D, 5D, 6D and 10D) and illuminated (2D1L, 3D1L, 4D1L, 5D1L, 6D1L and 10D1L) seedlings of Ws and PR48.2N were grown as previously described (sections 3.4, 3.6 and 3.7). The seedlings were harvested for RNA extraction with Concert Plant RNA Reagent and the total RNA (15 µg) was subjected to RNA-gel-blot analysis as previously described (section 3.5), using ³²P-labelled probes generated from nuclear photosynthesis genes, namely *RBCS-1A*, *LHCB1.2* and *HEMA1* encoding glutamyl-tRNA reductase that accomplishes the first step in the tetrapyrrole biosynthesis pathway (Ilag *et al.*, 1994), and seed protein genes, namely *CRA1*, *OLEO2* and *LEA76* (section 2.6.9). These probes were previously described (sections 3.3, 3.5, 3.10 and 3.11) except the probe for *HEMA1*, which is likely to cross-hybridise to another member of the *HEMA* family, *HEMA2* by their close sequence similarity (Kumar *et al.*, 1996). Hybridisation signals from the RNA-gel blots shown in Figures 3.19 and 3.21 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were normalised to *ACT7* signal to account for differences in the loading of total RNA (section 2.6.7) (Figures 3.20 and 3.22). Ethidium bromide-stained rRNAs were shown as loading controls in Figures 3.19 and 3.21.

The results of the experiment showed that the transcript abundance of nuclear photosynthesis and seed protein genes in PR48.2N seedlings subjected to various lengths of dark treatment were lower than the wild-type seedlings (Figures 3.19 and 3.20). Transcripts of all genes tested, except *HEMA*, were 3- to 5-fold less in dark-grown PR48.2N seedlings than the wild-type Ws seedlings. *HEMA* showed only slightly less transcripts in dark-grown PR48.2N seedlings compared to the wild type. In wild-type Ws seedlings, transcript abundance of *RBCS*, *CRA1* and *LEA76* decreased from 2D to 10D. It was previously reported that transcript abundance of *RBCS* decreases in

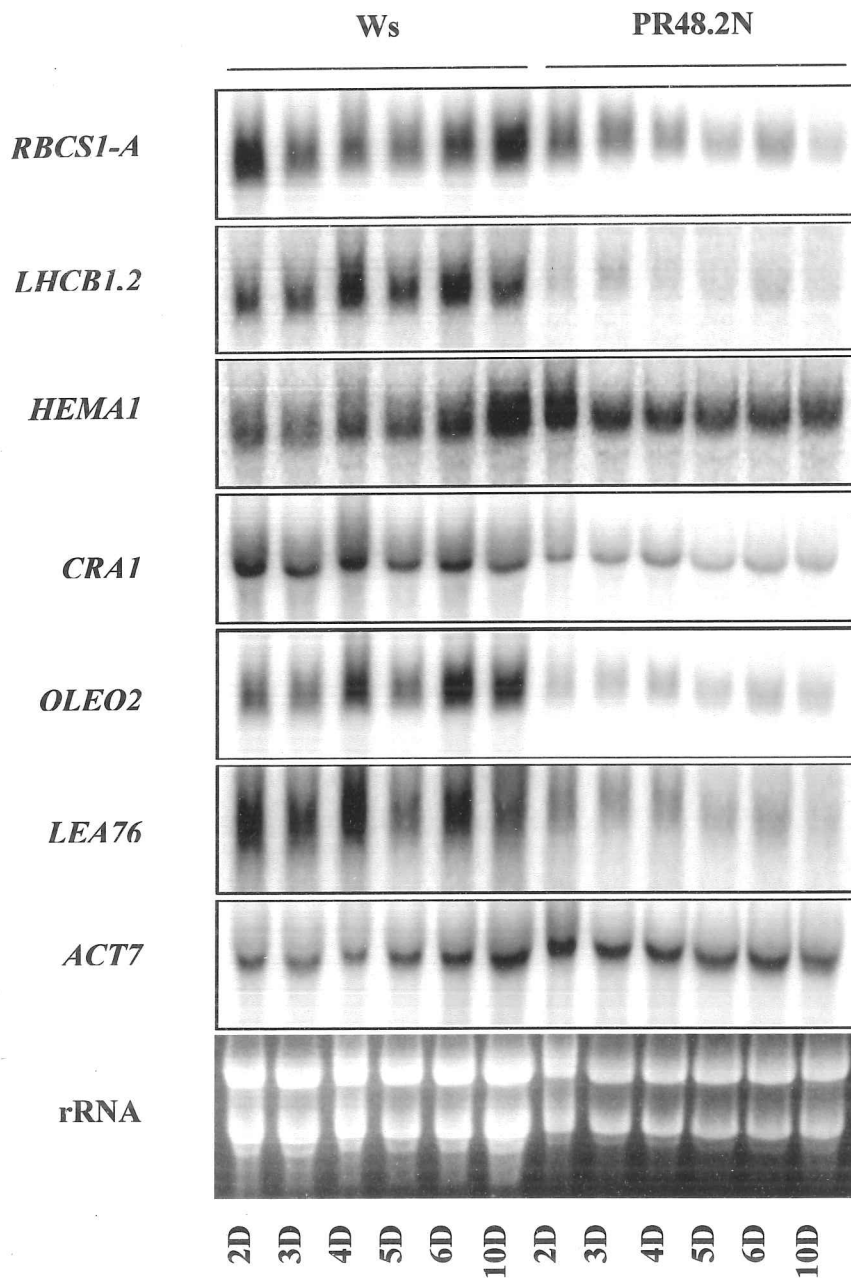


Figure 3.19 Transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type Ws and PR48.2N seedlings after 2, 3, 4, 5, 6 or 10 days in the dark.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D), 3 (3D), 4 (4D), 5 (5D), 6 (6D) or 10 (10D) days in the dark. Total RNA was extracted from the seedlings using Concert Plant RNA Reagent and 15 μ g of the total RNA was subjected to RNA-gel-blot analysis using 32 P-labelled probes from nuclear genes encoding photosynthesis proteins, RBCS-1A, LHCB1.2 and HEMA1, and seed proteins, CRA1, OLEO2 and LEA76. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

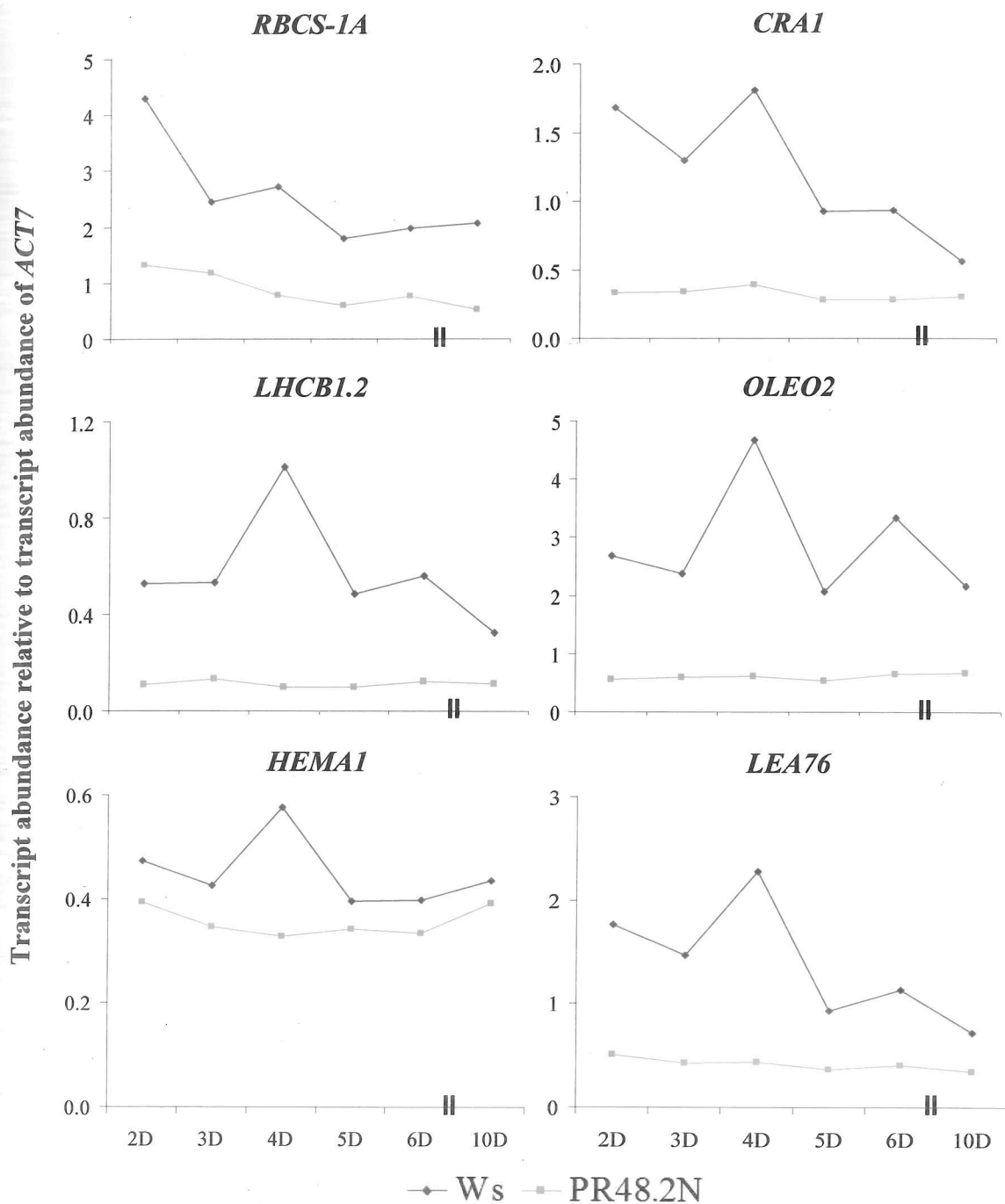


Figure 3.20 Quantification of the transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type *Ws* and *PR48.2N* seedlings after 2, 3, 4, 5, 6 or 10 days in the dark.

Hybridisation signals from the RNA-gel blots shown in Figure 3.19 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardised to *ACT7* signal to account for differences in the loading of total RNA. Abbreviations are as given in the legend of Figure 3.19.

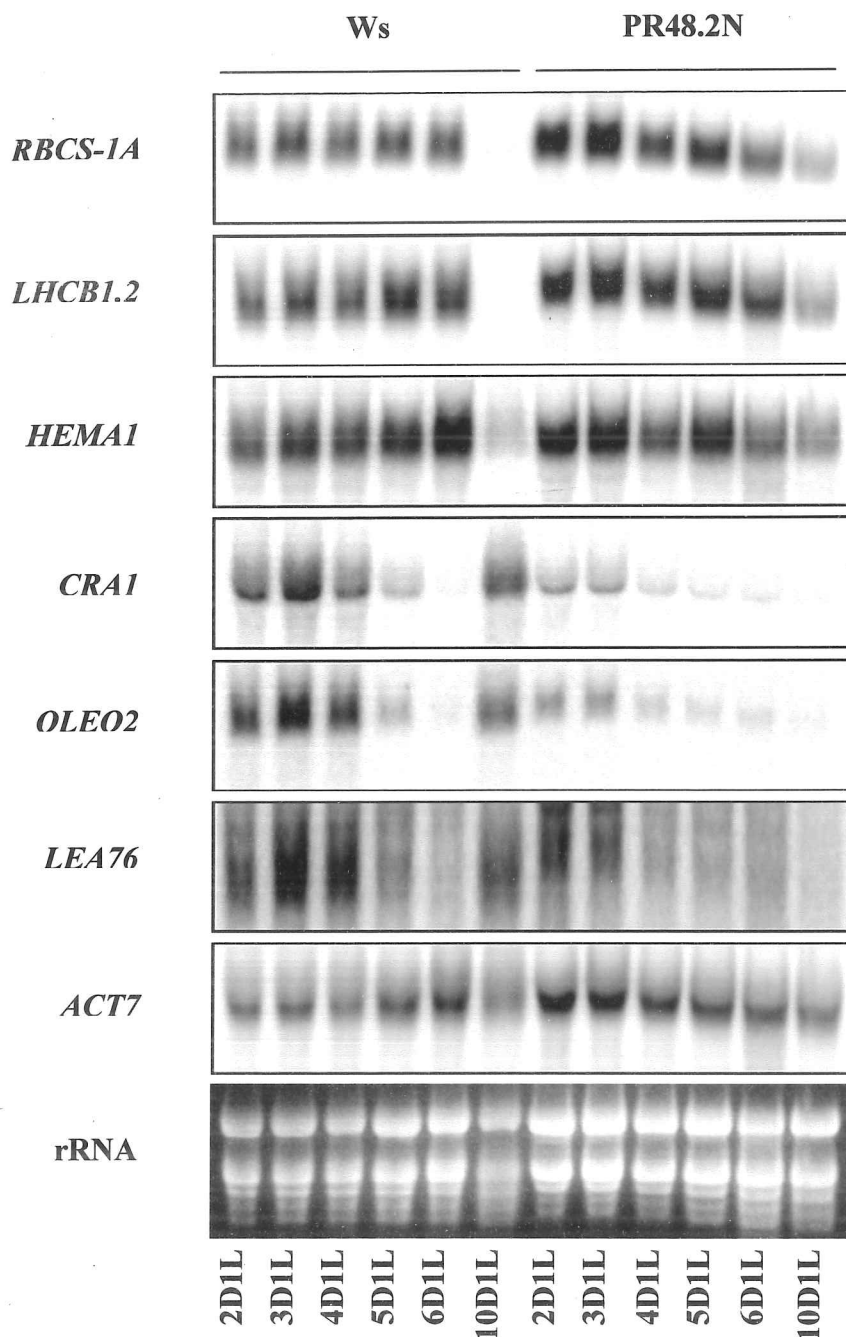


Figure 3.21 Transcript abundance of nuclear genes encoding photosynthesis and seed proteins in wild-type Ws and PR48.2N seedlings after 2, 3, 4, 5, 6 or 10 days in the dark followed by 1 day in the light.

Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 2 (2D1L), 3 (3D1L), 4 (4D1L), 5 (5D1L), 6 (6D1L) or 10 (10D1L) days in the dark followed by 1 day in the light. Total RNA was extracted from the seedlings using Concert Plant RNA Reagent and 15 μ g of the total RNA was subjected to RNA-gel-blot analysis using 32 P-labelled probes from nuclear genes encoding photosynthesis proteins, RBCS-1A, LHCB1.2 and HEMA1, and seed proteins, CRA1, OLEO2 and LEA76. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

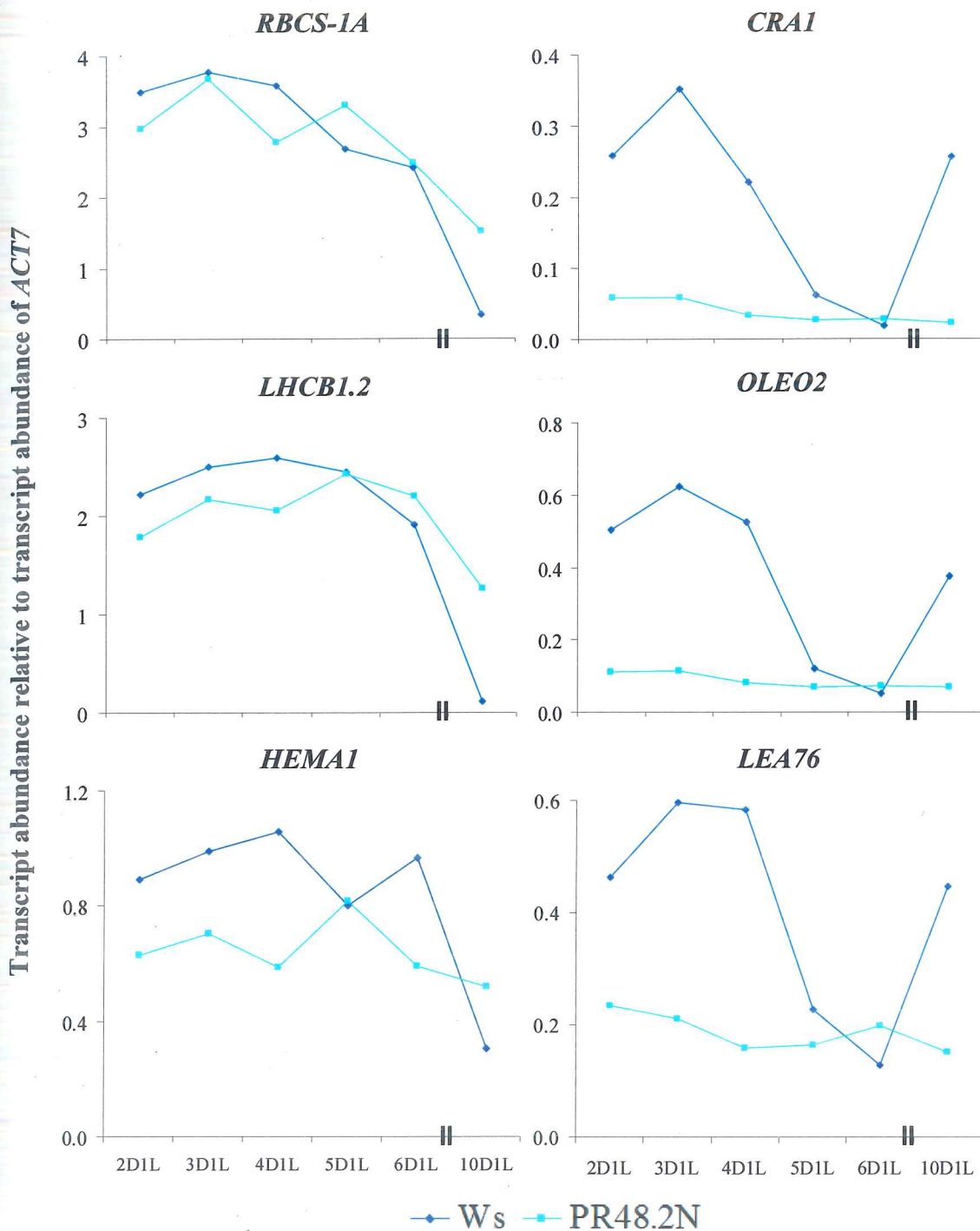


Figure 3.22 Quantification of the transcript abundance of nuclear genes encoding photosynthesis proteins and seed proteins in wild-type Ws and PR48.2N seedlings after 2, 3, 4, 5, 6 or 10 days in the dark followed by 1 day in the light.

Hybridisation signals from the RNA-gel blots shown in Figure 3.21 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardised to *ACT7* signal to account for differences in the loading of total RNA. Abbreviations are as given in the legend of Figure 3.21.

seedlings when the length of etiolated growth increases (Dedonder *et al.*, 1993), but some transcripts of seed proteins, *CRA1* and *LEA76*, displayed a similar pattern. Interestingly, there was a transcript peak observed at 4D for all nuclear photosynthesis and seed protein genes tested in dark-grown wild-type Ws seedlings, but not in PR48.2N seedlings growing in the dark.

Transcript abundance of nuclear photosynthesis and seed protein genes in illuminated PR48.2N seedlings after being subjected to various lengths of dark treatment were different from the wild-type Ws seedlings (Figures 3.21 and 3.22). In wild-type Ws seedlings, transcript abundance of nuclear photosynthesis genes, *RBCS*, *LHCBI* and *HEMA* increased, peaked at 3D1L or 4D1L, and decreased after that. In PR48.2N seedlings, transcripts of nuclear photosynthesis genes followed the similar trend, except that they were generally lower than the wild type from 2D1L to 4D1L and peaked around 5D1L before decreasing after that. However in the illuminated PR48.2N seedlings after prolonged dark treatment (10D1L), transcript abundance of nuclear photosynthesis genes was higher in the PR48.2N seedlings than the wild-type Ws.

Transcript abundance of seed protein genes, *CRA1*, *OLEO2* and *LEA76*, in illuminated dark-grown wild-type Ws increased, peaked at 3D1L, and decreased after that. The amounts of the seed protein transcripts increased again after prolonged dark treatment. At 10D1L, the transcripts reached approximately the same levels as in seedlings at 2D1L. Transcript abundance of seed protein genes, *CRA1*, *OLEO2* and *LEA76* in illuminated dark-grown PR48.2N seedlings decreased slightly from 2D1L to 10D1L. It was always lower than the wild-type transcript profile except at 6D1L when PR48.2N level was slightly higher or similar to the wild type.

Transcript abundance of nuclear photosynthesis and seed protein genes was affected in both dark-grown and greening PR48.2N seedlings. Dark-grown PR48.2N seedlings contained lower amounts of transcripts encoding nuclear photosynthesis and seed proteins compared to the wild type. When illuminated, transcripts of seed protein genes remained lower except at 6D when wild-type seedlings also showed low levels of transcripts, whereas transcripts of nuclear photosynthesis genes were lower for illumination after short periods of darkness but higher for illumination after prolonged darkness.

3.13 Consensus elements in upstream regions of highly affected genes in PR48.2N

From the microarray analysis presented in section 3.8, a large number of genes showed highly altered transcript abundance in PR48.2N seedlings. These highly affected genes, with higher or lower transcript abundance in PR48.2N compared to the wild type, may be co-regulated and therefore may share some common *cis*-elements in their upstream regions. By searching for and identifying these *cis*-regulatory elements, it might be possible to infer some information on the function of the gene that was mutated in PR48.2N.

Genes that showed very high or low transcript abundance in PR48.2N seedlings by at least a factor of 10 compared to wild-type seedlings were searched for common motifs located within a 1 kb region upstream of the translation start site. Approximately 20 genes with transcript levels 10-fold, or more, higher in PR48.2N relative to wild-type Ws and approximately 280 genes with transcript levels 10-fold, or more, lower in PR48.2N relative to wild-type Ws were subjected to the "Find Potential Regulatory Sequences" tool in GeneSpring using sequences of the *Arabidopsis thaliana* genome obtained from the NCBI Genome database, as described in section 2.6.10.2.

There were no common *cis*-elements found in the upstream regions of the genes showing higher transcript levels in PR48.2N seedlings relative to wild-type Ws. However, seven sequences of possible common *cis*-regulatory elements were found in the upstream regions of the genes showing lower transcript abundance in PR48.2N seedlings relative to wild-type Ws (Table 3.3). These elements passed the stringent P-value cut-off at 1×10^{-10} . All except one of these sequences contained an ACGT core and the most prominent sequences were the CACGTG-related elements. The frequency of CACGTG was high. It was observed in 37.6 % of the genes analysed, with a false probability of 7.6×10^{-15} . The sequence would be expected to occur in 8.0 % of the genes if the nucleotide sequence was random (random rate). The observed frequency of CACGTG occurring in the promoters of genes not in the list was 16.6 %. Two CACGTG-related elements, namely ACACGTG and CACGTGT, showed high frequencies of 23.7 % and 22.9 % respectively, with low false probabilities of 1.46×10^{-14} and 4.69×10^{-13} . ACACGTG and CACGTGT also had low random rates of 2.8 % and 2.7 % respectively, and observed probabilities occurring relative to the genes not in the

Table 3.3 Sequences enriched in 1-kb upstream regions of genes with 10-fold lower transcript levels in PR48.2N seedlings relative to wild-type Ws.

Upstream sequences of genes that showed 10-fold lower transcript abundance in PR48.2N seedlings relative to wild-type Ws were subjected to the "Find Potential Regulatory Sequences" tool in GeneSpring. The criteria for the search were: 6-8 nucleotides without any point discrepancies within 10-1000 nucleotides upstream from the translation start site of each gene. In all statistical analysis relative to upstream regions of other (unselected) genes, the cut-off P-value was set at 1×10^{-10} .

Sequence	Observed (%)	Observed in other genes (%)	Random rate (%)	P-value
ACGTGT	109/279 (39.1)	16.6	15.0	3.8×10^{-15}
CACGTG	105/279 (37.6)	15.7	8.0	7.6×10^{-15}
ACACGTG	66/279 (23.7)	6.8	2.8	1.5×10^{-14}
CACGTGT	64/279 (22.9)	6.8	2.7	4.7×10^{-13}
ACGTGTC	55/279 (19.7)	5.3	2.7	1.6×10^{-12}
CGTGTC	77/279 (27.6)	10.1	7.9	2.1×10^{-12}
ACACGT	102/279 (36.6)	16.9	16.0	3.2×10^{-11}

list of 6.8 %. The ACGT-containing elements were similar to ABA response elements (ABRE) (Guiltinan *et al.*, 1990; Skriver *et al.*, 1991; Shen *et al.*, 1993) that contain an ACGT core (Michel *et al.*, 1993). The prominent CACGTG-related elements were similar to one of the most typical ABRE previously identified by promoter and binding assays (Guiltinan *et al.*, 1990; Michel *et al.*, 1993; Shen *et al.*, 1993).

One element, CGTGTC, identified from the promoter analysis did not contain an ACGT core. CGTGTC was found in 27.6 % of the genes analysed, with false probability of 2.1×10^{-12} and random rate of 7.9 %. The observed frequency of CGTGTC occurring in the promoters of genes not in the list was 10.1 %. This element is required for ABA regulation (Kao *et al.*, 1996) and shares a common sequence with a *cis*-element, coupling element 3 (CE3) ACGCGTGTCTC, which functions in concert with the ACGT-containing ABRE to regulate ABA-inducible gene expression (Shen *et al.*, 1996; Hobo *et al.*, 1999).

For the 131 genes with CACGTG or/and CGTGTC elements in the upstream regions, about half of them contained only one copy of either one of the elements and the other half of them contained multiple copies of either one of the elements or combinations of both elements in various numbers (Figure 3.23).

3.14 ABA content of PR48.2N

PR48.2N seedlings showed lower transcript levels of genes involved in late embryogenesis and seed maturation in relative to wild-type Ws (sections 3.8, 3.9 and 3.10) and ABA-responsive *cis*-elements were found in the upstream regions of these genes (section 3.13). The endogenous ABA content in plants begins to rise and reach the highest level during the middle and late periods of seed development to regulate a number of physiological events, namely the maturation events of embryo development such as induction of seed dormancy, accumulation of nutritive reserves, and acquisition of desiccation tolerance (for reviews, Rock and Quatrano, 1995; Leung and Giraudat, 1998). Genes encoding either seed storage proteins or LEA proteins thought to be involved in desiccation tolerance are positively regulated by ABA (for review, Chandler and Robertson, 1994). Expression of seed storage protein or LEA protein genes in plant mutants deficient in ABA biosynthesis, which contain lower ABA content compared to

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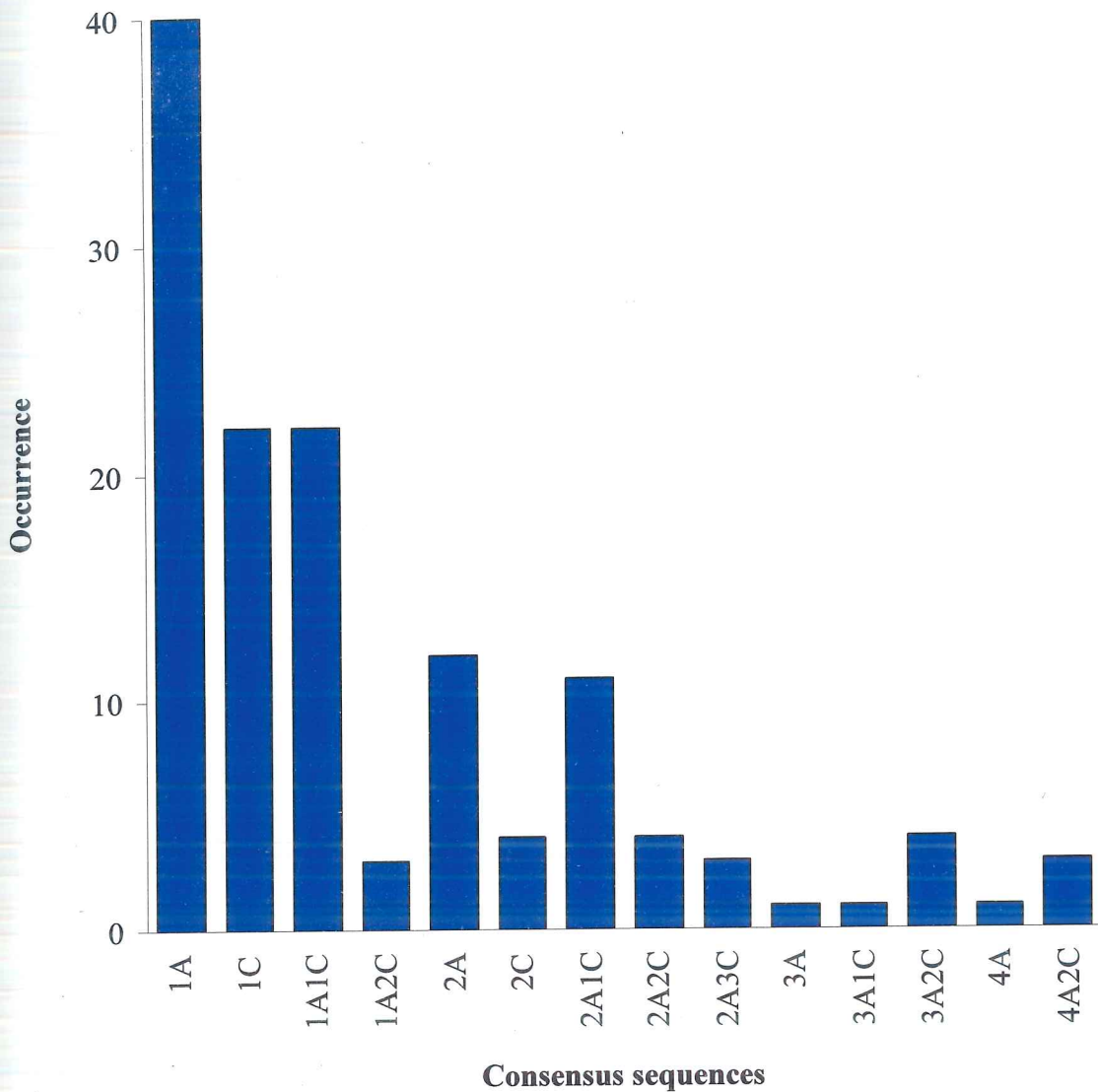


Figure 3.23 Occurrence of CACGTG (A) and CGTGTC (C) sequences in upstream regions of genes that showed 10-fold lower transcript levels in PR48.2N seedlings relative to wild-type Ws and contained the consensus sequences.

Single or multiple copies of either or both of the CACGTG (A) and CGTGTC (C) sequences were observed in 1-kb upstream regions of 131 genes that showed 10-fold lower transcript abundance in PR48.2N seedlings relative to wild-type Ws and contained the consensus sequences. Numbers indicate copy numbers of the consensus sequences; for instance, 1A1C means one copy of CACGTG and one copy of CGTGTC were observed in the upstream regions of the genes.

wild type, is down-regulated, although not as much as in ABA-insensitive mutants (Koornneef *et al.*, 1989; Kriz *et al.*, 1990; Meurs *et al.*, 1992; Finkelstein, 1993; Paiva and Kriz, 1994; Parcy *et al.*, 1994). Genes encoding oleosin proteins are expressed late in seed development coinciding with the expression of LEA genes and therefore may have a similar regulation pattern to LEA genes (for review, Murphy, 1993). Expression of oleosins also appears to be positively regulated by ABA (Hatzopoulos *et al.*, 1990; Plant *et al.*, 1994). Since ABA regulates the expression of the seed protein genes during seed development, the low transcript abundance of seed protein genes observed in PR48.2N seedlings might be due to altered ABA content of the mutant seedlings.

To establish if the ABA content of PR48.2N seedlings was altered, the ABA content of wild-type Ws and PR48.2N seedlings was measured and compared. The concentration of ABA in seedling tissue was determined by radioimmunoassay using the binding of a monoclonal antibody against (S)-*cis,trans*-ABA, as initially described in Quarrie *et al.* (1988), and performed entirely by J.C. Theobald (Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK) without knowing the identity of the samples during the process (section 2.7.3). Wild-type Ws and PR48.2N seeds were sterilised, plated, stratified and light-treated, as described in sections 2.2.1 and 2.2.2. The seeds were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the dark followed by 2 days in the light. The seedling samples were taken at this stage instead of the middle and late period of seed development which contain the highest level of ABA (Rock and Quatrano, 1995) because the phenotype of PR48.2N, differential transcript abundance of nuclear photosynthesis and seed protein genes, was discovered by RNA-gel-blot (sections 3.3 and 3.5) and microarray analyses (section 3.8) in seedlings grown for 5 days in the dark followed by 2 days in the light.

After 7 days, the whole seedlings (500-1000) were harvested and ground to a fine powder in a mortar with a pestle in liquid nitrogen. The powder was freeze-dried using an Edwards Super Modulyo Freeze-dryer before being sent to Lancaster. Three replicates for each line were prepared and 2-4 measurements were performed for each replicate. The measurements for each replicate were averaged. All measurements of ABA concentration were standardised to the dry weight of seedling tissue (μg of ABA/g of seedling dry weight).

The concentrations of ABA obtained from the wild-type Ws and PR48.2N seedlings were typical for *Arabidopsis* seedlings (JC Theobald, personal communication). The ABA contents of wild-type Ws samples ranged from 0.36 to 0.40 μg ABA/g seedling dry weight, whereas for PR48.2N samples, the range was 0.38 to 0.43 μg /g (Figure 3.24). There was no significant difference of ABA content between the wild-type Ws and PR48.2N seedlings. Therefore, PR48.2N did not appear to be a mutant in ABA biosynthesis, at least in 7-day-old seedlings. The mutant phenotypes of the developing PR48.2N seedlings were therefore unlikely to be related to their endogenous ABA level.

3.15 ABA sensitivity of PR48.2N

ABA-insensitive mutants show down-regulated expression of seed storage protein or LEA protein genes to a greater extent than ABA-deficient mutants (Koornneef *et al.*, 1989; Kriz *et al.*, 1990; Meurs *et al.*, 1992; Finkelstein, 1993; Paiva and Kriz, 1994; Parcy *et al.*, 1994). *aba-insensitive* (*abi*) mutants have been isolated as mutants that germinate in an inhibitory level of exogenous ABA for germination of wild-type seeds (Koornneef *et al.*, 1984). In *Arabidopsis*, there are five *abi* mutant loci (*abi1*, *abi2*, *abi3*, *abi4* and *abi5*) falling in two classes, *abi1-abi2* and *abi3-abi5*, based on their responses to altered water balance: *abi1* and *abi2* are semi-dominant mutations resulting in excessive transpiration, whereas *abi3* resembles wild-type in its water relations and acts in a seed-specific pathway with *abi4* and *abi5* (for review, Rock and Quatrano, 1995). ABI3, ABI4 and ABI5 regulate seed storage protein and LEA genes during late embryogenesis and *abi3* mutants especially show severely reduced accumulation of seed storage proteins and LEA proteins (for review, Fujiwara *et al.*, 2002). Since genes involved in late embryogenesis and seed maturation showed lower transcript levels in PR48.2N seedlings relative to wild-type Ws (sections 3.8, 3.9 and 3.10) but not in seed development (section 3.11), the ABA sensitivity or responsiveness of PR48.2N and wild-type seeds was examined by determining the percentage of seeds showing germination on media containing various concentrations of ABA.

Wild-type Ws and PR48.2N seeds were sterilised and were sown on 0.7 % agar with half-strength MS containing 0 (no-treatment control), 0.5, 1, 2.5, 5 and 10 μM ABA, and allowed to germinate in the light for ten days after the stratification. Seed

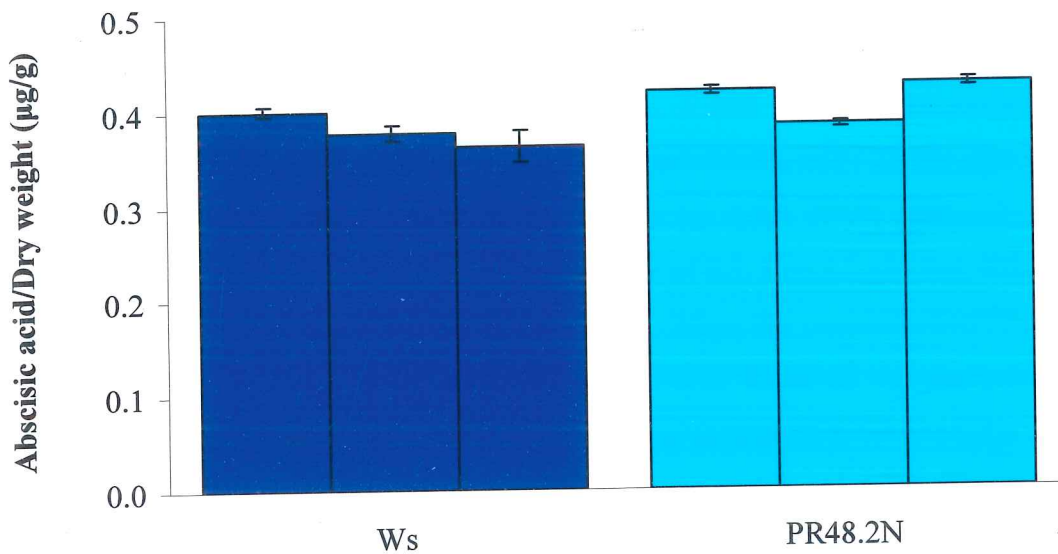


Figure 3.24 Abscisic acid (ABA) content of wild-type Ws and PR48.2N seedlings grown for 5 days in the dark followed by 2 days in the light.

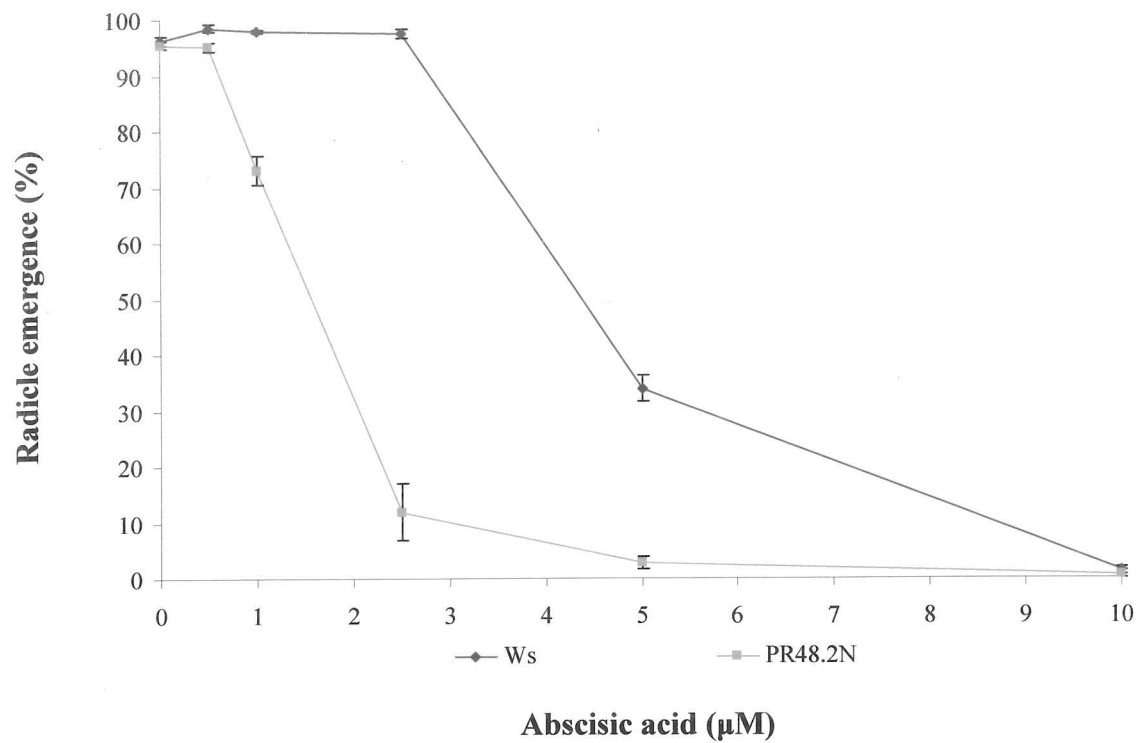
Wild-type Ws and PR48.2N seedlings were grown on 0.7 % agar with half-strength MS for 5 days in the dark followed by 2 days in the light and freeze-dried. Abscisic acid (ABA) was extracted and measured by radioimmunoassay. The entire process of extraction and measurement was performed by J.C. Theobald (Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK). Three replicates were prepared for each line and 2-4 measurements were performed for each replicate. Results are means \pm SEM for 2-4 replicates. Amounts of ABA were expressed on a basis of seedling dry weight.

germination, defined by radicle emergence, is inhibited by ABA (for review, Bewley, 1997). The percentage of seeds that germinated (radicle emergence observable to the naked eye) was determined. Three replicates for each line and treatment were performed. Average percentage of germination and standard errors were calculated from the replicates (section 2.7.2).

Radicle emergence of PR48.2N seeds was not inhibited by ABA lower than 0.5 μM . The germination of PR48.2N seeds in the presence of 0.5 μM (95 %) was similar to the no-ABA control, which was approximately 96 % (Figure 3.25). When the concentration of ABA was increased to 1, 2.5, 5 and 10 μM , the germination of PR48.2N seeds was decreased to 73.0, 11.9, 2.8 and 0.6 %, respectively. For wild-type Ws seeds, radicle emergence was not inhibited by ABA lower than 2.5 μM . The germination of wild-type Ws seeds in the presence of 0.5, 1 or 2.5 μM was similar to the no-ABA control, which was approximately 96 %. When the concentration of ABA was increased to 5 and 10 μM , the germination of wild-type Ws seeds was decreased to 33.8 and 1.3 %, respectively. The greatest difference in germination between the wild-type Ws and PR48.2N seeds was observed in the presence of 2.5 μM ABA. There was an 8-fold decrease in germination of PR48.2N seeds compared to the wild-type Ws.

The number of seedlings with fully open cotyledons also served as an estimate of the difference in ABA inhibitory effect on the germination of PR48.2N and wild-type seeds since the seeds that germinate slower also grow to the stage of fully-open-cotyledon slower. The number of wild-type Ws seedlings with fully open cotyledons was similar to the no-ABA control when the ABA concentration was lower than 1 μM (Figure 3.25). The number of Ws seedlings with fully open cotyledons was fewer when the ABA concentration was increased to 2.5 μM and was down to zero when the ABA concentration was increased to 5 and 10 μM . For PR48.2N, there were hardly any seeds grown to the stage with fully open cotyledons even in the presence of 0.5 μM . When the concentration of ABA was increased to 1, 2.5, 5 and 10 μM , there was no seedling with fully open cotyledons observed. The greatest difference of the percentage of seedlings with fully open cotyledons between wild-type Ws and PR48.2N was achieved in the presence of 1 μM ABA.

A



B

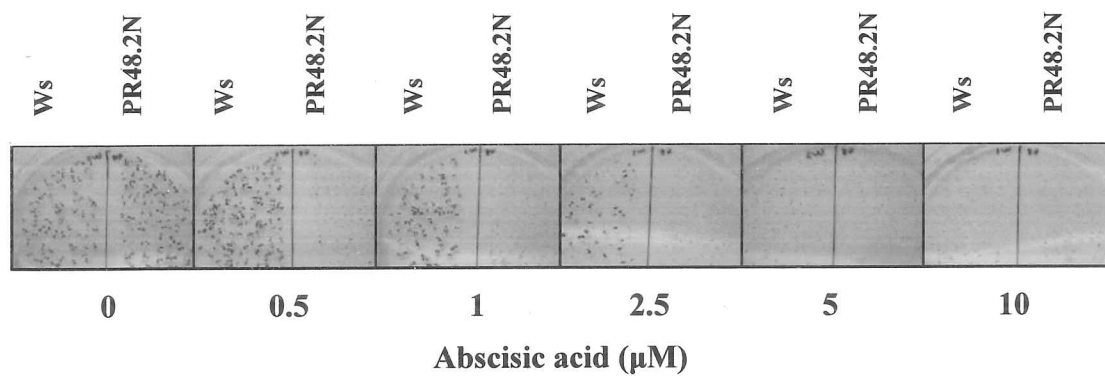


Figure 3.25 Dose-response of germination to abscisic acid (ABA) of wild-type Ws and PR48.2N seeds after 10 days in the light.

Wild-type Ws and PR48.2N seeds were sown on 0.7 % agar with half-strength MS with various concentrations of abscisic acid (ABA), namely 0, 0.5, 1, 2.5, 5 and 10 μM , and allowed to germinate in the light for 10 days. A) Percentage of seeds showing radicle emergence was determined for more than 70 seeds. Results are means \pm SEM for 3 replicates. B) Seedlings with fully open cotyledons. Images were taken using a Canon EOS 300D digital camera.

3.16 Discussion

PR48.2N was isolated as a mutant that expressed a GFP reporter gene under the control of a *RBCS* promoter in the presence of norflurazon and lincomycin. PR48.2N had distinct phenotypes of altered transcript abundance of nuclear photosynthesis genes (sections 3.3 and 3.5) and greening ability (sections 3.4 and 3.6) compared to the wild type and other putative *gun1*-like mutants and therefore was selected for further characterisation. PR48.2N seedlings showed 2-fold higher transcript abundance of nuclear photosynthesis genes, *RBCS* and *LHCBI*, compared to wild type with or without treatments of norflurazon or lincomycin using RNA-gel-blot analysis (section 3.5) and this raised a question of whether the mutant was involved in plastid-to-nucleus signalling. The *gun1*-like phenotype manifested in norflurazon- or lincomycin-treated PR48.2N seedlings could be due to higher transcript levels of nuclear photosynthesis genes in PR48.2N seedlings. However, PR48.2N was considered to be an interesting mutant for further study because no *Arabidopsis* mutant had been reported to have higher transcript levels of nuclear photosynthesis genes under normal growth conditions.

Another prominent mutant phenotype of PR48.2N was its altered greening ability during the transition from dark to light (sections 3.4 and 3.6). Pigment analysis of PR48.2N seedlings illuminated for a 16-h photoperiod after being subjected to various lengths of dark treatment demonstrated that the mutant line accumulated less chlorophyll than wild type after short periods of darkness (2-4 days) but showed an enhanced ability to green after prolonged dark treatments (5-10 days). Unlike PR48.2N, *gun1-1* showed a reduced ability to green after prolonged dark treatments (Susek *et al.*, 1993; Mochizuki *et al.*, 1996). This suggests that PR48.2N may be defective in a negative regulatory system repressing chlorophyll accumulation after prolonged darkness, whereas GUN1 may be a component positively regulating the function. The mutated gene in PR48.2N may also encode a component positively regulating chlorophyll accumulation after a short period of darkness. The greening processes after a short and prolonged darkness may be regulated by different mechanisms.

The mutation in PR48.2N affected the ultrastructure of etioplasts in dark-grown seedlings and of chloroplasts during the process of greening (section 3.7). After a short

period of darkness such as 2D, etioplasts in wild-type seedlings contained prolamellar bodies with long primary thylakoids and differentiated into developed chloroplasts with thylakoid membranes and starch grains when illuminated. However, 2D etioplasts of PR48.2N cotyledons appeared to be less developed, and chloroplasts with fewer thylakoid membranes and no starch grains were formed when illuminated. After a period of prolonged darkness such as 10D, the opposite was the case. PR48.2N etioplasts contained differentiating prolamellar bodies, although still smaller than the prolamellar bodies in 2D wild-type etioplasts, and differentiated into developed chloroplasts with thylakoid membranes and starch grains when illuminated. However, etioplasts in wild-type cotyledons appeared to be much more arrested and chloroplasts with fewer thylakoid membranes were formed when illuminated.

Microarray analysis indicated that a group of seed protein genes involved in late embryogenesis and seed maturation encoding seed storage proteins, oleosins and LEA proteins showed much lower abundance in PR48.2N seedlings (sections 3.8, 3.9 and 3.10) but not in the developing seeds at different stages (section 3.11). There was no apparent difference between PR48.2N and the wild type in the transcript abundance of *CRA1*, *OLEO2* and *LEA76* during the stages of seed development: early embryogenesis (1-5 DAF), seed maturation (8-11 DAF), late embryogenesis (17-21 DAF) and dry seeds (>21 DAF). However, a slight lag appeared to occur in accumulating transcripts of *CRA1* and *OLEO2* in PR48.2N seedlings at the stage of 8-11 DAF corresponding to seed maturation since PR48.2N showed slightly lower transcript abundance of *CRA1* and *OLEO2* during this stage when abundance of the transcripts increases (Parcy *et al.*, 1994).

The kinetics of transcript accumulation of *CRA1*, *OLEO2* and *LEA76* at different seed development stages were similar to findings of some similar studies in *Arabidopsis* previously reported. During early embryogenesis in *Arabidopsis* (1-5 DAF), transcripts encoding seed storage proteins, an oleosin and LEA proteins are not detectable (Parcy *et al.*, 1994) while fatty acid, amino acid and protein contents are low (Baud *et al.*, 2002). During seed maturation (8-11 DAF), transcript levels of genes encoding seed storage proteins, an oleosin and LEA proteins are increasing but the transcripts encoding LEA proteins are not detectable (Parcy *et al.*, 1994). At the same time, fatty acid and protein contents of the developing seeds are increasing (Baud *et al.*, 2002). During late

embryogenesis (17-21 DAF), transcript levels of genes encoding seed storage proteins, an oleosin and LEA proteins reach a plateau (Parcy *et al.*, 1994) while fatty acid and protein contents achieve the highest levels as well (Baud *et al.*, 2002).

CRA1, one of the 12S seed storage protein genes, was demonstrated to have much lower transcript abundance in PR48.2N seedlings and in the light when examined further by RNA-gel-blot analysis (section 3.10). Light grown 7-day-old *Arabidopsis* seedlings also contain fewer transcripts of other seed storage protein, oleosin and LEA protein genes (MacLean, 2005). ABA content of seedlings has been demonstrated to accumulate in the dark and decrease on illumination (Williams *et al.*, 1994; Weatherwax *et al.*, 1996; Riemann *et al.*, 2003) and genes encoding seed storage and LEA proteins are positively regulated by ABA (Chandler and Robertson, 1994). *NEGATIVELY PHYTOCHROME REGULATED (NPR)* genes in duckweed (*Lemna gibba*), which is related to LEA protein genes (Okubara *et al.*, 1993), are negatively regulated by phytochrome action (Okubara and Tobin, 1991; Okubara *et al.*, 1993) but positively regulated by ABA (Williams *et al.*, 1994). The transcription of *NPR* genes is increased in the dark (Okubara and Tobin, 1991) when the level of endogenous ABA is increased (Williams *et al.*, 1994; Weatherwax *et al.*, 1996). Therefore, increase of transcript levels of genes encoding seed storage and LEA proteins in the dark may be regulated by the increased endogenous ABA content. A DOF transcription factor expressed in developing endosperm of maize, barley and wheat (*Triticum aestivum*) is involved in the activation of seed storage proteins (Vicente-Carbajosa *et al.*, 1997; Mena *et al.*, 1998) and its DNA-binding activity can be regulated by light-dependent development (Yanagisawa and Sheen, 1998). In nature, many plants depend on light for seed germination (Casal and Sánchez, 1998). Upon seed germination, seed storage or reserves such as proteins and lipids, accumulated and stored in protein and oil bodies during seed development and maturation, are degraded rapidly by hydrolytic enzymes to provide nutrients for growth of embryos and seedlings that have not started photosynthesis yet (Pernollet, 1978; Huang, 1992; Fujiwara *et al.*, 2002; Penfield *et al.*, 2006b). This was probably why the transcripts encoding seed storage proteins were decreased by the light, which could serve as an environmental cue to start germination. It is already known that transcripts encoding seed storage proteins and LEA proteins are decreased in amount when seeds start to germinate (Higgins, 1984; Bewley, 1997).

Illuminated wild-type seedlings accumulated more chlorophyll and transcripts of nuclear photosynthesis and seed protein genes than PR48.2N seedlings after experiencing a short period of darkness. Under this condition, wild-type chloroplasts contained more thylakoid membrane and starch grains. After prolonged darkness, illuminated PR48.2N seedlings had higher chlorophyll content and transcripts of nuclear photosynthesis genes than the wild type. However, wild-type Ws seedlings accumulated higher amounts of transcripts encoding seed proteins at 10D1L compared to PR48.2N seedlings. Similarly, chloroplasts in PR48.2N seedlings at 10D1L contained more thylakoid membrane and starch grains. The low levels of chlorophyll content and the transcripts of nuclear photosynthesis genes in wild-type seedlings after prolonged darkness could indicate a state of starvation to up-regulate some mechanisms to accumulate more nutritive reserves such as storage proteins and lipids. The mutated gene in PR48.2N may encode a regulatory component in these mechanisms. Chloroplasts in PR48.2N seedlings grown in prolonged darkness appeared to have more starch grains. At 10D1L, PR48.2N accumulated more chlorophyll and transcripts of nuclear photosynthesis genes but not transcripts of seed protein genes. This physiological state could trigger the seedlings to intensify the activity of photosynthesis resulting in more starch grains.

There was a peak of transcript abundance of nuclear photosynthesis and seed protein genes observed in the wild-type Ws seedlings after growing in the dark for 4 days (section 3.12), which is possibly similar to a transient increase in the transcript abundance of *LHCBI* and *RBCS* found in early development of dark-grown *Arabidopsis* seedlings reported by Brusslan and Tobin (1992). This increase was not affected by external factors and therefore thought to be regulated by endogenous development (Brusslan and Tobin, 1992). This light-independent transient increase of transcripts has also been observed for *RBCS* in dark-grown seedlings of cucumber (*Cucumis sativus*) (Walden and Leaver, 1981) and oilseed rape (*Brassica napus*) (Fiebig *et al.*, 1990).

From this study, it was found that a number of seed protein genes such as *CRA1*, *OLEO2* and *LEA76* also showed early light-independent developmental transcript accumulation. The *Arabidopsis* mutant PR48.2N showed lower abundance of the transcripts encoding nuclear photosynthesis or seed protein genes and was found to be defective in this early light-independent developmental transcript accumulation. This

early light-independent developmental transcript accumulation of nuclear photosynthesis or seed protein genes may play a role in the greening process since PR48.2N seedlings showed altered greening phenotypes. The product encoded by the gene mutated in PR48.2N may represent a component of this early light-independent developmental programme.

Transcript abundance of plastid genes has also been observed to follow a similar developmental pattern in the dark. Many plastid genes showed transcript accumulation in the dark in mustard, especially the *trnK* gene for lysyl-tRNA, which shows a transient increase of transcript around 2-3 days in the dark (Fiebig *et al.*, 1990). The early light-independent developmental programme may involve both the plastid and nucleus since plastid transcripts reach a peak in the same leaf sections that show peaks of *RBCS* and *LHC* transcripts in barley (Rapp and Mullet, 1991). Transcripts of plastid and nuclear genes encoding the transcription and translation apparatus are co-regulated during early development and in response to light and plastid-to-nucleus signals in tobacco and *Arabidopsis* (MacLean, 2005). Plastid-to-nucleus signalling responding to the state of plastid protein synthesis occurs during early seedling development (Oelmüller *et al.*, 1986; Bajracharya *et al.*, 1987; Gray *et al.*, 1995) and tetrapyrrole intermediates only function as signal molecules at an early developmental stage of chloroplast development (Gadjieva *et al.*, 2005). Thus, plastid-to-nucleus signalling may be involved in this early light-independent developmental programme.

The promoter regions of the seed protein genes that showed lower transcript levels in PR48.2N relative to wild-type Ws shared some ACGT-containing *cis*-elements similar to ABA response elements (ABRE) (Guiltinan *et al.*, 1990; Skriver *et al.*, 1991; Shen *et al.*, 1993), which contain an ACGT core (Michel *et al.*, 1993) (section 3.13). However, elements similar or identical to the ACGT-containing ABRE especially the palindromic CACGTG G-box identified in section 3.13 have also been identified as *cis*-regulatory elements in the promoters of various genes regulated by a variety of environmental and physiological signals (Guiltinan *et al.*, 1990; Oeda *et al.*, 1991; Williams *et al.*, 1992; Michel *et al.*, 1993). These genes respond to environmental and physiological cues, such as visible (Giuliano *et al.*, 1988; Kao *et al.*, 1996) or UV (Schulze-Lefert *et al.*, 1989) light, auxin (Liu *et al.*, 1994), jasmonic acid (Mason *et al.*, 1993) and salicylic acid (Qin *et al.*, 1994), and cell cycle (Nakayama *et al.*, 1989). The

presence of a second *cis*-element in the vicinity of the G-box, namely CE1 and CE3, has been demonstrated to play an important role to determine the ABA response specificity (Shen and Ho, 1995; Shen *et al.*, 1996). However, ABRE and the coupling elements are functionally equivalent as they are interchangeable (Hobo *et al.*, 1999). This is consistent with the fact that only multiple copies of ABRE can confer ABA-responsiveness (Shen *et al.*, 1993; Shen and Ho, 1995; Vasil *et al.*, 1995). Since both ACGT-containing and CGTGTC CE3-like elements were identified in the upstream regions of genes with lower transcript levels in PR48.2N relative to wild-type Ws, the product encoded by the mutated gene in PR48.2N may play a role in an ABA-related pathway but not other G-box mediated responses.

The ABA content of PR48.2N seedlings was not significantly different to the wild type (section 3.14) and, thus PR48.2N is not likely to be an ABA-deficient mutant. However, the germination of mutant seeds was more sensitive to inhibition by ABA than the wild type (section 3.15). There are a large number of ABA-hypersensitive mutants, affecting a wide range of plant functions (for review, Finkelstein *et al.*, 2002). They include *enhanced response to aba 1 (era1)* (Cutler *et al.*, 1996), *ethylene-insensitive 2 (ein2; allelic to era3)* (Alonso *et al.*, 1999; Beaudoin *et al.*, 2000; Ghassemian *et al.*, 2000), *hyponastic leaves 1 (hyl1)* (Lu and Fedoroff, 2000), *aba hypersensitive 1 (abh1)* (Hugouvieux *et al.*, 2001), *supersensitive to aba and drought 1 (sad1)* (Xiong *et al.*, 2001a), *fiery 1 (fry1)* (Xiong *et al.*, 2001b), *triacylglycerol 1 (tag1)* (Lu and Hills, 2002), *rho of plants 10 (rop10)* (Zheng *et al.*, 2002), *aba-hypersensitive germination (ahg)* (Nishimura *et al.*, 2004), and, more recently, *arabidopsis heterotrimeric g-protein β subunit 1 (agb1)* (Pandey *et al.*, 2006). So far, no ABA-hypersensitive mutant is known to have similar phenotypes to PR48.2N, although *det2*, an *Arabidopsis* mutant hypersensitive to ABA inhibition of germination, exhibits a light-grown phenotype when grown in darkness which could be related to the phenotype of enhanced greening ability of PR48.2N (Steber and McCourt, 2001). In addition, ABI4 appears to play a role in a plastid-to-nucleus signalling pathway responding to plastid gene expression similar to GUN1 (Nott *et al.*, 2006). Expression of seed storage or LEA protein genes have been shown to be down-regulated in developing seeds of mutants insensitive to ABA inhibitory effect on germination (Finkelstein and Somerville, 1990; Kriz *et al.*, 1990; Nambara *et al.*, 1992; Finkelstein, 1993, 1994; Paiva and Kriz, 1994; Parcy *et al.*, 1994; Nambara *et al.*, 2000). In contrast, PR48.2N,

which contained fewer transcripts of seed protein genes in 7-day-old seedlings (section 3.8), showed hypersensitivity to the ABA inhibitory effect on germination (section 3.15). Therefore the components that are responsible for ABA sensitivity during germination, such as components encoded by the mutated genes in those ABA-insensitive mutants and PR48.2N, may also regulate events at different stages of plant development, namely in germinating seeds and in developing seedlings.

In conclusion, the product encoded by the gene mutated in PR48.2N may play a role in the transition from heterotrophic to photoautotrophic growth during early seedling development. In mustard seedlings, activities of marker enzymes of glyoxysomal function (heterotrophy) and leaf-peroxisomal function (photoautotrophy) of peroxisomes are affected in norflurazon-treated seedlings but only the activities of marker enzymes of glyoxysomal function are affected in chloramphenicol-treated seedlings (Bajracharya *et al.*, 1987). Also, studies of *Arabidopsis cue* mutants have suggested that plastid signals contribute to control of *LHC* expression and the greening process (López-Juez *et al.*, 1998). In order to understand further the nature of the gene mutated in PR48.2N, genetic analysis of PR48.2N is necessary and is discussed in the next chapter.

Chapter 4
Genetic analysis of
the PR48.2N mutant

4. Genetic analysis of the PR48.2N mutant

4.1 Introduction

In the previous chapter, PR48.2N was identified as a mutant altered in the process of transition from heterotrophic to photoautotrophic growth during early seedling development. It showed a number of distinct phenotypes, such as altered transcript abundance of nuclear genes encoding photosynthesis and seed proteins, enhanced greening ability and hypersensitivity to ABA inhibition of germination, compared to the wild type. In order to understand further the nature of the gene mutated in PR48.2N, an attempt to identify the precise location of the mutation in the genome was made using PCR-based genetic mapping techniques and is described in this chapter.

Prior to any genetic mapping, it is important to determine whether the mutation is dominant or recessive by examining the phenotypes of the F1 offspring of crosses between the mutant and a wild-type line. For PR48.2N, this analysis will have to be performed on a F2 population because it is not easy to do phenotypic analysis of individual F1 seedlings. The mutated gene in an uncharacterised mutant may be identified by establishing allelism with other known mutants. The analysis can be done by crossing the mutants and examining the phenotypes of F1 or F2 offspring. If the mutations are not allelic, double mutants can be isolated from the F2 offspring and characterised to study the genetic relation between the mutations such as epistasis. Possible allelism between the putative *gun1*-like mutants as described in Chapter 3 and *gun1-1* should be established.

For genetic mapping, the mutant should be crossed with a wild-type line of a different ecotype to obtain the F2 seeds for the analysis so that co-segregation of the mutant phenotype with DNA-based polymorphisms can be examined. In order to produce a F2 mapping population to map a mutation in a line of the Ws background, the *Ler* ecotype can be crossed with the Ws mutant line because the *Ler* ecotype was previously reported to be distantly related to the Ws ecotype among the commonly used *Arabidopsis* ecotypes (Hardtke *et al.*, 1996). Ws in combination with *Ler* enabled the greatest number of restriction fragment length polymorphism (RFLP) markers to be

used to distinguish the ecotypes (Liu *et al.*, 1996) but for other markers such as CAPS (Hauser *et al.*, 1998; Baumbusch *et al.*, 2001), SSLP (Hauser *et al.*, 1998), duplex analysis (Hauser *et al.*, 1998) and amplified fragment-length polymorphism (AFLP) (Peters *et al.*, 2001), Ws in combination with Col enabled the greatest number of markers to be utilised to distinguish the ecotypes. For the amplification refractory mutation system (ARMS), both Ws x *Ler* and Ws x Col combinations have similar numbers of markers that detect polymorphisms between the ecotypes (Hauser *et al.*, 1998).

Various PCR-based co-dominant molecular markers are available for mapping mutations using a F2 mapping population. These markers are useful to determine homozygosity or heterozygosity of the mapping lines, detectable in all developmental stages, and not affected by environmental factors. Cleaved amplified polymorphic sequence (CAPS), simple sequence length polymorphism (SSLP) and single nucleotide polymorphism (SNP) are especially easy-to-use PCR-based co-dominant molecular markers. CAPS is a procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers as described in Konieczny and Ausubel (1993). PCR primers are designed to amplify polymorphic genomic regions and the PCR products are then digested with specific restriction enzymes and separated on an agarose gel. The polymorphisms are detected by examining the difference in the size of DNA fragments due to absence or presence of a restriction site. The use of SSLP markers in *Arabidopsis* mapping capitalising on microsatellite loci was initially reported in Bell and Ecker (1994). These microsatellite repeat sequences are usually polymorphic in different ecotypes because of variations in the number of repeat units. Specific primers are used to amplify a genomic region that contains a polymorphic microsatellite sequence and the size of the amplified product will vary depending on the number of repeats present in a given ecotype. An allele-specific co-dominant PCR-based SNP mapping procedure in *Arabidopsis* was described in Drenkard *et al.* (2000). For a SNP marker distinguishing the different ecotypes used in a genetic mapping, allele-specific primers with specific mismatches at the 3' end were designed to preferentially amplify one of the single nucleotide variations. However under normal PCR conditions, different mismatches located at the 3' end are extended with different efficiencies by *Taq* polymerase and, therefore, an additional base pair change was introduced within the last four bases of some of the primers (Kwok *et al.*, 1990).

4.2 Genetic nature of the mutation in PR48.2N

Prior to the PCR-based genetic mapping of the mutated gene in PR48.2N, the genetic nature of PR48.2N was investigated to determine whether the mutation was dominant or recessive and whether the mutation was in a nuclear gene or a chloroplast gene. The genetic analysis may be done on both F1 and F2 individuals but F2 analysis shows some advantages over F1 analysis because artificial crosses result in small amount of F1 seeds and analysis on the next generation is always needed. F2 seeds from the crosses can be obtained in large number and therefore many analyses can be performed on the same F2 seed population with sufficient sample size. Phenotypes of PR48.2N were mainly seedling characters and, hence, many seeds were required to provide sufficient material for the experimental procedures. The genetic analysis was performed on a F2 population produced from a cross between PR48.2N and a wild type of a different ecotype, *Ler*, so that F2 mapping populations could also be selected from the F2 population as described in section 4.4.

PR48.2N in *Ws* background was crossed with the wild-type *Ler* ecotype, PR48.2N (female) x *Ler* (male), as described in section 2.3. Two crosses were successfully made and 20-30 seeds were obtained from each cross. Since there was the possibility of self pollination during the process of crossing, F1 seeds from the crosses were grown and the heterozygosity of the F1 individuals were confirmed by PCR using molecular markers. The cross between the *Ws* and *Ler* backgrounds in this case was confirmed with one of the CAPS markers described in section 4.4, M235, resulting in two fragments, 0.309 and 0.225 kb, for *Ws* and a 0.534 kb fragment for *Ler* after *Hind*III digestion on the PCR products (section 2.5.6.1). Molecular confirmation with the M235 marker performed on two putative F1 lines, 1 and 2, is shown in Figure 4.1. They both showed heterozygosity for the marker suggesting that the lines were genuine F1 lines of the cross.

The F1 seedlings were allowed to grow to maturity in soil and self to obtain F2 seeds (section 2.2.2) for analysis to confirm the segregation pattern in the F2 population of the expression of the GFP reporter gene originally carried by PR48.2N. The seeds of wild-type *Ws*, PR48.2N and the F2 seeds from line 2, one of the F1 lines of PR48.2N x *Ler* cross as shown in Figure 4.1, were sterilised, plated and stratified as described in

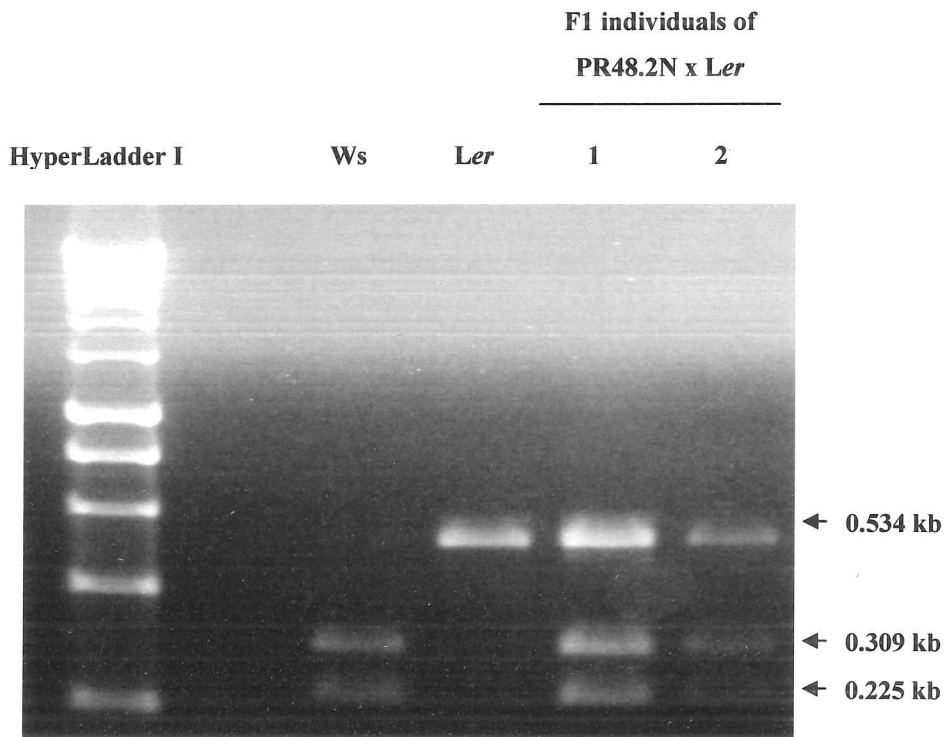


Figure 4.1 Molecular confirmation of PR48.2N crosses with wild-type *Ler*.

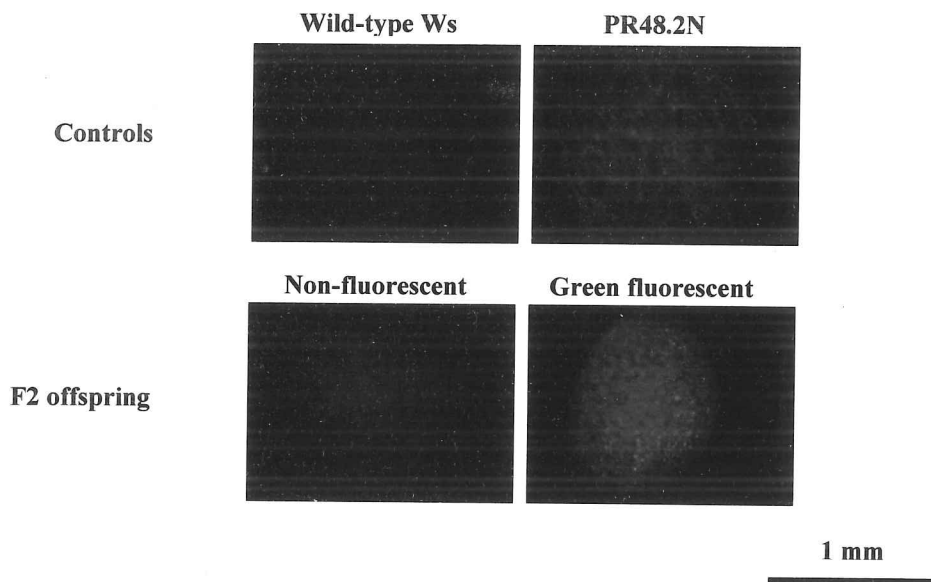
Heterozygosity of F1 individuals from crosses between PR48.2N (*Ws* background) and wild-type *Ler* were confirmed with a CAPS marker, M235, resulting in two fragments, 0.309 and 0.225 kb, for *Ws* and a 0.534 kb fragment for *Ler* after *Hind*III digestion.

sections 2.2.1 and 2.2.2. The seeds were germinated on 0.7 % agar with half-strength MS without any inhibitors of plastid function and grown for 5 days in the light. After 5 days, approximately 100 individual whole seedlings were picked, wet mounted on slides covered with cover slips, and viewed using a Nikon Optiphot 2 epifluorescence microscope with a filter set suitable for the 395 nm excitation and 507 nm emission peaks of GFP5 (Siemering *et al.*, 1996) (section 2.9.2).

The seedlings were observed to show either a non-fluorescent or a green fluorescent phenotype. From the total 105 seedlings scored, 30 (28.6 %) seedlings showed a non-fluorescent phenotype and 75 (71.4 %) seedlings showed a green fluorescent phenotype (Figure 4.2). A two-tailed chi-square test was used to test the observed distributions of phenotypes against the expected 1:3 (non-fluorescent : green fluorescent) distribution for a dominant gene. A P-value lower than the 5 % level was considered significant (*) and P-value higher than the 5 % level was considered not significant (^{ns}) (see section 2.10). The observed phenotypic distribution was not significantly different from the expected distribution at the 5 % level. Thus the GFP reporter gene appeared to segregate genetically as a dominant gene in the F2 population, confirming the hybrid nature of the F1 seedling and that a suitable F2 population had been produced for further analysis.

The inheritance of the mutation in PR48.2N was determined by the GFP expression in the presence of norflurazon or lincomycin in the same F2 population. The GFP detection experiment was repeated exactly except that the seeds of wild-type Ws, PR48.2N and the F2 seeds were germinated on 0.7 % agar with half-strength MS either with 5 μ M norflurazon (LN) or 0.5 mM lincomycin (LL). In these norflurazon- and lincomycin-treated F2 populations, the non-fluorescent or green fluorescent phenotypes were determined by the segregations of two genes, namely the mutated gene in PR48.2N and the GFP reporter gene. Figure 4.3 shows the Punnett square to predict the probability of genotypes and phenotypes of norflurazon- or lincomycin-treated F2 offspring from the PR48.2N x *Ler* cross if the mutation was dominant or recessive. In the case that the mutation in PR48.2N was dominant, all F2 individuals of the cross that were homozygous or heterozygous for the mutant allele in PR48.2N should express GFP except where the GFP reporter gene was absent. Therefore, the non-fluorescent

A



B

Phenotype of F2 offspring (number)		
Non-fluorescent	Green fluorescent	P-value
30 (28.6 %)	75 (71.4 %)	0.3658 ^{ns}

Figure 4.2 Phenotypes of F2 offspring from a PR48.2N x wild-type *Ler* cross in the absence of the inhibitors of plastid functions.

A) Non-fluorescent and green fluorescent phenotypes of 5-day-old F2 offspring from a PR48.2N x wild-type *Ler* cross in the absence of the inhibitors of plastid functions. Seeds were germinated on 0.7 % agar with half-strength MS and grown for 5 days in the light. Cotyledons of the seedlings were viewed for fluorescence using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 950 digital camera. B) Numbers of the F2 offspring showing non-fluorescent and green fluorescent phenotypes. The distribution of phenotypes of the F2 offspring was tested against the expected 1:3 (non-fluorescent : green fluorescent) distribution using two-tailed chi-square test. ^{ns} Not significant at 5 % level.

Dominant

	PG	Pg	pG	pg
PG	PPGG	PPGg	PpGG	PpGg
Pg	PPGg	PPgg	PpGg	Ppgg
pG	PpGG	PpGg	ppGG	ppGg
pg	PpGg	Ppgg	ppGg	ppgg

Non-fluorescent : Green fluorescent = 7:9

Recessive

	PG	Pg	pG	pg
PG	PPGG	PPGg	PpGG	PpGg
Pg	PPGg	PPgg	PpGg	Ppgg
pG	PpGG	PpGg	ppGG	ppGg
pg	PpGg	Ppgg	ppGg	ppgg

Non-fluorescent : Green fluorescent = 13:3

Figure 4.3 Punnett squares to predict the probability of genotypes and phenotypes of norflurazon- or lincomycin-treated F2 offspring from a PR48.2N x wild-type *Ler* cross if the mutation in PR48.2N was dominant or recessive.

Highlighted genotypes were predicted to express GFP protein in the presence of norflurazon or lincomycin (green fluorescent) and all the others were predicted to show non-fluorescent phenotype. P = wild-type allele of mutated gene in PR48.2N and G = GFP gene.

Dominant

	PG	Pg	pG	pg
PG	PPGG	PPGg	PpGG	PpGg
Pg	PPGg	PPgg	PpGg	Ppgg
pG	PpGG	PpGg	ppGG	ppGg
pg	PpGg	Ppgg	ppGg	ppgg

Non-fluorescent : Green fluorescent = 7:9

Recessive

	PG	Pg	pG	pg
PG	PPGG	PPGg	PpGG	PpGg
Pg	PPGg	PPgg	PpGg	Ppgg
pG	PpGG	PpGg	ppGG	ppGg
pg	PpGg	Ppgg	ppGg	ppgg

Non-fluorescent : Green fluorescent = 13:3

Figure 4.3 Punnett squares to predict the probability of genotypes and phenotypes of norflurazon- or lincomycin-treated F2 offspring from a PR48.2N x wild-type *Ler* cross if the mutation in PR48.2N was dominant or recessive.

Highlighted genotypes were predicted to express GFP protein in the presence of norflurazon or lincomycin (green fluorescent) and all the others were predicted to show non-fluorescent phenotype. P = wild-type allele of mutated gene in PR48.2N and G = GFP gene.

and green fluorescent phenotypes were predicted to segregate in a 7:9 ratio. In the case that the mutation in PR48.2N was recessive, only F2 individuals that were homozygous for the mutant allele in PR48.2N should express GFP except where the GFP reporter gene was absent. Therefore, the non-fluorescent and green fluorescent phenotypes were predicted to segregate in a 13:3 ratio.

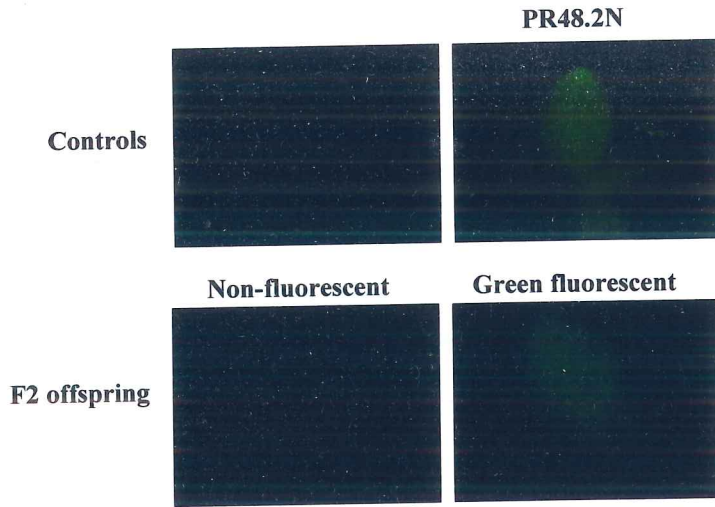
For the lincomycin-treated F2 offspring from the PR48.2N x *Ler* cross, 78 (75 %) seedlings out of the total 104 seedlings scored showed a non-fluorescent phenotype and 26 (25 %) seedlings showed a fluorescent phenotype (Figures 4.4A and B). When the phenotypic distribution of the lincomycin-treated F2 seedlings from the PR48.2N x *Ler* cross was tested against the expected dominant and recessive distributions as shown in Figure 4.3, the observed distribution was not significantly different from the expected recessive distribution at the 5 % level but significantly different from the expected dominant distribution at the 1 % level (Figure 4.4B). Therefore the GFP-expressing phenotype of PR48.2N in the presence of lincomycin appeared to be caused by a recessive mutation.

For the norflurazon-treated F2 offspring, 63 (59.4 %) seedlings out of the total 106 seedlings scored showed a non-fluorescent phenotype and 43 (40.6 %) seedlings showed a green fluorescent phenotype. When the phenotypic distribution of the norflurazon-treated F2 seedlings from the PR48.2N x *Ler* cross was tested against the expected dominant and recessive distributions, the observed distribution was significantly different from the expected distributions at the 1 % level. However, the number of seedlings showing a non-fluorescent phenotype was higher than the number of seedlings showing a fluorescent phenotype. Therefore it was more likely that the phenotype of PR48.2N in the presence of norflurazon was caused by a recessive mutation. That there were more fluorescent seedlings than the expected number in the recessive model could probably be due to poor discrimination between low-level GFP expression and high background fluorescence of wild-type seedlings.

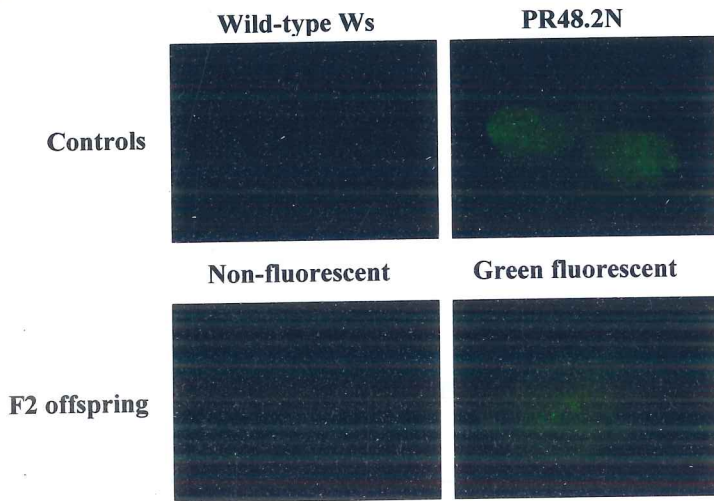
The inheritance pattern of the enhanced greening ability of PR48.2N after prolonged darkness was also investigated in the F2 population of the PR48.2N x *Ler* cross. The F2 seeds from the PR48.2N x *Ler* cross and the seeds of wild-type Ws and PR48.2N were prepared as previously described. The seeds were germinated on 0.7 %

A

Norflurazon



Lincomycin



1 mm

B

Treatment	Phenotype of F2 offspring (number)		P-value	
	Non-fluorescent	Green fluorescent	Dominant	Recessive
Norflurazon	63 (59.4 %)	43 (40.6 %)	0.0009 **	<0.0001 **
Lincomycin	78 (75.0 %)	26 (25.0 %)	<0.0001 **	0.1025 ^{ns}

Continued

C

Phenotype of F2 offspring (number)		P-value	
Wild type	Greening after 6D1L	Dominant	Recessive
151 (70.6 %)	63 (29.4 %)	<0.0001 **	0.1337 ^{ns}

Figure 4.4 Phenotypes of F2 offspring from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin (A and B) or after being grown for 6 days in the dark followed by 1 day in the light (6D1L) (C).

A) Non-fluorescent and green fluorescent phenotypes of 5-day-old F2 offspring from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin. Seeds were germinated on 0.7 % agar with half-strength MS either with 5 μ M norflurazon or 0.5 mM lincomycin and grown for 5 days in the light. Cotyledons of the seedlings were viewed for fluorescence using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 950 digital camera. B) Numbers of the F2 offspring treated with norflurazon or lincomycin showing non-fluorescent and green fluorescent phenotypes. The distributions of phenotypes of the F2 offspring were tested against the expected dominant and recessive distributions of F2 offspring from a PR48.2N x wild-type *Ler* cross as shown in Figure 4.3 using two-tailed chi-square test. ^{ns} Not significant at 5 % level; ** Significant at 1 % level. C) Numbers of the F2 offspring turning green or not after being grown for 6 days in the dark followed by 1 day in the light (6D1L). The distribution of phenotypes of the F2 offspring was tested against the expected 3:1 (wild type : greening) distributions using two-tailed chi-square test. ^{ns} Not significant at 5 % level; ** Significant at 1 % level.

agar with half-strength MS and grown for 6 days in the dark followed by 1 day in the light (6D1L). After 6D1L, the numbers of F2 seedlings with yellow or green cotyledons were counted. All the wild-type Ws seedlings had yellow cotyledons, whereas all the PR48.2N seedlings greened. From the total of 214 F2 seedlings scored, 151 (70.6 %) seedlings had yellow cotyledons, whereas 63 (29.4 %) seedlings turned green (Figure 4.4C). When this phenotypic distribution was tested against the expected dominant (yellow : green = 1:3) and recessive (yellow : green = 3:1) distributions, the observed distribution was not significantly different from the expected recessive distribution at the 5 % level but significantly different from the expected dominant distribution at the 1 % level, suggesting that the enhanced greening phenotype also resulted from a recessive mutation.

Since two of the mutant phenotypes of PR48.2N appeared to behave as recessive characters, it may be concluded that the mutation is in a nuclear gene rather than in a gene in one of the organelle genomes. Therefore, reciprocal crosses were not necessary for genetic studies on PR48.2N.

4.3 Genetic relation between *gun1-1* and PR48.2N

PR48.2N was isolated as a mutant from a collection of putative *gun1-like* mutants. Therefore it was important to establish the genetic relation between PR48.2N and the previously isolated *gun1-1*. There were two possible genetic relations between the genes: the mutants could contain two different alleles of the same gene or the mutations could be in two different genes and therefore were not allelic. F2 individuals of a cross between *gun1-1* and PR48.2N were examined for green fluorescence in the presence of norflurazon or lincomycin. The GFP expression in the presence of the inhibitors would be expected to segregate differently in the F2 population if the mutations in *gun1-1* and PR48.2N were allelic or not.

For the analysis, a *gun1-1* (female) x PR48.2N (male) cross was made as described in section 2.3. Four crosses were successfully made and 20-30 seeds were obtained from each cross. Seeds from putative F1 lines of the cross were grown and confirmed as F1 by PCR for an SSLP marker, G3883-1.4, producing a 1.4 kb fragment for Col and a 0.7 kb fragment for Ws (section 2.5.6.2). Two putative F1 lines, 1 and 2,

confirmed with the SSLP PCR are shown in Figure 4.5. The F1 seedlings were allowed to grow to maturity in soil (section 2.2.2) and self to obtain F2 seeds for this analysis. The seeds of wild-type Ws, PR48.2N and the F2 seeds from line 1, one of the F1 lines of the *gun1-1* x PR48.2N cross as shown in Figure 4.5, were prepared as previously (section 4.2) and germinated on 0.7 % agar with half-strength MS either with 5 μ M norflurazon (LN) or 0.5 mM lincomycin (LL). The GFP detection experiment as described in section 4.2 was repeated exactly.

The F2 seedlings in this experiment showed three distinct phenotypes, namely non-fluorescence, green fluorescence and bright green fluorescence (Figure 4.7A). For norflurazon-treated F2 offspring from the *gun1-1* x PR48.2N cross, 92 (80 %) seedlings out of the total 115 seedlings scored showed a non-fluorescent phenotype, whereas 18 (20 %) individuals showed a green fluorescent phenotype. Among the individuals showing green fluorescence, there were 5 (4 %) seedlings showing bright green fluorescence that was approximately 2- to 3-fold brighter compared to the fluorescent seedlings (Figure 4.7). For the lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross, 90 (79 %) seedlings out of the total 114 seedlings scored showed the non-fluorescent phenotype, whereas 18 (21 %) individuals showed green fluorescence. Again, there were 6 (5 %) seedlings having bright green fluorescence.

Figure 4.6 shows a Punnett square to predict the probability of genotypes and phenotypes of norflurazon or lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross if the recessive mutations were not allelic. Since mutation in PR48.2N behaved as a recessive mutation as discussed in section 4.2 and *gun1-1* is a recessive mutation as reported previously (Susek *et al.*, 1993), predictions for both phenotypic distributions of allelism or non-allelism were based on the nature of recessive mutants. In the case that the mutations in *gun1-1* and PR48.2N were not allelic, the genes mutated in PR48.2N and *gun1-1*, and the GFP reporter gene would be segregating in the norflurazon or lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross. Therefore, the non-fluorescent and green fluorescent phenotypes were predicted to segregate in a 43:21 ratio in the norflurazon or lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross. If the mutations in *gun1-1* and PR48.2N were allelic, all F2 individuals of the cross between the mutants would be *gun1* mutants and only the GFP

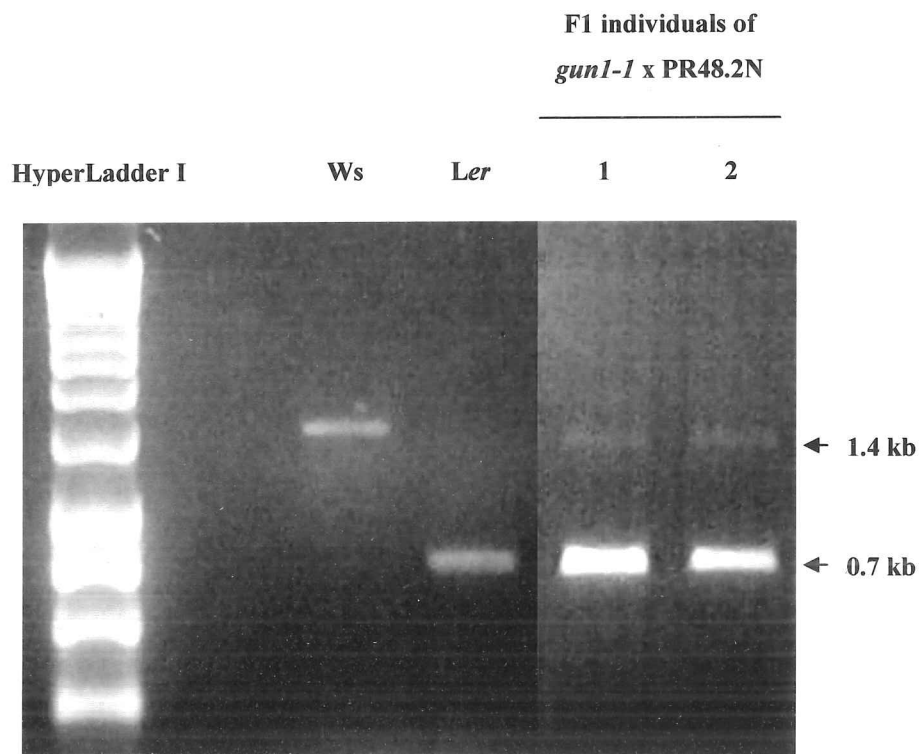


Figure 4.5 Molecular confirmation of PR48.2N crosses with *gun1-1*.

Heterozygosity of F1 individuals from crosses between *gun1-1* (Col background) and PR48.2N (Ws background) were confirmed using an SSLP marker, G3883-1.4, resulting in a 1.4 kb fragment for Col and a 0.7 kb fragment for Ws.

	NPG	NPg	NpG	Npg	nPG	nPg	npG	npg
NPG	NNPPGG	NNPPGg	NNPpGG	NNPpGg	NnPPGG	NnPPGg	NnPpGG	NnPpGg
NPg	NNPPGg	NNPPgg	NNPpGg	NNPpgg	NnPPGg	NnPPgg	NnPpGg	NnPpgg
NpG	NNPpGG	NNPpGg	<u>NNppGG</u>	<u>NNppGg</u>	NnPpGG	NnPpGg	<u>NnppGG</u>	<u>NnppGg</u>
Npg	NNPpGg	NNPpgg	<u>NnppGg</u>	NNppgg	NnPpGg	NnPpgg	<u>NnppGg</u>	Nnppgg
nPG	NnPPGG	NnPPGg	NnPpGG	NnPpGg	<u>nnPPGG</u>	<u>nnPPGg</u>	<u>nnPpGG</u>	<u>nnPpGg</u>
nPg	NnPPGg	NnPPgg	NnPpGg	NnPpgg	<u>nnPPGg</u>	nnPPgg	<u>nnPpGg</u>	nnPpgg
npG	NnPpGG	NnPpGg	<u>NnppGG</u>	<u>NnppGg</u>	<u>nnPpGG</u>	<u>nnPpGg</u>	<u>nnppGG</u>	<u>nnppGg</u>
npg	NnPpGg	NnPpgg	<u>NnppGg</u>	Nnppgg	<u>nnPpGg</u>	nnPpgg	<u>nnppGg</u>	nnppgg

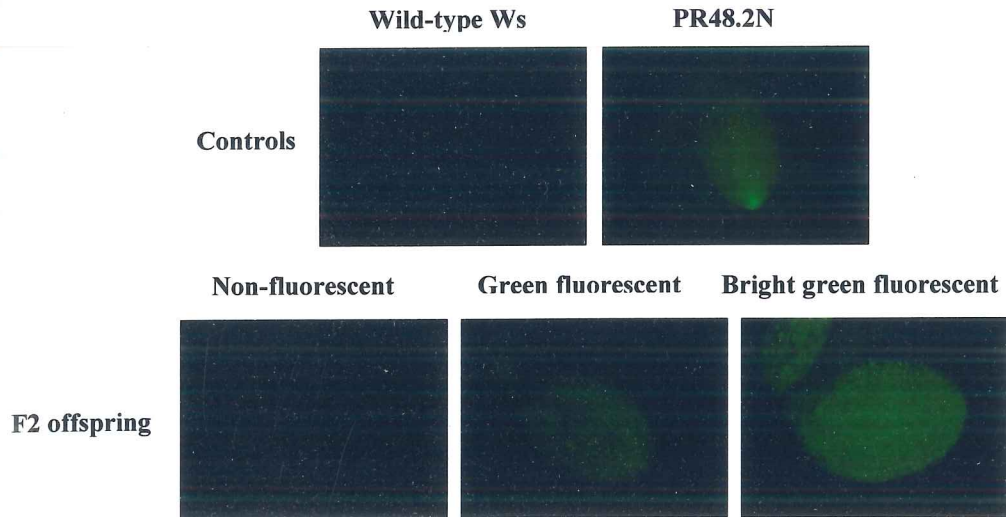
Non-fluorescent : Green fluorescent = 43:21

Figure 4.6 Punnett squares to predict the probability of genotypes and phenotypes of norflurazon or lincomycin-treated F2 offspring from a *gun1-1* x PR48.2N cross if the mutations were non-allelic.

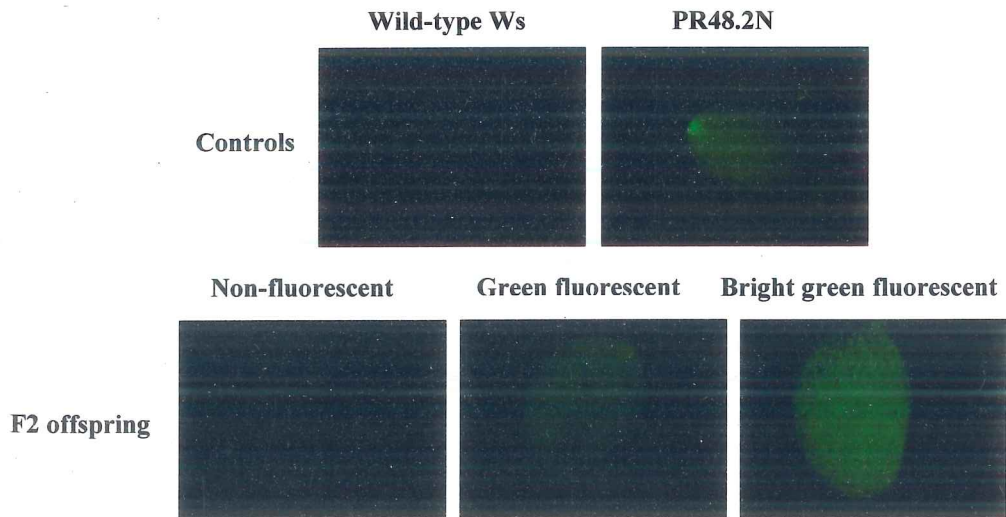
Highlighted genotypes were predicted to express GFP protein in the presence of norflurazon or lincomycin (green fluorescent) and all the others were predicted to show a non-fluorescent phenotype. Underlined genotypes were predicted to be double mutants of *gun1-1* and PR48.2N. N = *GUN1-1* allele, P = wild-type allele of the mutated gene in PR48.2N and G = GFP gene.

A

Norflurazon



Lincomycin



1 mm



Continued

B

Treatment	Phenotype of F2 Offspring (number)			P-value	
	Non-fluorescent	Green fluorescent	Bright green fluorescent	Allelic	Non-allelic
Norflurazon	92 (80 %)	18 (16 %)	5 (4 %)	<0.0001 **	0.0029 **
Lincomycin	90 (79 %)	18 (16%)	6 (5 %)	<0.0001 **	0.0093 **

Figure 4.7 Phenotypes in F2 offspring from a *gun1-1* x PR48.2N cross treated with norflurazon or lincomycin.

A) Non-fluorescent, green fluorescent and bright green fluorescent phenotypes of 5-day-old F2 offspring from a *gun1-1* x PR48.2N cross treated with norflurazon or lincomycin. Seeds were germinated on 0.7 % agar with half-strength MS either with 5 μ M norflurazon or 0.5 mM lincomycin and grown for 5 days in the light. Cotyledons of the seedlings were viewed for fluorescence using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 950 digital camera. B) Numbers of the F2 offspring treated with norflurazon or lincomycin showing non-fluorescent, green fluorescent and bright green fluorescent phenotypes. The distributions of phenotypes of the F2 offspring were tested against the expected allelic (non-fluorescent : green fluorescent = 1:3) and non-allelic distributions of F2 offspring from a *gun1-1* x PR48.2N cross as shown in Figure 4.6 using two-tailed chi-square test. ** Significant at 1 % level.

reporter gene would segregate in the F2 population in a dominant pattern as shown in section 4.2. The non-fluorescent and green fluorescent phenotypes were expected to segregate in 1:3 ratio in the norflurazon or lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross if the recessive mutations were allelic.

The observed phenotypic distributions of norflurazon- and lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross were similar. About 80 % of the F2 seedlings showed a non-fluorescent phenotype, whereas only 20 % of the seedlings showed green fluorescence. There were also 5-6 % norflurazon or lincomycin-treated F2 seedlings showing bright green fluorescence. When the phenotypic distribution of norflurazon-treated F2 offspring from the *gun1-1* x PR48.2N cross was tested against the expected distributions of allelism and non-allelism, the observed distribution was significantly different at the 1 % level from the expected distributions, although the P-value for testing against the non-allelic distribution, namely 0.0029, was much higher (Figure 4.7B). Similarly when the phenotypic distribution of lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross was tested against the expected distributions of allelism and non-allelism, the observed distribution was significantly different at the 1 % level from the expected distributions although the P-value for testing against the non-allelic distribution, namely 0.0093, was much higher.

These observed phenotypic distributions were significantly different from the expected distributions for both allelism and non-allelism because there were too many non-fluorescent seedlings. However, the observed phenotypic distributions seemed to fit better into the expected phenotypic distributions of non-allelism compared to the expected phenotypic distributions of allelism because the P-values for testing against the non-allelic distribution and the numbers of seedlings expressing the non-fluorescent phenotype were higher. Since the observed phenotypic distributions of norflurazon- and lincomycin-treated F2 offspring from the *gun1-1* x PR48.2N cross did not fit into any expected distributions, the genes mutated in *gun1-1* and PR48.2N may be interacting.

The possible interaction between the genes mutated in *gun1-1* and PR48.2N could also be supported by the existence of some seedlings showing bright green fluorescence in the F2 population from the *gun1-1* x PR48.2N cross. The fluorescent seedlings with different brightness were not due to the homozygosity or heterozygosity

of the GFP reporter gene because F2 seedlings with brighter fluorescence were not observed in the analysis in section 4.2. These seedlings with bright fluorescence constituted 5-6 % of the F2 population from the *gun1-1* x PR48.2N cross. This was close to the expected probability of the presence of double mutants in the non-allelic distribution of phenotypes (Figure 4.6), which was 3 in 64. Therefore, these seedlings with bright green fluorescence may be double mutants of *gun1-1* and PR48.2N. The fact that these putative double mutants showed bright green fluorescence, which was about 2- to 3-fold brighter than the average green fluorescent seedlings, suggests a possibility that *GUNI-1* and the gene mutated in PR48.2N were two different genes encoding products that were synergistically responsible for the phenotype of decreased transcript abundance of nuclear photosynthesis genes in the presence of norflurazon and lincomycin.

In summary, the mutations in *gun1-1* and PR48.2N were likely to be not allelic. They might be in two different genes encoding products that are synergistically responsible for the phenotype of decreased transcript abundance of nuclear photosynthesis genes in the presence of inhibitors of plastid functions such as norflurazon and lincomycin.

4.4 Genetic mapping of PR48.2N

This section describes an attempt to map the precise location of the mutation in PR48.2N in the *Arabidopsis* genome using PCR-based markers. As the mutation in PR48.2N was a recessive mutation, the F2 mapping population should constitute the homozygotes of the allele of the mutated gene in PR48.2N selected from the F2 population of the PR48.2N x *Ler* cross as described in section 4.2. Among the phenotypes investigated as described above, GFP expression in the presence of norflurazon and enhanced greening ability at 6D1L were chosen as the phenotypic markers to select for homozygotes of PR48.2N from the F2 population of the PR48.2N x *Ler* cross. The F2 mapping lines were not selected using lincomycin because it was much more difficult to rescue the F2 seedlings from the treatment. It was also much more difficult to rescue the F2 seedlings from the 6D1L treatment.

Genetic mapping to locate the mutation in PR48.2N to a chromosome arm using the F2 mapping populations selected from the F2 population of a PR48.2N x *Ler* cross (section 4.2) was performed. The mapping lines were rescued from treatments of norflurazon or 6D1L in the selection process and allowed to grow to maturity in soil (section 2.2.2). Twenty four F2 mapping lines selected as green fluorescent individuals in the presence of norflurazon (GFP-selected) and five F2 mapping lines selected as greened individuals at 6D1L (greening-selected) (section 4.2) were used in this analysis. DNA was extracted from the mature leaves of the F2 mapping lines as described in section 2.5.1.

In a preliminary round of genetic mapping, fifteen CAPS markers, namely G2395, UFO, GAPB, NPR1 and PAB5 for chromosome I, RNS1 and GPA1 for chromosome II, G4711 and PUR5 for chromosome III, GA1.1, TGCAPS2 and PRHA for chromosome IV, and N97067, LMYC6 and G2368 for chromosome V, and 2 SSLP markers, namely BIO2 for chromosome II and NGA76 for chromosome I, were used with 24 GFP-selected F2 mapping lines. These markers were approximately 30 cM apart from one another covering all 10 arms of *Arabidopsis* chromosomes (Figure 4.8). CAPS and SSLP PCR procedures were performed on the DNA extracted from the F2 mapping lines as described in sections 2.5.6.1 and 2.5.6.2. Electrophoresis gels for the products of CAPS and SSLP PCR of *Ws*, *Ler* and 24 GFP-selected F2 mapping lines are shown in Figure 4.9.

Molecular markers genetically linked to the mutation in PR48.2N should show a strong bias against the *Ler* allele and in favour of *Ws* allele, whereas unlinked markers should show no such bias. The linked markers should co-segregate with the mutation and therefore show fewer recombination events between the markers and the mutation. In addition, the markers tightly linked to the GFP transgene would be expected to show low recombination frequencies, which is about 33 %, but not as low as the recombination frequencies shown by the markers tightly linked to the mutation because the GFP-selected F2 mapping lines were selected as seedlings showing green fluorescence in the presence of norflurazon, so the lines should be homozygous or heterozygous for the dominant GFP reporter gene but homozygous for the recessive mutation in PR48.2N (section 4.2). A GFP transgene had been identified in a region on chromosome II that is approximately 2400 kbp north of GPA1 (A Cottage and E Mott,

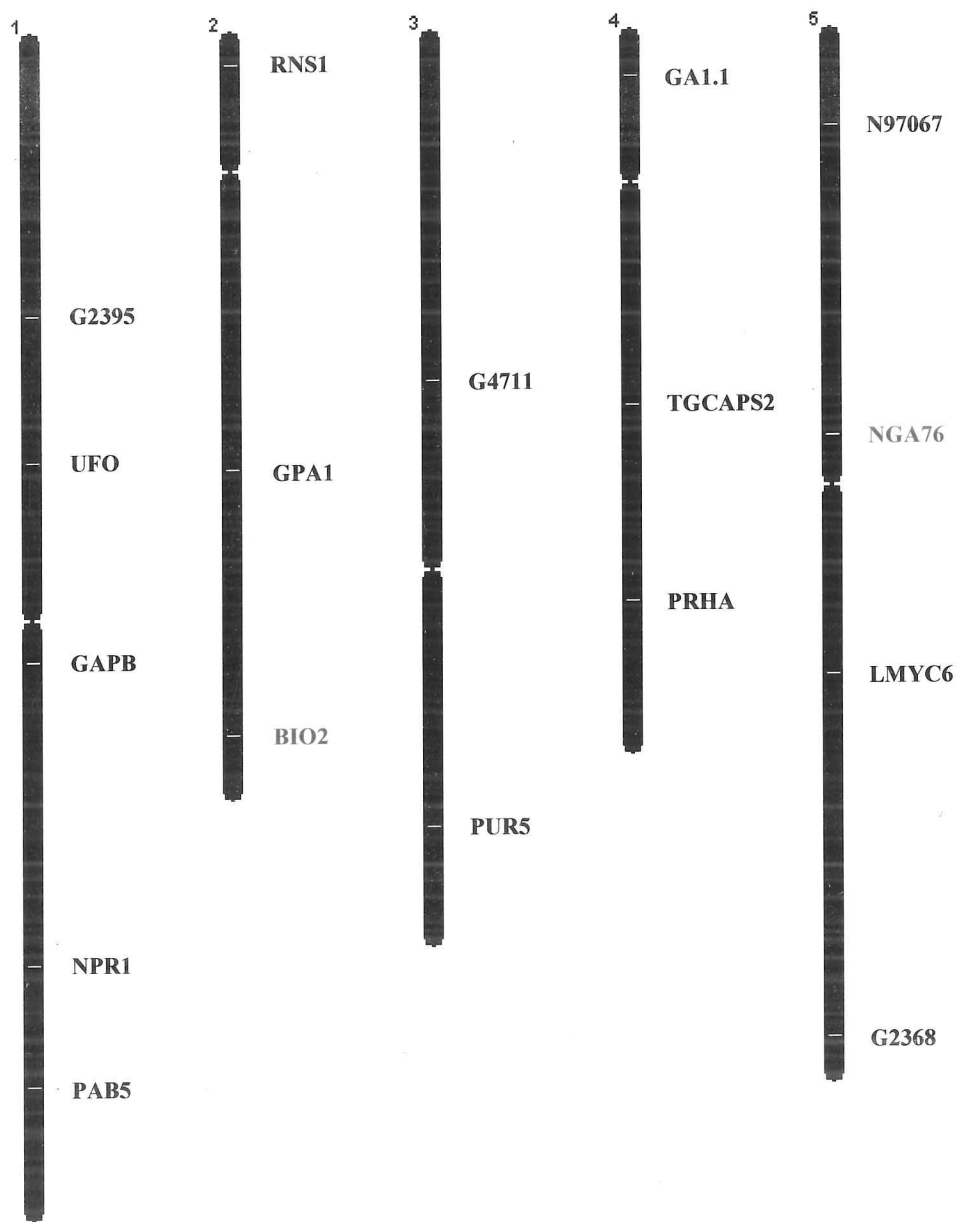


Figure 4.8 PCR-based molecular markers for mapping mutations to *Arabidopsis* chromosome arms.

Seventeen PCR-based molecular markers belonging to CAPS (coloured black) and SSCP (coloured purple) were selected for mapping mutations to *Arabidopsis* chromosome arms. These markers are approximately 30 cM apart from one another covering all 10 arms of *Arabidopsis* chromosomes.

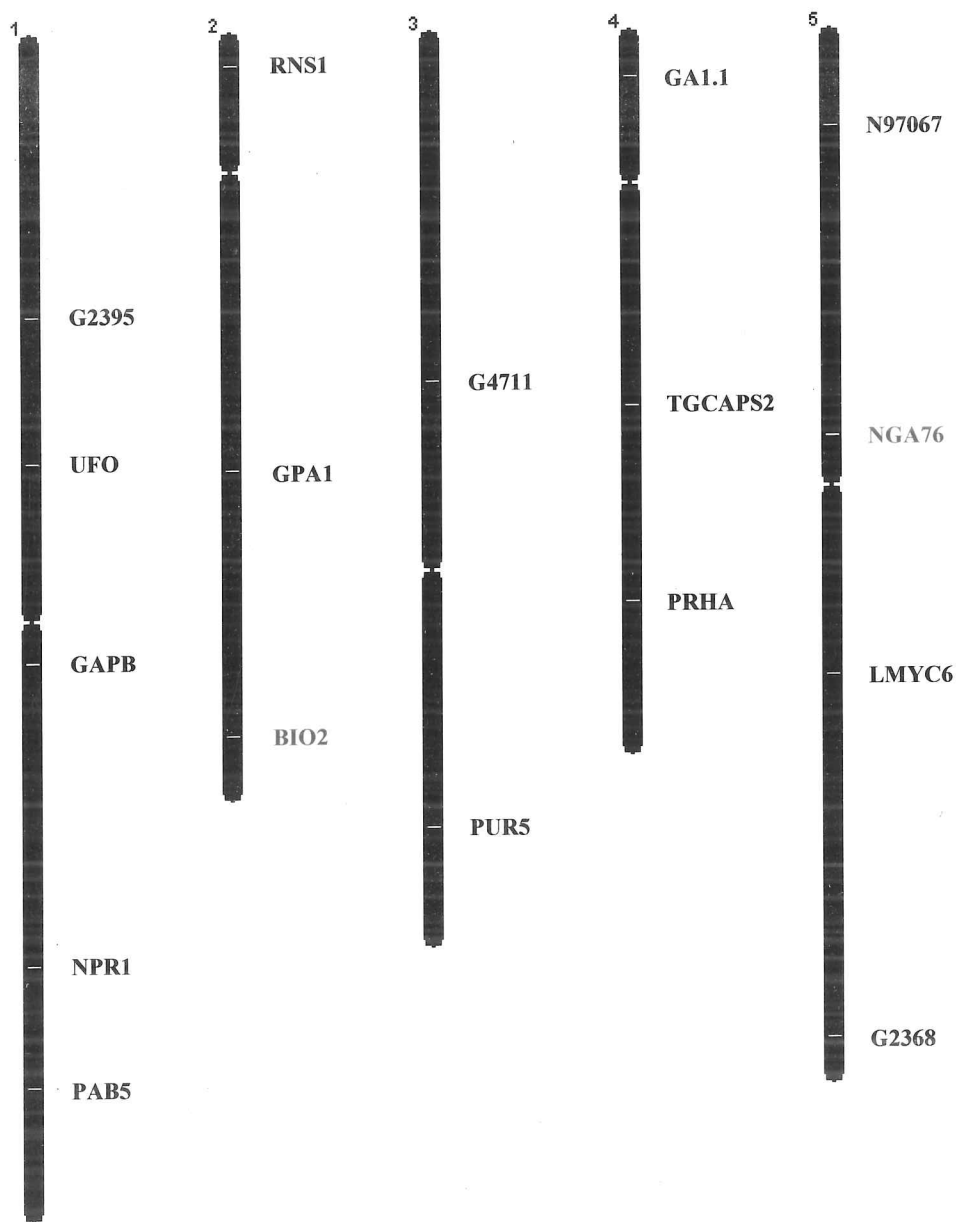
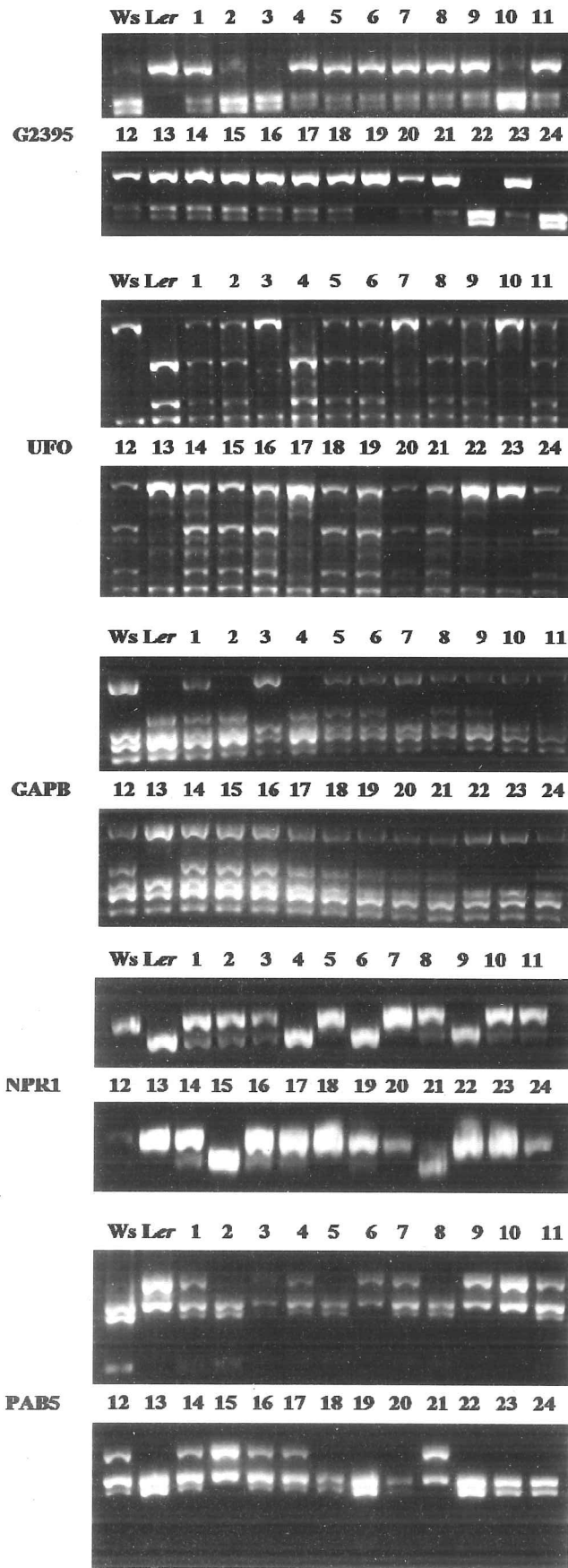


Figure 4.8 PCR-based molecular markers for mapping mutations to *Arabidopsis* chromosome arms.

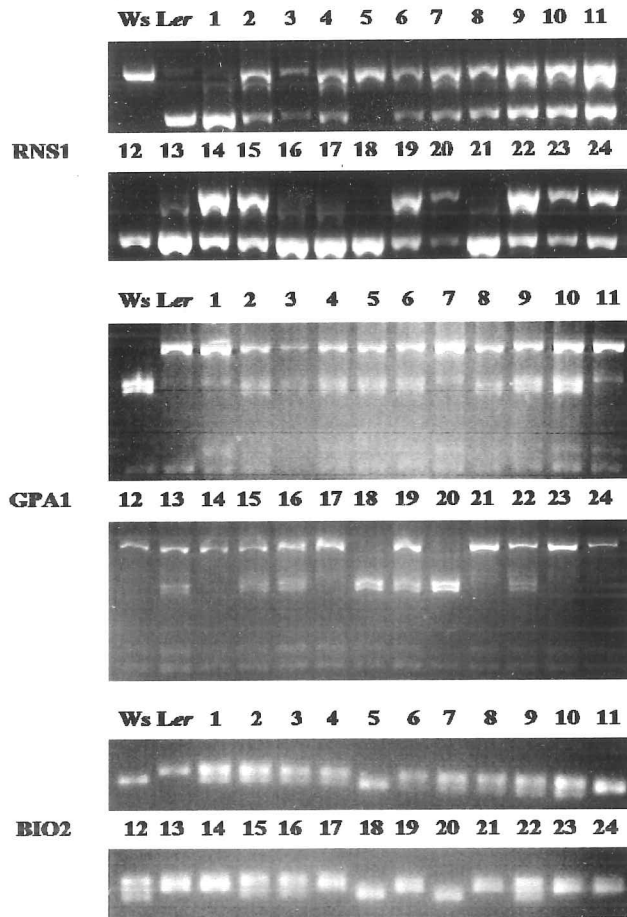
Seventeen PCR-based molecular markers belonging to CAPS (coloured black) and SSCP (coloured purple) were selected for mapping mutations to *Arabidopsis* chromosome arms. These markers are approximately 30 cM apart from one another covering all 10 arms of *Arabidopsis* chromosomes.

Chromosome I

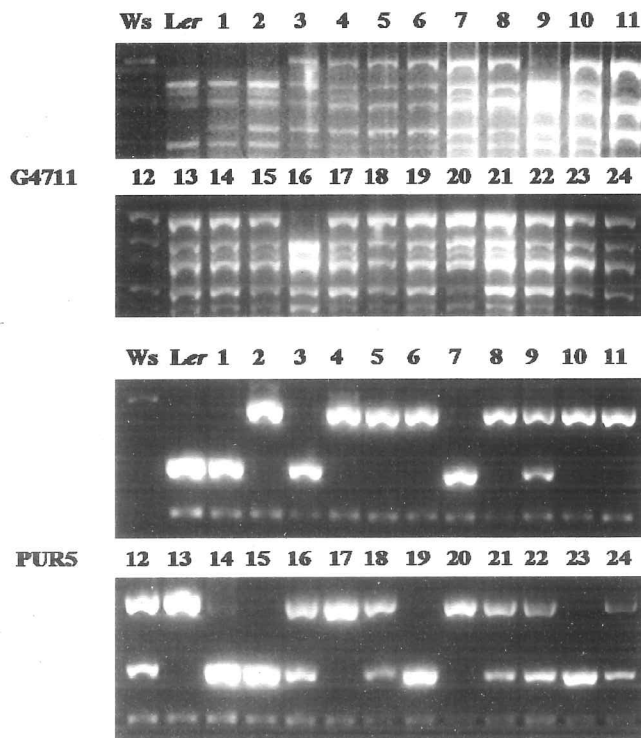


Continued

Chromosome II

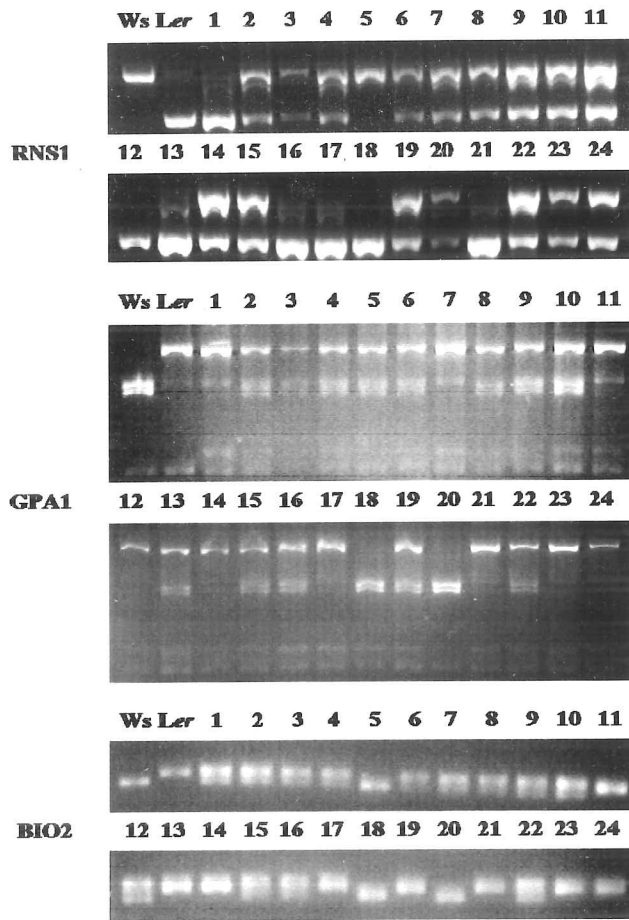


Chromosome III

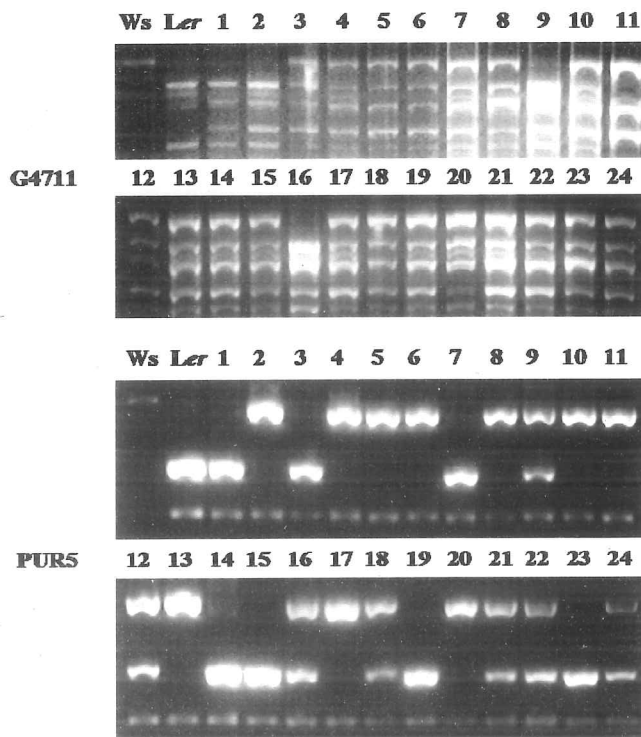


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Chromosome II

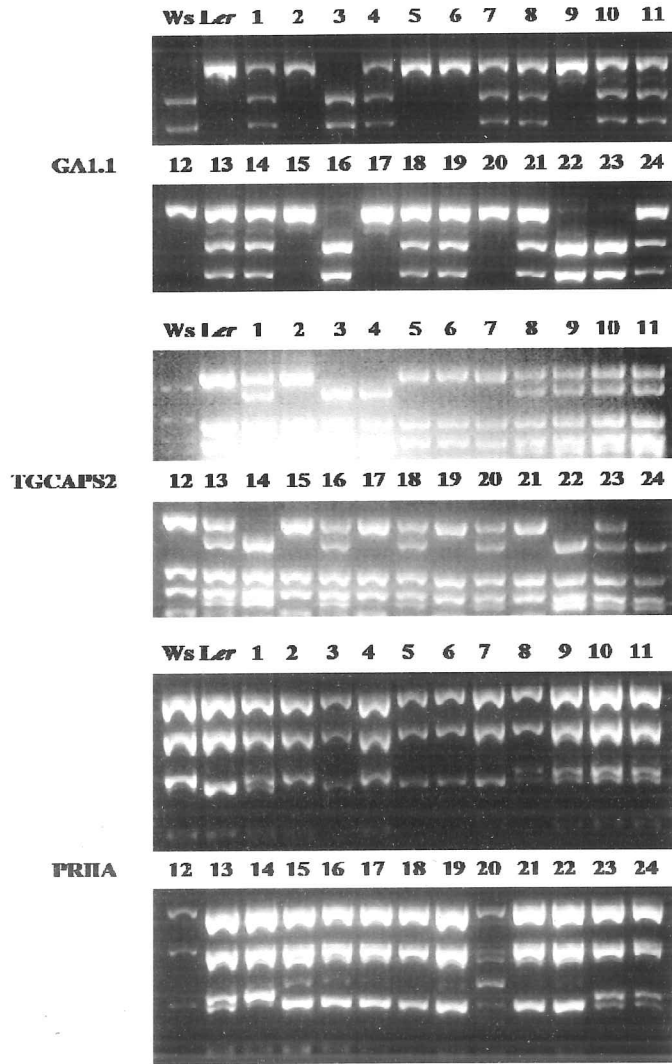


Chromosome III



Continued

Chromosome IV



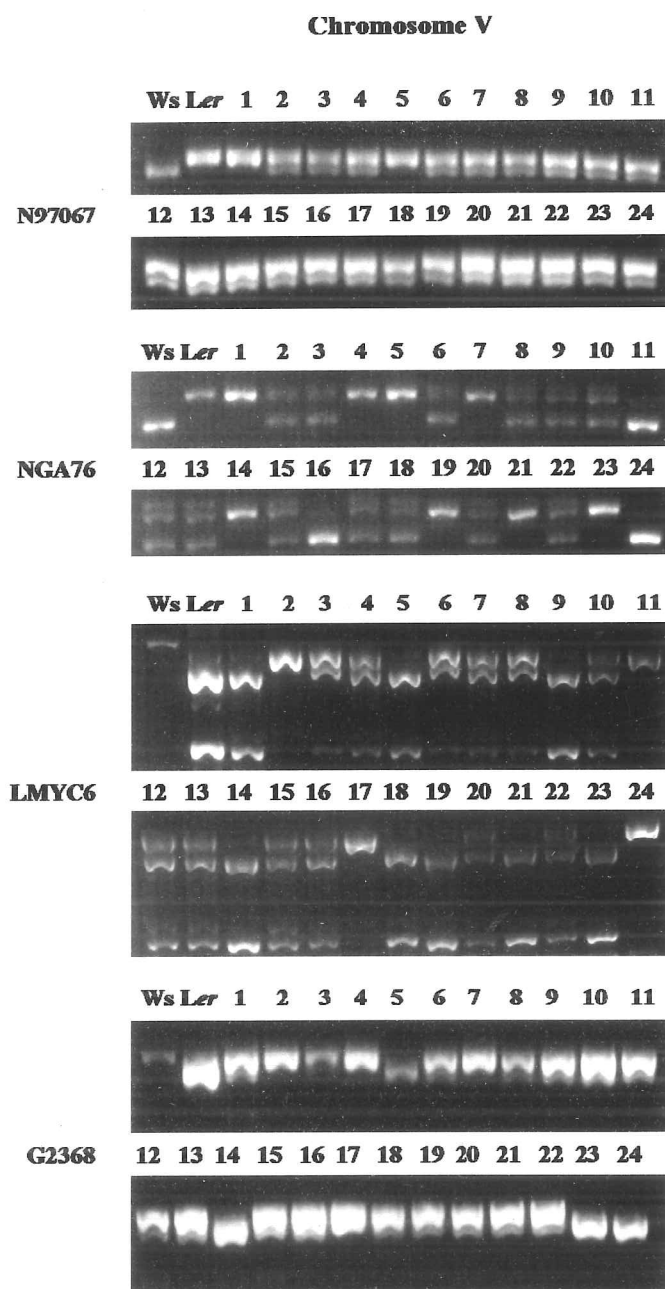


Figure 4.9 Genetic mapping of the mutation in PR48.2N using PCR-based molecular markers.

PCR-based genetic mapping of the mutation in PR48.2N using seventeen CAPS and SSLP markers covering five *Arabidopsis* chromosomes as shown in Figure 4.8 on a GFP-selected F2 mapping population (24 individuals) from a PR48.2N x wild-type *Ler* cross. Restriction enzymes and fragment sizes were provided in Appendix I.

personal communication) by a PCR walking procedure (Cottage *et al.*, 2001). Frequencies of recombination between the mutation in PR48.2N and the molecular markers were calculated as the percentages of *Ler* alleles present for the markers over the total number of chromosomes examined.

Genotypes of the GFP-selected F2 mapping lines for the PCR-based molecular markers and genetic linkages (recombination percentages) of the markers to the mutation in PR48.2N are tabulated in Table 4.1. The highest recombination frequency, namely 70.8 %, was shown by a CAPS marker, PRHA, on chromosome IV. In contrast, the lowest recombination frequency, namely 37.5 %, was shown by a CAPS marker, UFO, on chromosome I. Another region on chromosome III represented by a CAPS marker, PUR5, also had a low recombination frequency of 43.8 %. A region on chromosome V represented by N97067 showed an interesting enrichment of heterozygosity with 22 heterozygotes out of 24 F2 mapping lines analysed. In theory, the maximum recombination frequency for an unlinked marker segregating randomly should be 50 %. However, there were a number of molecular markers showing recombination frequencies higher than 50 % in this experiment. This is likely to be due to two possible reasons: first the small sample size (24 F2 mapping lines) and second, there may be some advantages provided by the *Ler* alleles in some chromosome regions for the plants to survive better, leading to over-representation of *Ler* alleles in those regions.

Since two regions, one on chromosome I and the other one on chromosome III, showed the lowest recombination frequencies, more molecular markers around those regions were used. Fewer mapping lines (12-24 lines) were used for a quicker analysis. For M235 to the north of UFO on chromosome I, the recombination frequency increased to 41.8% (24 mapping lines) compared to UFO and the recombination frequency shown by 7G6 to the south of UFO on chromosome I decreased to 32.1 % (14 mapping lines) compared to UFO. For PRC6 and AP3-LINKED that were near PUR5 on chromosome III, both CAPS markers displayed similar recombination frequencies, namely 40.6 % and 43.8 % (16 mapping lines), compared to PUR5 (Figures 4.10A, B, 4.11A and B; Table 4.2). This suggests that the region on chromosome I was more likely to be the region that contains the mutation.

Table 4.1 Genotypes of GFP-selected F2 mapping individuals for seventeen PCR-based molecular markers and genetic linkages (recombination percentages) of the markers to the mutation in PR48.2N.

Genotypes of twenty four GFP-selected F2 mapping individuals from a PR48.2N x wild-type *Ler* cross for seventeen CAPS and SSLP markers covering five *Arabidopsis* chromosomes were determined as shown in Figure 4.9. Recombination percentages of the markers to the mutation in PR48.2N were calculated as the percentages of *Ler* alleles present for the markers over the total number of chromosomes examined. W = homozygote of *Ws* allele, H = heterozygote and L = homozygote of *Ler* allele.

Marker	Recombination percentage (%)	F2 mapping individual (genotype)																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chromosome I:																									
G2395	41.7	H	W	W	H	H	H	H	H	H	W	H	H	H	H	H	H	H	L	H	H	W	H	W	
UFO	37.5	H	H	W	L	H	H	W	H	H	W	H	H	W	H	H	H	W	H	H	H	W	W	H	
GAPB	39.6	H	L	W	L	H	H	W	H	H	W	W	H	W	H	H	H	H	H	H	H	W	W	H	
NPR1	45.8	H	H	H	L	W	L	W	H	L	H	H	H	W	H	L	H	H	W	H	W	L	W	H	
PAB5	39.6	H	W	H	H	W	L	H	W	L	L	H	H	W	H	L	H	H	W	W	W	L	W	W	
Chromosome II:																									
RNS1	62.5	L	H	H	H	W	H	H	H	H	H	L	L	H	H	L	L	L	H	H	L	H	H	H	
GPA1	64.6	L	H	H	H	H	H	L	H	H	H	L	L	H	L	H	H	L	W	H	W	L	H	L	
BIO2	60.4	H	H	H	H	W	H	H	H	H	H	L	H	L	L	H	H	L	W	L	W	L	H	L	
Chromosome III:																									
G4711	56.3	L	L	H	H	H	H	H	H	L	H	W	H	H	H	H	L	H	H	H	H	H	H	H	
PUR5	43.8	L	W	L	W	W	W	L	W	H	W	W	H	W	L	L	H	W	H	L	W	H	H	L	

Continued

Chromosome IV:

GA1.1	58.3	H	L	W	H	L	L	H	H	L	H	H	L	H	H	L	W	L	H	H	L	H	W	W	H
TGCAPS2	58.3	H	L	W	W	L	L	L	H	H	H	H	L	H	W	L	H	L	H	L	H	L	W	H	W
PRHA	70.8	H	W	L	W	L	L	L	H	H	H	H	L	H	W	L	L	L	L	L	L	L	L	H	H

Chromosome V:

N97067	54.2	L	H	H	H	L	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H	H
NGA76	60.4	L	H	H	L	L	H	L	H	H	H	W	H	H	L	H	W	H	H	L	H	L	H	L	W
LMYC6	58.3	L	W	H	H	L	H	H	H	L	H	W	H	H	L	H	H	W	L	L	H	L	H	L	W
G2368	50.0	H	W	H	W	L	H	W	H	H	H	H	H	H	L	H	H	W	H	H	H	H	H	L	L

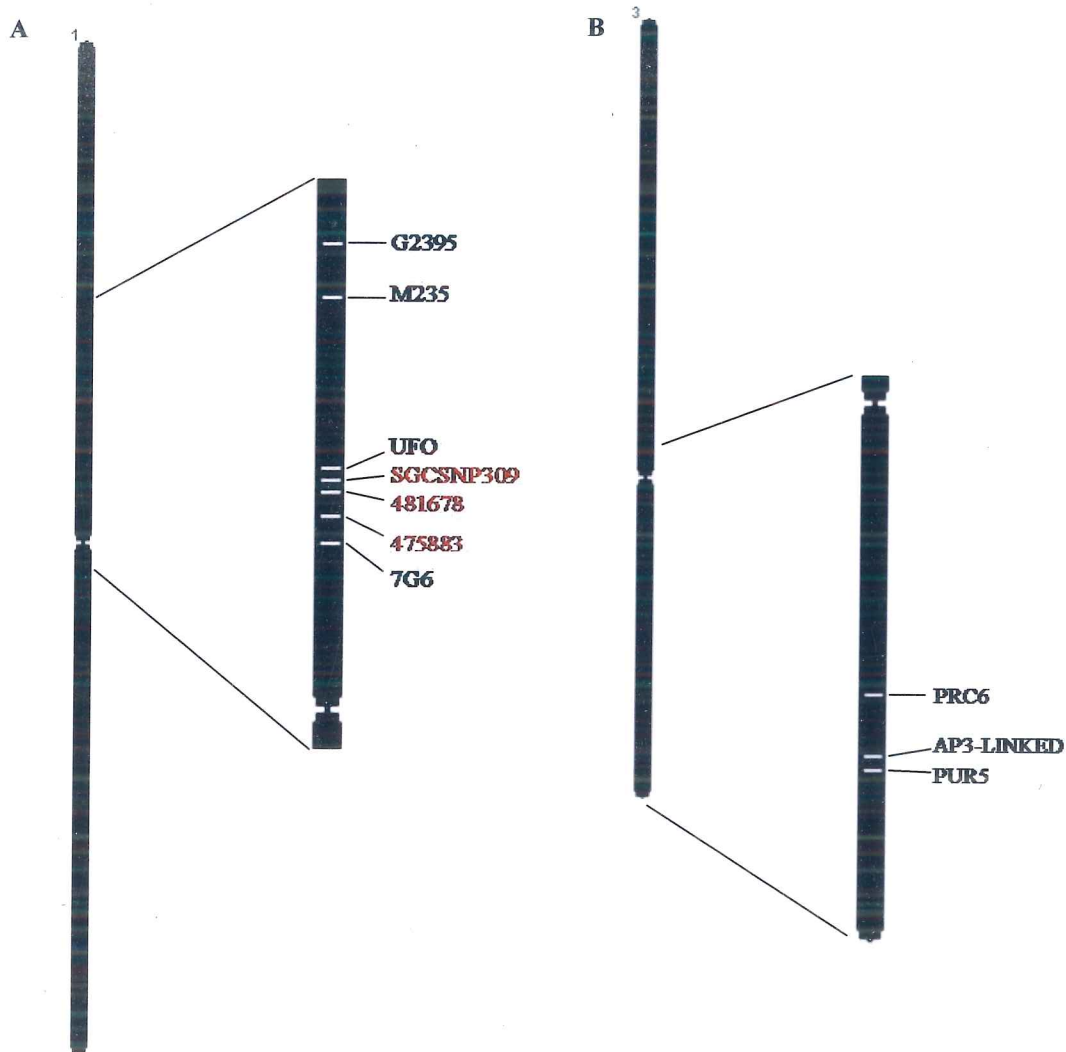
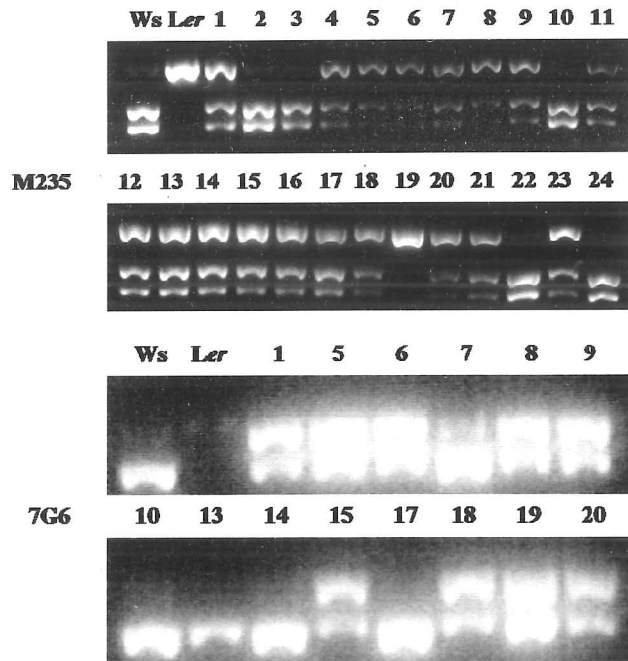


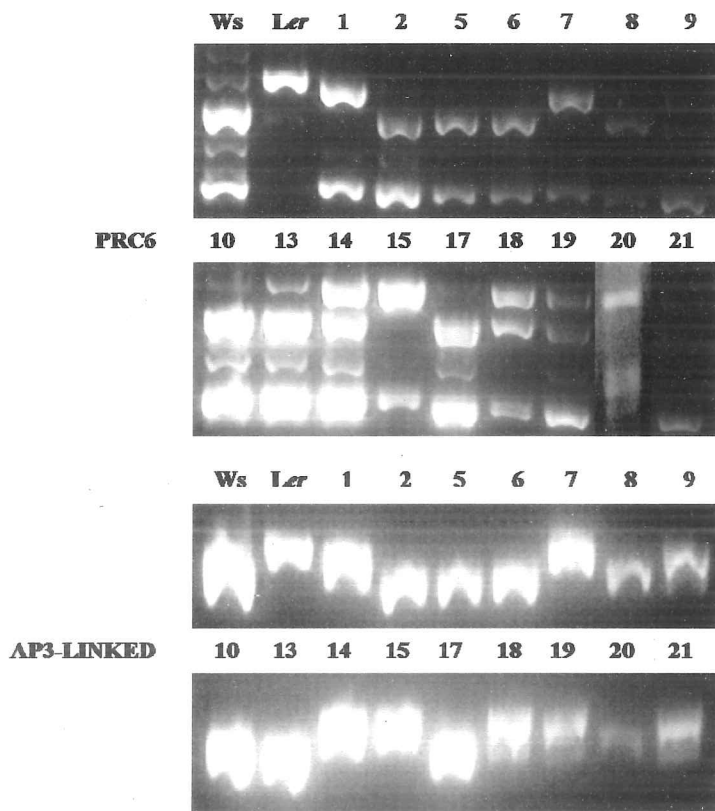
Figure 4.10 PCR-based molecular markers for mapping the mutation in PR48.2N in possible regions of *Arabidopsis* chromosomes I (A) and III (B).

A) Five additional PCR-based CAPS (coloured black) or SNP (coloured red) molecular markers were selected for mapping the mutation in PR48.2N in a possible region of *Arabidopsis* chromosome I near G2395 and UFO as shown in Figure 4.8. B) Two additional CAPS markers were selected for fine-mapping the mutation in PR48.2N in a possible region of *Arabidopsis* chromosome III around PUR5 as shown in Figure 4.8.

A



B



Continued

C

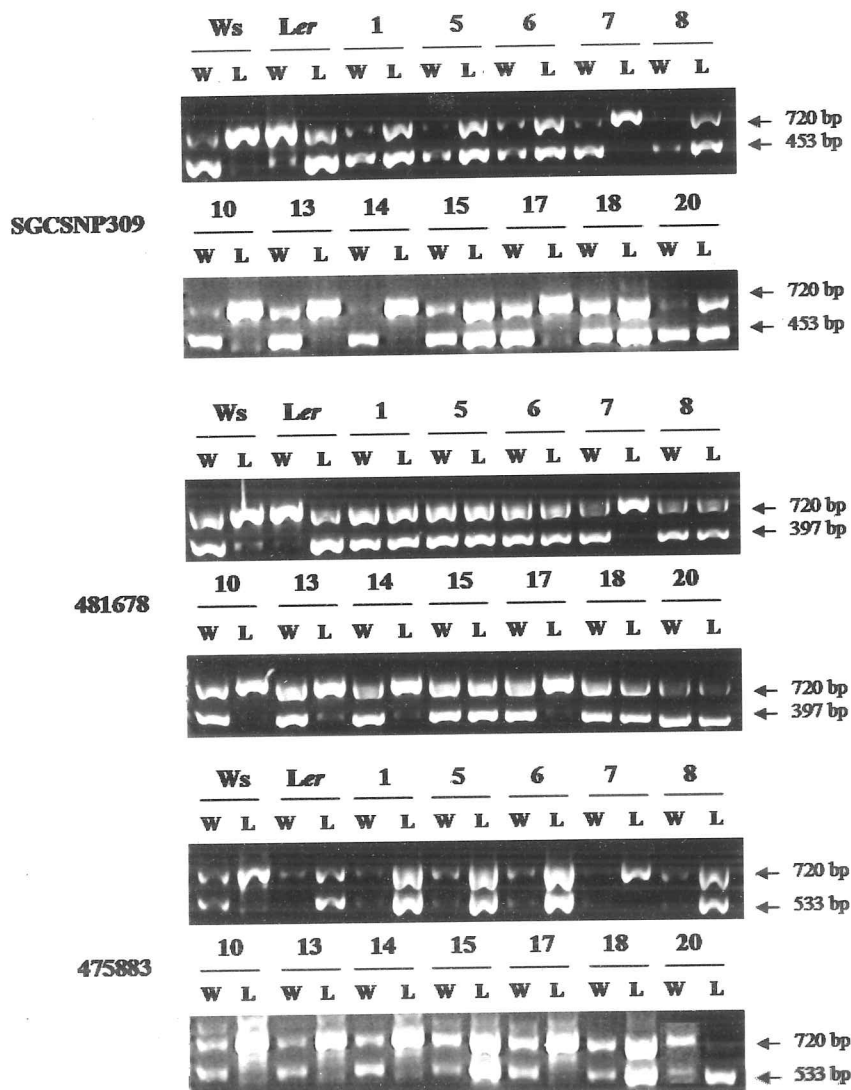


Figure 4.11 Genetic mapping of the mutation in PR48.2N in possible regions of *Arabidopsis* chromosomes I and III using CAPS and SNP markers.

PCR-based genetic mapping of the mutation in PR48.2N using CAPS markers in a possible region of chromosomes I (A) and III (B) as shown in Figure 4.10 on a GFP-selected F2 mapping population from a PR48.2N x wild-type *Ler* cross. Restriction enzymes and fragment sizes were provided in Appendix I. C) Genetic mapping of the mutation in PR48.2N in the possible region of chromosome I using SNP markers as shown in Figure 4.10. Lanes labelled W were products of PCR reactions with primers specific to *Ws* alleles, whereas lanes labelled L were products of PCR reactions with primers specific to *Ler* alleles. An internal control of 720 bp, an uncut product of AP3-LINKED CAPS marker, was included.

Table 4.2 Genotypes of GFP-selected F2 mapping individuals for seven PCR-based molecular markers covering candidate regions on chromosomes I and III, and genetic linkages (recombination percentages) of the markers to the mutation in PR48.2N.

Genotypes of GFP-selected F2 mapping individuals (12 to 24 individuals) from a PR48.2N x wild-type *Ler* cross for seven CAPS and SNP markers covering two candidate regions on chromosomes I and III were determined as shown in Figure 4.11. Recombination percentages of the markers to the mutation in PR48.2N were calculated as the percentages of *Ler* alleles present for the markers over the total number of chromosomes examined. W = homozygote of *Ws* allele, H = heterozygote and L = homozygote of *Ler* allele. Hyphens denote data unavailable.

Marker	Recombination percentage (%)	F2 mapping individual (genotype)																							
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
Chromosome I:																									
M235	41.7	H	W	W	H	H	H	H	H	H	W	H	H	H	H	H	H	H	L	H	H	W	H	W	
SGCSNP309	29.2	H	-	-	-	H	H	W	H	-	W	-	-	W	W	H	-	W	H	-	H	-	-	-	
481678	29.2	H	-	-	-	H	H	W	H	-	W	-	-	W	W	H	-	W	H	-	H	-	-	-	
475883	29.2	H	-	-	-	H	H	W	H	-	W	-	-	W	W	H	-	W	H	-	H	-	-	-	
7G6	32.1	H	-	-	-	H	H	W	H	H	W	-	-	W	W	H	-	W	H	H	H	-	-	-	
Chromosome III:																									
PRC6	40.6	L	W	-	-	W	W	L	W	H	W	-	-	W	H	L	-	W	H	H	L	H	-	-	
AP3-LINKED	43.8	L	W	-	-	W	W	L	W	H	W	-	-	W	L	L	-	W	H	H	L	H	-	-	

One of the candidate regions with the lowest recombination frequency was between UFO (37.5 %) and 7G6 (32.1 %). In order to analyse further this region, three SNP markers, namely SGCSNP309, 481678 and 475883, covering the region were analysed with the F2 mapping population (Figure 4.10). The SNP mapping procedure was performed using the DNA extracted from 12 GFP-selected F2 mapping lines (section 2.5.6.3). The electrophoresis gels for the products of SNP PCR of *Ws*, *Ler* and the F2 mapping lines are shown in Figure 4.11C. All the SNP markers analysed showed the same recombination frequency, which was 29.2 % (Table 4.2). In theory, genetic mapping using these numbers of molecular markers and mapping lines should be able to identify a region with much lower recombination frequency. However in this analysis, molecular markers that were closely linked to PR48.2N were not successfully identified.

In order to investigate if the unsuccessful mapping was due to the robustness of the phenotype used to select the mapping individuals, another five F2 individuals selected as greened individuals at 6D1L (greening-selected) (section 4.4) were used to repeat some of the molecular markers mentioned above. The regions with low recombination frequencies on chromosomes I and III represented by 7G6 and PUR5, and a heterozygote-enriched region on chromosome V represented by N97067 were re-analysed with the greening-selected F2 individuals (DNA was extracted from pooled F3 seedlings of the greening-selected F2 individuals [section 2.5.1]). The regions on chromosome I (7G6) and III (PUR5) showed high recombination frequencies, which were 40 % and 30 % respectively (Figure 4.12; Table 4.3), whereas the recombination frequency for the region on the north arm of chromosome V (N97067) was 0 % with the five mapping lines. This suggests that the mutation in PR48.2N that provided the enhanced greening ability may be genetically linked to N97067 on the north arm of chromosome V.

4.5 Co-segregation analysis of PR48.2N phenotypes

PR48.2N had a number of mutant phenotypes compared to the wild type, but the mutant line had not been back-crossed to its parental line to clear background mutations generated by the random mutagenesis. Therefore, a co-segregation analysis of the phenotypes was needed to investigate if the phenotypes observed in PR48.2N resulted from the same mutation.

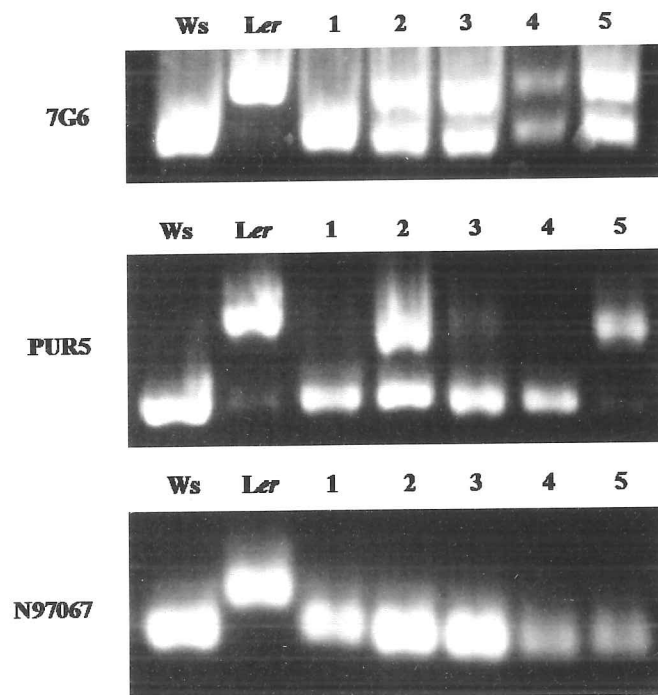


Figure 4.12 Genetic mapping of the mutation in PR48.2N using PCR-based molecular markers on greening-selected F2 mapping individuals.

PCR-based genetic mapping of the mutation in PR48.2N using three CAPS markers representing three candidate regions on chromosomes I (7G6), III (PUR5) and V (N97067) on a greening-selected F2 mapping population (5 individuals) from a PR48.2N x wild-type *Ler* cross. Restriction enzymes and fragment sizes were provided in Appendix II.

Table 4.3 Genotypes of greening-selected F2 mapping individuals for three PCR-based molecular markers representing candidate regions on chromosomes I, III and V, and genetic linkages (recombination percentages) of the markers to the mutation in PR48.2N.

Genotypes of five greening-selected F2 mapping individuals from a PR48.2N x wild-type *Ler* cross for three CAPS markers representing three candidate regions on chromosomes I (7G6), III (PUR5) and V (N97067) were determined as shown in Figure 4.12. Recombination percentages of the markers to the mutation in PR48.2N were calculated as the percentages of *Ler* alleles present for the markers over the total number of chromosomes examined. W = homozygote of Ws allele, H = heterozygote and L = homozygote of *Ler* allele.

Marker	Recombination percentage (%)	F2 mapping individual (genotype)				
		1	2	3	4	5
Chromosome I:						
7G6	40	W	H	H	H	H
Chromosome III:						
PUR5	30	W	H	W	W	L
Chromosome V:						
N97067	0	W	W	W	W	W

Five GFP-selected F2 mapping individuals (lines 20-24) and five greening-selected F2 mapping individuals (lines 1-5) as used for genetic mapping described in section 4.4 were allowed to grow to maturity in soil and self (section 2.2.2). F3 seeds were then collected from the lines for this analysis. The distributions of four PR48.2N phenotypes, namely GFP expression in the presence of norflurazon or lincomycin, enhanced greening ability after prolonged darkness, decreased transcripts of seed protein genes, and hypersensitivity to ABA inhibition of germination, were investigated with the F3 individuals.

4.5.1 GFP-expressing phenotype in the presence of norflurazon and lincomycin

The first part of this analysis was to study the distributions of non-fluorescent and green fluorescent phenotypes of F3 populations from the GFP- and greening-selected F2 mapping individuals. The seeds of wild-type Ws and PR48.2N together with F2 seeds from the PR48.2N x *Ler* cross, F3 seeds from five GFP-selected F2 mapping individuals, and F3 seeds from five greening-selected F2 mapping individuals were prepared, germinated in the presence of norflurazon or lincomycin, and examined for fluorescence as previously described (sections 4.2 and 4.3). Microphotographic images of cotyledons were taken using a Nikon Coolpix 8700 digital camera (section 2.9.2). Green fluorescence from the cotyledons was quantified as the average green pixel intensity from the microphotographic images using MetaMorph software. Frequency histograms to show the distribution of GFP intensities were drawn using Microsoft Excel 2002 software (section 2.9.3).

Both norflurazon- and lincomycin-treated wild-type Ws seedlings formed a unimodal histogram corresponding to low intensity of green fluorescence (Figure 4.13). Wild-type Ws seedlings do not carry the GFP reporter gene and therefore the histograms represented a background value. Both norflurazon- and lincomycin-treated PR48.2N seedlings that were expressing GFP formed a unimodal distribution at a higher fluorescence intensity than wild-type Ws. The frequency histograms of fluorescence of cotyledons of individual norflurazon- or lincomycin-treated seedlings from the F2 population of a PR48.2N x *Ler* cross displayed a different distribution. Unlike the unimodal distributions of fluorescence of norflurazon- and lincomycin-treated wild-type Ws and PR48.2N, norflurazon- and lincomycin-treated F2 individuals from the

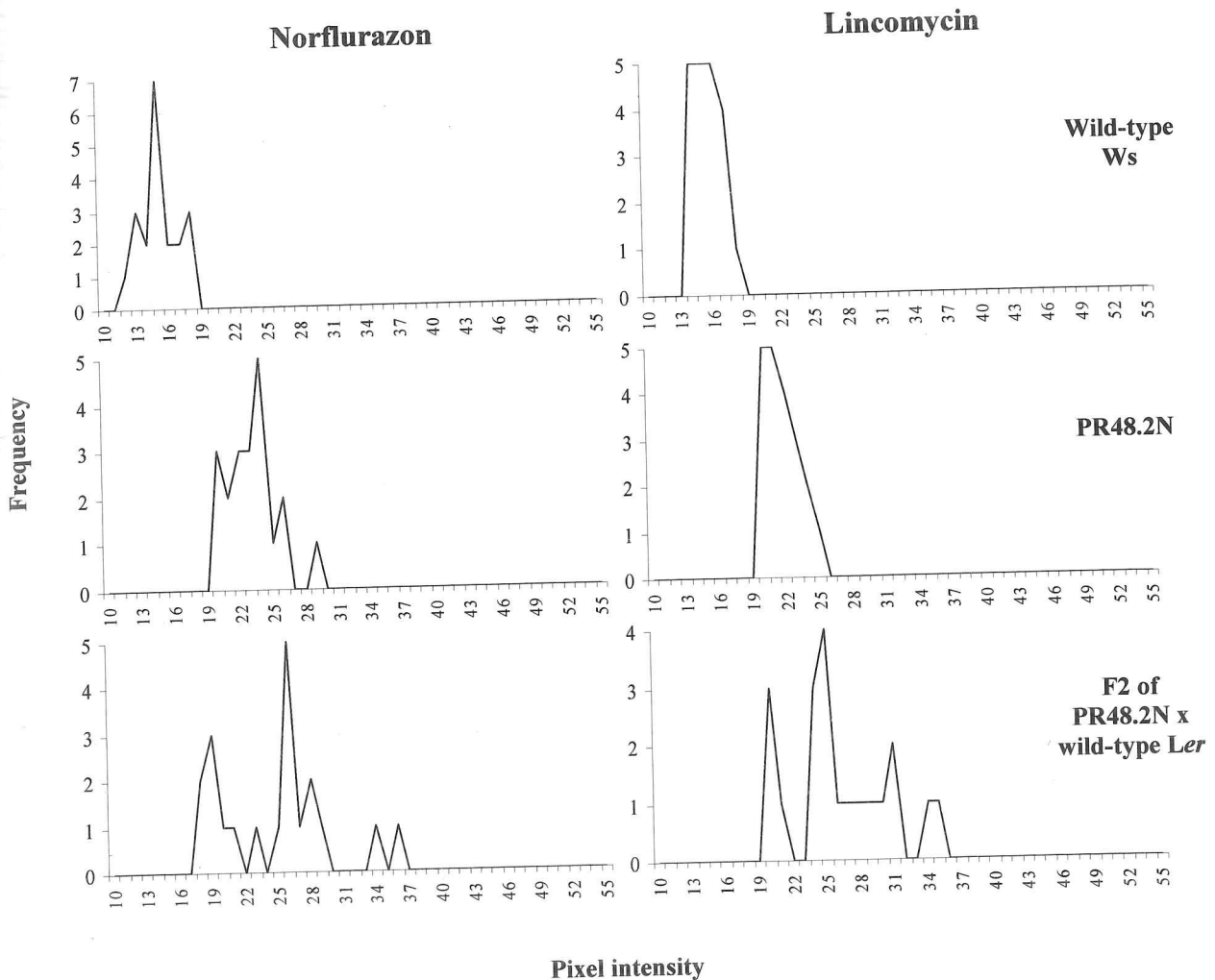


Figure 4.13 Frequency histograms of green fluorescence intensity of PR48.2N and F2 offspring from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin.

Frequency histograms of green fluorescence intensity detected from twenty 5-day-old cotyledons of PR48.2N and F2 offspring from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 8700 digital camera and green fluorescence intensity was quantified from the images using MetaMorph software version 4.01. Histograms were drawn using Microsoft Excel 2002 software.

PR48.2N x *Ler* cross showed a multimodal distribution in the frequency histograms. In these multimodal distributions, a number of peaks, major or minor, were observed in the phenotypic distributions of green fluorescence suggesting that the F2 population was a heterogeneous population with a broad range of intensities. However the distribution did not agree with any known genetic ratio that might reflect a possible segregating F2 population that was descended from heterozygous parents (F1 offspring of PR48.2N x *Ler* cross).

Figure 4.14 shows frequency histograms of green fluorescence of norflurazon- or lincomycin-treated F3 offspring from five F2 mapping individuals from a PR48.2N x wild-type *Ler* cross, selected as fluorescent seedlings in the presence of norflurazon. All five norflurazon- and lincomycin-treated F3 populations from the GFP-selected F2 mapping individuals showed multimodal distributions in the frequency histograms of green fluorescence. These multimodal distributions may suggest that the F3 populations were heterogeneous populations with a broad range of fluorescence intensities. However, the distributions did not indicate any known genetic nature of the F2 individuals. The fluorescent phenotype of seedlings in the presence of norflurazon appears not to be a robust phenotype for genetic analysis. Previous analyses to determine whether the mutation in PR48.2N was dominant or recessive (section 4.2) and the genetic mapping for the mutation in PR48.2N with the F2 mapping individuals selected as fluorescent seedlings in norflurazon (section 4.4) also produced uninterpretable segregation patterns.

Figure 4.15 shows frequency histograms of green fluorescence of norflurazon- and lincomycin-treated F3 offspring from five F2 mapping individuals from a PR48.2N x wild-type *Ler* cross, selected as greened seedlings after being grown for 6D1L. All five norflurazon- and lincomycin-treated F3 populations from the greening-selected F2 mapping individuals showed a similar distribution of green fluorescence to PR48.2N. These F3 populations from the greening-selected F2 mapping individuals appeared to be homogenous populations of the GFP-expressing phenotype suggesting that the five greening-selected F2 mapping individuals were homozygous for the mutation in PR48.2N. There was no obvious difference between the phenotypic distributions of norflurazon- and lincomycin-treated F3 offspring from the greening-selected F2

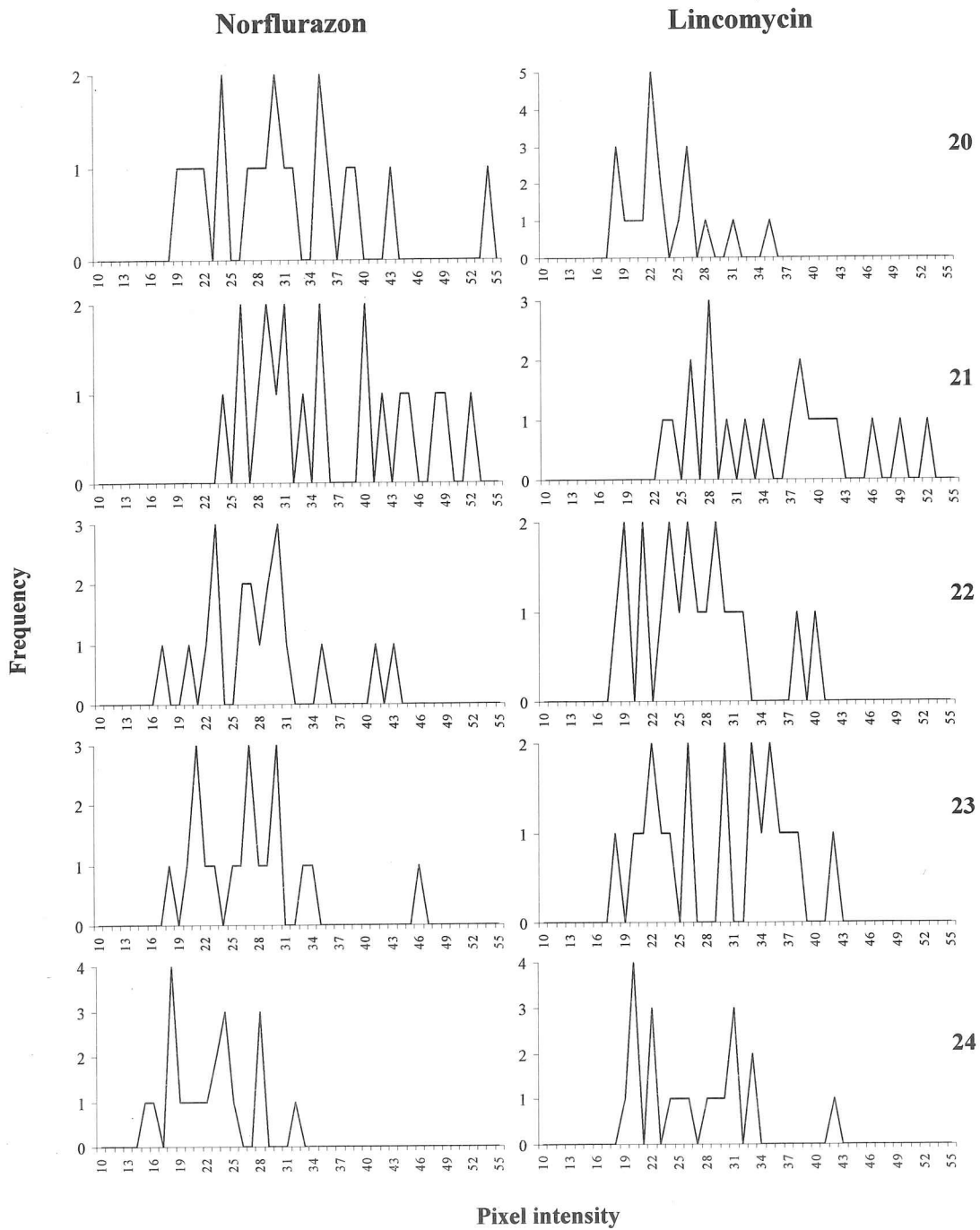


Figure 4.14 Frequency histograms of green fluorescence intensity of F3 offspring from five GFP-selected F2 mapping individuals from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin.

Frequency histograms of green fluorescence intensity detected from twenty 5-day-old cotyledons of F3 offspring from five GFP-selected F2 mapping individuals (20-24 as shown in Figures 4.9 and 4.11) from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 8700 digital camera and green fluorescence intensity was quantified from the images using MetaMorph software version 4.01. Histograms were drawn using Microsoft Excel 2002 software.

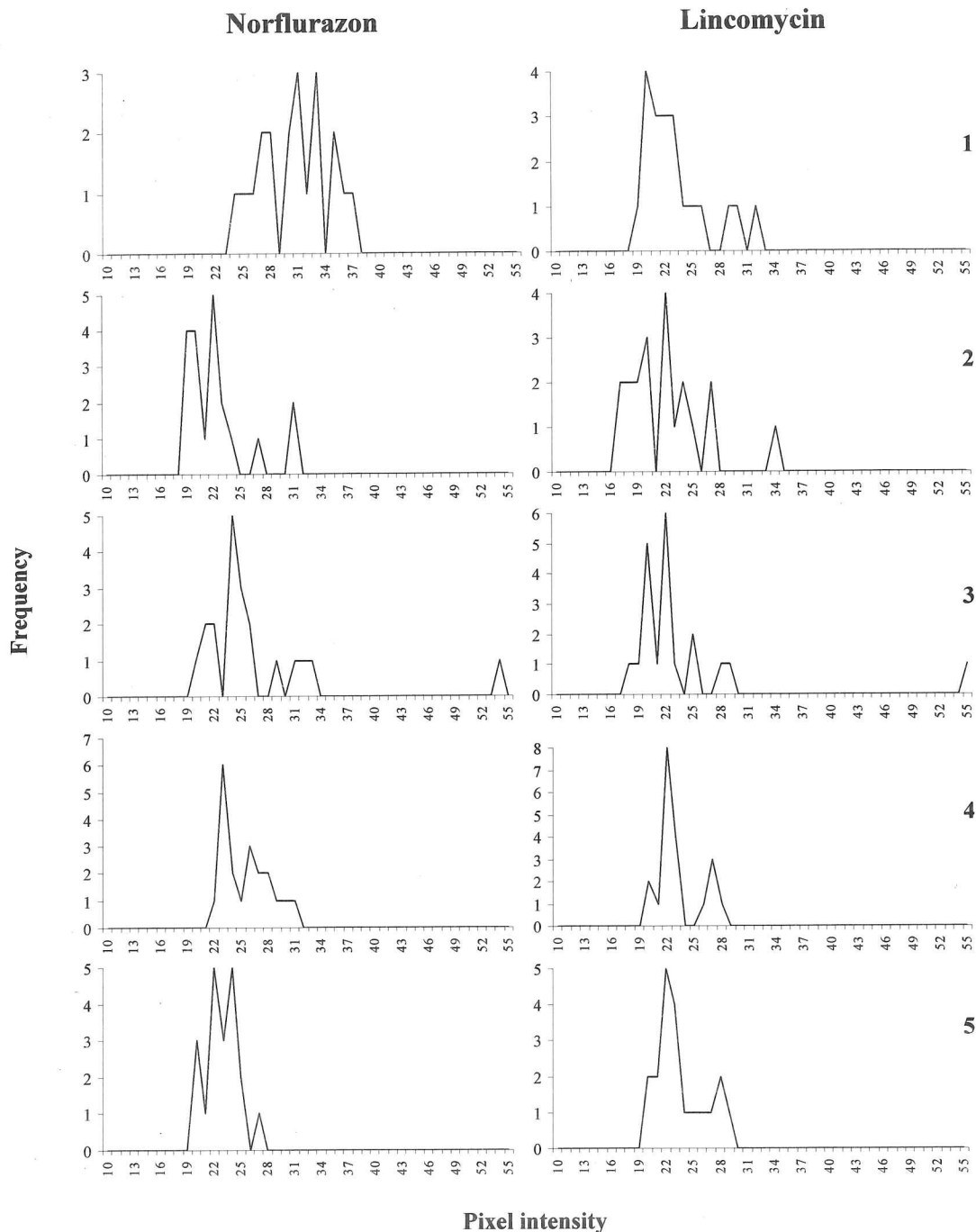


Figure 4.15 Frequency histograms of green fluorescence intensity of F3 offspring from five greening-selected F2 mapping individuals from a PR48.2N x wild-type *Ler* cross treated with norflurazon or lincomycin.

Frequency histograms of green fluorescence intensity detected from twenty 5-day-old cotyledons of F3 offspring from five greening-selected F2 mapping individuals (1-5 as shown in Figure 4.12) from a PR48.2N x wild-type *Ler* cross using a Nikon Optiphot 2 epifluorescence microscope. Images were taken using a Nikon Coolpix 8700 digital camera and green fluorescence intensity was quantified from the images using MetaMorph software version 4.01. Histograms were drawn using Microsoft Excel 2002 software.

mapping individuals. This may suggest that the phenotypes of GFP expression in the presence of norflurazon or lincomycin were genetically linked to each other.

Since the phenotypic distributions of the five GFP-selected mapping individuals were difficult to interpret, the co-segregation analysis was carried on with the greening-selected mapping individuals.

4.5.2 Enhanced greening ability after prolonged darkness

The distributions of wild-type and enhanced greening phenotypes after 10 days in the dark followed by 1 day in the light (10D1L) of the F3 populations from the greening-selected F2 mapping individuals were examined. 10D1L was chosen as the treatment for this analysis because it produced a clear-cut phenotype of enhanced greening ability after prolonged darkness in PR48.2N compared to the wild type (section 3.6). The seeds of wild-type Ws and PR48.2N together with the F3 seeds from five greening-selected F2 mapping individuals were prepared as previously described (section 4.2). The seeds were germinated on 0.7 % agar with half-strength MS and grown for 10 days in the dark followed by 1 day in the light. After the treatment, the numbers of seedlings with yellow cotyledons and with greened cotyledons were counted.

All wild-type Ws seedlings failed to green after 10D1L and almost all (83.3 %) PR48.2N seedlings greened after 10D1L (Table 4.4). For F3 offspring from the greening-selected F2 mapping individuals, almost all (92.2-100 %) of the seedlings greened after 10D1L. These F3 populations were similar to the population of homozygous PR48.2N seedlings and therefore the five greening-selected F2 mapping individuals were likely to be homozygous of the mutation in PR48.2N responsible for the greening-after-10D1L phenotype.

4.5.3 Transcript abundance of seed protein genes

The PR48.2N phenotype of decreased transcript abundance of seed protein genes was examined in the F3 populations of the greening-selected F2 mapping individuals. The seedlings from the F3 populations were grown in continuous darkness

Table 4.4 Number of greened seedlings after being grown for 10 days in the dark followed by 1 day in the light (10D1L) of F3 offspring from greening-selected F2 mapping lines of a PR48.2N x wild-type *Ler* cross.

Seedlings of wild-type *Ws*, PR48.2N and F3 offspring of five greening-selected F2 mapping lines from a PR48.2N x wild-type *Ler* cross were grown on 0.7 % agar with half-strength MS for 10 days in the dark followed by 1 day in the light (10D1L). Number of greened seedlings was determined after the treatment.

Line	Number of seedlings greened after 10D1L	Total seedling number	Percentage of greened seedlings (%)
<i>Ws</i>	0	50	0.0
PR48.2N	45	54	83.3
F3 offspring of greening-selected F2 mapping line:			
1	50	50	100.0
2	47	51	92.2
3	64	69	92.8
4	54	57	94.7
5	57	61	93.4

for 7 days, pooled for total RNA extraction and subjected to RNA-gel-blot analysis using a probe for *CRA1* encoding a 12S seed storage protein as previously described (section 3.5). Seedlings of Ws and PR48.2N were also grown in continuous darkness for 7 days and included in the analysis as controls.

The dark-grown seedlings of PR48.2N showed about 6-fold lower *CRA1* transcripts compared to the dark-grown seedlings of wild-type Ws (Figures 4.16 and 4.17). This was consistent with the results described in section 3.10, which also showed approximately 6-fold lower *CRA1* transcripts in the dark-grown seedlings of PR48.2N compared to the dark-grown seedlings of wild-type Ws. The pooled F3 populations showed *CRA1* transcript levels slightly lower or higher than PR48.2N but none showed a transcript abundance as high as the wild-type Ws. The highest *CRA1* transcript abundance of the pooled F3 populations was approximately 2-fold higher than the PR48.2N seedlings and the lowest *CRA1* transcript abundance was approximately 3-fold lower than the PR48.2N seedlings.

This analysis demonstrated that all F3 populations showed *CRA1* transcript abundance lower than the wild-type Ws seedlings, suggesting that the F3 populations were likely to be PR48.2N mutant seedlings. However, this analysis was not able to identify the phenotype of separate individuals in a population and was therefore not able to eliminate the possibility of segregation of wild-type and mutant phenotypes. Since a segregating population would contain a portion of homozygotes showing a phenotype of decreased transcript abundance of *CRA1*, the pooled *CRA1* transcripts of a segregating population should be lower than the wild-type seedlings to some extent.

4.5.4 Hypersensitivity to ABA

The PR48.2N phenotype of hypersensitivity to ABA inhibition of germination was also examined in the F3 populations of the greening-selected F2 mapping individuals. The seeds of wild-type Ws and PR48.2N together with the F3 seeds from five greening-selected F2 mapping individuals were prepared as previously (section 4.2) and allowed to germinate on 0.7 % agar with half-strength MS containing 0 (no-treatment control) and 2.5 μ M ABA in the light for ten days (section 2.7.2). The number of seeds showing radicle emergence observable by the naked eye was then determined.

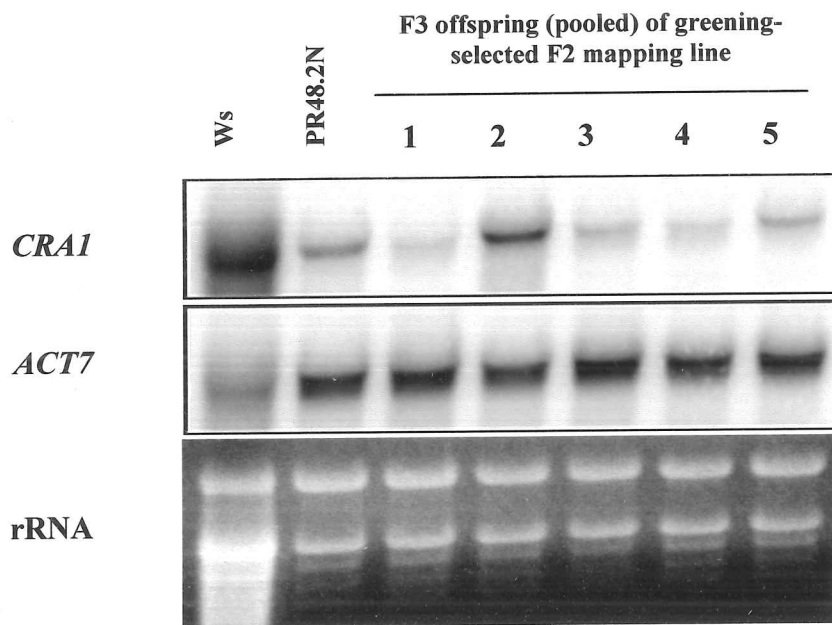


Figure 4.16 Transcript abundance of a 12S seed storage protein gene, *CRA1*, in dark-grown F3 offspring (pooled) of greening-selected F2 mapping lines from a PR48.2N x wild-type *Ler* cross.

Seeds of wild-type Ws, PR48.2N and F3 offspring (pooled) of five greening-selected F2 mapping lines from a PR48.2N x wild-type *Ler* cross were germinated on 0.7 % agar with half-strength MS and grown in continuous darkness for 7 days. Total RNA was extracted from the seedlings using Concert Plant RNA Reagent and 7 μ g of the total RNA was subjected to RNA-gel-blot analysis using a 32 P-labelled probe from *CRA1* encoding a 12S seed storage protein. Signal from a probe for *ACT7* hybridised on the blot and ethidium bromide-stained ribosomal RNAs (rRNAs) are shown as loading controls.

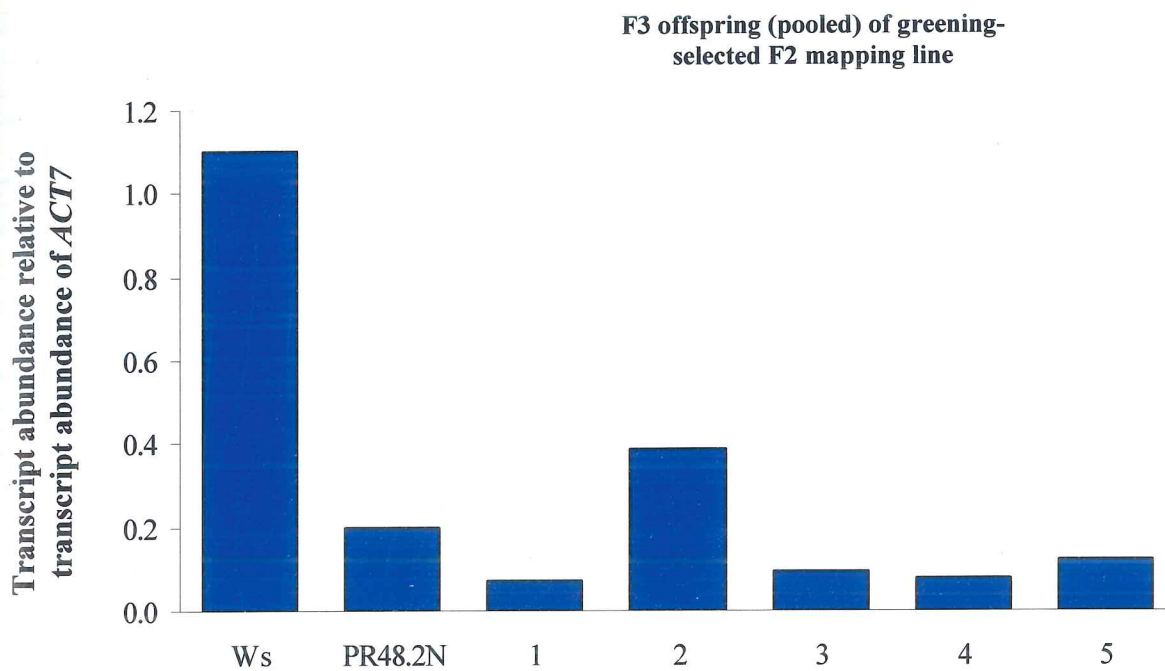


Figure 4.17 Quantification of transcript abundance of a 12S seed storage protein gene, *CR11*, in F3 offspring (pooled) of greening-selected F2 mapping lines from a PR48.2N x wild-type *Ler* cross.

Hybridisation signals from the RNA-gel blots shown in Figure 4.16 were quantified using a Typhoon 8600 Variable Mode Imager. Arbitrary units given by the imaging system to represent the intensities of the signals were standardised to *ACT7* signal to account for differences in the loading of total RNA.

The concentration of ABA for this diagnostic test was set at 2.5 μ M because the germination of wild-type Ws and PR48.2N showed the greatest difference in the presence of 2.5 μ M ABA after 10 days in the light (section 3.16).

The no-ABA controls showed that all seeds germinated well, with germination ranging from 86.7 to 100 % (Table 4.5). Almost all (82.1 %) wild-type Ws seeds germinated in the presence of 2.5 μ M ABA after 10 days in the light, whereas all PR48.2N seeds failed to germinate in the presence of 2.5 μ M ABA after 10 days in the light. All F3 seeds failed to germinate in the presence of 2.5 μ M ABA after 10 days in the light suggesting that these F3 populations were populations of PR48.2N mutant lines and the greening-selected F2 mapping individuals were homozygous for the mutation in PR48.2N.

PR48.2N phenotypes such as expression of the GFP reporter gene in the presence of norflurazon or lincomycin, enhanced greening ability after prolonged darkness, lower transcript levels of seed protein genes, and hypersensitivity to ABA inhibitory effect on germination appeared to co-segregate in the lines selected as greened individuals at 6D1L from a F2 population of a PR48.2N x *Ler* cross.

4.6 Discussion

GFP expression in the presence of norflurazon, one of the PR48.2N phenotypes, was unfortunately proved not to be a robust phenotype for genetic analysis. Genetic mapping with F2 mapping individuals selected using the phenotype was not successful (section 4.4). It could possibly be due to poor discrimination between low-level GFP expression and high background fluorescence of wild-type seedlings. Thus, there might be a mixture of other genotypes besides the desired homozygotes of the mutated gene in PR48.2N in the F2 mapping population. This possibility was supported by the observations that there were more fluorescent seedlings present in the norflurazon-treated F2 population of a PR48.2N x *Ler* cross than the expected number in the recessive model (section 4.2) and the enrichment of heterozygotes in the GFP-selected F2 mapping population for the region on chromosome V that may be linked to the enhanced greening ability of PR48.2N (section 4.4). F3 populations of the GFP-selected mapping individuals also showed unexplained phenotypic distributions of green

Table 4.5 Number of germinated seeds (radicle emergence) of F3 offspring from greening-selected F2 mapping lines of a PR48.2N x wild-type *Ler* cross after being grown for 10 days in the light in the presence or absence of 2.5 μ M abscisic acid (ABA).

Seeds of wild-type *Ws*, PR48.2N and F3 offspring of five greening-selected F2 mapping lines from a PR48.2N x wild-type *Ler* cross were sown on 0.7 % agar with half-strength MS in the presence or absence of 2.5 μ M abscisic acid (ABA) and allowed to germinate in the light. Number of seeds germinated (radicle emergence) was determined after 10 days.

Line	0 μ M ABA			2.5 μ M ABA		
	Number of seeds germinated	Total seed number	Percentage (%)	Number of seeds germinated	Total seed number	Percentage (%)
<i>Ws</i>	29	30	96.7	23	28	82.1
PR48.2N	29	29	100.0	0	27	0.0
F3 offspring of greening-selected F2 mapping line:						
1	83	83	100.0	0	56	0.0
2	65	65	100.0	0	54	0.0
3	68	68	100.0	0	112	0.0
4	47	49	95.9	0	37	0.0
5	75	75	100.0	0	57	0.0

fluorescence in the presence of norflurazon and lincomycin (section 4.5.1). Mutant phenotypes that are not distinct from the wild-type phenotypes can complicate phenotype selection for genetic mapping. For instance, *weil* (*weak ethylene-insensitive 1*) mutants in *Arabidopsis* had to be mapped using F3 mapping populations instead of F2 mapping populations because of its weak phenotype (Alonso *et al.*, 2003).

The phenotype of GFP expression in the presence of norflurazon was chosen as the means of selecting the mapping population because of the ease of rescuing seedlings from the treatment. Selection on lincomycin was not favoured because of the difficulty of rescuing seedlings from the treatment. This could be due to the fact that these lines were initially isolated as a putative *gun* mutant using norflurazon (section 3.1) and therefore they could possibly show some degree of insensitivity toward norflurazon treatment. Although no plant mutant resistant to norflurazon has been reported, phytoene desaturase mutants of the cyanobacterium *Synechococcus* are resistant to norflurazon (Chamovitz *et al.*, 1990; Linden *et al.*, 1990; Chamovitz *et al.*, 1991).

Genetic mapping with the GFP-selected F2 mapping population was not successful as the lowest recombination frequency obtained from the analysis was 29.2 % between UFO and 7G6 on chromosome I. The GFP transgene could have been inserted in this region because the mapping individuals were all homozygotes of the *Ws* allele or heterozygotes with the recombination frequency close to 33 %, which is the expected recombination frequency for the transgene region. However, a GFP transgene was identified in a region on chromosome II that is approximately 2400 kbp north of GPA1 (A Cottage and E Mott, personal communication) by a PCR walking procedure (Cottage *et al.*, 2001).

Since the phenotype of GFP expression in the presence of norflurazon was not a robust phenotype for selecting an F2 mapping population, a more robust PR48.2N phenotype such as the enhanced greening ability was needed for the genetic mapping. The five greening-selected F2 mapping individuals behaved as homozygotes of the mutated gene in PR48.2N. The F3 populations of these lines appeared to be homogeneous populations of individuals showing high intensities of green fluorescence in the presence of norflurazon or lincomycin, greening after 10D1L, low transcript

abundance of *CRA1*, and hypersensitivity to the inhibitory effect of ABA on germination. Hence, these phenotypes were likely to be genetically linked.

Chapter 5
Final discussion

5. Final discussion

PR48.2N isolated as a putative *gun1*-like mutant was demonstrated to be a mutant line showing distinct phenotypes in transcript levels of nuclear photosynthesis genes by RNA-blot analysis and the greening process after a dark treatment (sections 3.3 and 3.4). PR48.2N also showed altered transcript levels of seed protein genes (sections 3.8, 3.9 and 3.10) and sensitivity to the inhibitory effect of ABA on germination (section 3.15). These phenotypes were observed to co-segregate in a F2 segregating population (section 4.5).

5.1 PR48.2N and plastid signalling

PR48.2N seedlings showed altered levels of *RBCS* and *LHCBI* transcripts compared to the wild type depending on the length of darkness the seedlings experienced before illumination. Illuminated PR48.2N seedlings grown in the dark for a short period of time showed slightly lower amounts of *RBCS* and *LHCBI* transcripts, whereas, after longer periods of darkness, illuminated PR48.2N seedlings contained higher amounts of *RBCS* and *LHCBI* transcripts (sections 3.5 and 3.12). This may suggest that the gene mutated in the line is a negative regulator of nuclear photosynthesis gene expression after a long period of darkness.

Arabidopsis cue (*cab-underexpressed*) mutants have been demonstrated to show lower transcript abundance of *RBCS* and *LHCBI* in the light that is opposite to the PR48.2N phenotype (Li *et al.*, 1995; López-Juez *et al.*, 1998; Vinti *et al.*, 2005). Similar to a subset of *cue* mutants such as *cue3*, *cue6* and *cue8*, PR48.2N was shown in section 3.7 to have defective etioplasts after being grown in continuous darkness for 2 days (Vinti *et al.*, 2005). Analysis of the response of *LHCBI* transcripts and chlorophyll accumulation to phytochrome-activating light pulses in *cue3* and *cue8* has demonstrated that plastid development is necessary for normal phytochrome-mediated regulation of gene expression associated with photosynthesis (Vinti *et al.*, 2005). This may be supported by the observations on PR48.2N that light-induced nuclear photosynthesis gene transcripts and chlorophyll accumulation were lower in illuminated 2D seedlings of PR48.2N that possessed defective development of plastids (sections 3.6, 3.7 and 3.12). Similarly, light-induced nuclear photosynthesis gene transcripts and chlorophyll

accumulation were lower in illuminated 10D seedlings of wild-type Ws that showed under-development of plastids compared to the PR48.2N.

The PR48.2N line was isolated as a putative *gun1*-like mutant (sections 3.1 and 3.2) and PR48.2N seedlings showed more transcripts of nuclear photosynthesis genes in the presence of plastid function inhibitors such as norflurazon and lincomycin (section 3.5). However, it is not clear whether PR48.2N showed a *gun1*-like phenotype implying defective plastid signalling because higher transcript levels of nuclear photosynthesis genes were also observed without the inhibitors. However the genes mutated in *gun1* and PR48.2N appeared to interact for the phenotype of lower transcript levels of nuclear photosynthesis genes in the presence of norflurazon or lincomycin, supported by the presence of putative double mutant (section 4.3). Confirmation of the putative double mutant and further elucidation of the interaction will be possible when the mutated genes are identified.

5.2 PR48.2N and seed protein genes

Microarray analysis, described in section 3.8, identified another group of genes showing altered transcript abundance in PR48.2N seedlings. Genes involved in late embryogenesis and seed maturation, including genes encoding seed storage proteins, LEA proteins and oleosins, were found to show much lower transcript levels in PR48.2N seedlings. The transcripts of this group of genes are abundant in immature or dry seeds (Delseny *et al.*, 2001). However in this study, their transcript levels were affected in PR48.2N seedlings but not the developing seeds (sections 3.8, 3.10 and 3.11) suggesting that the seed protein genes may be regulated in seedling development.

One of the seed protein genes, *CRA1* encoding a 12S seed storage protein, showed much higher transcript abundance in dark-grown seedlings than in the light-grown seedlings suggesting the gene is light-regulated (section 3.10). With the findings that transcript levels of seed protein genes were altered in greening PR48.2N seedlings after various lengths of dark periods (section 3.12), the question whether seed protein genes are involved in the light-controlled greening process could be investigated further. However, the abundance of the seed proteins in greening seedlings should be examined first by western-blot analysis. It would also be interesting to perform

comparative proteomics between PR48.2N and the wild type using two-dimensional gel electrophoresis and mass spectrometry.

Illuminated PR48.2N seedlings after various lengths of darkness showed fewer transcripts of seed protein genes than the wild type except at 6D1L. Interestingly, illuminated wild-type seedlings after being grown in 10-day darkness accumulated high amount of seed protein gene transcripts. This induction could be caused by the low levels of nutritive reserves after the long darkness. Illuminated PR48.2N seedlings after being grown in 10-day darkness failed to accumulate transcripts of seed protein gene but their chloroplasts contained more starch grains (sections 3.7 and 3.12). The low levels of nutritive reserves could have instead triggered more photosynthesis activity in PR48.2N seedlings since the seedlings were likely to possess more developed machinery of photosynthesis at 10D1L.

PR48.2N seedlings also displayed altered transcript levels of seed protein genes in the dark. Similar to transcripts of nuclear photosynthesis gene, seed protein gene transcripts were present in the dark-grown seedlings with a light-independent induction occurred during the first few days of early seedling development (section 3.12). Dark-grown PR48.2N seedlings however had low levels of the gene transcripts and were devoid of the light-independent transcript peak. In the dark, the transcript profiles of both nuclear photosynthesis and seed protein genes appeared to be similar and, thus co-regulation could be possible. These transcript levels in the dark might affect the greening process of seedlings when illuminated since PR48.2N seedlings showed altered greening phenotypes.

Genes affected in PR48.2N such as those encoding either seed storage proteins or LEA proteins are positively regulated by ABA (Chandler and Robertson, 1994). An attempt to identify consensus *cis*-acting elements in the upstream regions of genes highly affected in PR48.2N resulted in several ACGT-containing sequences similar to ABA response elements and coupling element 3-like sequences (section 3.13). However PR48.2N seedlings did not show altered content of endogenous ABA but PR48.2N seeds appeared to be hypersensitive to the ABA inhibitory effect on germination (sections 3.14 and 3.15). Since PR48.2N that was hypersensitive to ABA during germination accumulated fewer transcripts of seed protein genes during seedling

development, the regulation of seed protein genes in seedlings may be independent of the ABA perception during germination. The product encoded by the mutated gene in PR48.2N may be required to regulate seed protein genes in seedlings positively.

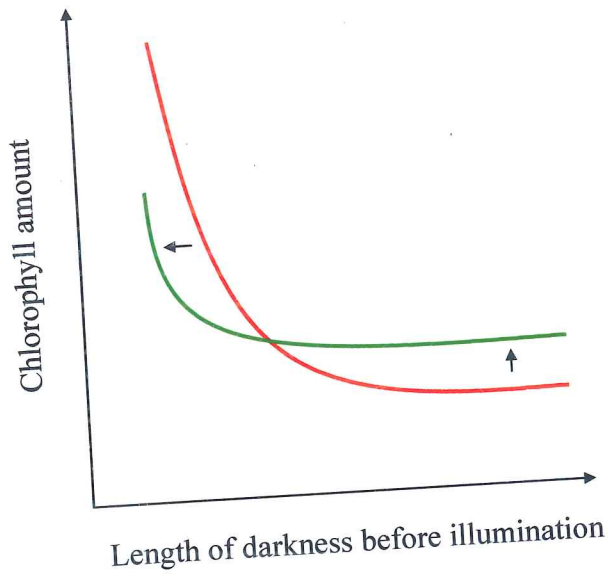
5.3 PR48.2N and the greening process

PR48.2N had a prominent phenotype in the greening process after growth in various lengths of darkness. PR48.2N seedlings showed differences in chlorophyll accumulation (sections 3.4 and 3.6), plastid ultrastructure (section 3.7) and transcript levels of nuclear photosynthesis and seed protein genes (section 3.12) during the greening process. Therefore, the gene mutated in PR48.2N may play a role in the transition from heterotrophic to photoautotrophic growth during early seedling development. PR48.2N greened less after a short period of darkness but displayed an enhanced greening ability after prolonged darkness (section 3.6). Unlike *gun1-1*, which is a loss-of-greening-ability mutant (Susek *et al.*, 1993; Mochizuki *et al.*, 1996), PR48.2N appeared to be a gain-of-greening-ability mutant after long periods of darkness.

Compared to the wild type, illuminated PR48.2N seedlings accumulated less chlorophyll and transcripts of nuclear photosynthesis genes after experiencing short periods of darkness, such as 2 days, but more after long periods of darkness, such as 10 days. It may be postulated that the greening processes after short and long periods of darkness are differentially regulated. However, another possibility could be that the mutation in PR48.2N caused a "shift" in the accumulation kinetics of chlorophyll and transcripts of nuclear photosynthesis genes during the greening process after various lengths of dark periods as shown in the schematic representations in Figure 5.1.

The altered greening process in PR48.2N seedlings appeared to be associated with their phenotypes in the dark. PR48.2N cotyledons contained under-developed etioplasts compared to the wild type after being grown in the dark for 2 days but better-developed etioplasts in continuous darkness for 10 days. There could be a lag in etioplast development in dark-grown PR48.2N so that PR48.2N etioplasts develop slower than the wild type. This postulation may explain the "shift" in the accumulation kinetics of chlorophyll and transcripts of nuclear photosynthesis genes during the

A



B

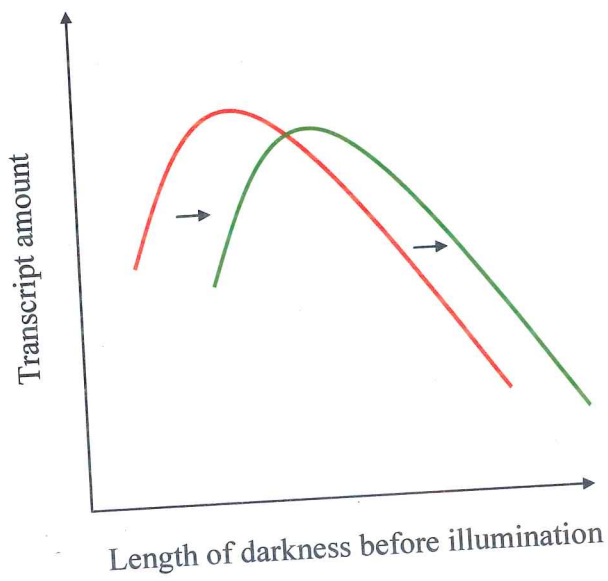


Figure 5.1 Accumulation kinetics of chlorophyll (A) and transcripts of nuclear photosynthesis genes (B) in illuminated wild-type Ws and PR48.2N seedlings after being grown in various lengths of darkness.

Accumulation kinetics of chlorophyll (A) and transcripts of nuclear photosynthesis genes (B) in illuminated wild-type Ws and PR48.2N seedlings after being grown in various lengths of darkness as in Figures 3.9 and 3.22 are summarised in the schematic representations. Red lines (—) represent wild-type Ws and green lines (—) represent PR48.2N.

greening process associated with less chlorophyll and transcripts of nuclear photosynthesis genes in 2D1L PR48.2N seedlings but more in 10D1L when compared to the wild type. Better-developed etioplasts were found to be associated with better-greened seedlings.

Another dark phenotype of PR48.2N was its altered transcript levels of nuclear photosynthesis genes. Transcripts of nuclear photosynthesis are present in dark-grown seedlings with a light-independent induction of the transcript levels during the first few days of early seedling development (Brusslan and Tobin, 1992). Dark-grown PR48.2N seedlings showed fewer transcripts of the nuclear photosynthesis genes and were devoid of the light-independent transcript induction (section 3.12). This defect in PR48.2N seedlings might contribute to their altered pattern of greening.

Seed and seedling are two important developmental stages in plants. Developing seeds accumulate nutritive reserves for germination and early seedling development, whereas developing seedlings establish machinery for photosynthesis. The regulatory components responsible for these developmental phases may overlap during the early seedling development. ABI3, ABI4 and ABI5 appear to be regulatory components during seed development, germination and early seedling development. These proteins regulate expression of seed protein genes during seed development (Finkelstein and Somerville, 1990; Kriz *et al.*, 1990; Nambara *et al.*, 1992; Finkelstein, 1993, 1994; Paiva and Kriz, 1994; Parcy *et al.*, 1994; Nambara *et al.*, 2000), ABA sensitivity (Koorneef *et al.*, 1984) and lipid mobilisation (Penfield *et al.*, 2006a) during germination. During early seedling development, ABI4 may be responsible for responses of photosynthesis genes to sugar, ABA (Acevedo-Hernández *et al.*, 2005) and plastid-to-nucleus signalling responding to the state of plastid protein synthesis (Nott *et al.*, 2006). ABI3 has also been reported to play a role in plastid differentiation in dark-grown *Arabidopsis* seedlings (Rohde *et al.*, 2000).

DET2, GUN1 and the product encoded by the mutated gene in PR48.2N may play a role in the regulation of seedling development. Mutants of all these three products are hypersensitive to ABA inhibitory effect on germination (*det2*, Steber and McCourt, 2001; *gun1*, A Cottage and E Mott, personal communication; PR48.2N, section 3.15) in contrast to *abi* mutants. Perhaps the components that regulate seed

development, such as *ABI3*, *ABI4* and *ABI5*, confer ABA sensitivity to prevent germination of seeds, whereas the components that regulate seedling development reverse the process. *DET2*, a steroid 5 α -reductase involved in brassinosteroid biosynthesis, is involved in photomorphogenesis during seedling development (Chory *et al.*, 1991; Li *et al.*, 1996). *GUN1*, a component involved in a plastid-to-nucleus signalling pathway, and the product encoded by the mutated gene in PR48.2N regulate the greening process of seedlings (Susek *et al.*, 1993; Mochizuki *et al.*, 1996; sections 3.4 and 3.6).

In order to test this hypothesis, genetic analyses and expression profiles of *ABI3*, *ABI4*, *ABI5*, *DET2*, *GUN1* and the gene mutated in PR48.2N should be elucidated. Genetic relations between the mutated gene in PR48.2N and *DET2* and *GUN1* should be established in order to investigate whether these components are acting in the same pathway or interacting pathways as the hypothesis suggest. Double mutants of *det2*, *gun1* and the mutated gene in PR48.2N should show a mutant phenotype, for instance ABA hypersensitivity, similar to any one of the monogenic mutants if the components are acting in the same pathway or an enhanced mutant phenotype for interacting pathways. If the components are acting in independent pathways, the mutant phenotype of the double mutants should be quantitatively additive. The mutated gene in PR48.2N may synergistically interact with *GUN1* since the putative double mutants showed an enhanced expression of a GFP transgene in the control of a photosynthesis gene promoter (section 4.3). When the mutated genes in *gun1* and PR48.2N are identified, the expression profiles of seed development components, such as *ABI3*, *ABI4* and *ABI5*, and seedling development components, such as *DET2*, *GUN1* and the product encoded by the mutated gene in PR48.2N, should be examined in developing seeds and seedlings. The hypothesis predicts that the expression of the components is specific to their respective developmental stages but overlapping at the early seedling development.

5.4 PR48.2N and genetic mapping

It is essential to identify the gene that is mutated in PR48.2N to elucidate further its function. The recessive mutation may be in a region on chromosome V of

Arabidopsis (section 4.4). However, the mapping was not successful with a F2 mapping population that was selected using GFP expression in the presence of norflurazon. A robust PR48.2N phenotype such as greening at 6D1L is needed to map the region on chromosome V in order to identify the mutated gene. PR48.2N was produced by random mutagenesis that can introduce more than one mutation in the genome. A cosegregation analysis described in section 4.5 showed that PR48.2N phenotypes were likely to be linked. PR48.2N line should be back-crossed to its parental line to eliminate the background mutations. The genetic mapping should be continued with more molecular markers on the chromosome arm and larger mapping population. Genetic complementation will help to map the mutation further when a region of reasonably small size is identified.

The identification of the mutated gene in PR48.2N can be confirmed by sequencing and complementation. It can then be followed by a thorough expression study to establish when, where and under what conditions the gene is expressed using northern blotting, promoter analysis, *in situ* hybridisation, western blotting and immunohistology. Various biochemical assays to characterise the activity and structure of the protein can be carried out. It will also be important to establish the possible interaction components using techniques such as two-hybrid system, co-precipitation and pull-down assays. With other related genes, the process that the gene is involved may be thoroughly characterised especially its role in the plastid-to-nucleus signalling pathway.

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Appendices

Appendix I - Probes generated by PCR for RNA-gel-blot analysis

Probe (AGI code for the gene template)	Primer sequence	Product size (bp)	Annealing temperature (°C)	Approximate transcript size (nt)
<i>CRA1</i> (At5g44120)	CRA1P1 5'-CAGCACTCTCAACAGTTACG-3'	436	52	1700
	CRA1P2 5'-GCTGTGAGTCAAAGTGGTCT-3'			
<i>HEMA1</i> (At1g58290)	HEMA1P1 5'-CAGCACGGGTTTACAATGTG-3'	362	52	2200
	HEMA1P2 5'-ATTCTCAAGGTCTCGCTCA-3'			
<i>LEA76</i> (At3g15670)	LEA15670P1 5'-ACAGCCAGTCAGCTAAAG-3'	436	52	1100
	LEA15670P2 5'-TGTCTTCTCCTCAGTAGCC-3'			
<i>LHCB1.2</i> (At1g29910)	LHCBF 5'-CGTCCGTTAGTCACCAAAA-3'	849	55	1100
	LHCBR 5'-TCACTTCCGGGAACAAAGT-3'			
<i>OLEO2</i> (At5g40420)	OLEO40420P1 5'-GTTGATTGGAGTCCCTGTC-3'	406	52	950
	OLEO40420P2 5'-AGTCTCATGACCCTTAGTGG-3'			
<i>RBCS-1A</i> (At1g67090)	RBCSL 5'-GGCTTCCTCTATGCTCTCCTC-3'	503	55	950
	RBCSR 5'-CTGATGCATTGGACTTGACG-3'			

Appendix II - Molecular markers for genetic mapping

Cleaved Amplified Polymorphic Sequences (CAPS)

Marker	Map position (bp)	Primer sequence	Number of cut(s) (size of products in bp)	Restriction enzyme
Chromosome I:				
G2395	7300914	G2395F 5'-GGTTCATCACCAAGCTCC-3'	Ws, 1 (183, 153); Ler, 0 (336)	<i>Xba</i> I
		G2395R 5'-CTCCTGTCTCCAGTCCC-3'		
M235	8166580	M235P1 5'-AGTCCACAACAATTGCAGCC-3'	Ws, 1 (309, 225); Ler, 0 (534)	<i>Hind</i> III
		M235P2 5'-GAATCTGTTTCGCCTAACGC-3'		
UFO	11037377	UFOF 5'-AAGGCATCATGACTGTGGTTTTTC-3'	Ws, 1 (983, 316); Ler, 2 (600, 383, 316)	<i>Taq</i> I
		UFOR 5'-GTGGCGTTTCAGACGGAGAGG-3'		
7G6	12285352	7G6P1 5'-GTTTTACTTAGTCCAATGGTAG-3'	Ws, 1 (200, 200); Ler, 0 (400)	<i>Acc</i> I
		7G6P2 5'-GGGTCTGGTTATGCCGTGAAG-3'		
GAPB	16130205	GAPBF 5'-GGCACTATGTTTCAGTGCTG-3'	Ws, 3 (605, 284, 225, 174); Ler, 4 (350, 284, 255, 225, 174)	<i>Dde</i> I
		GAPBR 5'-TCTGATCAGTTGCAGCTATG-3'		
NPR1	23856783	NPR1F 5'-GTTGATGTACATAAATATATATTACAAATC-3'	Ws, 0 (615); Ler, 1 (536, 79)	<i>Aha</i> I
		NPR1R 5'-CCGGGTGTAAAGATAGCACCTT-3'		
PAB5	26997132	PAB5F 5'-GATGCAACCGCCGCAGCCATAGCGATAAGA-3'	Ws, 3 (360, 320, 165, 40); Ler, 2 (485, 360, 40)	<i>Mbo</i> II
		PAB5R 5'-AGTATCATCAAAATCGAGAGATTG-3'		
Chromosome II:				
RNS1	873663	RNS1F 5'-GACCAAACCAATCTCCATGAAC-3'	Ws, 0 (1070); Ler, 1 (540, 530)	<i>Ava</i> I
		RNS1R 5'-ATCTTATAACCCTCAAAATCAC-3'		
GPA1	11204074	GPA1F 5'-GGGATTTGATGAAGGAGAAC-3'	Ws, 2 (705, 680, 209); Ler, 1 (1385, 209)	<i>Afi</i> III
		GPA1R 5'-ATTCCTTGGTCTCCATCATC-3'		

Chromosome III:				
G4711	8979868	G4711F 5'-CCTGTGAAAAACGACGTGCAGTTTC-3'	Ws, 0 (1500); Ler, 1 (1000, 500)	HindIII
		G4711R 5'-ACCAAATCTTCGTGGGGCTCAGCAG-3'		
PRC6	19042051	PRC6P1 5'-GAAAAAGGTAAAAGAATGGCGAG-3'	Ws, 1 (1400, 600); Ler, 0 (2000)	SacI
		PRC6P2 5'-ACGGTAAGTTACACAATGGAGA-3'		
AP3-LINKED	20137524	AP3-LP1 5'-GCGTAAAGCGATAAGTGC-3'	Ws, 1 (650); Ler, 0 (720)	Sau3AI
		AP3-LP2 5'-AGTGACGAACCACGATTCG-3'		
PUR5*	20398432	PUR5F 5'-AAACCTTTCCTCCTTT-3'	Ws, 2 (524, 132, 37); Ler, 3 (264, 260, 132, 37)	RsaI
		PUR5R 5'-GATGTAGACCTTGCTGAAAA-3'		
Chromosome IV:				
GA1.1	1242594	GA1.1F 5'-CCGGAGAATCGTACGGTAC-3'	Ws, 1 (707, 527); Ler, 0 (1196)	BsaBI
		GA1.1R 5'-AAGCTTCGAACTCAAGGTTTC-3'		
TGCAPS2	9600882	TGCAPS2F 5'-TGTCGATATGCGACCAGTACC-3'	Ws, 3 (340, 250, 160, 100); Ler, 2 (500, 250, 200)	DdeI
		TGCAPS2R 5'-CAGGCTTAGACCCAATTGACC-3'		
PRHA	14650874	PRHAF 5'-CTTGTTTCGCACTGCTCCACC-3'	Ws, 3 (778, 530, 348, 30); Ler, 4 (778, 530, 300, 50, 30)	DdeI
		PRHAR 5'-GCGGAAGAAGAACTCTGTTCG-3'		
Chromosome V:				
N97067	2521971	N97067F 5'-GCCCATCACCACATTC-3'	Ws, 1 (200, 20); Ler, 0 (220)	HinfI
		N97067R 5'-GGATTGCTGATACATTGC-3'		
LMYC6	16541175	LMYC6F 5'-GCACGTGGCGTATACTG-3'	Ws, 0 (1320); Ler, 1 (950, 370)	HindIII
		LMYC6R 5'-CTTGCTCATAGAGACTCGC-3'		
G2368*	25791322	G2368PD1 5'-AAGCTTTTGAATAGGACAGCA-3'	Ws, 0 (519); Ler, 1 (470, 49)	HindIII
		G2368PD2 5'-TGGTCTTCTTGGTTAGGAAA-3'		

* The primer sequences for these markers were modified in this study.

Simple Sequence Length Polymorphism (SSLP)

Marker	Map position (bp)	Primer sequence	Product size (bp)
Chromosome II:			
BIO2	18019881	BIO2F 5'-TTAACAGAAACCCAAAGCTTTC-3'	Ws (200);
		BIO2R 5'-TGACCTCCTCTCCATGGAG-3'	Ler (209)
Chromosome V:			
NGA76	10418614	NGA76F 5'-AGGCATGGGAGACATTTACG-3'	Ws (200);
		NGA76R 5'-GGAGAAAATGTCACTCTCCACC-3'	Ler (300)

Single Nucleotide Polymorphism (SNP)

Marker	Map position (bp)	Primer sequence*	Product size (bp)
Chromosome I:			
SGCSNP309	11250429	SGCSNP309C1 5'-TTAGTCCTGACGGTACGCCG-3'	453
		SGCSNP309L1 5'-TTAGTCCTGACGGTACGCCA-3'	
		SGCSNP309P2 5'-CACACAGATGATCAGAATCG-3'	
481678	11415173	481678C1 5'-TCAGATCGGGTAAACTAGCA-3'	397
		481678L1 5'-TCAGATCGGGTAAACTAGCG-3'	
		481678P2 5'-GCTCCTCCTACCTTCAAAG-3'	
475883	11845204	475883C1 5'-GAGAAACGAACAATCATAAG-3'	533
		475883L1 5'-GAGAAACGAACAATCATAAC-3'	
		475883P2 5'-CGTGCGCTTTAGCTCTCTCT-3'	

*Primer sequences with names containing C1 were specific for the Ws allele, whereas primer sequences with names containing L1 were specific for the Ler allele.

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