Effects of different signalling pathways on regulation of *GLK* GARP transcription factors in *Arabidopsis thaliana*

Ekaterina Ponomareva

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Ottawa-Carleton Institute of Biology
Faculty of Science
University of Ottawa

Abstract

GLK1 and *GLK2* transcription factors have been suggested to be involved in the regulation of chloroplast development, organic nitrogen signaling, disease resistance and circadian rhythmicity (Waters et al. 2009; Gutiérrez et al. 2008; Savitch et al. 2007; Sprott et al. 2010). This implies that multiple factors may play roles in regulation of *GLK* genes. In the present study, transcriptional regulation of *GLK1* and *GLK2* in *Arabidopsis* by various endogenous and environmental stimuli was investigated with the objective of elucidating the primary signalling pathway affecting expression of these two genes.

Collectively, results of *GLK1* and *GLK2* expression in response to the experimental treatments of *Arabidopsis* point to the regulation of the two genes by changes in photosynthetic metabolism and reactive oxygen species (ROS) levels, and by organic nitrogen signalling. Changes in ROS levels and organic nitrogen signalling may also affect the two genes indirectly by interfering with or altering photosynthetic metabolism.

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List of Abbreviations

ABA	Abscisic acid
ARRs	*Arabidopsis* Response Regulators
BR	Brassinosteroid
CAB1	Chlorophyll *a/b*-binding protein
Chl *a/b*	Ratio of chlorophyll *a* to chlorophyll *b*
C_T	Threshold cycle
DBMIB	2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone
DCMU	3-(3',4'-dichlorophenyl)-1,1'-dimethylurea
2-DOG	2-Deoxy-D-glucose
DTT	Dithiothreitol
EF1α	Elongation factor 1 alpha
ETR	Electron transport rate
EV	Empty vector
Fd	Ferredoxin
GA	Glyceraldehyde
GA_3	Gibberellic acid
GARP	***G****OLDEN2*, *Arabidopsis* ***AR****Rs* B-class, and *Chlamydomonas* ***P****SR1*; refers to transcription factor family or DNA binding domain
GBFs	G-box-binding bZIP transcription factors
GCT domain	GOLDEN2 C-terminal domain
GFP	Green Fluorescent Protein
Gln	Glutamine
Glu	Glutamate
G-6-P	Glucose-6-phosphate
GS	Glutamine Synthetase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
gun1-5	*Genomes uncoupled* mutants 1-5
H_2O_2	Hydrogen peroxide
HR	Hypersensitive Response
HXK	Hexokinase
IAA	Indoleacetic acid (Auxin)
JA	Jasmonic acid
MSX	Methionine sulfoximine
MV	Methyl viologen (Paraquat; N,N'-Dimethyl-4,4'-bipyridinium dichloride)
$NADP^+$	Nicotinamide-adenine-dinucleotidephosphate
NLS	Nuclear Localization Signal
PC	Plastocyanin

PG	Phosphatidylglycerol
PGP	Phosphatidylglycerolphosphate
pgp1	*Arabidopsis* phosphatidylglycerolphosphate synthase mutant
ppi2	*Arabidopsis Plastid protein import2* mutant
PR	Pathogenesis-related gene
PSI	Photosystem I
PSII	Photosystem II
Psm	*Pseudomonas syringae* pv. *maculicola*
Pst	*Pseudomonas syringae* pv. *tomato*
pv.	Pathovar
PQ	Plastoquinone
RbcL	Large subunit of ribulose-1,5-bisphosphate carboxylase
RbcS	Small subunit of ribulose-1,5-bisphosphate carboxylase
ROS	Reactive oxygen species
SA	Salicylic acid
ZT	Zeitgeber time

CHAPTER 1 INTRODUCTION

1.1 Overview

This research project aimed to investigate regulation of *GLK1* and *GLK2* transcription factors in the model plant *Arabidopsis thaliana*. *Golden 2-like*, or *GLK*, transcription factors are known to be involved in chloroplast development, and have also been implicated in nitrogen signalling, disease defence, and regulation of plant circadian clocks (Fitter et al. 2002; Waters et al. 2009; Gutiérrez et al. 2008; Savitch et al. 2007; Sprott et al. 2010).

The function of the *GLK* genes in several plant species has been studied by determining their transcript accumulation profiles in different tissues and analyzing the phenotype of knockouts and overexpressors, as well as by determining *GLK* gene targets (Rossini et al. 2001; Fitter et al. 2002; Savitch et al. 2007; Waters et al. 2009). However, their regulation has not been the focus of investigation. Thus, the present study investigated regulation of *GLK1* and *GLK2* in *Arabidopsis* in response to a variety of endogenous and exogenous stimuli. Transcript levels of the two genes were measured following experimental treatments of *Arabidopsis* that were designed to induce various signalling pathways. The signalling pathways were: retrograde chloroplast-to-nucleus-, sugar-, nitrogen-, pathogen-, phytohormone- and cold response signalling pathways.

The following Introduction will provide background information on *GLK* transcription factors, including their structure and role in photosynthetic development and other plant processes. The current state of knowledge on transcriptional regulation of *GLK* genes will be reviewed, providing a rationale and hypotheses for the present research project.

1.2 *GLK* transcription factors

Transcription factors are DNA-binding proteins that regulate expression of their target genes through repression or activation of transcription. Transcription factors may function in response to environmental stimuli or may be restricted to certain developmental stages, tissues or cell types. Transcriptional regulation of gene expression plays a major role in many biological processes including growth and development, progression through the cell cycle, and responses to changes in the environment (Riechmann et al. 2000; Zhang 2003). In the model plant *Arabidopsis*, more than 1,700 or 5% of genes are believed to encode transcription factors. Less than 10% of them have been genetically characterized (Riechmann and Ratcliffe 2000).

1.2.1 Discovery of *GLK* transcription factors

Chloroplast development is essential for the proper functioning of all photosynthetic cells. Although much is known about the components and assembly of the photosynthetic apparatus, very few regulatory genes have been identified (Fitter et al. 2002). Over the past two decades, *GLK* transcription factors have emerged as important regulators of chloroplast development and expression of nuclear photosynthesis-related genes in land plants, including both C_3 and C_4 species (Fitter et al. 2002; Waters et al. 2009; Waters and Langdale 2009).

In C_3 plants such as rice and *Arabidopsis*, carbon fixation occurs in mesophyll cells, while bundle sheath cells do not perform photosynthesis. In C_4 plants such as maize, carbon is fixed in mesophyll cells and shuttled to bundle sheath cells. The cells are arranged according to Kranz anatomy, with C_4 mesophyll cells forming a ring around C_4 bundle sheath cells surrounding the vein (Edwards and Walker 1983). Mesophyll cells that are not

in direct contact with bundle sheath cells, however, develop C_3 photosynthesis (Langdale et al. 1988). Thus, in the C_4 plant maize three photosynthetic cell types are found: C_4 bundle sheath, C_4 mesophyll and C_3 mesophyll. C_3 photosynthetic development appears to be the ground state, with C_4 differentiation occurring in response to light-induced signals (Langdale et al. 1988; Langdale and Nelson 1991).

GLK genes were independently discovered in maize by two groups and named *Golden2* (*G2*) and *Bsd1* (Jenkins 1926; Langdale and Kidner 1994). Mutation in *G2* was shown to produce golden colour in leaves, while mmutation in *Bsd1* resulted in a variegated phenotype with green revertant sectors on a pale green background (Jenkins 1926; Langdale and Kidner 1994). At the subcellular level, mutation in *Bsd1* disrupted chloroplast development and resulted in the loss of C_4 photosynthetic enzymes in bundle sheath cells. Chloroplasts contained rudimentary lamellae that did not span the length of the plastid. C_4 mesophyll cells, however, were unaffected by disruption of *Bsd1* (Langdale and Kidner 1994).

In dark-grown mutant seedlings, etioplast development was also disrupted in bundle sheath cells but unaffected in mesophyll cells. However, photosynthetic enzyme accumulation was reduced in both bundle sheath and mesophyll cells. Based on these data, it was suggested that the *Bsd1* gene is involved in morphological differentiation of chloroplasts in a light-independent and bundle sheath-specific manner. Photosynthetic gene expression is influenced by *Bsd1* in both mesophyll and bundle sheath cells in the dark (C_3 state), and exclusively in bundle sheath cells in the light (C_4 state). Thus, it was proposed that the *Bsd1* gene action is important in both cell types in the C_3 state, and is restricted to bundle sheath cells after induction of the C_4 state. *bsd1* was subsequently shown to be allelic

to *g2* (Langdale and Kidner 1994). The initial discovery led to the identification of a homologous maize gene, *Golden2-like* (*ZmGLK1*), as well as homologous genes in rice, *Arabidopsis*, and the moss *Physcomitrella patens* (Rossini et al. 2001; Fitter et al. 2002; Yasumura et al. 2005).

1.2.2 GARP transcription factor family

Based on the structure of their DNA binding domain, *GLK* genes belong to the plant-specific GARP family of transcription factors, which in *Arabidopsis* comprises 56 members (Riechmann et al. 2000; Guo et al. 2005). The GARP motif is classified as a MYB-like domain and is related to the MYB domain (Riechmann et al. 2000; Refer to Figure 1.1). MYB transcription factors control many important processes, such as development, metabolism and plant responses to biotic and abiotic stresses (Dubos et al. 2010).

The GARP transcription factor family was named after the maize ***G****OLDEN2* gene, *Arabidopsis* ***ARR*** B-class genes, and *Chlamydomonas* ***P****SR1* (Riechmann et al. 2000). *Arabidopsis* response regulators (ARRs), divided into type A and type B, are related to prokaryotic response regulators of two-component systems and are involved in the histidine to aspartate phosphorelay signal transduction system. Type B ARRs are believed to be nuclear-localized transcription factors activated through phosphorylation of their receiver domains, and are known to be involved in cytokinin signalling (Mizuno 2004). *Chlamydomonas PSR1* is a regulator of phosphorus metabolism (Wykoff et al. 1999).

The GARP family also includes *Arabidopsis PHR1*, a gene homologous to *PSR1*, which is involved in phosphate starvation signaling (Rubio et al. 2001), and genes of the *KANADI* transcription factor family, which regulate organ polarity in lateral organs of *Arabidopsis* (Eshed et al. 2001). Several GARP family members regulate vascular tissue

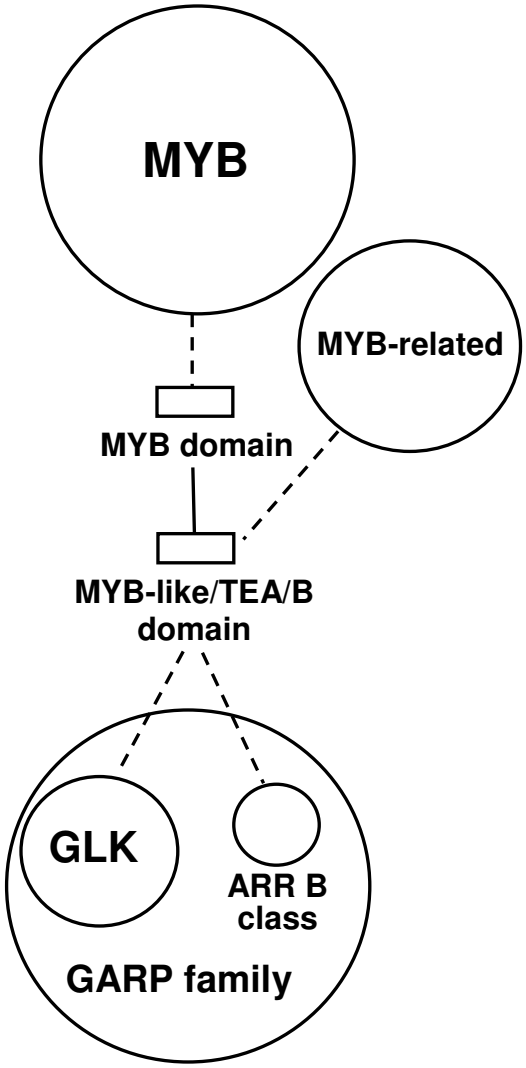


Figure 1.1 GARP transcription factor family and its relationship to other *Arabidopsis* transcription factor families. Gene families are indicated by circles and DNA-binding domains are represented by rectangles. Adapted from Riechmann et al. (2000).

development, for example *APL* (*ALTERED PHLOEM DEVELOPMENT*), involved in phloem and xylem histogenesis (Bonke et al. 2003). Other GARP transcription factors, *LUX ARRHYTHMO* and *PSEUDO RESPONSE REGULATOR*s (*PRR*s), regulate circadian rhythmicity (Hazen et al. 2005; Mizuno and Nakamichi 2005).

1.3 Structure of *GLK* genes

1.3.1 *GLK1* and *GLK2*

Although *GLK* genes in several plant species are classified as *GLK1* or *GLK2*, there is no definition of structure differentiating between the two genes. After the discovery of *GLK* genes in maize, homologs found in other plants were named *GLK1* or *GLK2* based on their sequence similarity to the maize *ZmGLK1* or *G2*, respectively (see for example Rossini et al. 2001; Fitter et al. 2002; Yasumura et al. 2005).

The nucleotide sequence identity between the full-length coding sequences of *Arabidopsis GLK1* and *GLK2* is 54.5% (NCBI accession numbers *AY028367* and *AY028368*, respectively). The sequence identity between the full-length peptides is 50.4%, while the two conserved regions, the GARP and GCT domains, exhibit higher amino acid sequence identity between the two proteins (90% and 79%, respectively) (Bravo-Garcia et al. 2009).

Based on the biased distribution of particular amino acids, GLK proteins can be divided into three regions. The N-terminal portion of GLK peptides is rich in acidic residues. The middle region, which includes the GARP domain, is basic, and the C-terminal region is proline-rich (Tamai et al. 2002). The different domains of GLK1 and GLK2 are shown in Figure 1.2 and described in more detail in Sections 1.3.3 and 1.3.4.

1.3.2 Nuclear localization signal and GARP domain

Consistent with its proposed role as a transcription factor, the maize *G2* was found to possess a nuclear localization signal (NLS) (Hall et al. 1998). Nuclear localization of G2 was demonstrated using *G2–GUS* reporter gene fusions in onion epidermal cells. Gene sequence analysis revealed that the NLS is located in the first exon of *G2* (Hall et al. 1998).

Several lines of evidence suggest that the GARP domain binds DNA. For example, analysis of *GLK* sequences revealed that the GARP domain is of the helix-loop-helix (HLH) structure, which had been shown to bind DNA in a number of transcription factors (Massari and Murre 2000; Rossini et al. 2001). In addition, the GARP domain of *GLK* genes shares some sequence similarity with eukaryotic DNA-binding TEA domains. The GARP domain is also highly similar to the B motif of type B ARRs (Imamura et al. 1999; Rossini et al. 2001), which was shown to be responsible for their DNA-binding activity (Sakai et al. 1998, 2000; Imamura et al. 1999; Hosoda et al. 2002).

Experimental evidence also confirmed that GLK proteins bind DNA. Chromatin immunoprecipitation experiments revealed direct association of GLK1 with several photosynthesis-related genes (Waters et al. 2009). However, GLK1 binding sites could not be defined experimentally using either bacterial-1-hybrid screens or gel shift assays coupled with random binding site selection. It was thus proposed that GLK1 binds DNA in a non-sequence-specific manner, and needs interacting partners to recognize specific *cis*-elements in promoters of its target genes (Waters et al. 2009). Such potential interacting partners of GLK proteins are G-box-binding bZIP transcription factors (GBFs) (Tamai et al. 2002; Waters et al. 2009). Yeast-two-hybrid and *in vitro* binding assays found AtGLK1 and AtGLK2 to interact with Pro-rich activation domains of GBF1 and GBF3 (Tamai et al.

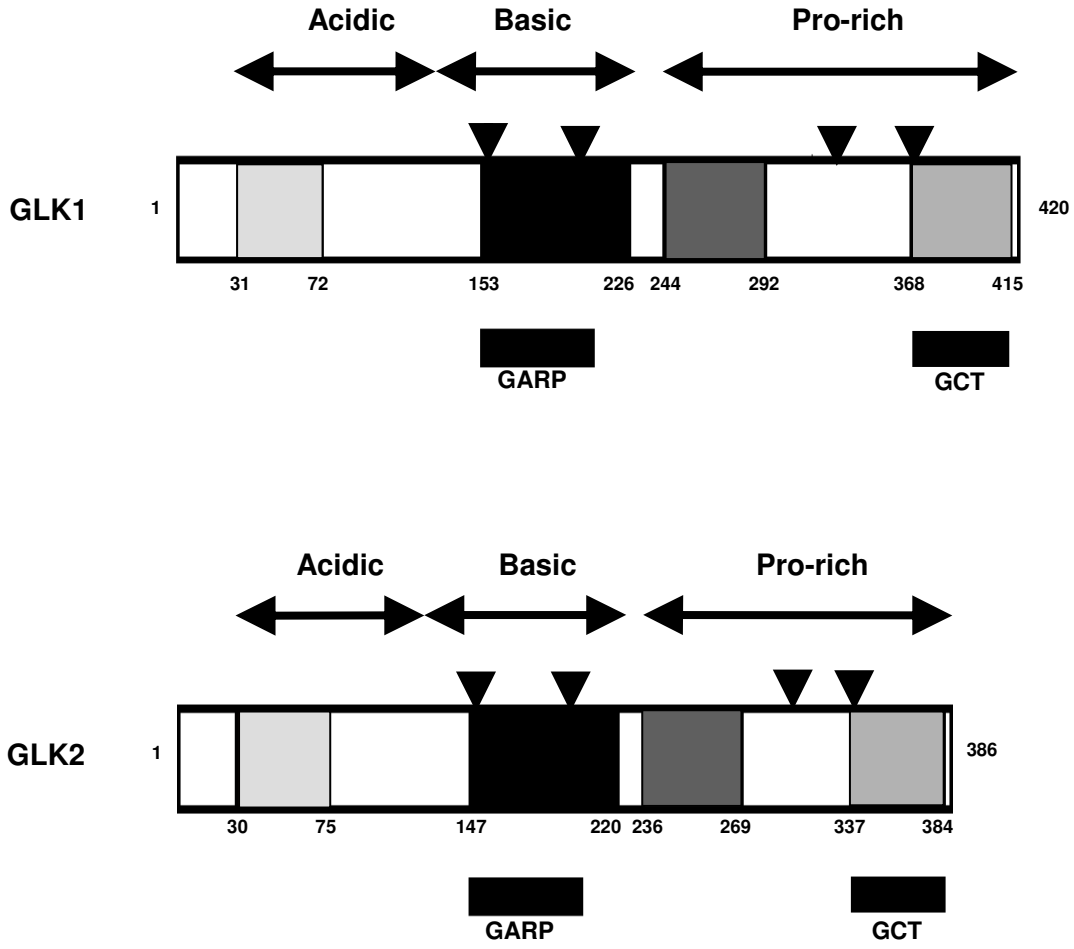


Figure 1.2 Schematic representation of *Arabidopsis* GLK1 and GLK2 peptides. Well-conserved regions are represented with filled-in rectangles. Amino acid positions are shown with numbers. Black triangles mark the position of introns. Adapted from Tamai et al. (2002).

2002). AtGLK1 was shown to bind with the Pro-rich regions of GBF1 and GBF3, and AtGLK2 was shown to bind to the Pro-rich region of GBF1. GBFs have been implicated in the regulation of light-responsive genes (Baum et al. 1999; Kircher et al. 1998; Schindler et al. 1992; Terzaghi et al. 1997; Wellmer et al. 2001). GBF3 has additionally been suggested to be involved in the regulation of genes responsive to abscisic acid (ABA) (Lu et al. 1996). As both *GLK* genes and GBFs are transcription factors, their ability to interact may indicate that they are able to act as part of a multiprotein complex formed on the promoter of target genes (Tamai et al. 2002).

1.3.3 N- and C- terminal regions

The N-terminal portion of GLK proteins was found to play a role in transactivation of transcription in yeast (Rossini et al. 2001; Tamai et al. 2002). Full length maize G2 or ZmGLK1 proteins induced expression of the reporter gene in a yeast GAL4 transactivation assay (Rossini et al. 2001). The N-terminal portions of the proteins, spanning the acidic N-terminus and the GARP domain, were sufficient to transactivate transcription. In contrast, the C-terminal portions, which include the proline-rich region and the GCT box, did not transactivate transcription in yeast (Rossini et al. 2001). The transactivating activity of *Arabidopsis* GLK1 in yeast was also mapped to the N-terminal portion of the protein (Tamai et al. 2002).

Although the yeast assays showed transactivation of transcription by the N-terminal portion of GLK proteins, both the N- and C-terminal portions seem to play a role in this process in higher plants. Thus, in transient assays in *Arabidopsis* cells, both the N-terminal and C-terminal portions of AtGLK1 were capable of inducing transcription of the reporter gene (Tamai et al. 2002).

The C-terminal end of *GLK* peptides has a highly conserved region, known as the GOLDEN2 C-terminal (GCT) box (Rossini et al. 2001). The GCT box, which had not been identified previously and could not be found in yeast and animal databases, was found to play a role in homo- and heterodimerization of the maize G2 and ZmGLK1 proteins. A yeast-two-hybrid assay revealed that the C-terminal peptides of G2 and ZmGLK1 interacted with either of the full-length proteins. However, they did not interact with the N- or C-terminal regions alone, indicating that either the interacting region spans the two regions or the secondary structure of the truncated peptides does not allow for dimerization in the yeast-two-hybrid system (Rossini et al. 2001).

1.4 The role of *GLK* genes in chloroplast development

1.4.1 *ZmGLK1* and *G2* in maize

Following the discovery of *G2*, the role of *GLK* genes in maize was investigated by determining transcript accumulation profiles of *G2* and *ZmGLK1* in different types of photosynthetic tissue (Rossini et al. 2001). *G2* transcripts were present in all photosynthetic cells in the C_3 tissue of etiolated seedlings, but became restricted to C_4 bundle sheath cells (absent from C_4 mesophyll) in C_4 tissue of fully-developed plants. Based on these data it was proposed that *G2* is a general regulator of chloroplast development in C_3 tissue, but specific regulator of chloroplast development in bundle sheath cells in C_4 tissue. *ZmGLK1* expression complemented that of *G2*, with transcripts preferentially accumulating in C4 mesophyll cells, and to a lesser extent in C_3 mesophyll and C_4 bundle sheath cells. It was thus suggested that *G2* and *ZmGLK1* have distinct roles in C_4 differentiation of maize (Rossini et al. 2001).

The phenotypes of *g2* maize mutants correlated with the *G2* transcript accumulation

profiles with respect to their chloroplast structure and photosynthetic enzyme accumulation (Cribb et al. 2001). *G2* mutants were pale green with revertant sectors. In C_4 bundle sheath cells, chloroplasts were reduced in size compared to wild type and contained rudimentary lamellae. In C_4 mesophyll cells, however, chloroplasts were indistinguishable from wild type with respect to their structure and size. Finally, in C_3 etiolated leaves, chloroplast development was also perturbed; unlike in wild-type cells, etioplasts did not contain prolamellar bodies and had a different internal membrane organization (Cribb et al. 2001).

Mutations in *G2* did not affect the expression of several photosynthetic enzymes, as their transcripts accumulated in the absence of the *G2* transcript. However, C_3 mesophyll cells in etiolated mutant plants had reduced transcript levels of *RbcS* and *RbcL*, encoding the small and large subunits of Rubisco, respectively, and no Rubisco protein was detected. Based on these findings, it was proposed that the primary role of *G2* is to regulate chloroplast development in both C_3 and C_4 tissue and that the effect on Rubisco in C_3 tissue is an indirect consequence of perturbed plastid development. A possible explanation for the different effects of perturbed plastids in the two tissue types is that plastid competence is more important for photosynthetic enzyme expression in C_3 tissue, while in C_4 tissue light signals may exert more influence (Cribb et al. 2001).

1.4.2 *OsGLK1* and *OsGLK2* in rice

GLK genes were also found in the C_3 plant rice (Rossini et al. 2001). Based on amino acid sequence identity, *OsGLK1* and *OsGLK2* were determined to be orthologs of the maize *ZmGLK1* and *G2*, respectively. The transcript accumulation profiles of *OsGLK1* and *OsGLK2* were found to overlap. However, there was some temporal separation between the two genes, as *OsGLK1* transcripts were more highly expressed during the early stages of

development, while *OsGLK2* transcript levels were maintained throughout development. It was suggested that the overlapping transcript accumulation profiles of *OsGLK1* and *OsGLK2* indicate functional redundancy of the two genes in C_3 plants. Conversely, transcript accumulation profiles of *ZmGLK1* and *G2* in maize and *g2* mutant phenotypes suggest that the evolution of C_4 plants involved specialization of the two genes for function in different photosynthetic cell types (Rossini et al. 2001).

Consistent with the proposed role of *GLK* genes in chloroplast development, ectopic overexpression of *OsGLK1* in non-green rice cells resulted in the regeneration of green callus and development of functional chloroplasts (Nakamura et al. 2009). Microarray analysis revealed that a large number of nuclear genes encoding chloroplast components as well as chloroplast-encoded genes were upregulated in the *GLK1*-overexpressing callus compared to wild type (Nakamura et al. 2009). Transient expression of *OsGLK1* in rice green tissue protoplasts also resulted in increased transcript levels of several selected photosynthetic genes (Zhang et al. 2011).

1.4.3 *AtGLK1* and *AtGLK2* in *Arabidopsis*

In *Arabidopsis*, both *AtGLK1* and *AtGLK2* transcripts were found to accumulate in the most photosynthetically active tissues (rosette and cauline leaves) (Fitter et al. 2002). In other tissues (cotyledons, shoots, flowers), *AtGLK2* transcripts were found to predominate. Furthermore, only *AtGLK2* was detected in roots and siliques. *AtGLK1* accumulation profiles were regulated by light, with transcript levels increasing during the light period of the diurnal cycle. In contrast, *AtGLK2* transcript accumulation was found to be regulated by the circadian clock, and enhanced by light. Based on these findings it was proposed that

"AtGLK1 acts to boost photosynthetic capability in tissues with a high photosynthetic workload" (Fitter et al. 2002).

In *Arabidopsis*, single *glk* mutants were indistinguishable from wild type in their overall appearance, thylakoid stacking, transcript levels of several photosynthetic genes, and chlorophyll levels, suggesting that *GLK1* and *GLK2* function is at least partially redundant (Fitter et al. 2002).

In contrast, double *glk* mutants were pale green and had reduced transcript levels of several photosynthetic enzymes. Chloroplasts in the double mutants were about 50% smaller than in wild type and had perturbed thylakoid stacking, reduced levels of granal lamellae and lower chlorophyll content (Fitter et al. 2002). Etioplasts of dark-grown *glk1 glk2* seedlings contained smaller prolamellar bodies than wild type, indicating that chlorophyll precursor levels were reduced in the mutants even in the dark (Waters et al. 2009). As expected, double *glk* mutants accumulated decreased amounts of the chlorophyll precursor protochlorophyllide, and exhibited a general reduction of flux through the chlorophyll biosynthesis pathway. In agreement with these findings, *glk1 glk2* plants contained decreased levels of transcripts of chlorophyll biosynthesis genes (Waters et al. 2009).

The perturbed thylakoid development of the double mutant was attributed to loss of Photosystem II (PSII) complexes, as levels of the D1 polypeptide, a representative structural protein from PSII, were reduced. Expression of several members of the LHCP gene family, which encode proteins that hold the chlorophyll molecules within the thylakoid membranes, was also lower than in wild type (Fitter et al. 2002).

Expression of *AtGLK1* or *AtGLK2* in the mutant *Atglk1 Atglk2* background resulted in reversal of the mutant phenotype, restoring chlorophyll levels and transcript levels of

LHCB6, a representative photosynthesis-related gene encoding a monomeric protein of the PSII antenna (Waters et al. 2008). Transcription of a number of other photosynthesis-related genes such as chlorophyll biosynthetic enzymes and antenna proteins was also increased in constitutive *GLK1* and *GLK2* overexpressors of *Arabidopsis*. Transcript levels of genes encoding enzymes of the Calvin cycle were, however, unaffected (Waters et al. 2009).

GLK targets were found to include a number of nuclear photosynthesis-related genes (Waters et al. 2009). For example, *GLK* induction in an inducible expression system followed by transcriptome analysis resulted in increased levels of transcripts for chlorophyll biosynthetic enzymes and light harvesting and electron transport genes. Chromatin immunoprecipitation experiments revealed that many of these genes are direct targets of GLK1. This finding, however, could not be confirmed by bacterial-1-hybrid screens or gel shift assays coupled with random binding site selection, which was suggested to indicate that GLK proteins need various interacting partners to bind DNA in a sequence-specific manner (Waters et al. 2009).

1.4.4 *PpGLK1* and *PpGLK2* in *Physcomitrella patens*

In the moss *P. patens*, no obvious differences in transcript accumulation profiles between the two *GLK* genes were observed (Yasumura et al. 2005). One possible explanation for this redundancy is that since the genes are expressed during the haploid stage of the life cycle, there may be additional selective pressure to maintain duplicate copies. Alternatively, the redundancy may be explained if the *P. patens GLK* genes were duplicated recently, which is supported by the fact that the degree of sequence conservation is much higher between the two *P. patens* genes than between the two *Arabidopsis GLK* genes (Yasumura et al. 2005).

The effects of *glk* mutations on the phenotype of *P. patens* were similar to those seen in *Arabidopsis* (Yasumura et al. 2005). Single *Ppglk* mutants were indistinguishable from wild type in their overall appearance, chlorophyll levels and chloroplast ultrastructure. In contrast, double *Ppglk1 Ppglk2* mutants were pale green, exhibited perturbed thylakoid stacking and accumulated reduced transcript levels of several photosynthetic genes (Yasumura et al. 2005).

When *PpGLK1* was introduced into the *Arabidopsis* double *glk* mutant, the mutant phenotype was partially reversed. Chlorophyll levels and levels of several photosynthetic enzymes increased compared to the double mutant. This partial rescue and the similar effects of *GLK* mutations in *Arabidopsis* and *P. patens* suggest that GLK function is conserved to some extent in bryophytes and angiosperms. As bryophytes diverged from the rest of the land plans more than 400 million years ago and are the most distantly related group to the angiosperms, *GLK* appears to be one of the most ancient conserved regulatory mechanisms in plants (Yasumura et al. 2005).

However, the cross-species complementation was found to be unidirectional, as expression of *AtGLK1* in *P. patens Ppglk1 glk2* double mutants failed to rescue the mutant phenotype (Bravo-Garcia et al. 2009). In addition, expression of *AtGLK1* in *glk1 glk2* mutants of *Arabidopsis* under the control of the *PpGLK* promoters also did not complement the mutant phenotype. These results imply that although *GLK* genes are involved in chloroplast development in both *P. patens* and *Arabidopsis*, the genes have diverged over the course of evolution, and have acquired specialized functions in bryophytes and angiosperms (Bravo-Garcia et al. 2009).

1.4.5 *GLK* genes act cell-autonomously

GLK genes were found to direct chloroplast development in a cell-autonomous manner, as determined by sector analysis and *AtGLK1* overexpression in *Arabidopsis* under the control of different tissue-specific promoters (Waters et al. 2008). Sector analysis was carried out by examining the boundary between wild-type sectors and mutant mesophyll tissue in a chimeric *Atglk1 Atglk2* double mutant transformed with a construct encoding a GFP-AtGLK1 fusion protein (GFP, Green Fluorescent Protein). Confocal microscopy revealed that in wild type tissue chloroplasts were larger than in mutant tissue and contained more chlorophyll, as determined by chlorophyll autofluorescence. There was a clear distinction between the two tissue types at the boundary, with no intermediate cellular phenotypes or chloroplasts of different phenotypes within a single cell (Waters et al. 2008).

The cell-autonomous function of *GLK* genes was also shown using tissue-specific promoters to express *AtGLK1* and *AtGLK2* in epidermis or phloem of *Atglk1 Atglk2* mutant *Arabidopsis*. In two separate experiments *GLK* expression in the epidermis under the control of different epidermis-specific promoters failed to rescue the mutant phenotype. There was limited rescue in plants with *GLK* expression driven by the phloem-specific promoter, although it was believed to be due to phloem unloading, rather than inherent properties of *Arabidopsis GLK* genes. The cell-autonomous function of *GLK* genes was suggested to be important for fine-tuning photosynthesis to the specific requirements of individual cells under varying environmental conditions (Waters et al. 2008).

1.4.6 The role of *GLK* genes in photosynthetic acclimation

In nature plants are faced with frequently changing environmental conditions. Fluctuations in the amount of incident light create the need for plants to adjust their

photosynthetic rate in order to balance the energy harvested by the photosynthetic electron transport chain with the rate of carbon assimilation. Under limiting light conditions, plants grow broader and thinner leaves (Anderson et al. 1995; Weston et al. 2000), decrease the ratio of chlorophyll *a* to chlorophyll *b* (Chl *a*/*b*) and develop larger grana (Weston et al. 2000). The opposite changes occur in plants grown under high light conditions. This process of long-term acclimation to light environment is believed to be initiated by redox signals from the chloroplast, generated at the photosynthetic electron transport chain due to an imbalance of energy between the light and dark reactions of photosynthesis (Pfannschmidt et al. 2009). Although the details of the resultant chloroplast-to-nucleus signalling pathways are largely unknown, redox signals ultimately affect expression of transcription factors and other genes associated with the acclimation response (Bonardi et al. 2005; Pfannschmidt et al. 2009).

Based on the phenotype of *glk* mutants and overexpressors, it was proposed that *GLK* genes are involved in long-term photosynthetic acclimation (Waters and Langdale 2009). *Arabidopsis glk1 glk2* double mutants display certain characteristics seen in plants grown under high light, such as non-stacked thylakoids, reduced levels of photosynthetic complexes and high Chl *a*/*b* ratio (Fitter et al. 2002, Waters et al. 2009). Overexpression of *GLK* on the mutant background reverses this phenotype (Waters et al. 2008, 2009). As discussed above (Section 1.3.3), GLK1 was also found to target a number of photosynthesis-related nuclear genes, such as LHC and enzymes of the chlorophyll biosynthetic pathway, but not genes encoding enzymes of the Calvin cycle (Waters et al. 2009). In addition, *GLK* gene expression was found to be regulated by retrograde chloroplast-to-nucleus signalling (Waters et al. 2009). Taken together, these data suggest that *GLK* genes are involved in the

adjustment of the rate of light reactions of photosynthesis as part of long term photosynthetic acclimation response (Waters and Langdale 2009). Under the proposed model, *GLK* expression increases in response to low light, leading to upregulation of photosynthesis-related nuclear genes and increased rate of the light-dependent reactions of photosynthesis. Conversely, high light conditions result in lower *GLK* levels, causing a reduction in transcript levels of photosynthesis-related nuclear genes and a decreased rate of the light-dependent reactions of photosynthesis (Waters and Langdale 2009).

1.5 The role of *GLK* genes in nitrogen signalling

Both *AtGLK1* and *AtGLK2* were found to be induced in a global analysis of gene expression in response to organic nitrogen. Predictions of regulatory interactions among the affected genes, together with a previously described multinetwork of gene interactions in *Arabidopsis*, were used to generate a network of genes responsive to organic nitrogen (Gutiérrez et al. 2007, 2008). The identified transcription factors were ranked based on the number of predicted regulatory interactions with their targets. Thus, with 46 connections, *GLK1* was considered to be one of the "master" regulators of organic nitrogen signalling, ranking third after a MYB family transcription factor (At1g74840), with 51 connections, and the central clock gene *CCA1* (At2g46830), with 47 connections. *GLK2* was predicted to have 19 connections and ranked 12th among the identified regulators of organic nitrogen signalling (Gutiérrez et al. 2008).

1.6 The role of *GLK* genes in disease defence

Surprisingly, *AtGLK1* overexpression in wild type *Arabidopsis* affected transcription of a number of defence-related genes, as well as conferred resistance to the plant fungal pathogen *Fusarium graminearum* (Savitch et al. 2007). In a microarray analysis of gene expression in an overexpressor of *GLK1* under the control of the double 35S promoter, 194 genes were upregulated and 146 genes were downregulated more than two-fold. Most highly upregulated were defence-related genes as well as genes of enzymatic function known to be involved in biotic stress responses (Savitch et al. 2007).

1.7 The role of *GLK* genes and plant circadian clocks

Recent findings suggest that *GLK* genes may be involved in the regulation of circadian clocks (Sprott et al. 2010). Transcriptome analyses in *Arabidopsis* revealed that a large number of genes were affected similarly in *GLK* knockouts and overexpressors. Both *GLK* knockouts and overexpressors also exhibited changes in the time of induction of many genes, with genes typically induced in the morning having peak expression in the evening, and vice versa. To further explore the possibility that *GLK* genes may be involved in the regulation of circadian clocks, the major clock related genes were monitored for diurnal changes in expression in *GLK1* OE, *GLK2* OE, *glk1* KO, *glk2* KO and *glk1 glk2* KO. Thus, *CCA1*, *LHY*, *APRR3*, *APRR5*, *APRR7*, *APRR9*, *TOC1*, *GI*, and *PCL1* were found to be differentially affected in diurnal amplitude or time of appearance. Therefore, disruption of either *GLK1* or *GLK2* altered circadian parameters of a number of genes (Sprott et al. 2010).

1.8 Transcriptional regulation of *GLK* genes

Most findings on transcriptional regulation of *GLK* genes relate to their regulation by retrograde chloroplast-to-nucleus signalling (Kakizaki et al. 2009; Pesaresi et al. 2009; Waters et al. 2009; Inaba 2010). In addition, the plant hormone brassinosteroid (BR) has been shown to suppress *GLK* genes in *Arabidopsis* (Yu et al. 2011). Numerous other factors have been found to affect *GLK* expression, although these studies did not focus specifically on *GLK* genes (Jiao et al. 2003; Cao et al. 2006; Kim et al. 2006; Tepperman et al. 2006; Jung et al. 2007; Lee et al. 2007; Soitamo et al. 2008; Bozso et al. 2009).

1.8.1 Retrograde chloroplast-to-nucleus signalling

Genes encoding chloroplast components are located in both the nuclear and chloroplast genomes. Less than 5% of chloroplast proteins are encoded within the organelle, and as a result, chloroplast multiprotein complexes are mosaics of chloroplast- and nuclear-encoded proteins (Leister 2003). Thus, their assembly and maintenance requires coordination of chloroplast and nuclear gene expression. Retrograde chloroplast-to-nucleus signalling plays an important role in this process, relaying to the nucleus information related to the developmental or metabolic state of the chloroplast. Retrograde signals may be generated in the chloroplast as a result of chlorophyll biosynthesis by the tetrapyrrole pathway, chloroplast protein synthesis, production of ROS, or changes in redox state of the organelle (Kleine et al. 2009).

1.8.2 Redox state of the photosynthetic electron transport chain and plastid protein import

Recent evidence suggests that *GLK* expression may be affected by retrograde signals from the chloroplast to the nucleus. For example, *GLK1* expression was suppressed in the

state-transition mutant *psad1*, which has overreduced photosynthetic electron transport chain under high light conditions (Pesaresi et al. 2009). *GLK1* was also suppressed in the *Arabidopsis plastid protein import2* (*ppi2*) mutant, which lacks the chloroplast protein import receptor atToc159 and generates retrograde chloroplast signals leading to downregulation of nuclear photosynthesis-related genes (Kakizaki et al. 2009). *GLK1* overexpression on the *ppi2* mutant background partially reversed the effect of the mutation, resulting in increased expression of some photosynthesis-related genes and a pale green phenotype, as opposed to the albino phenotype of *ppi2* (Kakizaki et al. 2009).

The repression of *GLK1* by retrograde signalling arising from defective plastid protein import may be mediated by *GUN1*, a chloroplast protein which has been shown to integrate signals from tetrapyrrole biosynthesis, plastid gene expression, redox changes and plastid protein import (Koussevitzky et al. 2007; Kakizaki et al. 2009). Thus, treatment with the tetrapyrrole biosynthesis inhibitor norflurazon repressed *GLK1* to a greater extent in wild type than in the *gun1* mutant (Kakizaki et al. 2009). In addition, *GLK1* levels were higher in the *gun1 ppi2* double mutant than in the *ppi2* single mutant. Based on these data, it was proposed that chloroplast retrograde signals arising from defective plastid protein import upregulate *GUN1*. *GUN1* in turn downregulates *GLK1*, resulting in suppression of nuclear photosynthesis-related genes (Kakizaki et al. 2009; Inaba 2010).

1.8.3 Tetrapyrrole biosynthesis and chloroplast protein synthesis

In addition, *GLK1* and *GLK2* transcription was found to be affected by retrograde chloroplast signals generated as a result of perturbations in tetrapyrrole levels and plastid gene expression, as was shown in a study that used *genomes uncoupled* (*gun*) mutants and the herbicide norflurazon (Koussevitzky et al. 2007). Norflurazon inhibits carotenoid

biosynthesis and causes photooxidative damage (Breitenbach et al. 2001), resulting in the repression of nuclear photosynthesis-related genes (Batschauer et al. 1986). In *gun* mutants, however, nuclear photosynthesis-related gene expression is not affected by norflurazon (Susek et al. 1993). GUN2, GUN3, GUN4 and GUN5 are involved in chlorophyll biosynthesis and their respective mutants exhibit perturbations in the levels of tetrapyrrole intermediates (Davis et al. 1999; Muramoto et al. 1999; Kohchi et al. 2001; Mochizuki et al. 2001; Larkin et al. 2003). The precise function of GUN1 is currently unknown, but it appears to be involved in a different chloroplast signalling pathway than the other GUN proteins. Thus, in the *gun1* mutant, unlike in wild type plants or *gun2-5* mutants, expression of photosynthesis-related nuclear genes was not lost in response to treatment with the plastid protein synthesis inhibitor lincomycin (Gray et al. 2003). In addition, the nuclear-encoded gene *Lhcb* was repressed in the *gun1* mutant in response to high light to a lesser extent than in wild type plants. It was thus suggested that GUN1 is a "master switch," integrating plastid signals derived from chlorophyll biosynthesis, plastid gene expression and high light (Koussevitzky et al. 2007).

Publically available microarray data suggest that *GLK1* expression is regulated by retrograde signals from the chloroplast through the GUN1-mediated pathway. *GLK1* transcription was about two times higher in the *gun1* mutant than in wild type *Arabidopsis*. In addition, *GLK1* suppression by norflurazon was much less pronounced in the *gun1* mutant than in wild type plants. *GLK2* appears to be affected by chloroplast retrograde signals mediated by GUN1 and GUN2-5, as its transcription was repressed by norflurazon to a lower extent than in wild type in both the *gun1* and *gun5* mutants. (Koussevitzky et al. 2007; GEO Profiles accession number GDS3379, available at http://www.ncbi.nlm.nih.gov).

The *gun1 gun5* double mutant, which has a more pronounced phenotype than single *gun* mutants (Mochizuki et al. 2001), also exhibited reduced repression of *GLK1* and *GLK2* in response to either norflurazon or lincomycin (Waters et al. 2009). The extent of rescue was different for the two genes: *GLK2* repression was strongly reversed, while *GLK1* repression was weakly reversed in the double mutant. The differential regulation of transcription of *GLK1* and *GLK2* was suggested to be responsible for maintaining the duplicated gene pair, despite the seemingly similar role of the two genes in chloroplast development. *GLK1* and *GLK2* may thus act differently if each of them is co-expressed together with different sets of other genes (Waters et al. 2009).

Interestingly, the *glk1 glk2* double mutant was found to have a weak *gun*-like phenotype, as several genes known to be highly sensitive to plastid signals were repressed by lincomycin and norflurazon to a lower extent in *glk1 glk2* plants than in wild type. Overall, *GLK1* and *GLK2* were shown to be responsive to chloroplast retrograde signals, but to be regulated differently (Waters et al. 2009).

1.8.4 Other factors affecting *GLK* transcription

In addition to being regulated by retrograde chloroplast-to-nucleus signals, *GLK* gene expression is affected by a number of factors. For example, BR-derived signals were found to adversely affect chloroplast development in *Arabidopsis*, possibly through *GLK1* and *GLK2* (Yu et al. 2011). BES1, a transcription factor involved in BR signalling, was found to indirectly repress *GLK1* and *GLK2* expression. Gain-of-function *bes1-D* mutants had reduced *GLK1* and *GLK2* transcript levels, decreased chlorophyll content and enlarged plastoglobules in their chloroplasts. In addition, out of the 20 genes that were upregulated most strongly in response to *GLK1* and *GLK2* overexpression, seven were suppressed in the

bes1-D mutant (Yu et al. 2011; Waters et al. 2009). Based on these findings, it was proposed that BR suppression of chloroplast development in the dark may proceed through the negative regulation of *GLK* genes by BES1 (Yu et al. 2011).

GLK1 expression was also shown to be affected by pathogen infection in several plant species. In *Arabidopsis*, *GLK1* was repressed in response to infection with a number of bacterial pathogens: *Agrobacterium tumefaciens*, *P. syringae* pv. *tomato DC3000* (*Pst*), *Pst avrRpm1*, and *P. savastanoi* pv. *phaseolicola*. In addition, *GLK1* was repressed in *Arabidopsis* in response to *Myzus persicae* (aphids) infestation (Bozso et al. 2009). In the model legume *Medicago truncatula*, the *GLK1* homolog was repressed in response to *Pseudomonas syringae* pv. *syringae 61* (*P.s.*) and *P.s. hrcC*, which induce HR and basal response, respectively (Bozso et al. 2009). Conversely, *GLK1* was found to be upregulated in a global analysis of gene expression in tobacco (*Nicotiana benthamiana*) in response to HR induced by *P. syringae* pv. s*yringae* infection (Kim et al. 2006).

A search of publically available microarray data revealed that a number of other environmental and internal stimuli affect *GLK* transcription. For example, during *Arabidopsis* seedling development, both *GLK1* and *GLK2* were upregulated by white light (Jiao et al. 2003). *GLK2* responded to early light induction much more strongly than *GLK1* (Tepperman et al. 2006), and was also upregulated by blue light, while *GLK1* was not (Jiao et al. 2003). *GLK2* was also upregulated in response to light under low temperature treatment (Soitamo et al. 2008). Interestingly, promoter of *GLK2*, but not of *GLK1,* contains binding sites of the photomorphogenesis regulator *HY5* (Lee et al. 2007).

AtGLK1 and *AtGLK2* were also among the affected genes identified in a study of global regulation of gene expression by gibberellin (Cao et al. 2006). Gibberellin is a plant

hormone involved in seed germination, stem elongation and floral development. The binding of gibberellin to its receptor triggers degradation of DELLA repressors, which prevent various growth processes in the absence of the gibberellin signal. *AtGLK1* and *AtGLK2* were found to be gibberellin-downregulated and DELLA-upregulated (Cao et al. 2006).

The plant hormone methyl jasmonate was shown to downregulate transcription of a number of *Arabidopsis* genes encoding chloroplast components and other proteins involved in photosynthesis (Jung et al. 2007). Consistent with its role in chloroplast development, *AtGLK2* was also among the genes that were downregulated by methyl jasmonate treatment of *Arabidopsis* (Jung et al. 2007). Many other studies that did not focus specifically on *GLK* genes provide information on *GLK* transcription as part of analyses of global changes in gene expression under various environmental conditions.

1.9 Hypotheses and objectives of research

Although the function of *GLK* genes is beginning to emerge, primarily with respect to their role in photosynthetic development, one area of research which requires further investigation is the conditions under which *GLK* genes are expressed. The fragmented information on *GLK* expression that is available from various microarray analyses does not provide a complete picture of the regulation of these genes. The present study is a comprehensive investigation of *GLK* regulation, addressing the question of how various environmental and endogenous stimuli influence transcription of *GLK1* and *GLK2* in the model plant *Arabidopsis* (NCBI accession numbers At2g20570 and At5g44190, respectively). The objective is to elucidate the signalling pathways that regulate expression of these transcription factors.

The first hypothesis is that, although thought to be partially redundant with respect to chloroplast development (Fitter et al. 2002; Waters et al. 2009), *GLK1* and *GLK2* are differentially affected by various environmental and endogenous signals. Thus, *GLK1* and *GLK2* transcript levels are expected to be different in response to at least some stimuli applied to *Arabidopsis*. The second hypothesis is that although *GLK1* and *GLK2* transcription is affected by retrograde chloroplast-to-nucleus signals, as suggested by recent studies, different other factors and stimuli may influence *GLK* expression indirectly by producing chloroplast-to-nucleus retrograde signals. The testable prediction is that *GLK1* and *GLK2* transcription will be affected by various stimuli that do not directly induce chloroplast-to-nucleus retrograde signalling. In addition, *GLK* response to these stimuli is expected to be modulated by changes in redox state of the chloroplast, levels of ROS or by other known sources of chloroplast-to-nucleus retrograde signalling.

In order to address these hypotheses, various compounds/stimuli were tested for their effect on *GLK1* and *GLK2* transcription in wild type *Arabidopsis*, as well as in the *pgp1* mutant, which is deficient in phosphatidylglycerolphosphate (PGP) synthase (Xu et al. 2002). The *pgp1* mutant is characterized by reduced content of phosphotidylglycerol (PG) and exhibits developmentally-arrested chloroplasts under cold temperature conditions due to disruption of thylakoid membrane formation.

Arabidopsis leaves were treated with chemicals known to affect redox state of the chloroplast or the cell, nitrogen and its derivatives, sugars, pathogens and plant hormones. In addition, wild type and *pgp1* mutant *Arabidopsis* plants were acclimated to cold temperature for 5 weeks. *GLK* transcript levels were then analyzed by reverse transcription (RT) and polymerase chain reaction (RT-PCR) or quantitative PCR (qPCR).

1.9.1 Retrograde chloroplast-to-nucleus signalling

As mentioned above (Section 1.8.2), *GLK* expression was found to be affected by chloroplast retrograde signalling, including tetrapyrrole biosynthesis and plastid gene expression, demonstrated in experiments employing the plastid inhibitors norflurazon and lincomycin, respectively (Waters et al. 2009). In order to dissect the retrograde signalling pathway regulating *GLK* expression further, we focused on chloroplast redox signalling and chloroplast-derived ROS signalling, the two other known signal transduction pathways from the chloroplast to the nucleus (Pfannschmidt et al. 2003a).

In addition, we investigated the role of changes in redox state of the cytosol in regulating *GLK* expression. Changes in redox state of the cytosol have been proposed to be one of the ways of transmitting redox retrograde signals from the chloroplast to the nucleus (Mullineaux and Rausch 2005; Pfannschmidt et al. 2009). Altered redox state of the cytosol may affect transcription of nuclear genes through its influence on the activity of redox-sensitive regulatory proteins (Mullineaux and Rausch 2005).

Redox retrograde signals may be generated at several points of the chloroplast electron transport chain, or as a result of reduction of the stroma (Pfannschmidt et al. 2003a). At the electron transport chain, signals may be produced from changes in redox state of the plastoquinone (PQ) pool or ferredoxin (Fd). Various inhibitors of electron transport or of photosynthetic enzymes can be used to pinpoint the source of retrograde signals within the chloroplast (Pfannschmidt et al. 2003a). For the purposes of this study, 3-(3',4'-dichlorophenyl)-1,1'-dimethylurea (DCMU), 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB), methyl viologen (paraquat; N,N'-Dimethyl-4,4'-bipyridinium dichloride; MV), dithiothreitol (DTT), and glyceraldehyde (GA) were used. Each of these

chemicals has a distinct effect on the chloroplast, such as reduction or oxidation of the PQ pool or stroma or production of ROS.

Although the direct effect of the inhibitors used in this study is on the redox state of the chloroplast, they may also change the redox state of the cytosol through their effect on glutathione levels. Glutathione acts as a cellular redox buffer through its interconversion between its reduced (GSH) and oxidized (GSSG) forms (Rouhier et al. 2008). The first step in the synthesis of glutathione occurs in the chloroplast, and perturbations in photosynthesis may thus indirectly affect glutathione levels in the cytosol, altering its redox state (Mullineaux and Rausch 2005).

Therefore, experimental treatments with glutathione were carried out to determine whether changes in redox state of the cytosol affect *GLK* expression. Treatments with the chloroplast inhibitors in combination with GSH and GSSG were used to determine whether any effects seen with inhibitors result from changes in redox state of the cytosol. For the same purpose, treatments with glutathione were also performed in combination with other factors, such as sugars and pathogens (discussed below). It is our hope that results from this experiment will enable us to determine the site of generation of redox retrograde signals affecting *GLK* expression, whether this redox signalling proceeds through changes in redox state of the cytosol, and whether ROS plays a role in *GLK* regulation.

1.9.2 Sugars

The role of sugars in plants is not limited to their metabolism, as they also serve as signaling molecules involved in regulating growth and development, photosynthesis, secondary metabolism, and stress response (Rolland et al. 2002, 2006). Considering the numerous studies implicating *GLK* genes in chloroplast development, we propose that *GLK*

expression may be regulated by sugars. Thus, several sugars and sugar analogs were tested in *Arabidopsis* to dissect the sugar signalling pathway regulating *GLK* gene expression.

Negative feedback regulation of photosynthesis by carbon metabolites includes suppression of nuclear-encoded photosynthetic genes such as chlorophyll *a/b* binding protein (*CAB1*) and *RbcS* (Sheen 1990; Jang et al. 1997; Oswald et al. 2001). A number of nuclear-encoded photosynthetic genes are also repressed in *glk1 glk2* double mutants of *Arabidopsis* and, conversely, upregulated in constitutive and inducible *GLK* overexpressors (Fitter et al. 2002; Yasumura et al. 2005; Waters et al. 2009). The regulation of nuclear photosynthesis-related genes by sugars and *GLK* genes provides the rationale for testing the effects of sugars on *GLK* expression.

1.9.3 Nitrogen

Since *GLK1* was shown to be a central regulator of organic nitrogen signalling in *Arabidopsis* (Gutiérrez et al. 2008), and nitrogen is an essential macronutrient required for various plant growth and development processes, we decided to investigate in detail the role of nitrogen in *GLK1* and *GLK2* regulation. Numerous studies have shown that both inorganic and organic nitrogen serve as signals regulating the plant transcriptome (see for example Wang et al. 2007; Gifford et al. 2008; Gutiérrez et al. 2008). Nitrogen limitation was found to cause a reduction in growth and photosynthesis (Scheible et al. 2004; Peng et al. 2007). Resupplying nitrate-starved plants with nitrate resulted in changes in expression of a number of transcription factors, including those from the MYB and the G2-like GARP family (Scheible et al. 2004). In addition, nitrogen is required for tetrapyrrole biosynthesis, which is one of the sources of retrograde chloroplast-to-nucleus signals (Mochizuki et al. 2001; Larkin et al. 2003; Strand et al. 2003; Kleine et al. 2009).

Given the role of *GLK* genes in the regulation of photosynthetic gene expression and organic nitrogen signalling (Gutiérrez et al. 2008; Waters et al. 2009), as well as their responsiveness to chloroplast retrograde signals (Waters et al. 2009), the role of organic nitrogen in regulating *GLK* genes was investigated further. An inhibitor of organic nitrogen synthesis and several organic nitrogen derivatives were used to dissect the organic nitrogen signalling pathway responsible for regulating *GLK* expression in *Arabidopsis*.

1.9.4 Pathogens

Although most of the studies on *GLK* genes have focused on their role in chloroplast development, *AtGLK1* has also been implicated in disease defense (Savitch et al. 2007) and its transcript levels were affected by several pathogens, as discussed in Section 1.8. Pathogen infection of *Arabidopsis* may affect *GLK* transcript levels through the associated production of ROS and repression of photosynthesis (Chisholm et al. 2006; Berger et al. 2007). Generation of ROS in the chloroplast is one of the known pathways of retrograde signaling (Pfannschmidt et al. 2003a) and *GLK* expression was shown to be affected by retrograde signals from the chloroplast (Refer to Section 1.8.1). Repression of photosynthesis by pathogens may also be accompanied by downregulation of *GLK* transcription factors, as *GLK1* and *GLK2* have been shown to positively regulate a number of photosynthesis-related nuclear genes (Waters et al. 2009).

The possible role of *GLK* genes in disease defence was investigated by measuring *GLK1* and *GLK2* transcript levels *Arabidopsis* plants after infection with the nonhost *Arabidopsis* pathogen *P. syringae* pv. *maculicola* (*Psm*) and the well-studied host pathogen of *Arabidopsis P. syringae* pv. *tomato* carrying empty vector (EV), the *AvrRpm1*, or *AvrRpt2* avirulence genes.

In addition, we evaluated changes in *GLK* transcription in response to salicylic acid (SA), jasmonic acid (JA), and hydrogen peroxide (H_2O_2). SA and JA are hormones which are known to play distinct roles in defence against pathogens (Kunkel and Brooks 2002; Kachroo and Kachroo 2007). H_2O_2 is an important signalling molecule involved in the hypersensitive response (HR), a rapid death of cells at the site of pathogen infection (Ingle et al. 2006; Jones and Dangl 2006). Results of this experiment will be used to improve our understanding of the role of *GLK* genes in disease defence and their regulation in response to pathogen infection.

1.9.5 Hormones

Previously, the plant hormone BR was proposed to negatively regulate *GLK1* and *GLK2* expression in *Arabidopsis* (Yu et al. 2011; Refer to Section 1.8.4). In the present study, we tested BR and several other hormones for their effect on *GLK1* and *GLK2* expression. Plant hormones are important signalling molecules influencing many physiological processes such as growth, differentiation and development (Santner et al. 2009). In addition to SA and JA, used in the experiment with pathogens, the hormones used for experimental treatments of *Arabidopsis* were auxin (indoleacetic acid, IAA), cytokinin, gibberellic acid (GA_3), ABA, and BR.

SA and JA treatments may affect *GLK* expression through their involvement in pathogen defence pathways. Other hormones may affect *GLK* expression by influencing photosynthesis, chloroplast development, or plant circadian clock regulation, as *GLK* genes have been implicated in all of these processes (Savitch et al. 2007; Waters et al. 2009; Sprott et al. 2010). For example, auxin was shown to increase the net photosynthetic rate in *Brassica juncae* and chickpea (Ahmad et al. 2001; Hayat et al. 2009). Cytokinin has an

effect on photosynthesis through overreduction of the electron transport chain (Jordi et al. 2000; Wingler et al. 2005), and plays a role in chloroplast development (Werner and Schmülling 2009; Argueso et al. 2010). In addition, different plant hormones influence distinct circadian clock parameters such as period and amplitude (Hanano et al. 2006). The experiment with different hormones will be used to understand which growth, developmental, or pathogen pathways regulate *GLK* genes.

1.9.6 *pgp1* mutation and cold temperature

In addition to performing chemical treatments of *Arabidopsis*, an alternative way to investigate *GLK* regulation is through the use of mutants with altered *GLK* expression. As mentioned in Section 1.8, the *ppi2* mutant of *Arabidopsis*, which lacks the protein import receptor AtToc159, has reduced *GLK1* transcript levels (Kakizaki et al. 2009). Genetic studies indicated that *GLK1* seems to be downregulated in the *ppi2* mutant by GUN1, and in turn downregulates nuclear photosynthesis-related genes.

Another mutant with disrupted chloroplast development is the *pgp1* mutant, which is deficient in phosphatidylglycerol (PG), a glycolipid essential for proper structure and function of thylakoid membranes (Hagio et al. 2002; Xu et al. 2002; Krol et al. 2010). The *pgp1* mutant with a 30% decrease in PG content exhibited a phenotype indistinguishable from wild type at 20ºC, and a distinct yellow-white phenotype at 5ºC (Krol et al. 2010). The low-temperature phenotype was associated with a reduction in total leaf chlorophyll content and the number of stromal thylakoids per chloroplast, as well as a complete inhibition of granal stack formation.

Since at 5ºC the *pgp1* mutant has a similar phenotype to that of the *glk1 glk2* double knockout mutant, we hypothesized that the observed effect of exposure to cold temperatures

is mediated through a reduction in *GLK* transcript levels. In addition, we hypothesized that the downregulation of *GLK* genes is mediated by retrograde chloroplast-to-nucleus signals, and may thus be reversed by chemical inhibitors that block those signals. In order to test these hypotheses, wild type and *pgp1 Arabidopsis* plants were exposed to cold temperatures as well as DCMU, DBMIB, GSH and GSSG at 20ºC and at 5ºC. The use of the mutant is expected to provide a confirmation of results of experiments employing chemical treatments, as well as to help understand regulation of *GLK* genes in response to cold.

1.9.7 Gene regulatory networks

Although many different factors were tested for their effect on *GLK* expression, we hope to integrate the information gained into a comprehensive model that will reconcile the seemingly unrelated roles of *GLK* genes in chloroplast development, organic nitrogen and BR signalling, disease defence and circadian clock regulation.

The different signalling pathways examined form a complex network of interactions, such that some stimuli may affect *GLK1* and *GLK2* expression indirectly through their effect on other factors that regulate the two genes. For example, inorganic nitrogen taken up by plants from the soil is converted into the organic nitrogen derivative glutamate (Glu) (Miflin and Lea 1977), which is subsequently used for the synthesis of glutathione (Renneberg 1982). Glutathione may affect gene expression through its role as a cellular redox buffer or interaction with plant hormone and pathogen defence signalling pathways, among its many other roles in plants (Rouhier et al. 2008).

Such multiple effects of individual treatments on the plant may complicate result interpretation. Pleiotropy is a common phenomenon that is often unavoidable when investigating changes in gene expression in response to different stimuli. In particular, plant

hormones are known to have pleiotropic effects on plant growth and development through activation of large numbers of transcription factors (Santner et al. 2009). For example, auxin has been implicated in diverse processes in plants, including relay of environmental signals, regulation of branching in shoots and roots, and patterned cellular differentiation in meristems (Berleth et al. 2004). The various roles of auxin are mediated by the auxin-response factors (ARF) protein family, which in *Arabidopsis* comprises 22 members (Berleth et al. 2004).

The issue of pleiotropy as it relates to interpretation of results on *GLK* expression will be addressed in the discussion by considering various possible effects of individual treatments on *Arabidopsis*. For example, chloroplast inhibitors such as MV are expected to interfere not only with retrograde chloroplast-to-nucleus signalling generated at the electron transport chain, but also with carbon fixation (Ekkehard and Stitt 1989). Pathogens may affect gene expression through gene-for-gene interactions and as a result of increase in soluble sugar levels (Kunkel and Brooks 2002; Berger et al. 2007; Kachroo and Kachroo 2007). These and other multiple effects of different treatments will be considered together in order to understand *GLK* regulation.

To determine which of the possible pleiotropic effects of treatments are responsible for changes in *GLK* transcript levels, we used combinations of factors. For example, glutathione was used in combination with other factors to elucidate whether they regulate *GLK* genes through changes in redox state of the cell/chloroplast or through other unrelated mechanisms. An alternative way to deal with the issue of pleiotropy is to use mutants. Although mutations may also have pleiotropic effects, they can be used to complement results obtained with chemical treatments. Results of *GLK* expression analysis in the *pgp1*

mutant are expected to provide additional information on *GLK* regulation by chloroplast-to-nucleus signalling.

Overall, the experiments of the present study will add to the growing body of knowledge on *GLK* transcription factors, specifically, their regulation by environmental and endogenous stimuli. Since the known functions of *GLK* genes in chloroplast development and processes such as disease defence make them potential targets for improving plant productivity and disease resistance, studying *GLK* genes is important not only for accumulating basic knowledge but also for possible future applications to agriculture.

CHAPTER 2 MATERIALS AND METHODS

2.1 Plant growth conditions

Arabidopsis thaliana (ecotype Columbia-0) was grown under controlled conditions in a Conviron E15 cabinet. A 16-h photoperiod with a photosynthetic flux density (PFD) of 150 µmol photons m^{-2} s^{-1} and a constant temperature of 22°C were maintained. The plants were grown for 5 weeks on a mixture of Pro-Mix, soil and sand at a ratio of 1:1:1. They were watered one to two times per week as needed and fertilized with 20-20-20 (N:P:K) fertilizer every fourth watering starting when rosette diameter was approximately 1½". The relative humidity in the growth cabinet was 50%. Whole *Arabidopsis* plants or fully-developed non-senescent rosette leaves of 5-week old *Arabidopsis* plants were used in all experiments. In addition, for the cold signalling experiment, *Arabidopsis* plants grown at 20°C for 3 weeks were transferred to 5°C for 5 weeks to allow cold acclimation.

The *pgp1* mutant with a 30% reduction in PG content was kindly provided by N.P.A. Huner (University of Western Ontario, London, ON). Plants were grown for 5 weeks at 20°C or at 5°C, with all other conditions maintained as described above. In addition, some *pgp1* plants grown at 5°C were exposed to 20°C and continuous light for 48h.

2.2 Experimental treatments of *Arabidopsis*

In redox, cold, nitrogen, and sugar signalling experiments the chemicals were applied to detached *Arabidopsis* leaves by vacuum infiltration. The technique of vacuum infiltration itself had no effect on *GLK* transcript levels, as demonstrated by the control experiment using water (Figure 2.1A). Pathogen infection of whole *Arabidopsis* plants was performed by infiltrating leaves with bacterial suspensions using a needless syringe.

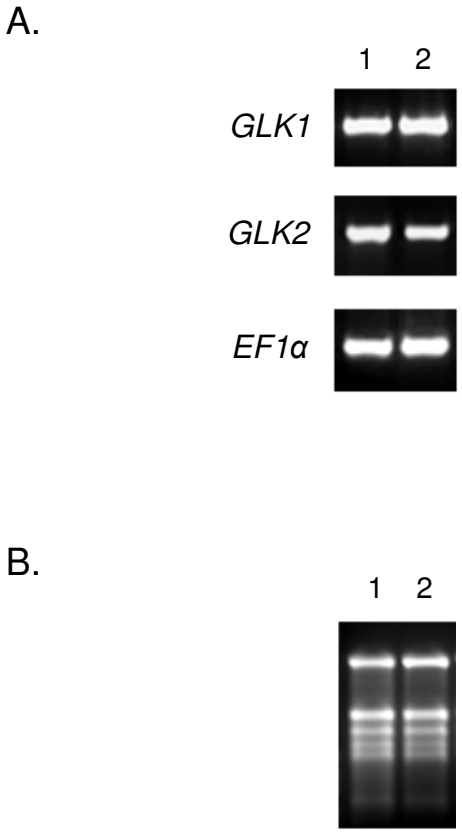


Figure 2.1 Effect of vacuum infiltration on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR analysis (A), and a representative denaturing formaldehyde gel analysis of RNA (B). (1) water-treated control; *Arabidopsis* plants were sprayed with water and leaves were collected after 24h of incubation from ZT4 to ZT4 the next day, (2) vacuum infiltration of *Arabidopsis* leaves with water; leaves were collected after 24h of incubation from ZT4 to ZT4 the next day. Two biological replicates, each with three RT-PCR replicates, were performed. Representative results are shown. Elongation factor 1α (*EF1α*) was used as housekeeping gene control.

Hormones were applied to whole *Arabidopsis* plants by spraying.

Duration of incubation was 4, 6, or 24h, depending on the particular experiment, as described below. Leaves were collected either in the early afternoon at ZT7 or ZT8, or in the evening, at ZT12 (ZT, Zeitgeber time, is time after the onset of light). The early afternoon and evening time points of collection were chosen in order to account for circadian variation in *GLK* transcript levels. Levels of both *AtGLK1* and *AtGLK2* were previously found to be highest during the light period and to decline at the end of the light period (Fitter et al. 2002). Our results confirmed that transcript levels of both *GLK1* and *GLK2* are diurnally controlled, with maximum level of transcript accumulation occurring during the morning hours (Figure 2.2). If a compound upregulates *GLK* transcription, the effect is expected to be more pronounced when the transcript levels are low, as in leaves collected in the evening. Conversely, downregulation of *GLK* may be easier to detect when transcript levels are high during the day.

Following experimental treatments, leaves were immediately frozen in liquid nitrogen and stored at -80°C for subsequent RNA extraction. Two biological replicates of each experiment were performed.

2.2.1 Chloroplast-to-nucleus retrograde signalling

The following chemicals were used to investigate the source of retrograde signals potentially affecting *GLK* expression: DCMU, DBMIB, MV, DTT, GA, GSH, and GSSG. The mode of action of these chemicals is illustrated schematically in Figure 2.3.

DCMU blocks electron transfer from the primary acceptor of PSII to the PQ pool in the electron transport chain, leading to oxidation of the PQ pool (Kirilovsky et al. 1994; Ralph et al. 2011). In contrast, DBMIB blocks electron transfer from the PQ pool to

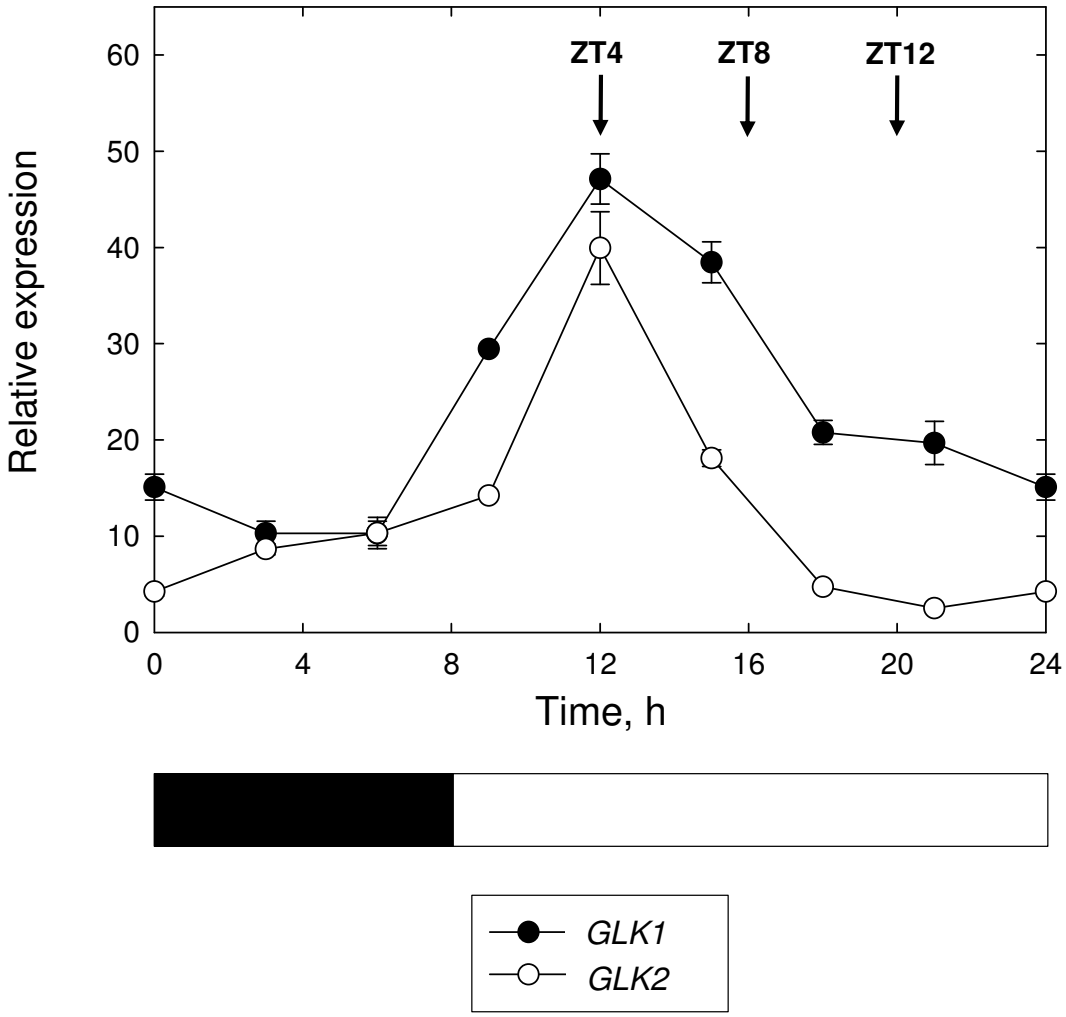


Figure 2.2 Diurnal rhythmicity of *GLK1* and *GLK2* expression in *Arabidopsis*, analyzed by qPCR. Leaves of wild type *Arabidopsis* plants were collected every 3h over a 24h period, starting at the onset of the dark period. Black bar represents the 8h dark period, and white bar represents the 16h light period. Two biological replicates, each with three qPCR replicates, were performed and the mean gene expression level was determined. The resultant values were normalized to the mean transcript levels of the housekeeping gene Elongation factor 1α (*EF1α*) and are represented by bars. Error bars represent standard error (SE). Abbreviations: ZT, Zeitgeber time.

cytochrome b6/f (Cyt b6/f), leading to overreduction of the PQ pool (Trebst 1974; Ralph et al. 2011). As changes in redox state of the PQ pool are a known source of retrograde chloroplast-to-nucleus signals (Pfannschmidt et al. 2003a), DCMU and DBMIB were used to determine whether *Arabidopsis GLK* genes are regulated by this mechanism.

MV accepts electrons from the early acceptors of Photosystem I (PSI) and reacts with oxygen, resulting in the production of the ROS superoxide ($\cdot O^{2-}$), and subsequently hydrogen peroxide (H_2O_2) (Salin 1988; Dan Hess 2000). Because it oxidizes the electron transport chain, MV may have a similar signalling effect to DCMU. However, additional signalling effects are possible through production of ROS, which are another known source of retrograde chloroplast-to-nucleus signals (Pfannschmidt et al. 2003a). In addition, MV application causes oxidation of glutathione (Law et al. 1983; Smith 1985), through induction of the ascorbate-glutathione cycle to detoxify H_2O_2 (Foyer and Noctor 2011). Oxidation of glutathione alters the redox state of the cell by altering the ratio of reduced to oxidized glutathione (GSH/GSSG). A number of important processes in plants have been shown to be under redox regulation, including gene expression, disease resistance, signal transduction and hormone signalling (Potters et al. 2010). Therefore, MV may affect *GLK* expression through several different pathways.

DTT is a strong reducing agent which has been used to reduce Fd, the final electron acceptor in the electron transport chain of photosynthesis (see for example Neumann and Drechsler 1984). The reduction of Fd results in signalling through the ferredoxin/thioredoxin (Fdx/Trx) system (Schurmann and Buchanan 2008). GA is a Calvin cycle inhibitor, blocking the regeneration of ribulose-1,5-bisphosphate (Slabas and Walker 1976; Ralph et al. 2011), and thus limiting the consumption of NADPH and ATP, which results in reduction of the

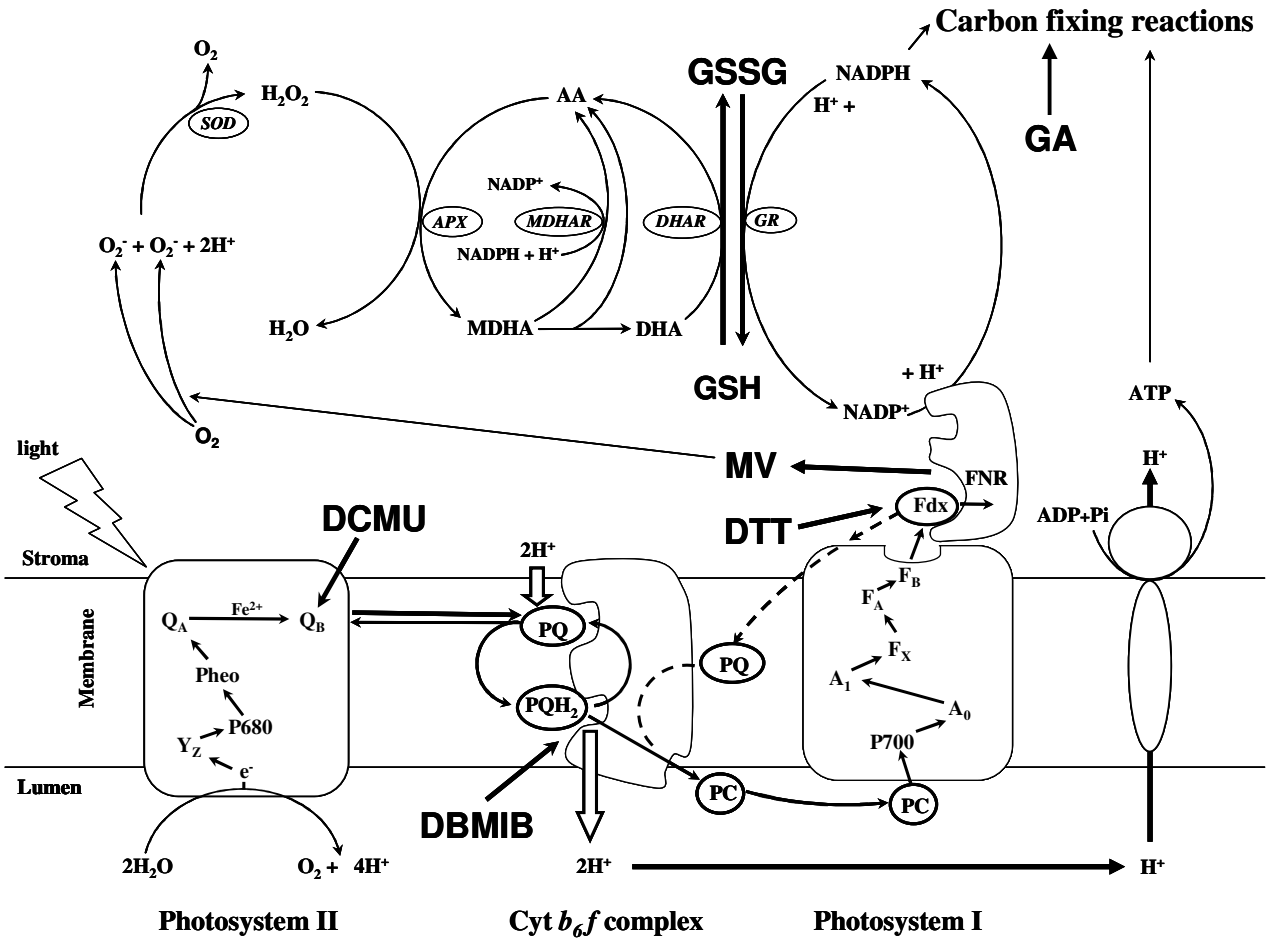


Figure 2.3 Schematic diagram illustrating the site of action of selected photosynthetic inhibitors and redox signalling compounds, showing photosynthetic electron transport chain and the ascorbate-glutathione cycle. Abbreviations: AA, ascorbic acid; APX, ascorbate peroxidase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; MDHA, monodehydroascorbate; MDHAR, monodehydroascorbate reductase; SOD, superoxide dismutase. Adapted from Noctor and Foyer (1998). Refer to text for explanation of the mode of action of the redox signalling compounds.

PQ pool and the chloroplast stroma.

Glutathione acts as a cellular redox buffer through its interconversion between reduced (GSH) and oxidized (GSSG) forms. In addition, it plays a role in diverse processes such as xenobiotic and heavy metal detoxification, plant-pathogen interactions and plant development (Rouhier et al. 2008). As mentioned above, glutathione also participates in detoxification of ROS, and changes in the GSH/GSSG ratio generate redox regulatory signals (Potters et al. 2010).

The final concentrations of the solutions were based on previous studies and were as follows: 10 μM DCMU (Karpinski et al. 1999; Yabuta et al. 2004), 0.24 μM DBMIB (Yabuta et al. 2004), 50 μM MV (Yuasa et al. 2001), 1 mM DTT (Kamauchi et al. 2005), 5 mM GA (Reddy et al. 1991), 5 mM GSH (Gomez et al. 2004; Yadav et al. 2005), and 5 mM GSSG (Gomez et al. 2004; Krügel et al. 2008). In addition, DCMU, DBMIB, MV, DTT and GA were used in combination with GSH or GSSG at the same concentrations as those listed above. Solutions containing DCMU and DBMIB were prepared in 0.1% ethanol; all other solutions were prepared in water. Experimental treatment of *Arabidopsis* leaves with 0.1% ethanol did not affect *GLK* transcript levels compared to water control, as determined by RT-PCR analysis (data not shown).

The combinatorial treatments with reduced and oxidized forms of glutathione were performed in order to assess whether any changes in *GLK* expression seen after application of DCMU, DBMIB, MV, GA or DTT are mediated through changes in redox state of the cell. It was expected that if redox state of the cell plays a role in *GLK* regulation, addition of GSH and/or GSSG would override the effects seen with the these chemicals.

Detached *Arabidopsis* leaves were vacuum-infiltrated with respective solutions under 65 cmHg pressure for 30 min, in 3 intervals of 10 min each. Infiltrated leaves were placed on Petri dishes with their adaxial surface facing up, and incubated with respective chemical solutions in the light at 150 μmol photons $m^{-2}s^{-1}$. The incubation was performed for 4h, from ZT4 to ZT8. Leaves were collected at ZT8, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.2.2 Sugar signalling

Sugars and sugar analogs tested for their effect on *GLK1* and *GLK2* expression were glucose, 2-deoxy-D-glucose (2-DOG), glucose-6-phosphate (G-6-P), sucrose, and mannitol. In addition, glucose and G-6-P were used in combinations with DTT, GSH, and GSSG.

In addition to their metabolic function, sugars in plants play an important role in signalling to regulate growth and development, photosynthesis, secondary metabolism, and stress response (Rolland et al. 2002, 2006). Glucose signalling is classified as hexokinase-independent, -dependent, or glycolysis-dependent, and can be dissected using glucose analogs (Refer to Figure 2.4). 2-DOG is a nonmetabolizable sugar analog which is transported into the cell and phosphorylated, but does not subsequently enter glycolysis. G-6-P is the first metabolite of glucose, produced by the phosphorylation of glucose by hexokinase (HXK) and can be used to determine whether the glucose-induced signal comes from glycolysis (Sheen et al. 1999; Rolland et al. 2001). Treatments with 2-DOG and G-6-P were performed to determine whether glucose-induced signalling affecting *GLK* expression is hexokinase-independent, hexokinase-dependent, or glycolysis-dependent. Sucrose was tested because it is the major translocated sugar in plants. It can signal either through a sucrose-specific pathway or a glucose signalling pathway after its breakdown into

monomeric sugars (Koch 1996; Sheen et al. 1999). Mannitol is a sugar alcohol, commonly used as a control for changes in osmotic pressure (Li et al. 2006; Kojima et al. 2007). In addition, mannitol may be catabolized to mannose by the plant enzyme mannitol dehydrogenase, which is activated during plant pathogen defence response (Jennings et al. 1998). Mannose may be used to produce fructose-6-phosphate, which may subsequently enter glycolysis. *Arabidopsis* does not synthesize mannitol, but is capable of taking up mannitol through hexose transporters (Reinders et al. 2005; Klepek et al. 2005). Although experimental evidence of the mannitol catabolism pathway in *Arabidopsis* is lacking, various enzymes in the pathway are predicted to exist in this plant based on annotated sequence data (Williamson et al. 1995; Tissier 2007; Maruta et al. 2008)

The final concentrations of the solutions were based on previous studies and were as follows: 330 mM glucose (Gibson and Graham 1999), 14.5 mM 2-DOG (Kojima et al. 2007), 30 mM G-6-P, 175 mM sucrose (Kojima et al. 2007), and 165 mM mannitol (Li et al. 2006). In addition, leaves were treated with solutions of 330 mM glucose in combination with 1 mM DTT, 5 mM GSH or 5 mM GSSG, as well as solutions of 30 mM G-6-P in combination with1 mM DTT, 5 mM GSH or 5 mM GSSG. All solutions were prepared in water.

Combinatorial treatments with DTT, GSH and GSSG were performed in order to determine whether sugar signalling affecting *GLK* expression is modulated by redox state of the chloroplast or the cell. In addition to signalling through the specific pathways mentioned above, sugars may signal by causing osmotic stress, which has been shown to produce ROS, which in turn alter cellular GSH/GSSG ratio upon detoxification (Borsani et al. 2001). Thus,

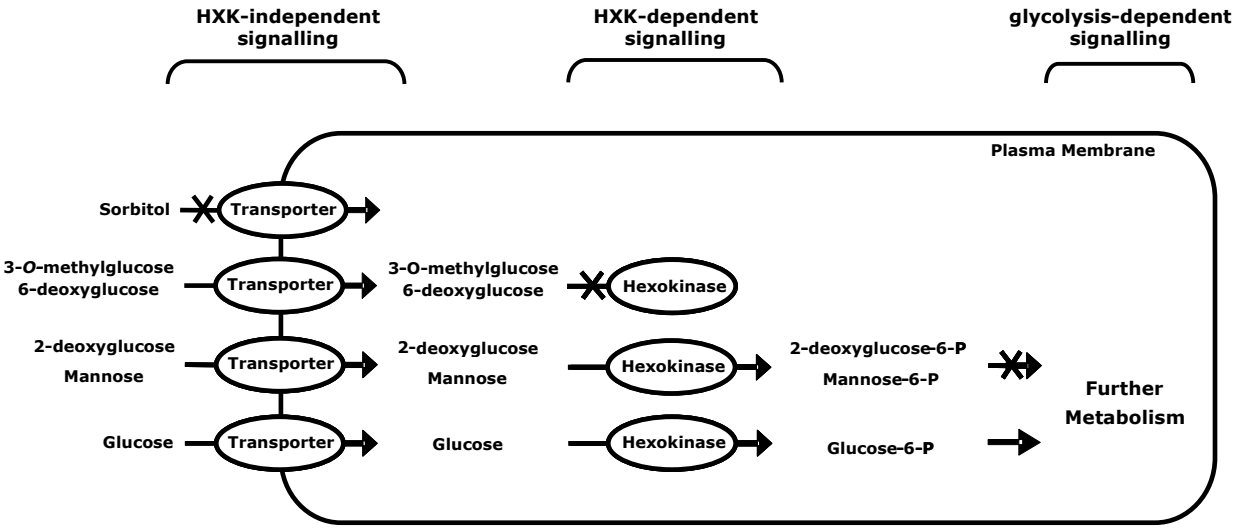


Figure 2.4 Schematic diagram illustrating sugar analog metabolism in plants. Crossed-out arrows indicate that the compound is not metabolized further. Sorbitol and mannitol are not transported into the cell, 3-*O*-methylglucose and 6-deoxyglucose are transported into the cell but not phosphorylated, and mannitol and 2-deoxyglucos are not metabolized following phosphorylation by hexokinase. Adapted from Gibson (2000).

if osmotic stress regulates *GLK* expression, addition of GSH or GSSG may reverse or exacerbate effects seen with sugar treatments.

DCMU, DBMIB, MV, or GA were inappropriate to use in combination with sugars, since they require light-driven electron transport to exert their effect on redox state of the chloroplast, while the sugar signalling experiment was performed in the dark. DTT, on the other hand, is capable of reducing the electron transport chain in the dark, and was thus used as a modulator of chloroplast redox state. Detached *Arabidopsis* leaves were vacuum-infiltrated with respective chemical solutions under 65 cmHg pressure for 30 min, in 3 intervals of 10 min each. Infiltrated leaves were placed on Petri dishes and incubated with respective chemical solutions in the dark for 4h or 24h. The 4h incubation was performed from ZT4 to ZT8, and the 24h incubations were performed from ZT4 to ZT4 the next day, and from ZT12 to ZT12 the next day. Following incubation, leaves were immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.2.3 Nitrogen signalling

In the nitrogen signalling experiment, glutamine (Gln), glutamate (Glu), methionine sulfoximine (MSX), GSH, and GSSG were used to treat *Arabidopsis* leaves. MSX is an inhibitor of glutamine synthetase (GS), an enzyme that participates in the GS/GOGAT system to convert ammonia into Gln (Miflin and Lea 1977; Evstigneeva et al. 2003; Refer to Figure 2.5).

Previously, GLK1 was found to be an important regulator of organic nitrogen signalling (Gutiérrez et al. 2008; Refer to Introduction). Thus, we investigated whether *GLK1* and *GLK2* gene expression is responsive to increased levels of organic nitrogen derivatives, and whether this regulation is dependent on the redox state of the cell.

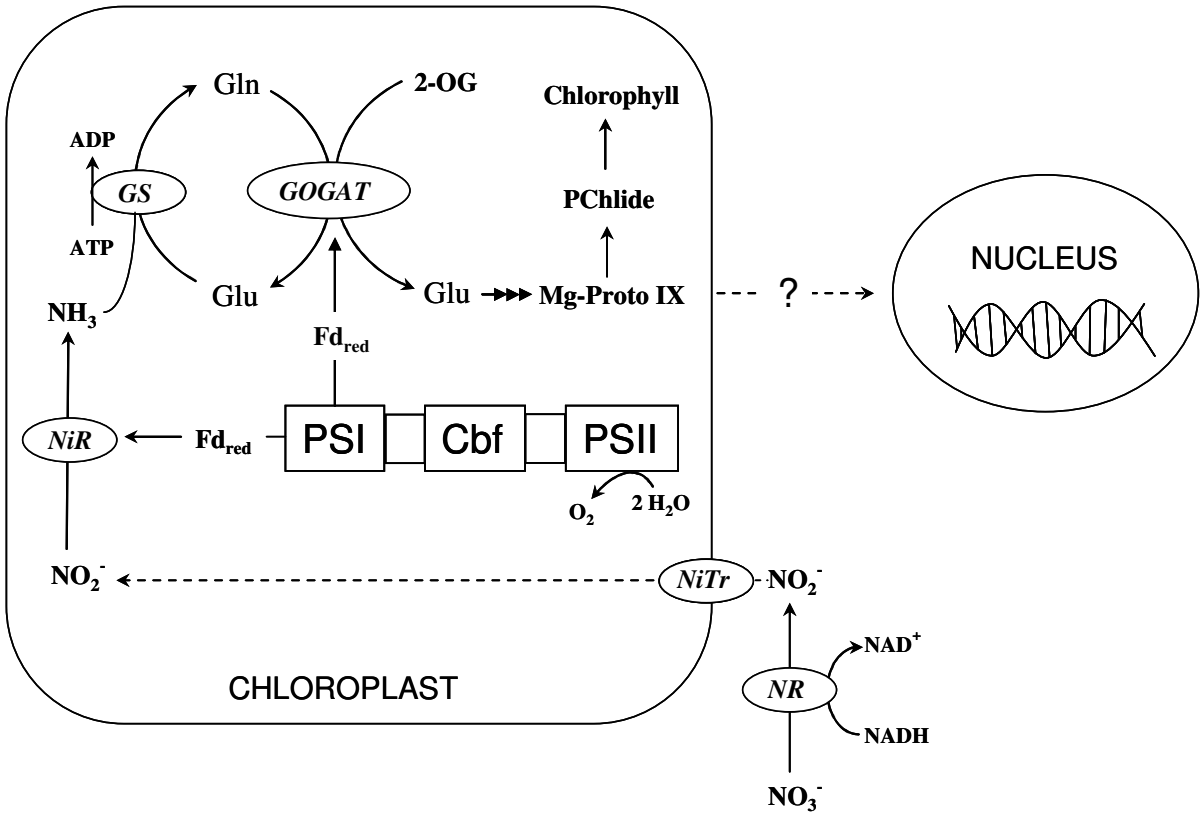


Figure 2.5 Schematic diagram illustrating nitrogen assimilation and chlorophyll biosynthesis in plants. Abbreviations: Cbf, cytochrome b_6f complex; Fd_{red}, reduced ferredoxin; Gln, glutamine; Glu, glutamate; GS, glutamine synthetase; GOGAT, glutamate synthase; Mg-ProtoIX, Mg-Protoporphyrin IX; NiR, nitrite reductase; NiTr, nitrite transporter; NR, nitrate reductase; 2-OG, 2-oxoglutarate; PChlide, protochlorophyllide; PSI and PSII, Photosystems I and II. Adapted from Foyer et al. (2011).

The final concentrations of the solutions were based on previous studies and were as follows: 5 mM Gln (Gifford et al. 2008), 5 mM Glu (Gutiérrez et al. 2008), 1 mM MSX (Gutiérrez et al. 2008), 1 mM MSX + 5 mM GSH, 1 mM MSX + 5 mM GSSG, 1 mM MSX + 5 mM Gln. All solutions were prepared in water. Combinations with glutathione were used to assess whether nitrogen signalling affects *GLK* expression through changes in redox state of the cell. Treatment with a combination of MSX and Gln was used to determine whether changes in *GLK* transcript levels in response to MSX were due to suppression of organic nitrogen synthesis.

Detached *Arabidopsis* leaves were vacuum-infiltrated with respective chemical solutions under 65 cmHg pressure for 30 min, in 3 intervals of 10 min each. Infiltrated leaves were placed on Petri dishes with their adaxial surface facing up, and incubated with respective chemical solutions in the light at 150 μmol photons $m^{-2}s^{-1}$. The incubation was performed for 4h, from ZT4 to ZT8. Leaves were collected at ZT8, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.2.4 Pathogen signalling

The following strains of bacteria were used: *Pseudomonas syringae* pv. *maculicola* ES4326 (*Psm*) and *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) carrying empty vector pVSP61 (control) or pVSP61 expressing avirulence gene *avrRpm1* or *avrRpt2*. *P. syringae* was used for infection of *Arabidopsis* because the genomes of both organisms have been sequenced and the *P. syringae–Arabidopsis* pathosystem has been well-characterized (Innes 1995). Since *GLK1* had previously been proposed to play a role in disease defence in *Arabidopsis* (Savitch et al. 2007), and had been suggested to be affected by pathogenesis (Kim et al. 2006; Bozso et al. 2009), we were interested in investigating whether and how

GLK gene expression is affected by pathogen infection. The two avirulence genes *avrRpm1* and *avrRpt2* both cause HR in *Arabidopsis*, but with different degrees of severity – the development of disease symptoms thus requires different duration of time after infection (Katagiri et al. 2002; Hou et al. 2009). Both *avrRpm1* and *avrRpt2* genes induce gene-for-gene resistance, in which the plant recognizes a pathogen effector protein encoded by an avirulence (*avr*) gene, through the action of the corresponding plant resistance gene (Katagiri et al. 2002). In order to determine whether signalling arising from gene-for-gene resistance response affects *GLK* expression, we infected *Arabidopsis* with strains of *P. syringae* carrying two different resistance genes, *avrRpm1* and *avrRpt2*. *P. syringae* carrying empty vector was used as a control to determine whether pathogen infection affects *GLK* expression independently of gene-for-gene resistance.

In order to investigate the pathogen signalling pathway affecting *GLK* expression, we tested the effect of exogenous application of SA, JA and H_2O_2. SA and JA are two key signalling molecules involved in plant defence against pathogens (Kunkel and Brooks 2002). The two signalling pathways are mutually antagonistic, with the SA pathway generally being induced in response to biotrophic pathogens and JA pathway in response to necrotrophic pathogens, insect herbivory and wounding (Kunkel and Brooks 2002).

H_2O_2 treatment was carried out as part of the pathogen signalling experiment because it is known to be produced in plants in a localized oxidative burst in response to pathogen attack (Apostol et al. 1989; Levine et al. 1994; Jabs et al. 1997). Although application of MV, which was used in the redox signalling experiment, also generates H_2O_2, the source of H_2O_2 production is different. MV results in H_2O_2 production inside the chloroplast, by accepting electrons from the early acceptors of PSI and reacting with

oxygen, in the process creating $\cdot O_2^-$, and subsequently H_2O_2 (Salin 1988). Pathogen-induced H_2O_2, however, is generated by a NADPH oxidase at the plant plasma membrane (Keller et al. 1998). Thus, exogenous treatment with H_2O_2 was performed in order to simulate its production in response to pathogen attack.

Pst avrRpm1 infection was also performed in combination with GSH, GSSG, DCMU and DBMIB treatments in order to test whether changes in redox state of the cell or the chloroplast can reverse any effect on *GLK* expression caused by pathogen infection. A reversal by one or more of these compounds would indicate that *GLK* gene expression is likely affected not directly by the plant recognition of the pathogen effector proteins, but by downstream signalling events.

Treatments with GSH/GSSG were performed because glutathione is known to play an important role in plant defence against pathogens (Noctor et al. 2011). It is well known that *P. syringae* infection induces an oxidative burst and programmed cell death (Ingle et al. 2006; Jones and Dangle 2006). Modifications in glutathione redox status have been suggested to modulate signalling cascades associated with programmed cell death in plants (Kranner et al. 2006; Foyer and Noctor 2011). Thus, increased oxidation of glutathione is indicative of oxidative stress in response to various conditions, such as ozone, cold and pathogen infection (Noctor et al. 2011). Therefore, given the role of glutathione in defence against pathogens, we tested whether changes in *GLK* expression in response to pathogens depend on glutathione redox state.

Pathogen inoculation was performed by infiltrating rosette leaves of a similar developmental stage from 5-week-old *Arabidopsis* plants with bacterial suspensions of $5x10^7$ cfu/ml in 10 mM $MgCl_2$ using a needleless 1-mL syringe, as described in Katagiri et

al. (2002). The plants were covered with a clear plastic dome and kept in the light at 150 μmol photons $m^{-2}s^{-1}$ until the time of collection of leaves. Inoculation with pathogens was performed at ZT6. Infected leaves were collected 6h later, at ZT12, after inoculation with *Pst* EV and *Pst avrRpm1*, and 24h later, at ZT6 the next day, after inoculation with *Pst avrRpt2*, *Psm* EV, *Psm avrRpm1* and *Psm avrRpt2*. Inoculated leaves were collected from plants, immediately frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction.

For the combinatorial treatments, *Arabidopsis* plants were inoculated with *Pst avrRpm1* and kept in the light for 1h from ZT6 to ZT7. Inoculated leaves were collected, vacuum infiltrated as described in Section 2.2.1 with 10 μM DCMU, 0.24 μM DBMIB, 5 mM GSH, or 5 mM GSSG from ZT7 to ZT8, and kept in respective solutions in the light at 150 μmol photons m^{-2} s^{-1} for additional 4h from ZT8 to ZT12. Following incubation, leaves were immediately frozen in liquid nitrogen and stored at -80ºC for subsequent RNA extraction.

Concentrations of the SA, JA and H_2O_2 solutions were based on previous studies and were as follows: 1 mM SA (Heidel and Baldwin 2004), 500 μM JA (Nagpal et al. 2005) and 10 mM H_2O_2 (Yabuta et al. 2004). Solutions of SA and H_2O_2 were prepared in water and JA solution was prepared in 1% methanol. Experimental treatment of *Arabidopsis* with 1% methanol did not affect *GLK* transcript levels compared to water control, as determined by RT-PCR analysis (data not shown). The chemicals were applied to whole *Arabidopsis* plants by spraying with respective solutions. After spraying with SA, JA and H_2O_2, plants were kept in the light at 150 μmol photons $m^{-2}s^{-1}$ for 4h from ZT4 to ZT8. Rosette leaves were

collected from plants, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.2.5 Phytohormone signalling

Phytohormones as a group were chosen for experimental treatments because of their importance and diverse effects in plants (Santner et al. 2009). As hormones are involved in virtually all aspects of the plant growth and development, and *GLK* genes have been suggested to be affected by BR and GA_3 signalling, we investigated in more detail effects of the major plant hormones.

The final concentrations of the solutions were based on previous studies and were as follows: 10 μM IAA (Nagpal et al. 2005), 20 μM cytokinin (Kiba et al. 2005), 100 μM GA_3 (Friedrichsen et al. 2002), 100 μM ABA (Friedrichsen et al. 2002; Larkindale and Knight 2002), 1 μM BR (Friedrichsen et al. 2002; Yin et al. 2002; Nemhauser et al. 2004). All solutions were prepared in water, except for cytokinin, which was prepared in 0.35% methanol, and GA_3, which was prepared in 0.1% ethanol. Experimental treatment of *Arabidopsis* with 0.35% methanol or 0.1% ethanol did not affect *GLK* transcript levels compared to water control, as determined by RT-PCR analysis (data not shown).

Phytohormones were applied to *Arabidopsis* by spraying whole plants with respective solutions. After spraying, plants were kept in the light at 150 μmol photons $m^{-2}s^{-1}$ for 4h or 24h. The 4h incubation was performed from ZT4 to ZT8, and the 24h incubations were performed from ZT4 to ZT4 the next day, and from ZT12 to ZT12 the next day. Rosette leaves were collected from plants, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.2.6 *pgp1* mutation and cold temperature signalling

The *pgp1* mutant of *Arabidopsis* is characterized by a reduction in levels of PG, a glycolipid which is an important constituent of thylakoid membranes (Hagio et al. 2002; Xu et al. 2002; Krol et al. 2010; Refer to Introduction). While the mutant is indistinguishable from wild type at room temperature, growth at 5°C results in a yellow-white phenotype, reduced chlorophyll content and inhibition of granal stack formation (Krol et al. 2010). The cold-induced phenotype of the *pgp1* mutant grown at 5°C could be reversed from yellow-white to green by a shift of the plants to 20°C in continuous light for 48 h (N.P.A Huner laboratory, unpublished).

In order to investigate whether the cold-induced phenotype of *pgp1* is associated with *GLK* suppression, we analyzed *GLK1* and *GLK2* transcript levels in five-week old wild type and *pgp1 Arabidopsis* grown at 20°C or at 5°C, as well as in *pgp1* shifted from 5°C to 20°C, as described above. Untreated leaves were collected at ZT7, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

In addition, leaves of wild type and *pgp1* mutant *Arabidopsis* grown at 20°C or at 5°C were treated with DCMU, DBMIB, GSH or GSSG. DCMU and DBMIB block electron flow through the photosynthetic electron transport chain, resulting in oxidation or reduction of the PQ pool, respectively (Trebst 1974; Kirilovsky et al. 1994; Ralph et al. 2011; Refer to Section 2.2.1). As the redox state of the PQ pool is a known site of generation of retrograde chloroplast-to-nucleus signals (Pfannschmidt et al. 2003a), the two inhibitors were used to assess whether it affects *GLK* gene regulation in response to PG deficiency and cold temperature conditions. Similarly, GSH and GSSG were used to address the question of how

redox state of the cell or the chloroplast modulates regulation of *GLK* under the conditions of the present experiment.

The final concentrations of the solutions were the same as in the chloroplast-to-nucleus signalling experiment: 10 μM DCMU, 0.24 μM DBMIB, 5 mM GSH, and 5 mM GSSG (Refer to Section 2.2.1). Solutions containing DCMU and DBMIB were prepared in 0.1% ethanol; solutions of GSH and GSSG were prepared in water. As stated above in Section 2.2.1, experimental treatment of *Arabidopsis* with 0.1% ethanol did not affect *GLK* transcript levels compared to water control, as determined by RT-PCR analysis (data not shown).

Detached *Arabidopsis* leaves were vacuum-infiltrated with respective chemical solutions under 65 cmHg pressure for 30 min, in 3 intervals of 10 min each. Infiltrated leaves were placed on Petri dishes with their adaxial surface facing up, and incubated with respective chemical solutions in the light at 150 μmol photons $m^{-2}s^{-1}$. The incubation was performed for 4h, from ZT3 to ZT7. Leaves were collected at ZT7, immediately frozen in liquid nitrogen, and stored at -80ºC for subsequent RNA extraction.

2.3 RT-PCR and qPCR analyses

RNA was isolated from frozen *Arabidopsis* tissue by guanidinium thiocyanate-phenol-chloroform extraction, using TRIzol reagent according to the manufacturer's instructions (Invitrogen, Burlington, ON, Canada) and DNase-treated according to the manufacturer's instructions using the DNA-free™ kit from Ambion (Streetsville, ON, Canada). RNA yield was quantified by measuring absorbance at 260 nm, and its purity was determined as the 260/280 nm ratio using Pharmacia Biotech GeneQuant RNA/DNA

Calculator (Cambridge, England). To check for quality and equal loading, RNA was run on 1% formaldehyde gel and visualized in Alphaimager under UV light (Figure 2.1B)

Reverse transcription (RT) was carried out using the Superscript II Reverse Transcriptase kit from Invitrogen (Burlington, ON, Canada), according to the manufacturer's instructions. Reactions containing RNA template, anchored Oligo dT, and water were incubated at 65°C for 5 min and cooled on ice. Following this, a mixture of RNase Inhibitor, Superscript II 5x buffer, dNTPs, DTT, and Superscript II RT was added to each tube and reactions were incubated at 42°C for 1 hour. Controls for genomic DNA contamination were carried out by performing duplicate reactions in which no Superscript II RT was present. I didn't do controls for every single sample. RT products were stored at -20°C and used for Polymerase Chain Reaction (RT-PCR) and quantitative RT-PCR (qPCR) reactions.

All RT-PCR reactions were carried out using *Taq* polymerase (Invitrogen, Burlington, ON, Canada). Amplification was carried out as follows: one cycle at 94ºC for 3 min for initial denaturation, followed by 30 cycles of: 94ºC for 30 sec for denaturation, 58ºC for 30 sec for annealing, and 72ºC for 1 or 2 min for extension; followed by 10 min of incubation at 72°C. The amplified products were run on 1% TAE-agarose gel and visualized in Alphaimager under UV light.

All qPCR reactions were carried out according to the manufacturer's instructions using Maxima® SYBR Green/ ROX qPCR Master Mix (2x) from Fermentas (Burlington, ON, Canada). Amplification was performed using MJ Research PTC-200 DNA Engine Thermal Cycler from Bio-Rad (Mississauga, ON, Canada). The cycling was carried out as follows: one cycle at 95ºC for 10 min for initial denaturation, followed by 40 cycles at 95ºC

for 20 sec for denaturation, 56ºC for 45 sec for annealing, and 72ºC for 45 sec for extension. The data were analyzed using Opticon Monitor™ Version 3.1 software (Bio-Rad Laboratories, Mississauga, ON, Canada).

Two to five technical replicates of RT-PCR reactions and three technical replicates of qPCR reactions were performed. The primers used in this project are listed in Table 2.2. *GLK* primers were designed such that full-length coding sequences were amplified by RT-PCR, and 3' end fragments were amplified by qPCR (Refer to Table 2.2). The primers were designed using DNASTAR Lasergene8 PrimerSelect software, and optimal primer pairs were chosen based on their predicted ability to amplify their targets with high sensitivity and specificity. With the exception of forward qPCR primer for *GLK1*, all the chosen primers anneal within exons. The forward qPCR primer for *GLK1* anneals at the splice junction between exon4 and exon5 (Figure 2.7). Such design prevents amplification of genomic DNA templates. However, no genomic DNA amplification was detected with any of the primer pairs. The size of amplicons from genomic and cDNA templates is predicted to be different and that the resulting RT-PCR products would appear as two separate bands on agarose gels, which was not the case in any of the experiments. In addition, control RT-PCR reactions did not reveal genomic DNA contamination. We did this with selected treatments, not every single treatment.

Elongation factor 1α (*EF1α*, At5g60390) was used for all RT-PCR and qPCR reactions as a control. Expression of *EF1α* did not vary significantly among samples in any of the experiments, as seen from C_T values, which reflect the number of cycles required to reach threshold fluorescence. Table 2.1 shows C_T values obtained in the cold signalling experiment. The difference between individual values in any set of samples was less than 1,

indicating that the experimental treatments did not significantly affect expression of this housekeeping control gene.

Ribosomal protein L2 (At2g18020) was used as an additional control for RT-PCR (data not shown). Actin (At2g42100) was found to be inappropriate as a control gene as transcript levels were found to be affected by the experimental treatments (Figure 2.6).

2.4 Chlorophyll fluorescence measurements

Effects of experimental treatments on redox state of the chloroplast, photosynthetic performance and energy partitioning were assessed by chlorophyll *a* fluorescence analysis. Measurements were performed on detached *Arabidopsis* leaves vacuum infiltrated as described in Section 2.2.1 with chemicals at the following final concentrations: 10 μM DCMU, 0.24 uM DBMIB, 50 uM MV, 1 mM DTT, 5 mM GA, 1 mM MSX, 5 mM GSH, and 5 mM GSSG. In addition, vacuum infiltration with DCMU, DBMI, MV, DTT, GA and MSX was performed in combination with 5 mM GSH or 5 mM GSSG, at the same concentrations as listed above. Solutions containing DCMU and DBMIB were prepared in 0.1% ethanol and all other chemical solutions were prepared in water. Following vacuum infiltration, leaves were placed in the respective chemical solutions in the dark for 30 min of dark adaptation before fluorescence measurements were taken.

Fluorescence measurements were also performed on leaves from *Arabidopsis* plants that were sprayed with 20 μM cytokinin and incubated in the light at 150 μmol m^{-2} s^{-1} for 4h, from ZT4 to ZT8. Cytokinin solution was prepared in 0.35% methanol. Following incubation in the light, leaves were collected and placed in the cytokinin solution in the dark for 30 min of dark adaptation before fluorescence measurements were taken.

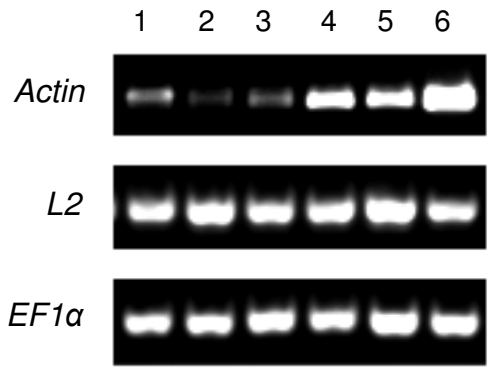


Figure 2.6 Effects of sugars and glucose analogs on expression of three different *Arabidopsis* housekeeping genes, determined by RT-PCR analysis. Following vacuum infiltration, leaves were incubated in solutions for 4h from ZT4 to ZT8. (1) water control, (2) 330 mM glucose, (3) 14.5 mM 2-DOG, (4) 30 mM G-6-P, (5) 175 mM sucrose, (6) 165 mM mannitol. The housekeeping genes were: L2, Ribosomal protein L2 (At2g18020); Actin (At2g42100); *EF1α*, Elongation factor 1α (At5g60390). Two biological replicates, each with three RT-PCR replicates, were performed. Representative results are shown.

Table 2.1 Threshold cycle (C_T) values of Elongation factor 1α (*EF1α*) expression from the cold signalling experiment. Abbreviations: WT, wild type; *pgp1*, *pgp1* mutant; SD, standard deviation.

Sample	C_T	SD
WT 20°C DCMU	16.49	0.17
WT 20°C DBMIB	16.22	0.19
WT 20°C GSH	16.63	0.05
WT 20°C GSSG	16.64	0.12
pgp1 20°C DCMU	15.87	0.25
pgp1 20°C DBMIB	16.13	0.18
pgp1 20°C GSH	16.16	0.08
pgp1 20°C GSSG	16.03	0.02
WT 5°C DCMU	16.37	0.3
WT 5°C DBMIB	16.66	0.26
WT 5°C GSH	16.69	0.27
WT 5°C GSSG	16.28	0.01
pgp1 5°C DCMU	15.23	0.14
pgp1 5°C DBMIB	15.77	0.13
pgp1 5°C GSH	15.59	0.24
pgp1 5°C GSSG	15.46	0.23

In addition, fluorescence was performed on infected leaves of plants inoculated with bacterial suspensions of $5x10^7$ cfu/ml *Pst* EV and *Pst avrRpm1* using a needleless 1-mL syringe. Bacteria were suspended in 10 mM $MgCl_2$. After inoculation, the plants were covered with a clear plastic dome and kept in the light at 150 µmol photons $m^{-2}s^{-1}$ from ZT4 to ZT10. Following incubation in the light, leaves were collected and placed in the bacterial suspensions in the dark for 30 min of dark adaptation before fluorescence measurements were taken. Finally, fluorescence was performed on leaves of cold stressed or cold-acclimated *Arabidopsis* plants, grown under conditions described in Section 2.1. Prior to performing fluorescence measurements, detached leaves were placed in water in the dark for 30 min of dark adaptation before fluorescence measurements were taken.

Fluorescence was determined using a PAM modulated fluorescence system. (Heinz Walz, Effletrich, Germany) (Schreiber et al. 1986), at 20°C and a PFD of 150 µmol photons m^{-2} s^{-1}. At least four leaves from different plants were used to measure fluorescence after each of the experimental treatments. Two biological replicates were performed. The average and standard error (SE) were calculated for each of the fluorescence parameter measured.

Chlorophyll fluorescence parameters were defined according to the nomenclature of van Kooten and Snel (1990) (Refer to Figure 2.8). F_o is defined as the lowest level of chlorophyll fluorescence after the weak pulse light which does not drive photosynthesis is turned on. F_m is measured as the maximum level of fluorescence after a flash of 10,000 µmol photons m^{-2} s^{-1} is applied, causing reaction centres to close and all the light energy to be given off as fluorescence. Following the first flash, actinic light of 150 µmol photons m^{-2} s^{-2}, or light capable of driving photosynthesis, is turned on. The resulting gradual increase in

Table 2.2 List of primers used in RT-PCR and qPCR analyses.

Type of PCR analysis	Gene	Locus Tag	Forward Primer Sequence	Reverse Primer Sequence	Expected size of amplicon
RT-PCR					
	GLK1	At2g20570	TCGGGGAAAGGTGAAGAAGTCGT	CAGCCATGGCCTCGTCAATACA	852 bp
	GLK2	At5g44190	TTTGCCGGATTTGGAGATAGATTC	AACTCCGTCATAACACCGTCAACC	930 bp
	EF1α	At5g60390	AGCCCCTTCGTCTTCCACTTCAG	TGGGCTCCTTCTCAATCTCCTTAC	444 bp
	L2	At2g18020	CGCAAAGGTCCGGCTAAGTTCC	GTACGCGTTTCCTGCCTTGAGC	492 bp
	Actin 5	At2g42100	GGTGCCCTGAGGTTCTTTTC	CAATCGCCCCTCCCTGTTC	442 bp
qPCR					
	GLK1	At2g20570	ACGAGATTTAGAGCACCGCCAGTT	AGCCATGGCCTCGTCAATACATCT	170 bp
	GLK2	At5g44190	TGGCATCAGCAACCACTCTATCCA	ATTGTCTTGTGGGAACACCGATGC	82 bp
	EF1α	At5g60390	TTCTCGATTGCCACACCTCTCACA	TCTCCTTACCAGAACGCCTGTCAA	82 bp

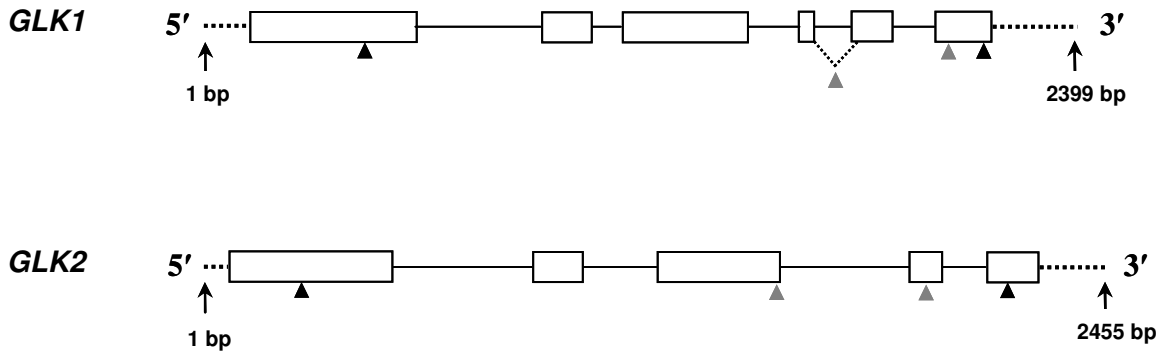


Figure 2.7 Structure of *Arabidopsis GLK1* and *GLK2* genes showing positions of RT-PCR and qPCR primers. Introns are shown as lines, exons as boxes and untranslated regions (UTRs) as dotted lines. Positions of primers used for RT-PCR are indicated by black arrowheads, and positions of qPCR primers are indicated by gray arrowheads.

photochemistry and nonphotochemical quenching processes is accompanied by a decline in chlorophyll fluorescence, until a steady state of photosynthesis, F_s, is reached.

Light pulses of 10,000 µmol photons m^{-2} s^{-1} and 800 ms duration applied with an interval of 40 sec result in the closing of reaction centres and transient increases of fluorescence, recorded as peaks on the fluorescence trace. F_m' is measured as the fluorescence at the peaks after the steady state F_s is reached. F_o' is determined as the minimal level of fluorescence after actinic light is turned off.

The maximum quantum efficiency of PSII photochemistry was estimated as F_v/F_m. The maximum quantum efficiency of PSII photochemistry at the light irradiance and temperature used was estimated as F_v'/F_m'. PSII excitation pressure, or the redox state of Q_A, which reflec ts the redox state of the photosynthetic electron transport chain, was calculated as $1 - q_L$, where $q_L = F_o/F_s \bullet (F_m' - F_s)/F_v$ (Kramer et al. 2004).

Photosynthetic yield of PSII, defined as the fraction of photons absorbed by the PSII antennae that are utilized by PSII photochemistry, was calculated as $\Phi_{PSII} = 1 - F_s/F_m'$. Non-photochemical quenching through ΔpH- and xanthophyll-regulated thermal dissipation was calculated as $\Phi_{NPQ} = F_s/F_m' - F_s/F_m$. Non-photochemical quenching through constitutive thermal dissipation was calculated as $\Phi_{f,D} = F_s/F_m$. All three photosynthetic parameters, Φ_{PSII}, Φ_{NPQ}, $\Phi_{f,D}$, were estimated according to the model described by Hendrickson et al. (2004).

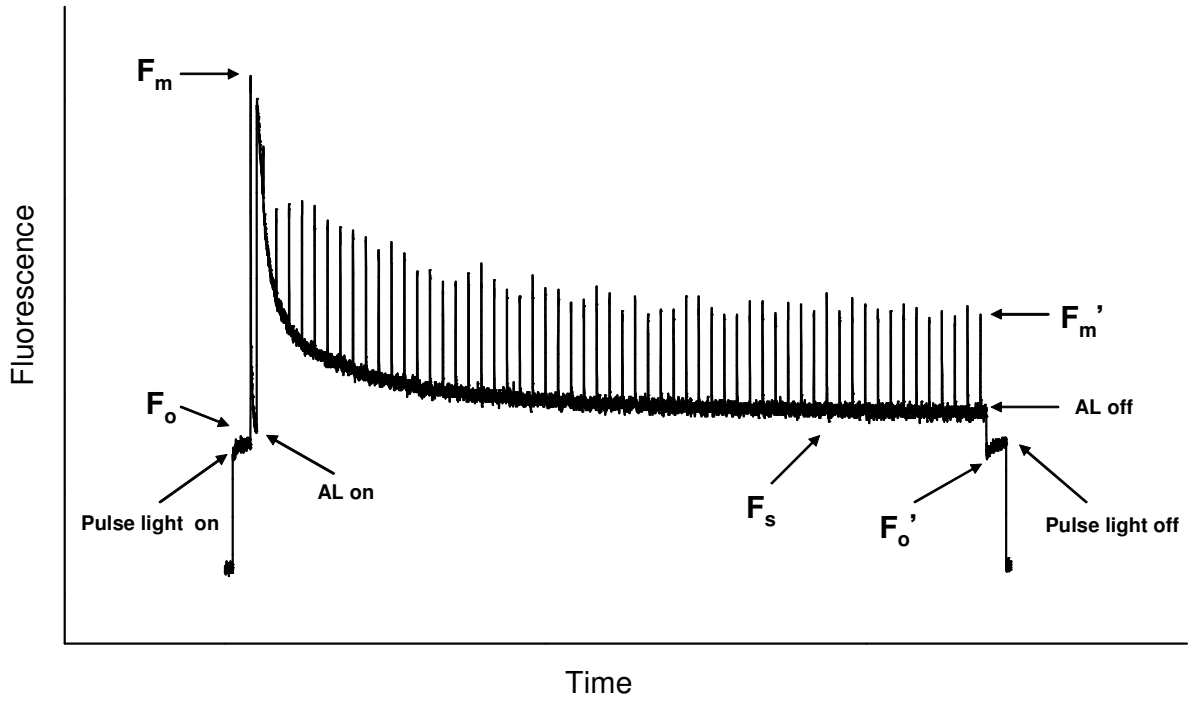


Figure 2.8 Typical PSII chlorophyll *a* fluorescence trace from a dark-adapted *Arabidopsis* leaf. F_o is the minimal fluorescence under a weak measuring light (pulse light). Fluorescence reaches its maximum level (F_m) upon illumination with a saturating light flash. Under continuous illumination with actinic light, fluorescence decreases gradually, as photochemical and non-photochemical energy dissipation processes increase, until a steady state level of fluorescence (F_s) is reached. Abbreviations: AL, actinic light.

CHAPTER 3 CHLOROPHYLL FLUORESCENCE

3.1 Chlorophyll fluorescence

Chlorophyll fluorescence analysis is a powerful tool to detect changes in plant photosynthetic performance (Ralph et al. 2011). As several lines of evidence suggest that signals regulating *GLK1* and *GLK2* expression in *Arabidopsis* originate in the chloroplast, it was of interest to determine whether the experimental treatments affect various chloroplast processes that may give rise to retrograde chloroplast-to-nucleus signalling. An additional purpose of fluorescence analysis was to verify whether chemicals that are commonly used in modulating photosynthetic parameters produced the intended effects in our experiment, as well as to investigate the mode of action of the less commonly used compounds.

Light incident on leaves is absorbed by photoactive pigments associated with multiprotein complexes in the thylakoid membrane of chloroplasts, known as Photosystems I and II (PSI and PSII, respectively). This initiates the transport of electrons from water through PSII, the PQ pool, cyt_{b6f} complex, and PSI, to the final electron acceptor Fd, which reduces nicotinamide-adenine-dinucleotidephosphate ($NADP^+$). In the process, ADP is phosphorylated to ATP. NADPH and ATP are subsequently used for carbon fixation in the Calvin cycle (Roháček 2002; Refer to Figure 2.3).

Most of the light energy absorbed by PSII drives photochemistry, resulting in electron transfer from the reaction centre to the primary quinone acceptor Q_A (Baker 2008). Photochemistry is in direct competition with loss of light energy to chlorophyll fluorescence and heat dissipation. Heat dissipation is known as nonphotochemical quenching. An increase in any of the three processes of photochemistry, fluorescence or nonphotochemical quenching is accompanied by a decrease in the other two. Thus, changes in chlorophyll can

fluorescence provide information on the photochemical and nonphotochemical processes of light energy utilization (Butler 1978; Baker 2008).

Nonphotochemical quenching of light energy is divided into antenna and reaction centre processes (Ort 2001; Ivanov et al. 2008). Antenna quenching involves the xanthophyll cycle, in which the diepoxide pigment violaxanthin is converted to the epoxide-free zeaxanthin (Ruban and Horton 1995). The process requires the formation of a pH gradient across the thylakoid membrane (Jahns and Holzwarth 2011). Although antenna quenching is recognized as the major photoprotective mechanism, reaction centre quenching may also contribute significantly to preventing damage to PSII, such as in plants acclimated to cold temperatures or excess light conditions (Ivanov et al. 2008).

Chlorophyll fluorescence quenching analysis can be used to assess photosynthetic performance of plants by measuring various photochemical and nonphotochemical parameters. The following commonly used fluorescence parameters were measured: maximum quantum yield of PSII (F_v/F_m), PSII excitation pressure (1-qL), effective quantum yield of PSII (Φ_{PSII}), nonphotochemical antenna quenching (Φ_{NPQ}), and nonphotochemical reaction centre quenching ($\Phi_{f,D}$). The following discussion considers the effects of different experimental treatments on photosynthetic performance of *Arabidopsis*, as revealed by chlorophyll fluorescence analysis.

In addition, chlorophyll fluorescence measurements were used to infer changes in the redox state of the PQ pool, Fd, and the stroma, as well as in the levels of ROS, all of which are capable of generating retrograde chloroplast-to-nucleus signals (Refer to Introduction; Kleine et al. 2009). The fluorescence measurements are reported in Table 3.1.

3.2 Treatment with glutathione

Glutathione, a low molecular weight antioxidant, is expected to affect photosynthetic performance through its well-established role in ROS detoxification. In the chloroplast, photoreduction of O_2 leading to production of ROS can occur at the PSII reaction centre, the PQ pool or PSI (Badger et al. 2000). The most significant source of reactive oxygen radicals is the Mehler reaction of PSI, which results in the production of O_2^- (Asada 1999; Badger et al. 2000). This ROS is dismutated to H_2O_2 and subsequently converted to water by the ascorbate-glutathione cycle (Noctor and Foyer 1998; Refer to Figure 2.3). Together, the Mehler reaction and the ascorbate-glutathione cycle comprise the water-water cycle (Asada 1999; Asada 2006).

Detoxification of ROS by glutathione may affect electron flow through the photosynthetic electron transport chain in two possible ways. The first of these is donation of electrons to PSI or PSII by ascorbate, which is reduced from monodehydroascorbate in the ascorbate-glutathione cycle (Noctor and Foyer 1998; Refer to Figure 2.3). Ascorbate is known to donate electrons to both PSI and PSII when electron transport is impaired. For example, it is oxidized by PSII when the water oxidase complex is inactivated by acidic pH, UV-B or heat (Katoh and San Pietro 1967; Mano et al. 2004).

The second effect of increased detoxification of ROS is acceleration of electron transport, as the water-water cycle serves as an alternative electron sink and enhances cyclic electron flow around PSI (Asada 2006; Murchie and Niyogi 2011). Donation of electrons to PSII by ascorbate is reflected by the decreased F_v/F_m value upon treatment with either GSH or GSSG (Table 3.1). Low F_v/F_m may indicate photoinhibitory stress and damage to PSII reaction centres (Roháček et al. 2008). However, the leaves were not exposed to light prior

to dark adaptation, ruling out photoinhibition as the cause of reduced F_v/F_m in this case. Thus, it seems likely that F_v/F_m decreased because of the inability of PSII reaction centres to open during dark adaption, due to the presence of excess electrons donated to PSII by ascorbate.

At the same time, nonphotochemical antenna quenching (Φ_{NPQ}) increased in response to glutathione (Table 3.1). This enhancement may be attributed to increased cyclic electron transport around PSI, allowing the establishment of pH gradient required for this heat dissipation process (Murchie and Niyogi 2011). $\Phi_{f,D}$ also increased, as an additional protective mechanism in response to excess light energy. On the other hand, the photochemical parameter Φ_{PSII} was lowered as a result of the competing nonphotochemical processes.

The similar effect of reduced and oxidized glutathione on F_v/F_m, Φ_{PSII}, and nonphotochemical quenching may be due to the maintenance of glutathione homeostasis. Previously, GSH levels in *Arabidopsis* leaves increased in response to both GSH and GSSG treatments, although to a greater extent following GSH treatment (Karpinska et al. 2000). The lower amounts of GSH in leaves following GSSG treatment compared to GSH treatment may explain the difference in the effect of the two forms of glutathione on the PSII excitation pressure, 1-qL. Thus, ROS detoxification may be more efficient upon GSH treatment, resulting in no change in 1-qL compared to control. Conversely, the elevated 1-qL following GSSG treatment may reflect slower rates of ROS detoxification.

Under the conditions of the present experiment, the main observed effects of glutathione on photosynthetic performance were reduced maximum and effective quantum yield of PSII, accompanied by increased nonphotochemical fluorescence quenching.

Table 3.1 Chlorophyll *a* fluorescence parameters measured using rosette leaves of 5-week-old *Arabidopsis* plants, following experimental treatments as described in text, reported as mean ± standard error (SE). Two biological replicates, each with at least four technical replicates, were performed.

	F_v/F_m	1-qL	qN	Φ_{PSII}	Φ_{NPQ}	$\Phi_{f,D}$
Control	0.784 ± 0.004	0.346 ± 0.019	0.348 ± 0.029	0.594 ± 0.018	0.150 ± 0.019	0.256 ± 0.008
GSH	0.685 ± 0.021	0.378 ± 0.015	0.685 ± 0.020	0.356 ± 0.029	0.325 ± 0.019	0.320 ± 0.015
GSSG	0.635 ± 0.037	0.555 ± 0.061	0.823 ± 0.015	0.193 ± 0.045	0.488 ± 0.020	0.320 ± 0.027
DCMU	0.483±0.024	1	0.358 ± 0.040	0	0.218 ± 0.027	0.782 ± 0.027
DCMU+GSH	0.400±0.046	1	0.840 ± 0.029	0	0.576 ± 0.068	0.424 ± 0.068
DCMU+GSSG	0.362±0.077	1	0.902 ± 0.005	0	0.528 ± 0.110	0.472 ± 0.011
DBMIB	0.804 ± 0.025	0.693 ± 0.089	0.823 ± 0.027	0.139 ± 0.041	0.614 ± 0.010	0.216 ± 0.030
DBMIB+GSH	0.738 ± 0.011	0.531 ± 0.079	0.902 ± 0.015	0.063 ± 0.010	0.633 ± 0.005	0.320 ± 0.020
DBMIB+GSSG	0.312 ± 0.030	0.400 ± 0.039	0.836 ± 0.027	0.122 ± 0.005	0.467 ± 0.034	0.383 ± 0.034
MV	0.775 ± 0.003	0.561 ± 0.038	0.736 ± 0.022	0.359 ± 0.034	0.386 ± 0.028	0.238 ± 0.010
MV+GSH	0.513 ± 0.108	1	0.898 ± 0.018	0	0.533 ± 0.059	0.467 ± 0.059
MV+GSSG	0.537 ± 0.071	1	0.898 ± 0.007	0	0.596 ± 0.012	0.404 ± 0.012
DTT	0.769 ± 0.009	0.451 ± 0.021	0.441 ± 0.026	0.507 ± 0.008	0.169 ± 0.013	0.324 ± 0.013
DTT+GSH	0.492 ± 0.082	0.386 ± 0.021	0.820 ± 0.053	0.120 ± 0.020	0.417 ± 0.058	0.463 ± 0.052
DTT+GSSG	0.766 ± 0.024	0.376 ± 0.030	0.636 ± 0.046	0.390 ± 0.013	0.288 ± 0.027	0.322 ± 0.023

Table 3.1 (Continued)

	F_v/F_m	1-qL	qN	Φ_{PSII}	Φ_{NPQ}	$\Phi_{f,D}$
Control	0.784 ± 0.004	0.346 ± 0.019	0.348 ± 0.029	0.594 ± 0.018	0.150 ± 0.019	0.256 ± 0.008
GA	0.820 ± 0.004	0.910 ± 0.033	0.846 ± 0.016	0.065 ± 0.025	0.650 ± 0.028	0.285 ± 0.011
GA+GSH	0.755 ± 0.019	0.973 ± 0.027	0.862 ± 0.023	0.020 ± 0.020	0.657 ± 0.018	0.322 ± 0.019
GA+GSSG	0.446 ± 0.058	0.779 ± 0.076	0.823 ± 0.008	0.055 ± 0.019	0.469 ± 0.031	0.476 ± 0.035
MSX	0.821 ± 0.011	0.350 ± 0.010	0.425 ± 0.020	0.650 ± 0.022	0.124 ± 0.011	0.226 ± 0.013
MSX+GSH	0.665 ± 0.031	0.422 ± 0.033	0.545 ± 0.031	0.359 ± 0.039	0.234 ± 0.020	0.407 ± 0.034
MSX+GSSG	0.744 ± 0.014	0.426 ± 0.021	0.580 ± 0.012	0.454 ± 0.012	0.246 ± 0.009	0.300 ± 0.007
***Pto* EV**	0.726 ± 0.008	0.423 ± 0.012	0.582 ± 0.012	0.487 ± 0.003	0.238 ± 0.005	0.275 ± 0.007
Pto avrRpm1	0.637 ± 0.034	0.301 ± 0.008	0.383 ± 0.013	0.480 ± 0.034	0.132 ± 0.007	0.379 ± 0.025
Cytokinin	0.769 ± 0.003	0.341 ± 0.017	0.391 ± 0.028	0.587 ± 0.009	0.128 ± 0.011	0.285 ± 0.007
Cold stress	0.739 ± 0.004	0.687 ± 0.012	0.736 ± 0.010	0.224 ± 0.015	0.450 ± 0.014	0.325 ± 0.007
Cold acclimation	0.773 ± 0.007	0.373 ± 0.031	0.618 ± 0.040	0.467 ± 0.032	0.273 ± 0.033	0.257 ± 0.006

The effect on PSII excitation pressure depended on whether reduced or oxidized glutathione was used, with GSSG but not GSH resulting in increased redox state of Q_A.

The increased redox state of Q_A reflects an increase in the redox state of the PQ pool. Thus, GSSG but not GSH treatment resulted in reduction of the PQ pool. Fd was likely reduced as a result of both treatments, as cyclic electron transport has been shown to result in reduction of Fd (Cleland and Bendall 1992). The glutathione pool and the stroma were likely reduced in response to GSH and oxidized in response to GSSG.

Despite of the known role of glutathione in ROS detoxification, enhanced GSH biosynthesis in transgenic tobacco plants has been found to increase oxidative stress, resulting in chlorosis or necrosis (Creissen et al. 1999). In addition, infiltration of *Arabidopsis* leaves with GSH or GSSG and subsequent exposure to light caused extensive photodamage (Karpinska et al. 2000). Similarly, pre-treatment of rice leaves with GSH enhanced their sensitivity to photodamage in low temperature conditions (Xu et al. 2000). The inhibitory effect of exogenous GSH on photosynthesis has been attributed to decreased expression of two cytosolic ascorbate peroxidase (APX) genes due to a disturbance in stress signal transduction brought about by increased GSH levels (Karpinski et al. 1997).

In the present study, the plants were not incubated in glutathione in the light prior to fluorescence measurements. However, we cannot rule out the possibility of oxidative stress after long-term treatments with glutathione, such as those performed prior to analysis of *GLK* transcript levels in response to glutathione, alone or in combinations with various chemicals.

3.3 Treatment with DCMU

DCMU, also known as diuron, is a herbicide that is the most commonly used inhibitor of photosynthetic electron transport in studies employing chlorophyll fluorescence analysis to evaluate plant photosynthetic performance (Ralph et al. 2011). DCMU blocks electron flow between PSII and PSI by competing with PQ for the Q_B binding site of PSII (Trebst 1986; Crofts et al. 1993; Figure 2.2). Therefore, electrons are held in the D1 protein of PSII, causing reduction of Q_A and oxidation of the downstream PQ pool (Kirilovsky et al. 1994; Durnford et al. 1998).

The decrease in F_v/F_m observed following DCMU treatment (Table 3.1) indicates that the herbicide interfered with the opening of PSII reaction centres during dark adaptation, thus lowering the maximum fluorescence F_m upon exposure to the high-intensity light flash following dark adaptation. As expected, 1-qL was equal to 1, which indicates that all PSII reaction centres were closed, Q_A was completely reduced, and the PQ pool was completely oxidized. The effective quantum yield of PSII, Φ_{PSII} decreased to zero, confirming that no photosynthesis was taking place as a result of blockage of photosynthetic electron transport. The drop in Φ_{PSII} was compensated by increased reaction centre quenching, $\Phi_{f,D}$ (Table 3.1).

Thus, DCMU was confirmed to result in reduction of Q_A and oxidation of the downstream components of the electron transport chain, including the PQ pool, Fd and the stroma. This was accompanied by increased heat dissipation from PSII reaction centres. However, despite reduction of PSII, DCMU has previously been shown to prevent singlet oxygen production and to protect PSII from photoinhibition (Nakajima et al. 1996; Rutherford and Krieger-Liszkay 2001; Fufezan et al. 2002).

3.4 Treatment with DCMU+GSH

DCMU blocks the electron transport chain, resulting in reduction of $Q_{A,}$ and inhibition of photochemical quenching (Kirilovsky et al. 1994; Durnford et al. 1998; Ralph et al. 2011), which is reflected by 1-qL being equal to 1 and Φ_{PSII} being equal to zero. Addition of GSH did not change either 1-qL or $\Phi_{PSII,}$ indicating that Q_B was still blocked by DCMU (Table 3.1). Additionally, DCMU+GSH caused the same decrease in F_v/F_m as that observed following DCMU treatment, indicating that the blockage of Q_B interfered with the opening of PSII reaction centres during dark adaptation.

Nonphotochemical antenna quenching, Φ_{NPQ}, increased in response to treatment with DCMU+GSH. This may be explained by the role of glutathione in ascorbate regeneration as part of the ascorbate-glutathione cycle (Noctor and Foyer 1998). Thus, electron donation from ascorbate to PSI seems to have caused cyclic electron flow around PSI. The resultant increase in ΔpH, as well as donation of electrons from ascorbate to violaxanthin de-epoxidase (Bratt et al. 1995), allowed for greater heat dissipation through antenna.

At the same time, compared to the DCMU treatment alone, DCMU+GSH treatment resulted in lower heat dissipation through the reaction centre, $\Phi_{f,D}$, although the value was still greater than in the untreated control. Therefore, it appears that activation of antenna quenching upon the addition of GSH allowed for decreased reaction centre quenching.

Overall, effects of the DCMU+GSH treatment are similar to those caused by DCMU. However, nonphotochemical antenna quenching was activated and reaction centre quenching was lower, which seems to be due to increased cyclic electron transport in response to GSH. In addition, increased cyclic electron transport likely resulted in reduction of the PQ pool and Fd.

Blockage of linear electron transport by DCMU is expected to affect redox state of the stroma by preventing photosynthetic $NADP^+$ reduction, while addition of GSH likely increased redox state of the glutathione pool. Glutathione is known to act as a cellular redox buffer (Rouhier et al. 2008), and is thus expected to at least partially reverse oxidation of the NADPH/$NADP^+$ pool. Thus, compared to the DCMU treatment, DCMU+GSH likely resulted in greater reduction of the stroma. As DCMU has previously been shown to prevent singlet oxygen production and GSH participates in ROS detoxification, the combination of DCMU+GSH likely caused no change or a decrease in ROS levels.

3.5 Treatment with DCMU+GSSG

DCMU+GSSG caused very similar changes in photosynthetic parameters as DCMU+GSH, indicating that reduced and oxidized glutathione activate the same photoprotective mechanisms when used in combination with DCMU (Refer to Section 3.2).

The similar effect of the two forms of glutathione is likely due to the maintenance of glutathione homeostasis in plants. As discussed in Section 3.2, GSH levels in *Arabidopsis* leaves have been found to increase in response to exogenous applications of either GSH or GSSG, although to a greater extent following GSH treatment (Karpinska et al. 2000).

Given the similar effects of DCMU+GSH and DCMU+GSSG on photosynthetic parameters, the two treatments are both expected to reduce the PQ pool and Fd, as well as to detoxify ROS. However, they may differ in their effect on redox state of the stroma. Compared to DCMU, DCMU+GSH is expected to cause a reduction of the stroma, while DCMU+GSSG is expected to oxidize the glutathione pool, leading to an overall oxidation of the stroma.

Overall, DCMU, DCMU+GSH, and DCMU+GSSG treatments caused reduction of $Q_{A.}$ All three treatments completely inhibited photochemistry, while enhancing nonphotochemical quenching of excess energy. Compared to the DCMU treatment, addition of either GSH or GSSG in combination with DCMU activated nonphotochemical antenna quenching, thus allowing for a decrease in reaction centre quenching.

3.6 Treatment with DBMIB

DBMIB is an artificial quinone which interferes with the Reiske iron-sulfur centre, thereby blocking electron transport through the cytochrome b_6f complex (Trebst 2007; Refer to Figure 2.3). DBMIB binds close to the Q_o binding site for plastoquinol, the reduced form of PQ (Cramer et al. 2006). This results in inhibition of reoxidation of PQH_2 and reduction of the PQ pool (Trebst 1980).

DBMIB acts as an effective quencher of excitation energy from the light harvesting antenna, resulting in a decrease in F_o level of chlorophyll fluorescence (Vasil'ev et al. 1998), which was confirmed by our results (data not shown). The decrease in F_o caused by artificial quinones has been shown to be due to increased non-photochemical quenching (Vasil'ev et al. 1998; Bukhov et al. 2003), reflected by the higher measured Φ_{NPQ} value (Table 3.1). Due to the approximately 4-fold increase in Φ_{NPQ}, reaction centre quenching, $\Phi_{f,D}$, did not change significantly.

However, illumination reversibly reduces DBMIB, resulting in a loss of its ability to act as a non-photochemical quencher (Bukhov et al. 2003). Increased reduction of DBMIB and the PQ pool was reflected by a higher PSII excitation pressure (1-qL), compared to untreated control leaves (Table 3.1).

The known role of DBMIB in blocking electron transport through the cytochrome b_6f complex was evident from increased reduction of Q_A and decreased effective quantum yield of PSII. Thus, DBMIB had a similar effect on 1-qL and Φ_{PSII} values as DCMU (Refer to Section 3.3).

One difference between the effect of DCMU and DBMIB on photosynthetic parameters is that the latter does not abolish the establishment of pH gradient. As a result, unlike DCMU, DBMIB caused an increase in non-photochemical antenna quenching without concomitant increase in reaction centre quenching. Another difference between the two inhibitors is that DBMIB blocks electron transport chain downstream of DCMU, resulting in reduction of PSII and the PQ pool and oxidation of the rest of the electron transport chain, while DCMU results in reduction of PSII only and oxidation of the rest of the electron transport chain.

Effects of DCMU and DBMIB on redox state of Fd and the stroma and production of ROS are expected to be similar. However, DBMIB possibly resulted in lower levels of ROS due to nonphotochemical antenna quenching, at least prior to loss of its ability to act as a quencher of excess excitation energy.

3.7 Treatment with DBMIB+GSH

Similarly to DBMIB alone, DBMIB in combination with GSH caused suppression of photochemistry and enhancement of nonphotochemical quenching of light energy (Table 3.1). Although the Q_o site of the cyt$_{b6f}$ complex was blocked by DBMIB, as indicated by increased 1-qL and decreased Φ_{PSII} values compared to the control, the suppression of electron transport was incomplete. Complete inhibition of electron flow results in 1-qL

being equal to 1 and Φ_{PSII} being equal to zero, as was observed for DCMU (Table 3.1). DBMIB, however, resulted in "leaky" blockage of the electron transport chain, which seems to have allowed for some cyclic electron transport in response to GSH.

Cyclic electron transport and increased ΔpH may account for even greater Φ_{NPQ} observed in response to DBMIB+GSH compared to treatment with DBMIB alone. Reaction centre quenching, $\Phi_{f,D}$, increased as an additional photoprotective mechanism. Overall, the effects of DBMIB and DBMIB+GSH treatments compared to the control are qualitatively similar, with the exception of increase in $\Phi_{f,D}$ in response to DBMIB+GSH.

Similarly to DBMIB, DBMIB+GSH caused reduction of the PQ pool, as indicated by the increased in 1-qL value compared to control. However, compared to DBMIB treatment, the PQ pool was not as strongly reduced, possibly due to increased electron transport due to the water-water cycle. Fd was likely more reduced following the DBMIB+GSH treatment compared to the DBMIB and control treatments, as a result of increased cyclic electron transport, which was suggested by the slightly higher Φ_{NPQ} value. The stroma was likely more reduced in response to DBMIB+GSH compared to DBMIB. Finally, ROS levels likely decreased as a result of the DBMIB+GSH treatment compared to the DBMIB treatment.

3.8 Treatment with DBMIB+GSSG

As discussed in Section 3.2, effects of glutathione on fluorescence parameters seem to be due to donation of electrons to PSII from ascorbate regenerated in the ascorbate-glutathione cycle, as well as increased cyclic electron transport as a result of enhanced Mehler reaction. Due to the maintenance of glutathione homeostasis, both reduced and

oxidized glutathione are expected to activate these processes. However, as mentioned previously, the amount of intracellular GSH following treatment with GSSG has been found to be lower than following treatment with GSH (Karpinska et al. 2000). As a result, treatment with DBMIB+GSSG seems to have resulted in a lower rate of electron donation from ascorbate to PSII and that of Mehler reaction, compared to the DBMIB+GSH treatment.

The lower rate of electron donation from ascorbate to PSII is reflected by a lower redox state of 1-qL, compared to the DBMIB+GSH treatment (Table 3.1). Reduced Mehler reaction and, consequently, reduced cyclic electron transport are reflected by reduced Φ_{NPQ}, and enhanced $\Phi_{f,D}$, as a compensatory mechanism. Therefore, compared to the DBMIB+GSH treatment, nonphotochemical quenching was redistributed in favour of reaction centre quenching, as antenna quenching was not as efficient. As nonphotochemical and photochemical energy quenching are in direct competition, the decrease in Φ_{NPQ} was accompanied by a slight increase of Φ_{PSII} in response to DBMIB+GSSG, compared to the DBMIB+GSH treatment.

Thus, although changes in fluorescence parameters in response to DBMIB+GSSG may be attributed to the action of DBMIB and increased intracellular GSH, the extent of the effects was lower compared to DBMIB+GSH treatment. Overall, compared to the untreated control, a decrease in photochemistry and an increase in nonphotochemical quenching were observed. Based on these results, it can be concluded that the PQ pool and Fd were reduced as a result of the DBMIB+GSSG treatment, for the same reasons as outlined above in the discussion on DBMIB+GSH (Refer to Section 3.7).

The stroma was likely oxidized compared to control, DBMIB and DBMIB+GSH treatments, because DBMIB is expected to result in oxidation of the NADPH/NADP+ pool, while GSSG is expected to result in oxidation of the glutathione pool. ROS levels were likely higher in response to the DBMIB+GSSG treatment compared to the DBMIB+GSH treatment, as cyclic electron transport and ROS detoxification seem to be reduced.

3.9 Treatment with MV

MV, also known as paraquat, is an artificial electron acceptor which competes with the final photosynthetic electron acceptor Fd for electrons from PSI (Dan Hess 2000). Reduced MV may react with oxygen, resulting in the production of superoxide ($\cdot O^{2-}$), and subsequently H_2O_2, ROS which are damaging to the chloroplast (Salin 1988). Application of MV is expected to oxidize the PQ pool, increase Φ_{PSII} and decrease non-photochemical quenching due to acceleration of electron transport (Ralph et al. 2011). However, we observed the opposite effects of MV on chlorophyll fluorescence parameters, with a decrease in the effective quantum yield (Φ_{PSII}) and increases in PSII excitation pressure (1-qL) and non-photochemical quenching (Φ_{NPQ}) (Table 3.1).

Our results are in agreement with those of Fan et al. (2009), who showed that at low concentrations of ≤50 μM, MV causes an inhibition of PSII photosynthetic parameters. It was suggested that higher concentrations of MV are required to transfer electrons from the acceptor side of PSI to oxygen. The concentration of MV used in the present study was 50 μM, which may explain the observed fluorescence measurements.

At low concentrations, MV was suggested to accelerate downhill electron transfers from PSII to the cyt$_{b6f}$ complex and from PSI to Fd. The resulting excessive charge

separation within PSII decreased F_v'/F_m', as was confirmed in the present experiment (data not shown; Fan et al. 2009).

Although the accelerated electron flow was shown to increase the rate of oxidation of Q_A, the rate of Q_A reduction increased to an even greater extent, resulting in the closing of PSII reaction centres. Since effective quantum yield is a product of the maximum quantum yield in the light (F_v'/F_m') and the proportion of open reaction centres, its measured value Φ_{PSII} also decreased. Non-photochemical quenching increased to compensate for the decreased photochemical dissipation of excess light energy (Fan et al. 2009). The same mechanism of accelerated downhill electron transfer in response to MV may be responsible for the changes in 1-qL, Φ_{PSII} and Φ_{NPQ} observed in the present experiment (Table 3.1).

Alternatively, MV action may be explained by its participation in the Mehler reaction. As discussed in Section 3.2, in addition to its role in preventing ROS damage to the chloroplast, the water-water cycle enhances cyclic electron transport around PSI, establishing ΔpH needed for nonphotochemical antenna quenching (Murchie and Niyogi 2011). Increased ΔpH and overreduction of the PQ pool by cyclic electron transport explain the observed values of the fluorescence parameters, including decreased Φ_{PSII} and increased 1-qL and Φ_{NPQ} (Table 3.1).

Although it is unclear which of the two alternatives presented above is the true explanation for the observed effect of MV, the herbicide resulted in a pronounced effect on photosynthetic performance, lowering effective quantum yield and increasing PSII excitation pressure and nonphotochemical antenna quenching.

As indicated by the increased 1-qL value compared to control, the PQ pool was reduced. Fd was also likely reduced, as a result of increased cyclic electron transport. The

stroma is expected to be oxidized, because MV interferes with reduction of $NADP^+$ by Fd (Trebst 2007). Finally, ROS levels are expected to increase, as reduced MV is known to react with oxygen and result in the production of superoxide (Salin 1988).

3.10 Treatment with MV+GSH

Compared to MV treatment, treatment with MV+GSH caused more pronounced changes in chlorophyll fluorescence parameters, including PSII excitation pressure (1-qL), effective quantum yield of PSII (Φ_{PSII}) and nonphotochemical antenna quenching (Φ_{NPQ}). In addition, F_v/F_m and $\Phi_{f,D}$ changed compared to the control, while they were unaffected by treatment with MV alone (Table 3.1).

As with glutathione treatment, F_v/F_m seems to have decreased in response to MV+GSH as a result of electron donation to PSII by ascorbate produced through the ascorbate-glutathione cycle (Katoh and San Pietro 1967; Mano et al. 2004; Refer to Section 3.2). Changes in the other photosynthetic parameters seem to be related to increased rate of Mehler reaction in response to the MV+GSH compared to MV alone. Since MV induces ROS production and GSH participates in ROS detoxification (Salin 1988; Noctor and Foyer 1998), the two chemicals together may increase the rate of the water-water cycle. Thus, cyclic electron transport seems to have increased to such an extent that linear electron transport was inhibited, as seen from complete reduction of Q_A (1-qL = 1) and inhibition of photochemical quenching (Φ_{PSII} = 0). Increased cyclic electron transport allowed for the establishment of pH gradient necessary for nonphotochemical quenching which compensated for the suppression of photochemical quenching of light energy. Both antenna

and reaction centre quenching were activated, as indicated by increased values Φ_{NPQ} and $\Phi_{f,D}$.

Overall, fluorescence analysis has shown that MV and GSH likely promote both ROS production and detoxification, which results in suppression of photochemistry and increase in nonphotochemical fluorescence quenching. The PQ and Fd seem to have been reduced as a result of increased cyclic electron transport. The stroma was likely more reduced in response to the MV+GSH treatment compared to MV alone. ROS levels likely decreased as a result of the MV+GSH treatment compared to the MV treatment.

3.11 Treatment with MV+GSSG

Treatment with MV+GSSG resulted in suppression of photochemistry and increase in nonphotochemical fluorescence quenching, with values not significantly different from those obtained following MV+GSH treatment. The same effect of reduced and oxidized glutathione on the background of MV may indicate that either of the two treatments caused an increase in intracellular GSH content that was sufficient to completely block Q_A and inhibit photochemistry, as well as to increase both reaction centre and antenna quenching of excess light energy.

Like treatment with MV+GSH, MV+GSSG caused reduction of the PQ pool and Fd. The stroma, however, was likely oxidized compared to either control, MV, or MV+GSH treatments, due to the addition of GSSG. Finally, ROS levels were likely the same as with MV+GSH, as no significant differences in photosynthetic parameters were observed between the MV+GSH and MV+GSSG treatments.

3.12 Treatment with DTT

DTT is a redox reagent that reduces Fd (Neumann and Drechsler 1984). Previously, it was found that in the absence of excessive light (at 100 to 200 μmol m^{-2} s^{-} PFD), DTT does not affect photosynthetic rate or quantum yield of PSII, but leads to increased susceptibility to photoinhibition in high light (Bilger and Björkman 1990). Under the conditions used (150 μmol m^{-2} s^{-} PFD), DTT treatment caused a 30% increase in Q_A reduction and a 15% decrease in the effective PSII quantum yield, Φ_{PSII}, suggesting that DTT has the expected effect on photosynthetic parameters and may cause pronounced photoinhibition at higher light intensities.

An additional consequence of DTT treatment is inhibition of de-epoxidation, which occurs as part of the photoprotective xanthophyll cycle (Long et al. 1994). Thus, because of the inhibitory effect of DTT on the xanthophyll cycle, the photosynthetic apparatus is unable to dissipate excess light energy through increased nonphotochemical antenna quenching, as was reflected by no change in the Φ_{NPQ} value (Table 3.1). As a result, excess light energy was dissipated through reaction centre quenching, demonstrated by the increased $\Phi_{f,D}$ value.

At the concentrations used in the present experiment, the overall effect of DTT was reduction of the electron transport chain, decrease in effective quantum yield of PSII and increased reaction centre quenching. PQ pool, Fd, and the stroma were likely all reduced. ROS production possibly occurred as a result of overreduction of the electron transport chain.

3.13 Treatment with DTT+GSH

As discussed in Section 3.12, effects of DTT on photosynthetic performance seem to

be due to reduction of Fd and resultant overreduction of the electron transport chain, as well as inhibition of violaxanthin de-epoxidation (Neumann and Drechsler 1984; Long et al. 1994). These changes, together with increased cyclic electron transport in response to GSH and the positive effect of ascorbate on violaxanthin de-epoxidation, seem to be responsible for the observed values of chlorophyll fluorescence parameters following the DTT+GSH treatment.

Following DTT+GSH treatment, PSII excitation pressure, 1-qL, did not change significantly compared to the control, and decreased slightly compared to the DTT treatment (Table 3.1). This decrease may be attributed to enhanced Mehler reaction and the water-water cycle due to detoxification of ROS by glutathione. Consequently, electron transport increased, as the water-water cycle is known to serve as an alternative electron sink (Badger et al. 2000).

Cyclic electron transport in response to GSH seems to be responsible for increased ΔpH and nonphotochemical antenna quenching, Φ_{NPQ}. While DTT is a violaxanthin de-epoxidation inhibitor, ascorbate generated from the ascorbate-glutathione cycle donates electrons to violaxanthin de-epoxidase, promoting antenna quenching (Bratt et al. 1995). Thus, Φ_{NPQ} did not change with DTT alone but increased after the DTT+GSH treatment.

Although reaction centre quenching does not depend on zeaxanthin formation, it was suggested to be activated by thylakoid protonation (Bukhov et al. 2001), which may explain the increase in the $\Phi_{f,D}$ value seen in response to DTT+GSH treatment compared to the DTT treatment. Φ_{PSII} decreased as a result of increase in the competing nonphotochemical quenching processes. F_v/F_m decreased compared to control, likely as a result of donation of

electrons from ascorbate to PSII and consequently the inability of PSII reaction centres to open during dark adaptation (Katoh and San Pietro 1967; Mano et al. 2004).

The main effects of DTT+GSH on fluorescence parameters were decreased photochemical quenching and increased nonphotochemical quenching, including both antenna and reaction centre quenching. Following the DTT+GSH treatment, no change in PQ pool redox state was detected compared to control, as indicated by the observed 1-qL value (Table 3.1). Due to increased cyclic electron transport, Fd was likely reduced by the DTT+GSH treatment to an even greater extent than by DTT alone. The DTT+GSH treatment is also expected to cause a greater reduction of the stroma than DTT alone, due to the exogenous GSH. Finally, ROS levels were likely lower than with DTT alone as a result of ROS detoxification by the glutathione-ascorbate cycle.

3.14 Treatment with DTT+GSSG

Results of fluorescence analysis following DTT+GSSG treatment suggest that cyclic electron transport was activated by the resulting increase in intracellular GSH content, as was suggested to occur following the DTT+GSH treatment (Refer to Section 3.13). However, the rate of cyclic electron transport seems to be slower in response to GSSG, compared to that induced by GSH in combination with DTT. This may explain why changes in Φ_{NPQ}, $\Phi_{f,D}$ and Φ_{PSII} were smaller as a result of treatment with DTT+GSSG compared to the DTT+GSH treatment (Table 3.1). In addition, F_v/F_m did not decrease compared to control, which may indicate that the amount of ascorbate generated in response to the exogenous GSSG was not sufficient to reduce PSII during dark adaptation. Thus, DTT+GSSG produced similar, although smaller, changes in most of the examined

photosynthetic parameters as DTT+GSH, which included decreased photochemical quenching and increased nonphotochemical quenching.

Similarly to the DTT+GSH treatment, DTT+GSSG treatment caused no changes in the PQ redox state compared to control, as indicated by the observed 1-qL value (Table 3.1). Fd was likely reduced as a result of enhanced cyclic electron transport. However, compared to the DTT+GSH treatment, DTT+GSSG likely resulted in oxidation of the stroma as a result of exogenous GSSG application. Finally, ROS levels may have been higher as a result of DTT+GSSG treatment compared to the DTT+GSH treatment, as ROS detoxification is expected to be less efficient upon addition of GSSG as opposed to GSH.

3.15 Treatment with GA

GA is a Calvin cycle inhibitor, which inhibits CO_2 fixation by blocking the conversion of triose-P to ribulose-1,5-bisphosphate (Stokes and Walker 1972). The inhibition of carbon fixation by GA results in increased reduction of the electron transport chain and a decrease in the electron transfer rate and photochemical quenching (Hakala et al. 2005). In the present study GA resulted in the expected overreduction of the electron transport chain and suppression of photosynthesis, which was reflected by increased 1-qL and decreased Φ_{PSII} values. Increased Φ_{NPQ} indicates enhanced dissipation of excess light energy by antenna quenching, compensating for decreased photochemical quenching.

Therefore, the observed fluorescence parameters confirmed that GA had the intended effect of blocking the Calvin cycle and preventing electron flow through the electron transport chain. Based on these results, it can be concluded that the PQ pool, Fd and stroma were reduced and ROS was produced at one or both photosystems.

3.16 Treatment with GA+GSH

As discussed in Section 3.15, GA inhibits carbon fixation, which causes overreduction of the electron transport chain, suppression of photochemistry and enhancement of nonphotochemical antenna quenching of chlorophyll fluorescence (Stokes and Walker 1972; Hakala et al. 2005). The main effects of GSH, alone and in combination with various chemicals, seem to be donation of electrons from ascorbate to PSII and enhancement of cyclic electron transport.

Enhancement of cyclic electron transport is expected to exacerbate the negative effects of GA on photosynthetic performance. Indeed, compared to treatment with GA, GA+GSH caused slightly greater reduction of the electron transport chain, reflected by increased 1-qL, and a slight suppression of effective quantum yield of PSII, Φ_{PSII} (Table 3.1). However, the changes were not statistically significant and Φ_{NPQ} did not change, as would be predicted to occur as a result of increased ΔpH due to enhancement of cyclic electron transport. Nevertheless, reaction centre quenching, $\Phi_{f,D}$, increased significantly following the GA+GSH treatment compared to both the control and GA treatments, suggesting that increased thylakoid protonation activated this photoprotective mechanism.

The lack of significant differences in 1-qL, Φ_{PSII} and Φ_{NPQ} between the GA and GA+GSH treatments may indicate that redox state of the electron transport chain and nonphotochemical antenna quenching were already at their maximum level following the GA treatment. As seen in Table 3.1, the electron transport chain was almost completely reduced and the maximum quantum yield of PSII was close to zero. Perhaps Φ_{NPQ} was also at its maximum following the GA treatment and could not be further enhanced by GSH.

Overall, with the exception of slight enhancement of $\Phi_{f,D}$, fluorescence parameters were similar between the GA and GA+GSH treatments, with an observed overreduction of the electron transport chain, inhibition of photochemistry and induction of nonphotochemical quenching.

Thus, effects on the redox state of the PQ pool, Fd and the stroma are also expected to be similar between the two treatments. ROS levels were possibly lower following the GA+GSH treatment compared to the GA treatment due to ROS detoxification by the ascorbate-glutathione cycle.

3.17 Treatment with GA+GSSG

Similarly to both GA and GA+GSH treatments, GA+GSSG caused a reduction of quantum yield of PSII and increases in PSII excitation pressure and nonphotochemical quenching of chlorophyll fluorescence. However, compared to the GA+GSH treatment, PSII excitation pressure, 1-qL, decreased, which may be due to slower regeneration of ascorbate in response to exogenous GSSG, compared to GSH. Thus, donation of electrons from ascorbate to PSII is expected to be reduced, resulting in a more oxidized PQ pool.

In addition, it seems that cyclic electron transport was also reduced in response to GSSG compared to GSH. This is evident from the observation that compared to the GA+GSH treatment, GA+GSSG resulted in lower Φ_{NPQ}, accompanied by higher Φ_{PSII}, and increased $\Phi_{f,D}$. Compared to control, GA+GSSG caused similar changes as GA and GA+GSH. However, treatments with GA in combination with reduced and oxidized forms of glutathione were different from each other in that GSSG caused greater photochemical

quenching and a redistribution of nonphotochemical quenching, with increased antenna quenching and reduced reaction centre quenching.

Thus, the PQ pool and Fd were likely reduced in response to GA+GSSG. The stroma is expected to be oxidized compared to the GA+GSH treatment due to exogenous GSSG application. Finally, ROS levels were likely higher than those following treatment with GA+GSH.

3.18 Treatment with MSX

MSX blocks organic nitrogen synthesis by inhibiting GS, an enzyme that participates in the GS/GOGAT system and uses ammonia to produce Gln (Miflin and Lea 1977; Evstigneeva et al. 2003; Refer to Figure 2.5). MSX application to photosynthetic organisms results in accumulation of ammonia, as was for example shown in cyanobacteria (Jeanfils and Loudeche 1986). Therefore, effects of MSX may be attributed to either inhibition of GS or resultant accumulation of ammonia. The use of GS mutants allows this distinction to be made. One study employing GS mutants found that a decrease in CO_2 fixation rate in the mutants was due to suppression of photorespiration, and not as a result of ammonia accumulation (Blackwell et al. 1987). Suppression of photorespiration was suggested to inhibit photosynthesis by reducing the amount of carbon returned to the Calvin cycle. However, we did not detect a decline in 1-qL or Φ_{PSII} values, indicating that MSX did not affect PSII excitation pressure or effective quantum yield.

At millimollar concentrations, ammonia has been shown to inhibit photosynthesis via uncoupling of electron transport (Larsen et al. 1981; Allen 1984). Uncouplers interfere with the establishment of ΔpH required for nonphotochemical antenna quenching (Krause and

Behrend 1986). However, in the present study, uncoupling of electron transport was not observed as Φ_{NPQ} did not decrease significantly (Table 3.1). The lack of inhibition of photosynthesis and antenna quenching may be due to insufficient duration of incubation or low concentrations of ammonia in the chloroplast. Thus, overall, MSX did not have a significant effect on any of the measured photosynthetic parameters.

Therefore, it can be concluded that MSX also did not affect redox state of the PQ pool, Fd, or the stroma. Additionally, it likely did not result in significant ROS production. Nitrogen assimilation is an alternative electron sink; however, under steady state conditions, it is not expected to make a significant contribution to the dissipation of excess light energy (Niyogi et al. 2000). Therefore, inhibition of nitrogen assimilation is not expected to cause significant overreduction of the electron transport chain or production of ROS.

3.19 Treatment with MSX+GSH

Since MSX alone had no significant effect on photosynthetic parameters, it is useful to compare effects of MSX+GSH/GSSG treatments to those of GSH and GSSG. Compared to GSH treatment, MSX+GSH caused greater reduction of the electron transport chain, reflected by increased 1-qL value, and a redistribution of energy partitioning between the two nonphotochemical heat dissipation processes, with an observed decrease in antenna quenching, Φ_{NPQ}, and an increase in reaction centre quenching, $\Phi_{f,D}$ (Table 3.1).

The observed differences between the two treatments may be due to production of ammonia as a result of MSX treatment (Refer to Section 3.18; Jeanfils and Loudeche 1986). Although MSX alone had no significant effect on chlorophyll fluorescence parameters, it resulted in changes in photosynthetic performance in combination with GSH, as GSH and

MSX+GSH treatments differed in their effects on fluorescence parameters, as described above.

Ammonia is known to act as an electron transport uncoupler (Larsen et al. 1981; Allen 1984) and is expected to result in decreased ΔpH, which may explain the observed suppression of nonphotochemical antenna quenching, Φ_{NPQ}, by MSX+GSH. Reaction centre quenching, $\Phi_{f,D}$, seems to have increased to compensate for reduced antenna quenching.

An additional effect of uncouplers such as ammonia is photoinhibition and decreased capacity for electron transport (Krause and Behrend 1986), which is reflected by the increased 1-qL value observed following MSX+GSH treatment compared to GSH treatment. Although 1-qL did not increase as a result of treatment with MSX alone, it may be possible that any ammonia produced as a result of blockage of nitrogen assimilation did not interfere with the flow of electrons under normal conditions, and the inhibitory effect became apparent under conditions of enhanced cyclic electron transport.

Thus, it seems that the MSX+GSH treatment caused increased cyclic electron transport and nonphotochemical energy quenching due to the enhancement of Mehler reaction by GSH. However, compared to GSH treatment, the uncoupling of electron transport by ammonia resulted in greater reduction of the electron transport chain and redistribution of nonphotochemical energy dissipation between antenna and reaction centre quenching. As a result of the processes described above, the PQ pool and Fd were likely reduced. The stroma was also likely reduced, as a result of GSH application. As discussed below, MSX+GSH also seems to have caused photoinhibition as a result of increased ROS production.

3.20 Treatment with MSX+GSSG

Effects of MSX+GSSG on chlorophyll fluorescence parameters were similar to those of MSX+GSH, in that compared to the control treatment, photochemical quenching was decreased and nonphotochemical quenching was enhanced (Table 3.1). However, suppression of photochemical quenching, Φ_{PSII}, was less pronounced, which was compensated by a smaller increase in reaction centre quenching, $\Phi_{f,D}$.

The lower value of Φ_{PSII} in response to MSX+GSH treatment compared to MSX+GSSG may be related to differences in ascorbate levels produced as a result of exogenous GSH and GSSG application, as donation of electrons from ascorbate to PSII affects photochemical quenching. However, no differences in 1-qL value were observed between the two treatments, indicating that excitation pressure of PSII did not change, in contrast to the expected effect of electron donation from ascorbate to PSII.

Alternatively, Φ_{PSII} may decrease as a result of increased nonphotochemical antenna quenching, as zeaxanthin accumulation has been linked to decreased photochemistry (Demmig-Adams 1990; Demmig-Adams and Adams 1996). However, Φ_{NPQ} was again the same in response to the MSX+GSH and MSX+GSSG treatments.

Therefore, it seems likely that the greater reduction in Φ_{PSII} in response to MSX+GSH compared to MSX+GSSG is related to photoinhibition. This is supported by the lower F_v/F_m (Table 3.1), which is known to decrease as a result of photoinhibition and the resultant destruction of PSII complexes.

The reason for greater photoinhibition in response to MSX+GSH compared to MSX+GSSG is unclear, as GSH participates in the detoxification of ROS which damage the thylakoid membrane (Noctor and Foyer 1998). Perhaps, as Karpinski et al. (1997) has

suggested, the oxidative damage in response to exogenous GSH is due to a disturbance in stress signal transduction brought about by the redox imbalance. Although treatment with either form of glutathione results in GSH accumulation, its levels are higher in response to exogenous GSH. Thus, it seems that GSH disrupted the cellular redox balance to a greater extent than GSSG.

Overall, the MSX+GSSG treatment resulted in a decrease in photochemical quenching and an increase in nonphotochemical quenching. However, compared to the MSX+GSH treatment, some differences in the magnitude of these effects were observed. These differences seem to arise from photoinhibition in response to MSX+GSH. Similarly to the MSX+GSH treatment, MSX+GSSG seems to have caused a reduction of the PQ pool and Fd. The stroma was likely oxidized as a result of exogenous GSSG application. It is possible that ammonia production resulted in generation of ROS; however, levels of ROS were likely lower than in response to the MSX+GSH treatment.

3.21 Treatment with *Pst* EV and *Pst avrRpm1*

Infection of plants with various pathogens is known to result in repression of photosynthesis. Chlorophyll fluorescence parameters such as F_v/F_m and Φ_{PSII} have been found to decrease following infection with biotrophic and necrotrophic pathogens, in both compatible and incompatible interactions (Balachandran et al. 1997; Nedbal et al. 2000; Chaerle and Van der Straeten 2001; Meyer et al. 2001; Soukupova et al. 2003; Berger et al. 2004; Chaerle et al. 2004; Berger et al. 2007). Specifically, both virulent and avirulent strains of *P. syringae* have been shown to decrease maximum quantum efficiency of PSII,

F_v/F_m (Berger et al. 2007). The two strains activated similar defence responses in the plant, but their induction by the avirulent strain was faster and stronger (Tao et al. 2003).

As seen in Table 3.1, the maximum quantum yield of PSII in the dark-adapted state, F_v/F_m, did not change significantly following infection with the virulent *P. syringae* strain, *Pst* EV, and decreased by 19% following infection with the avirulent *P. syringae* strain, *Pst avrRpm1*, indicating damage to PSII reaction centres. The effective quantum yield of PSII, $\Phi_{PSII,}$ decreased as a result of infection with both strains.

The increase in PSII excitation pressure, 1-qL, as a result of infection with *Pst* EV was likely related to the decreased photosynthetic efficiency due to damage to PSII reaction centres. However, following infection with *Pst avrRpm1*, 1-qL was 13% lower than in untreated control leaves, indicating a slight increase in oxidation of Q_A and possibly faster electron transport rate, ETR. Previously, ETR was found to increase in some plant-pathogen interactions, such as upon infection of tomato with *Botrytis cinerea* (Berger et al. 2004). In this pathosystem, ETR was stimulated in regions immediately adjacent to the developing lesions, which was suggested to provide additional energy for defence responses. Similarly, infection of wheat with *Mycosphaerella graminicola* caused increased ETR in uninfected regions of the leaf, presumably to compensate for suppression of photosynthesis in infected areas (Scholes and Rolfe 2009). It is likely that the same mechanisms resulted in the slightly decreased 1-qL observed following infection with *Pst avrRpm1*. The reason for the different effects of the virulent and virulent *P. syringae* strain on 1-qL is that, as mentioned above, avirulent strains induce a faster and stronger defence response, which seems to result in local activation in photosynthesis around the sites of infection.

Nonphotochemical antenna quenching, Φ_{NPQ}, increased after infection with *Pst* EV, but did not change significantly after infection with *Pst avrRpm1* (Table 3.1). This difference may be explained by the severity of pathogen-induced stress. Nonphotochemical antenna quenching serves to protect the photosynthetic apparatus against excess light energy (Schreiber 2004). However, oxidative stress and the resultant extensive tissue damage may compromise this photoprotective mechanism (Rolfe and Scholes 2010). Thus, it seems that *Pst avrRpm1* caused greater tissue damage to the plant. As a consequence, infection with *Pst avrRpm1* but not with *Pst* EV caused increased reaction centre heat dissipation, $\Phi_{f,D}$, to compensate for the lack of nonphotochemical antenna quenching (Table 3.1).

Fluorescence measurements have shown that, as expected, infection of *Arabidopsis* with *P. syringae* led to a suppression of photosynthesis. *Pst avrRpm1* seems to have caused a localized enhancement of electron transport rate, but its overall negative effect on photosynthetic performance was more pronounced compared to infection with *Pst* EV.

The two strains of *P. syringae* seem to have the opposite effect on redox state of the PQ pool, with *Pst avrRpm1* causing its oxidation and *Pst* EV causing its reduction. Since there was no inhibition of the electron transport chain downstream of the PQ pool, 1-qL also reflects redox state of Fd. Therefore, *Pst avrRpm1* and EV likely caused oxidation and reduction of Fd, respectively.

In addition, it may be concluded that following *Pst avrRpm1* infection, redox state of the stroma did not change. If, as previously suggested, increased electron transport provides additional energy for defence responses, the rate of the Calvin cycle would be expected to increase, avoiding overreduction of the stroma (Berger et al. 2004; Scholes and Rolfe 2009).

On the other hand, infection with *Pst* EV seems to have caused reduction of the stroma, as no increase in the electron transport rate was observed.

As mentioned above, both the avirulent and virulent strains of *P. syringae* have been shown to result in ROS production (Tao et al. 2003). Since avirulent strains cause faster and stronger defence response in the plant, levels of ROS are expected to be higher in response to infection with the avirulent strain, which was confirmed in by the decreased F_v/F_m in response to *Pst avrRpm1* and not *Pst* EV.

3.22 Treatment with cytokinin

Cytokinin is a phytohormone with several different known functions in plants, including regulation of cell division, promotion of shoot growth, and release from apical dominance (Chernyad'ev 2009). It also plays a role in chloroplast development, prevents chlorophyll degradation and slows down leaf and chloroplast senescence (Chernyad'ev 2009; Werner and Schmülling 2009; Argueso et al. 2010 and references therein). In addition, cytokinin protects photosynthetic machinery against various environmental stresses such as high or low temperature and drought (Chernyad'ev 2009). The protection is achieved through several different mechanisms, including increased synthesis of photosynthetic proteins and reprogramming of gene expression (Chernyad'ev 2009).

As can be seen in Table 3.1, vacuum infiltration of *Arabidopsis* leaves with cytokinin, followed by a 4h incubation at 150 μmol photons m^{-2} s^{-1} did not affect significantly any of the photosynthetic variables measured. As mentioned above, cytokinin has a protective effect on the chloroplast. For example, treatment of bean plants with a synthetic cytokinin protected chloroplast ultrustructure from the combined drought and high

temperature stress (Stoyanova and Yordanov 2000). Given that we did not find cytokinin to significantly affect photosynthetic parameters, it seems likely that the protective effect of this phytohormone is evident under stress, and not under normal physiological conditions. Since no changes in chlorophyll fluorescence were detected, it can be concluded that cytokinin had no effect on redox state of the PQ pool, Fd, or the stroma, and did not enhance ROS production.

3.23 Cold stress and acclimation

In annual cold tolerant plants such as *Brassica* and *Arabidopsis*, cold temperatures cause a short-term suppression of photosynthesis, followed by a strong recovery (Huner et al. 1993; Stitt and Hurry 2002). Moreover, exposure to low non-freezing temperatures results in freezing tolerance, a process known as cold acclimation (Thomashow 1999).

Our results confirmed suppression of photosynthesis by cold stress and a reversal of this suppression in cold acclimated *Arabidopsis* plants (Table 3.1). Effective quantum yield of PSII (Φ_{PSII}) decreased, while nonphotochemical antenna quenching (Φ_{NPQ}) increased compared to control under both cold stress and cold acclimation conditions. However, as expected, the magnitude of the changes was smaller in cold acclimated than in cold stressed plants. PSII excitation pressure (1-qL) and reaction centre quenching ($\Phi_{f,D}$) increased only in cold stressed plants, and not in cold acclimated plants.

Short-term (hours to days) suppression of photosynthesis by cold temperature stress is due to lower activity of enzymes, resulting in decreased utilization of photosynthetic assimilates (Ruelland et al. 2009). As a consequence of slower carbon assimilation, the electron transport chain becomes overreduced, which is reflected by the increased 1-qL

value. Nonphotochemical antenna quenching and PSII reaction centre quenching increase as photoprotective mechanisms to dissipate excess light energy (Krol et al. 1999; Ivanov et al. 2008; Ruelland et al. 2009). In addition, lower activity of ROS scavenging enzymes and overreduction of electron transport chain result in increased oxidative damage to the photosynthetic apparatus, further lowering PSII efficiency (Ruelland et al. 2009).

Over days to weeks of exposure to cold temperatures, *Arabidopsis* and other over-wintering herbaceous plants grow new better adapted leaves, which are responsible for the recovery of photosynthesis (Hurry et al. 1994, Strand et al. 1997). Cold acclimated plants are characterized by increased content and activity of a number of Calvin cycle enzymes (Hurry et al. 1994, 1995, 2000; Goulas et al. 2006), as well as increased capacity for ROS scavenging (Krol and Huner 1985; Streb and Feierabend 1999; Streb et al. 1999, 2003). As seen in Table 3.1, chlorophyll fluorescence parameters confirm the negative effect of cold stress on photosynthetic performance of *Arabidopsis* and demonstrate a recovery in cold acclimated plants.

Cold stress but not cold acclimation caused an increase in redox state of the PQ pool. However, as indicated by the observed changes in photosynthetic parameters, the recovery of photosynthesis in cold acclimated plants was not complete (Table 3.1). This suggests that both cold stressed and cold acclimated plants exhibit a reduction of Fd and the stroma and increased ROS production. As discussed above, the magnitude of the changes in photosynthetic parameters was lower in response to cold acclimation compared to cold stress treatment. Therefore, it can be concluded that the reduction of Fd and the stroma was less pronounced and less ROS was produced as a result of cold acclimation compared to cold stress conditions.

3.24 Summary of chlorophyll fluorescence in response to experimental treatments

As seen in Table 3.1, all of the experimental treatments that were analyzed using chlorophyll fluorescence resulted in changes in one or more fluorescence parameters, with the exception of MSX and cytokinin, which had no significant impact on photosynthetic performance. The lack of changes in photosynthetic performance due to inhibition of organic nitrogen synthesis by MSX may be explained by the fact that nitrogen assimilation does not contribute significantly to dissipation of excess light energy under steady state conditions (Niyogi et al. 2000). In addition, amounts of ammonia produced as a result of MSX application seem to be insufficient to result in significant electron uncoupling.

The lack of changes in photosynthetic performance in response to cytokinin may be due to its known protective effect on the photosynthetic apparatus under stress conditions, which may explain its lack of effect under the non-stress conditions of the present experiment.

Some treatments seem to have a negative effect on the photosynthetic apparatus by causing oxidative damage. Suppression of photosynthesis by MV, *P. syringae* and cold stress appears to be a consequence of the role of these treatments in the production of ROS.

The maximum quantum yield of PSII in the dark-adapted state, F_v/F_m, did not change compared to control in response to any of the treatments except GSH, GSSG, DCMU and *Pto avrRpm1*. Since, with the exception of cytokinin, leaves were not exposed to actinic light prior to fluorescence measurements, decrease in F_v/F_m cannot be attributed to damage to PSII reaction centres by excess light energy. Instead, suppression of this photosynthetic parameter may be caused by damage to PSII reaction centres in the absence of actinic light. GSH, GSSG, and *Pto avrRpm1* may act through this mechanism, as they seem to induce

oxidative stress in plants. Alternatively, decreases in F_v/F_m may be due to the inability of reaction centres to "relax", or open, during dark adaptation. This mechanism is relevant to the treatment with DCMU, as it blocks the electron transport chain at $Q_{A,}$ the primary quinone acceptor of PSII. As a result, electrons are trapped in PSII and cannot be transported out of reaction centres even during dark adaptation.

Fluorescence measurements indicate that most of the experimental treatments suppressed photochemical quenching, which is reflected by increased PSII excitation pressure (1-qL) and decreased effective quantum yield of PSII (Φ_{PSII}) (Table 3.1). As expected, the same treatments resulted in increased nonphotochemical heat dissipation, either through antenna or reaction centre quenching, or a combination of the two processes. It is not clear why reaction centre quenching was activated in some cases and not in others. In the case of DTT and *Pst avrRpm1*, increased reaction centre quenching seems to be related to the suppression of antenna quenching. DTT prevents antenna quenching by inhibiting de-epoxidation required for the xanthophyll cycle, while *Pst avrRpm1* interferes with this photoprotective mechanism by causing oxidative stress and extensive tissue damage (Long et al. 1994; Rolfe and Scholes 2010).

Based on results of chlorophyll fluorescence analysis, it was in most cases possible to evaluate effects of experimental treatments on redox state of the PQ pool, Fd and the stroma as well as levels of ROS, all of which may serve as a source of retrograde chloroplast-to-nucleus signals. Since MSX and cytokinin had no effect on photosynthetic parameters, they likely did not change redox state of the chloroplast or levels of ROS.

Most of the treatments reduced PQ pool and Fd, confirming their inhibitory effect on electron transport and overall photosynthetic performance. Redox state of the stroma in

response to different treatments varied. For some of the combinatorial treatments it was impossible to determine the overall change in redox state of the stroma, since the two chemicals had the opposite effect. One example of such treatment is DCMU+GSH. While DCMU oxidizes the stroma by blocking the electron transport chain, GSH reduces it through a transient increase in the GSH/GSSG ratio. Similarly, it is unclear whether detoxification of ROS by GSH/GSSG could reverse the increase in levels of ROS produced in response to most of the experimental treatments.

Overall, most of the experimental treatments resulted in suppression of photochemistry and activation of nonphotochemical processes, which was in some cases related to oxidative stress. In most cases, changes in redox state of the PQ pool, Fd and the stroma and levels of ROS could be inferred. Chlorophyll fluorescence analysis confirmed expected effects of the commonly used photosynthetic inhibitors, and, for some treatments, revealed specific effects under the conditions of the present study.

CHAPTER 4 CHLOROPLAST-TO-NUCLEUS SIGNALLING

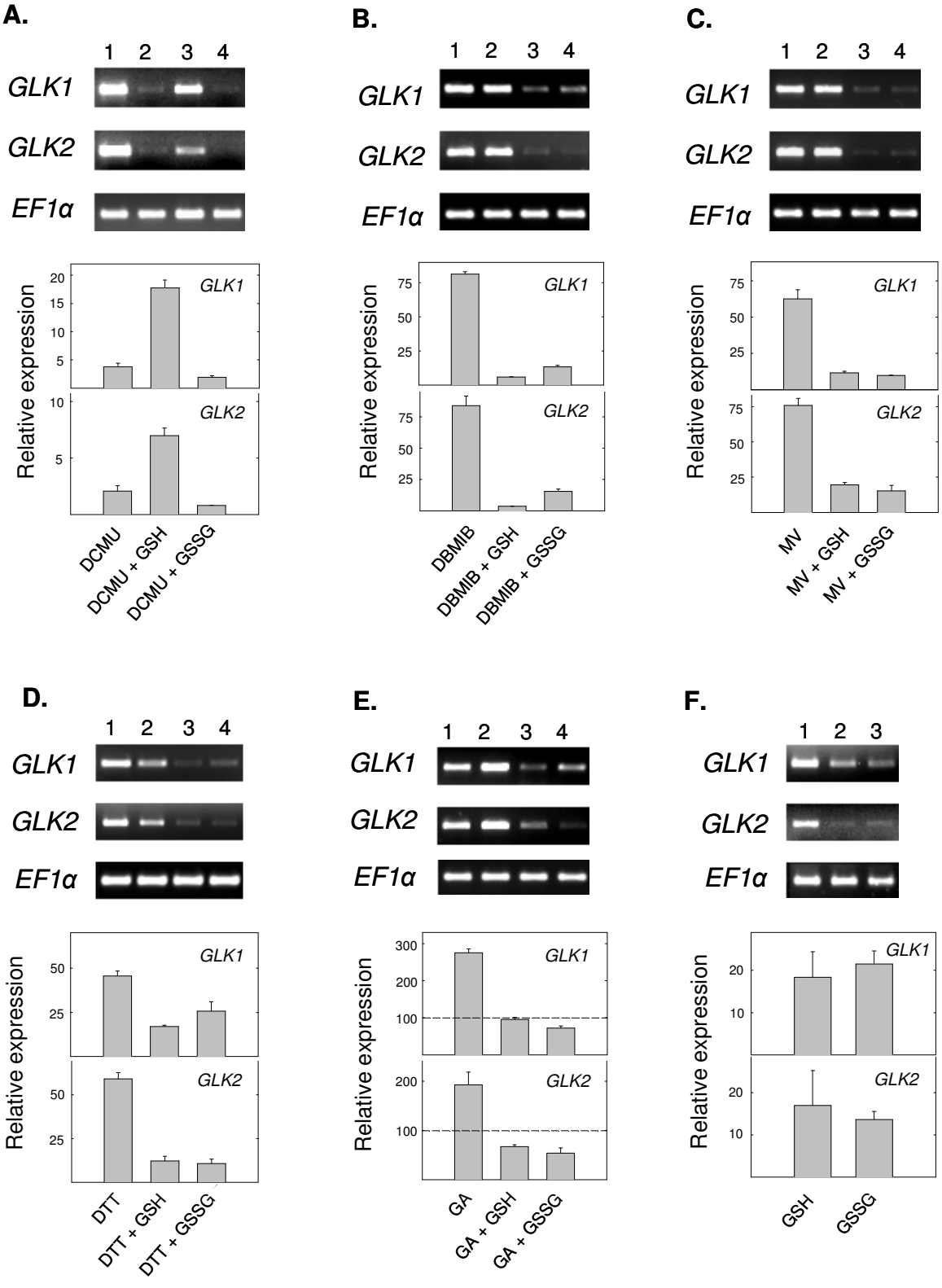
4.1 Chloroplast-to-nucleus signalling

GLK1 and *GLK2* have been suggested to be regulated by retrograde chloroplast-to-nucleus signalling, specifically tetrapyrrole biosynthesis and chloroplast protein synthesis (Waters et al. 2009; Refer to Introduction). In order to investigate whether the other known retrograde signalling pathways regulate *GLK* genes, we analyzed *GLK1* and *GLK2* expression in *Arabidopsis* in response to several different chemicals known to alter redox state of the chloroplast or the cell, to produce ROS, or to alter photosynthetic metabolism.

The main sources of redox signals arising from the chloroplast are changes in redox state of the PQ pool, Fd, or the stroma (Pfannschmidt et al. 2009). Excess ROS can damage the cell, but at the same time serve as important signalling molecules (Foyer and Noctor 2009). Changes in metabolite pools changes have also been recently implicated in signalling from the chloroplast to the nucleus (Weber and Fischer 2007; Pfannschmidt 2010). Effects of the chemicals on the electron transport chain and photosynthetic performance are presented in Table 3.1. Combined with results on *GLK1* and *GLK2* expression, these data will be used to elucidate the retrograde chloroplast-to-nucleus signalling pathways regulating the two genes.

Qualitatively, *GLK1* and *GLK2* responded similarly to the treatments that were used to modulate redox state of the chloroplast or the cell, in that both were suppressed by all the treatments except GA (Figure 4.1). With the exception of DCMU+GSH, treatments involving GSH/GSSG suppressed both *GLK1* and *GLK2* to a greater extent than treatments with the individual chemicals alone. Previously, the two genes were found to respond differently to both norflurazon and lincomycin treatments, which inhibit tetrapyrrole

Figure 4.1 Effects of selected photosynthetic inhibitors and redox signalling compounds on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR and qPCR analyses. Following vacuum infiltration, leaves were incubated in solutions for 4h from ZT4 to ZT8. **A.** (1) water control; (2) 10 μM DCMU, (3) 10 μM DCMU + 5 mM GSH, (4) 10 μM DCMU + 5 mM GSSG; **B.** (1) water control, (2) 0.24 μM DBMIB, (3) 0.24 μM DBMIB + 5 mM GSH; (4) 0.24 μM DBMIB + 5 mM GSSG; **C.** (1) water control, (2) 50 μM MV, (3) 50 μM MV + 5 mM GSH, (4) 50 μM MV + 5 mM GSSG; **D.** (1) water control, (2) 1 mM DTT, (3) 1 mM DTT + 5 mM GSH, (4) 1 mM DTT + 5 mM GSSG; **E.** (1) water control, (2) 5 mM GA, (3) 5 mM GA + 5 mM GSH, (4) 5 mM GA + 5 mM GSSG; **F.** (1) water control, (2) 5 mM GSH, (3) 5 mM GSSG. Two biological replicates were performed. For RT-PCR analysis, two to five replicates of RT-PCR were performed for each of the two biological replicates. Representative results are shown. Elongation factor 1α (*EF1α*) was used as housekeeping gene control. For qPCR analysis, three replicates of qPCR were performed for each of the two biological replicates. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE). Expression level of *GLK1* and *GLK2* in the water control was set to 100.


A.
1 2 3 4
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
DCMU
DCMU + GSH
DCMU + GSSG
B.
1 2 3 4
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
DBMIB
DBMIB + GSH
DBMIB + GSSG
C.
1 2 3 4
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
MV
MV + GSH
MV + GSSG
D.
1 2 3 4
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
DTT
DTT + GSH
DTT + GSSG
E.
1 2 3 4
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
GA
GA + GSH
GA + GSSG
F.
1 2 3
GLK1
GLK2
EF1α
Relative expression
GLK1
GLK2
GSH
GSSG

biosynthesis and plastid protein translation, respectively (Waters et al. 2009). Thus, it seems that *GLK1* and *GLK2* transcription is affected by several retrograde chloroplast-to-nucleus signalling pathways and some pathways regulate the two genes differentially, while others in a similar manner.

4.2 PQ pool

The commonly used herbicide DCMU caused a significant downregulation of *GLK1* and *GLK2* (Figure 4.1A). A slight downregulation of both genes by DBMIB was also observed (Figure 4.1B). In addition, pronounced downregulation of *GLK* genes by DBMIB was observed in the cold signalling experiment (Figure 9.2). Taken together, these results indicate that both DCMU and DBMIB suppress *GLK1* and *GLK2*. Since the two herbicides have the opposite effect on the redox state of the PQ pool (Pfannschmidt et al. 2009; Refer to Sections 3.3 and 3.6), similar changes in expression of *GLK* genes in response of DCMU and DBMIB suggest that the PQ pool does not play a role in their regulation.

Interestingly, DCMU+GSH caused a partial reversal of the suppression observed with DCMU (Figure 4.1A). No such reversal was observed with DBMIB+GSH compared to DBMIB. Since the main difference between the effect of DCMU and DBMIB on plants is related to changes in redox state of the PQ pool, it appears that this signalling mechanism does play a role in *GLK1* and *GLK2* regulation under certain conditions.

Microarray analysis has revealed that different sets of *Arabidopsis* nuclear genes are responsive to changes in redox state of the PQ pool at different light intensities (Adamiec et al. 2008). Out of the 50 genes whose expression in response to increased light irradiance was found to be reverted by DCMU, 24 were modulated at medium irradiance, 32 at high

irradiance and one at excessive irradiance (Adamiec et al. 2008).

Addition of GSH or GSSG together with DCMU seems to mimic an increased light intensity. As seen in Table 3.1, the DCMU+GSH/GSSG treatment caused an increase in nonphotochemical antenna heat dissipation, Φ_{NPQ}, which reflects an increase in the rate of cyclic electron transport. Thus, DCMU+GSH/GSSG is expected to cause a greater reduction of the PQ pool than DCMU alone. Although 1-qL was equal to 1 as a result of treatments with either DCMU or DCMU+GSH/GSSG, the parameter does not reflect the redox state of the PQ pool in this particular situation. Usually 1-qL, which indicates the redox state of Q_A, also reflects the redox state of the PQ pool. However, DCMU blocks the electron transport chain directly downstream of Q_A and upstream of the PQ pool (Refer to Figure 2.3). As a result, any increases in redox state of the PQ pool due to glutathione application were not reflected by 1-qL.

The difference between the effects of DCMU+GSH and DCMU+GSSG on *GLK* expression may be related to changes in redox state of the cellular glutathione pool. PQ-generated retrograde signalling was found to be abolished by the addition of GSH (Karpinski et al. 1997). It was suggested that the glutathione pool is responsible for transducing the signal from the PQ pool to the nucleus and that perturbations in the GSH/GSSG ratio may prevent this signalling.

4.3 ROS

Results of chlorophyll fluorescence analysis suggest that chloroplast-generated ROS are not responsible for the observed changes in *GLK1* and *GLK2* expression in response to the various treatments. For example, results with DCMU and DBMIB suggest that singlet

oxygen does not play a role in the observed *GLK* downregulation. Previously, DCMU was found to prevent singlet oxygen production and to protect PSII from photoinhibition (Nakajima et al. 1996; Rutherford and Krieger-Liszkay 2001; Fufezan et al. 2002). In contrast, overreduction of PSII by DBMIB causes generation of singlet oxygen, resulting in photodamage to PSII (Friso et al. 1993). Thus, since DCMU and DBMIB have the opposite effect on singlet oxygen production but similar effect on *GLK* expression, singlet oxygen does not appear to regulate *GLK1* or *GLK2*.

Similarly, a comparison of *GLK* expression in response to MV, DTT and GA indicates that H_2O_2/superoxide does not regulate the two genes. While each of these chemicals is expected to cause production of these ROS at PSI, MV and DTT were found to downregulate *GLK1* and *GLK2* while GA upregulated both genes (Figure 4.1C, D, E). Although less than two-fold downregulation of *GLK* genes was observed following 4h incubation in MV solution (Figure 4.1C), a time course analysis has shown a pronounced downregulation as a result of longer incubation periods (Figure 4.2). Therefore, it can be concluded that chloroplast-derived ROS generated either at PSII or PSI do not regulate *GLK1* or *GLK2*.

However, the possibility of regulation of *GLK* genes by ROS generated in the cytosol or other cellular compartments cannot be excluded. For example, ROS may be responsible for the downregulation of *GLK1* and *GLK2* by glutathione. While glutathione plays a major role in ROS detoxification (Noctor et al. 2011), transgenic tobacco plants overaccumulating glutathione were under increased oxidative stress, which was suggested to be caused by a disruption of the chloroplast's ability to sense redox changes (Creissen et al. 1999).

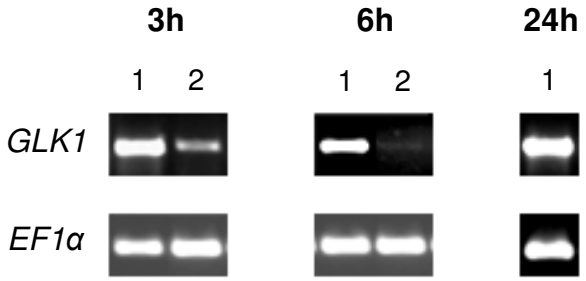


Figure 4.2 Effects of MV on *GLK1* expression in *Arabidopsis*, determined by RT-PCR analysis. Leaves were incubated in 50 μM MV solution starting at ZT4 for 3, 6, or 24h, as indicated, and collected at ZT7, ZT10, or ZT4 the next day, respectively. Two biological replicates, each with two replicates of RT-PCR, were performed. Representative results are shown. (1) water control, (2) 50 μM MV. Treatment with MV for 24h caused extensive tissue damage, such that RNA could not be extracted and, as a result, no RT-PCR or qPCR analysis was performed. Elongation factor 1α (*EF1α*) was used as housekeeping gene control.

4.4 Fd and the stroma

The observed decreases in *GLK* expression also do not seem to be related to changes in redox state of Fd or the stroma, as DCMU and DBMIB both oxidize Fd and downregulate *GLK* genes, while MV and DTT reduce Fd but still downregulate *GLK* genes (Figure 4.1A; Refer to Sections 3.3, 3.6, 3.9, 3.12).

Similarly, downstream targets of Fd do not appear to regulate *GLK1* and *GLK2*. DCMU, DBMIB and MV are expected to oxidize the stroma, including the NADPH/$NADP^+$ pool and thioredoxins (Trx). All three chemicals have a negative effect on *GLK* gene expression (Figure 4.1A, B, C). On the other hand, DTT and GSH are expected to reduce the stroma, but also downregulate *GLK1* and *GLK2*. Thus, changes in redox state of Fd or the stroma were not found to regulate expression of *GLK* genes.

4.5 Metabolites

Out of all the photosynthetic inhibitors tested, only GA caused upregulation of *GLK* genes (Figure 4.1E). Compared to most of the other compounds used, GA blocks photosynthesis downstream, inhibiting ribulose-1,5-bisphosphate regeneration in the Calvin cycle, as opposed to interfering with photosynthetic electron transport (Slabas and Walker 1976; Ralph et al. 2011). Thus, different treatments are expected to have different effects on photosynthetic metabolism.

Changes in pool sizes of metabolites have been suggested to be involved in retrograde chloroplast-to-nucleus signalling (Pfannschmidt 2010). Additionally, it was proposed that rather than a single metabolite, metabolite states or signatures can be sensed by the nucleus (Bräutigam et al. 2009; Pfannschmidt 2010).

Photosynthetic inhibitors are known to affect carbon fixation and to alter photosynthetic metabolism. For example, increased energization of the thylakoid membrane as a result of MV application has been shown to alter levels of various metabolic intermediates of the Calvin cycle and to disrupt control of sucrose and starch synthesis (Ekkehard and Stitt 1989). Chlorophyll fluorescence analysis revealed that all of the photosynthetic inhibitors tested for their effects on *GLK* expression alter photosynthetic performance. This includes increasing thylakoid membrane energization, reflected by increases in Φ_{NPQ} (Table 3.1). Thus, different photosynthetic inhibitors may have various effects on photosynthetic metabolism. Our results indicate that *GLK1* and *GLK2* are both responsive to sugar signals (Chapter 5). Taken together, results of the sugar and redox signalling experiment suggest that *GLK1* and *GLK2* expression is regulated by the availability and possibly composition of cellular metabolite pools.

GLK1 and *GLK2* expression in response to glutathione, alone or in combination with other chemicals, suggests that glutathione signalling overrides that induced by the various photosynthetic inhibitors. As seen in Figure 4.1F, when used alone, GSH and GSSG both downregulated *GLK* genes. In addition, with the exception of DCMU+GSH, all combinatorial treatments with glutathione decreased *GLK* expression compared to the respective chemicals alone (Figure 4.1A-F). The similar effect of GSH and GSSG may be due to the maintenance of glutathione homeostasis. For example, increased oxidation of glutathione is followed or accompanied by increases in the total glutathione pool (Noctor et al. 2011). Thus, total glutathione pool and not GSH/GSSG ratio seems to play a role in the regulation of *GLK1* and *GLK2* in *Arabidopsis*, at least within the time frame of 4h used in the present experiment.

One cellular role of glutathione which may be responsible for the observed effects on *GLK1* and *GLK2* expression is glutathionylation of proteins. Glutathione is known to interact with the Trx signalling system, which is normally activated by donation of electrons from Fd (Michelet et al. 2005). Glutathionylation of Thioredoxin f (TRXf) was found to interfere with its reduction by electrons from Fd, preventing subsequent activation of target enzymes (Michelet et al. 2005). TRXf is a key regulator of carbon fixation enzymes, such as fructose-1,6-bisphosphatase (Schürmann and Jacquot 2000; Michelet et al. 2005). Such inhibition of carbon fixation by glutathione is in agreement with the proposed role of photosynthetic metabolism in *GLK1* and *GLK2* regulation and explains their suppression by glutathione (Figure 4.1F).

CHAPTER 5 SUGAR SIGNALLING

5.1 Sugar signalling

Sugars control many important processes throughout the plant life cycle, such as growth and development, photosynthesis, secondary metabolism, and stress response (Rolland et al. 2002, 2006). Increased sugar levels enhance expression of genes involved in the synthesis of polysaccharides, storage proteins and pigments, as well as defence-related genes and genes involved in respiration (Koch 1996). Conversely, expression of photosynthetic genes encoding CAB1, plastocyanin (PC), and RbcS is repressed by elevated sugar levels (Xiao et al. 2000; Rolland et al. 2006).

The role of sugars in plants has traditionally been attributed to their metabolism. However, sugars may also serve as signalling molecules distinct from their function as metabolites (Smeekens and Rook 1997; Smeekens 1998; Lalonde et al. 1999; Roitsch 1999). The best-characterized sugar signalling pathway in plants involves HXK, an enzyme which phosphorylates glucose and fructose before they enter glycolysis. HXK has been found to possess both metabolic and signalling functions (Jang et al. 1997; Jang and Sheen 1997; Perata et al. 1997; Umemura et al. 1998; Pego et al. 1999). The metabolic and signalling functions of HXK can be uncoupled, as was shown in an experiment with transgenic *Arabidopsis* expressing sense and antisense *AtHXK* or yeast *HXK*. Overexpressors of *AtHXK* were hypersensitive, while underexpressors were hyposensitive to glucose. Plants expressing yeast *HXK* exhibited decreased sensitivity to sugar, despite a 5-fold increase in catalytic activity. This suggests that plant HXK possesses not only phosphorylating activity, but also signalling functions which cannot be replaced by yeast HXK (Jang et al. 1997).

Sucrose can be sensed in several ways: by a sucrose sensor at the plasma membrane not acting as a transporter; by a sucrose transporter; inside the cell through a sucrose-specific pathway; and after its breakdown to fructose and glucose by sucrose invertases or to UDP-glucose and fructose by sucrose synthase. Glucose and fructose may subsequently also serve as downstream signalling molecules (Sheen et al. 1999; Figure 5.1).

Glucose and fructose can signal without entering the cell by binding to specific receptors at the plasma membrane, or at several points in the pathway that involves entry into the cell. In this pathway, the signal may arise from glucose or fructose transport into the cell, from their phosphorylation by HXK, or from metabolites of glycolysis. Glucose and fructose signal transduction is thus classified as HXK-independent, HXK-dependent or glycolysis-dependent, respectively (Sheen et al. 1999; Rolland et al. 2001; Refer to Figure 2.4).

HXK-independent sugar signalling pathway was found to regulate several genes. For example, it was shown to be responsible for glucose induction of genes encoding chalcone synthase (CHS) and phenylalanine amonia-lyase1 (PAL1), as well as for glucose repression of the gene encoding asparagine synthase1 (AS1) (Xiao et al. 2000). HXK-dependent sugar signalling pathway is associated with repression of nuclear photosynthetic genes encoding chloroplast proteins such as Rubisco and light-harvesting complex proteins (Moore et al. 2003). Examples of such genes include *CAB1*, *PC* and *RbcS* (Xiao et al. 2000). Finally, the glycolysis-dependent signalling pathway was shown to induce pathogenesis-related (PR) genes *PR1* and *PR5* (Xiao et al. 2000).

Glucose analogs can be used to dissect the signalling pathway described above (Borsani et al. 2001; Refer to Materials and Methods). For example, as shown in Figure 2.4,

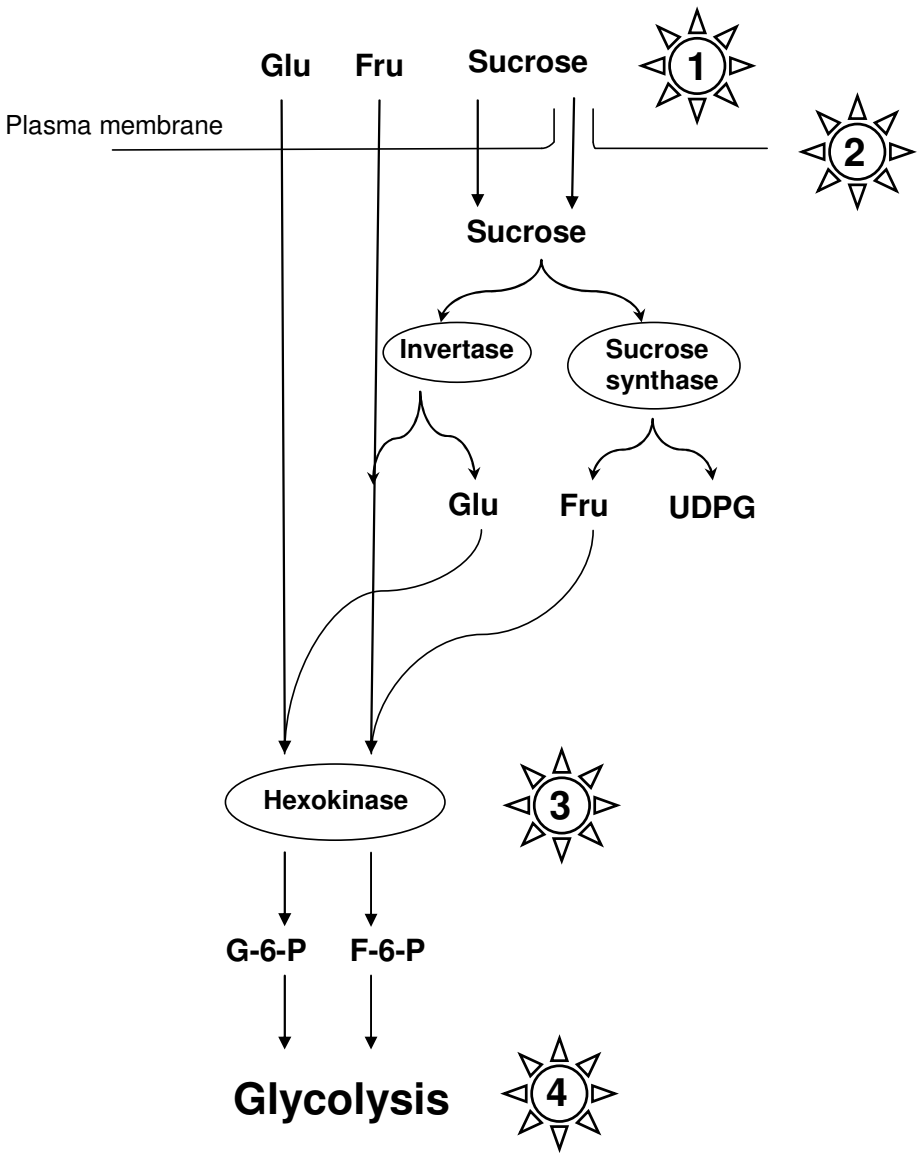


Figure 5.1 Schematic diagram illustrating the plant sugar signalling pathway involving the HXK glucose sensor. Enzymes are shown as ovals. Potential points of signalling are indicated by numbers 1-4. (1) recognition by plasma membrane receptors, (2) transport into the cell, (3) phosphorylation by HXK, (4) glycolysis and subsequent metabolism. Abbreviations: Glu, glucose; Fru, fructose; G-6-P, glucose-6-phosphate; F-6-P, fructose-6-phosphate. Adapted from Koch (1996).

the glucose analog 2-DOG is transported into the cell and phosphorylated but does not subsequently enter glycolysis. If the glucose-induced effect on gene expression is mimicked by 2-DOG, the signal is likely generated as a result of glucose phosphorylation by HXK. Similarly, if the glucose-induced effect is mimicked by the phosphorylated glucose derivative G-6-P, the signal may be attributed to glycolysis or subsequent metabolism.

5.2 Effects of sugar signalling on *GLK* and *GLK2* gene expression

Leaves were incubated in the dark for either 24h or 4h after vacuum infiltration and collected at three time points: at ZT8 after 4h incubation, and at ZT4 or ZT12 after 24h incubations. The two different time periods for the duration of incubation were chosen because we hypothesized that the effect of exogenous sugar application may vary in strength with duration of incubation of leaves in the sugar solutions. This idea is supported by a time course analysis of expression of senescence associated genes (*SAG*s) in rosette leaves from 4-week old *Arabidopsis* plants (Noh and Amasino 1999). While applications of exogenous glucose or sucrose consistently increased transcript levels of *SAG12* and *SAG13* compared to the water-treated control, the extent of upregulation varied over the 48h of incubation. Thus, glucose-induced expression of *SAG13* peaked at 24h, while its sucrose-induced expression peaked at 4 and 48 hours. (Noh and Amasino 1999). Thus, 4h and 24h of incubation were chosen in the hope of detecting changes in *GLK* expression when the effect of sugar treatments is most pronounced.

5.2.1 Effects of sugars on *CHS* expression

Glucose, sucrose, mannitol, 2-DOG and G-6-P were used to treat *Arabidopsis* leaves in order to dissect the sugar signalling pathway affecting *GLK* gene expression. The gene encoding the secondary metabolism enzyme chalcone synthase (CHS, At5g13930) was used as a control for HXK-independent regulation of gene expression by sugars. *CHS* is known to be upregulated by glucose in a HXK-independent manner (Xiao et al. 2000), which was confirmed by its response to the sugars and sugar analogs used in this study. As seen in Figure 5.2, glucose and sucrose treatments both caused an increase in *CHS* transcript levels. The positive effect of sucrose on *CHS* expression can be explained by its breakdown to glucose and fructose and the downstream signalling of these metabolites.

2-DOG caused no change in *CHS* transcript levels compared to the water control, showing that the glucose-induced signal affecting *CHS* expression is not HXK-dependent. G-6-P treatment also resulted in no change in *CHS* levels compared to wild type, indicating that the signal is not dependent on glycolysis. The only point of signalling by glucose that is not also common to the 2-DOG and G-6-P signalling pathways is glucose recognition by specific receptors at the plasma membrane. Therefore, the signal upregulating *CHS* was confirmed to be HXK-independent. The positive effects of glucose, sucrose and mannitol on *CHS* expression were more pronounced after 24h than after 4h of treatment, indicating that, as hypothesized, duration of plant exposure to the treatment plays a role in the experimental outcome.

5.2.2 Effects of sugars on *GLK1* and *GLK2* expression

GLK1 and *GLK2* responded to the experimental treatments differently. While *GLK1* was upregulated by glucose, sucrose and mannitol, *GLK2* was upregulated only by mannitol

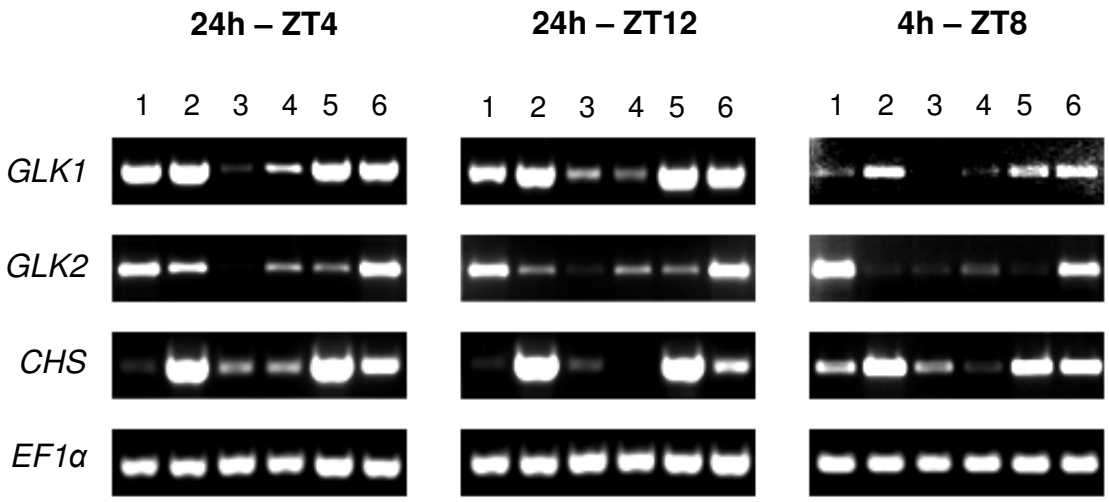
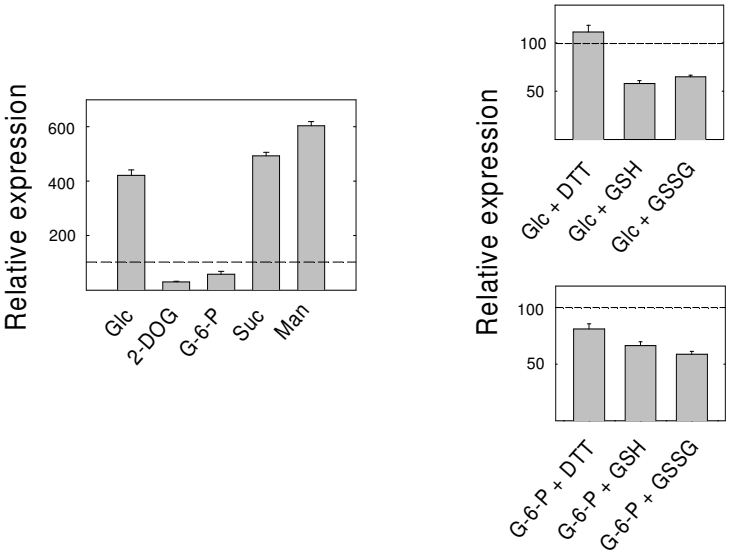


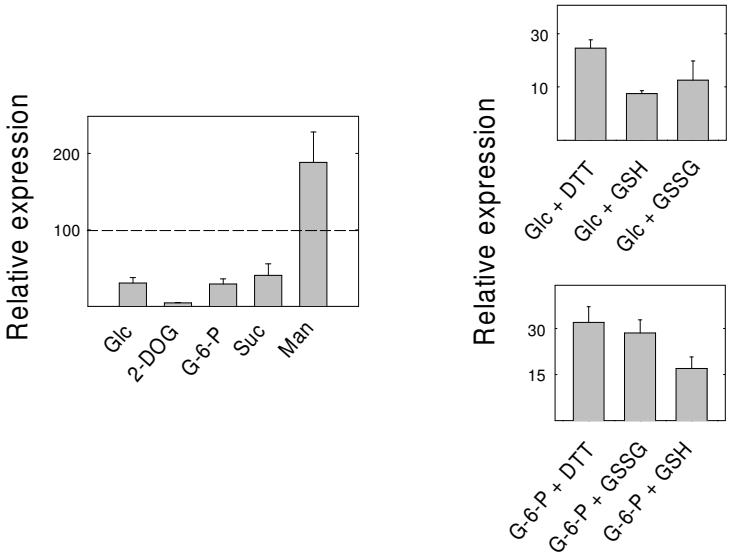
Figure 5.2 Effects of sugars and glucose analogs on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR analysis. Following vacuum infiltration, leaves were incubated in solutions for 24h from ZT4 to ZT4 the next day, for 24h from ZT12 to ZT12 the next day, and for 4h from ZT4 to ZT8. Time of collection is indicated. (1) water control, (2) 330 mM glucose, (3) 14.5 mM 2-DOG, (4) 30 mM G-6-P, (5) 175 mM sucrose, (6) 165 mM mannitol. The gene encoding chalcone synthase (*CHS*, At5g13930) was used as a positive control for HXK-independent signalling. Elongation factor 1α (*EF1α*) was used as housekeeping gene control. Two biological replicates, each with three replicates of RT-PCR, were performed. Representative results are shown. Abbreviations: ZT, Zeitgeber time.

Figure 5.3 Effects of sugars and sugar analogs in combination with redox signalling compounds on *GLK1* (A) and *GLK2* (B) expression in *Arabidopsis*, determined by qPCR analysis. Leaves were incubated in the dark for 4h from ZT4 to ZT8 in the following chemical solutions: water (control), 330 mM glucose, 14.5 mM 2-DOG, 30 mM G-6-P, 175 mM sucrose, 165 mM mannitol, 330 mM glucose + 1 mM DTT, 330 mM glucose + 5 mM GSH, 330 mM glucose + 5 mM GSSG, 30 mM G-6-P, 30 mM G-6-P + 1 mM DTT, 30 mM G-6-P + 5 mM GSH, 30 mM G-6-P + 5 mM GSSG. For qPCR analysis, three replicates of qPCR were performed for each of the two biological replicates. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE). Expression level of *GLK1* and *GLK2* in the empty vector control was set to 100.

A.


Relative expression
600
400
200
Glc
2-DOG
G-6-P
Suc
Man
Relative expression
100
50
Glc + DTT
Glc + GSH
Glc + GSSG
100
50
G-6-P + DTT
G-6-P + GSH
G-6-P + GSSG

B.


Relative expression
200
100
Glc
2-DOG
G-6-P
Suc
Man
Relative expression
30
10
Glc + DTT
Glc + GSH
Glc + GSSG
30
15
G-6-P + DTT
G-6-P + GSSG
G-6-P + GSH

(Figures 5.2 and 5.3). *GLK1* transcript levels decreased after 2-DOG and G-6-P treatments. *GLK2* was dowregulated by glucose, sucrose, 2-DOG and G-6-P.

GLK1 response displayed a similar profile in response to the treatments as that seen with *CHS*, suggesting that it is also upregulated by glucose in a HXK-independent manner. As seen from RT-PCR analysis, the upregulation of *GLK1* by glucose and sucrose after 24h was more pronounced at ZT12 than at ZT4. This is likely because *GLK1* transcript levels are lowest in the evening and thus upregulation is more pronounced compared to when transcript levels are higher, as in the morning and early afternoon (Refer to Section 2.2).

GLK2 was downregulated by glucose, 2-DOG and G-6-P. Downregulation by 2-DOG means that the glucose-induced downregulation is caused either by glucose phosphorylation by HXK or an upstream signal, which could be generated as a result of glucose reception at the plasma membrane or transport into the cell. Since 2-DOG is not metabolized after phosphorylation, the result rules out generation of the negative glucose signal as a result of glycolysis. Similarly, downregulation of *GLK2* by G-6-P rules out HXK-dependent signalling, as G-6-P is phosphorylated and thus does not serve as a substrate for HXK. Therefore, by the process of elimination, the repressive glucose-induced signal is generated as a result of glucose recognition at the plasma membrane or its transport into the cell. *GLK2* regulation by glucose can thus be classified as HXK-independent repression.

Thus, sugar signalling affecting *GLK* expression was found to be HXK-independent for both *GLK1* and *GLK2*. *GLK1* seems to be upregulated by glucose binding to glucose receptors at the plasma membrane, while *GLK2* seems to be downregulated by glucose as a result of its reception at the plasma membrane or transport into the cell.

Expression of both *GLK1* and *GLK2* was induced by treatment with mannitol (Figures 5.2 and 5.3). The observed effect may be due to the antioxidant properties of mannitol. Osmolytes such as mannitol are produced in plants in response to osmotic stress which may be caused by drought, high salinity or low temperature (Smirnoff 1998). In addition, experiments with transgenic tobacco accumulating mannitol have shown that it can act as an antioxidant by scavenging hydroxyl radicals (OH•) (Smirnoff and Cumbes 1989; Orthen et al. 1994; Shen et al. 1997).

GLK genes were downregulated in response to 4h treatments with H_2O_2, MV and SA (Figures 4.1 and 7.1), all of which are expected to generate ROS. Taken together with upregulation by mannitol, these results suggest a negative effect of ROS and a positive effect of antioxidant compounds on *GLK* gene expression.

Although *GLK1* and *GLK2* have been suggested to have equivalent functions (Waters et al. 2008, 2009), the two genes responded differently to glucose and sucrose treatments. *GLK1* was upregulated, while *GLK2* was suppressed by both sugars. Previously, it was shown that *GLK* genes respond differentially to plastid retrograde signals (Waters et al. 2009). Thus, if sugar signalling affects *GLK* expression by triggering retrograde chloroplast-to-nucleus communication, *GLK1* and *GLK2* would be expected to be differentially regulated by changes in sugar levels, as was observed in their response to glucose and sucrose.

The finding that *GLK1* was upregulated by glucose and sucrose is in agreement with a recent study which found *GLK1* to be one of the major regulators of organic nitrogen metabolism (Gutiérrez et al. 2008). Sugars induce expression of a number of genes involved in nitrogen metabolism, such as nitrate transporters, nitrate reductase, and GS (Koch 1996;

Lam et al. 1998; Lejay et al. 1999). Expression of the asparagine synthase gene *ASN1* is repressed by sugars (Lam et al. 1998). In *Arabidopsis*, some nitrogen metabolism genes, including *GS2* and *ASN1* seem to be regulated by glucose in a HXK-independent manner (Sheen et al. 1999). Like sugars, *GLK1* was predicted to suppress *ASN1* expression and enhance *GS* expression (Gutiérrez et al. 2008). These findings, together with the results from the present study indicating that *GLK1* is upregulated by glucose in a HXK-independent manner, suggest that sugars may affect expression of nitrogen metabolism genes through upregulation of *GLK1*.

5.3 Redox state of the cell and the chloroplast

Sugar signalling has previously been shown to be modulated by chloroplast redox signals (Oswald et al. 2001). In *Arabidopsis*, blocking of photosynthetic electron flux by the inhibitor DCMU following sugar depletion prevented increase in transcript levels of the nuclear photosynthesis-related genes *CAB1, PC* and *RbcS*. It was thus proposed that chloroplast-derived redox signalling can override regulation of nuclear photosynthesis-related genes by sugars (Oswald et al. 2001).

To verify whether sugar signalling affects *GLK* transcript levels through its effect on redox state of the cell or the chloroplast, glucose and G-6-P treatments were also performed in combination with GSH, GSSG, or DTT. The effects of sugars were evaluated in the dark to minimize interference of endogenous sugars produced by photosynthesis with the experimental treatments. Therefore, DCMU, DBMIB, MV, or GA could not be used in combination with sugars, since they require light-driven electron transport to exert their

effect on redox state of the chloroplast. DTT, on the other hand, acts as a strong reductant irrespective of light conditions, and was thus used as a modulator of chloroplast redox state.

Application of DTT in combination with glucose partially reversed the glucose-induced upregulation of *GLK1*, but did not affect transcript levels of *GLK2* compared to glucose treatment (Figure 5.3). Similarly, the G-6-P+DTT treatment did not affect *GLK1* or *GLK2* expression compared to G-6-P. Effects of DTT on plant metabolism were previously found to be similar to those observed in response to sugars, suggesting an overlap in thiol-disulfide and sugar signalling targets (Kolbe et al. 2006). With the exception of the effect of glucose+DTT treatment on *GLK1* expression, our results also demonstrate similar regulation of *GLK1* and *GLK2* by DTT and sugars. The antagonistic interaction between glucose and DTT with respect to *GLK1* may be related to the previously-described interplay between sugars and chloroplast-derived redox signals, such that redox signals override sugar-induced signalling (Oswald et al. 2001).

GSH or GSSG in combination with glucose or G-6-P had negative effects on *GLK1* expression (Figure 5.3). Glucose-induced upregulation of *GLK1* was reversed by both forms of glutathione. The observed similar changes in *GLK* gene expression in response to the reduced and oxidized forms of glutathione are likely due to the maintenance in plants of glutathione homeostasis (Figure 5.3; Refer to Section 4.5). It is possible that the greater suppression of *GLK1* and *GLK2* by sugars in combination with glutathione than by sugars alone was due to the interplay of redox and sugar signalling, as discussed above (Oswald et al. 2001). Perhaps the observed effects of GSH and GSSG in combination with sugars is related to glutathione-induced suppression of carbon fixation (Refer to Section 4.5).

Alternatively, as suggested in Section 4.3, glutathione may regulate *GLK* genes through generation of ROS.

CHAPTER 6 NITROGEN SIGNALLING

6.1 Nitrogen Signalling

Nitrogen is an essential macronutrient required for plant growth and development processes such as root and leaf growth, flowering, and seed germination (Maathuis 2009). Inorganic nitrogen is taken up by plants from the soil predominantly in the form of nitrate (NO_3^-), which is subsequently converted to nitrite (NO_2^-) and ammonia (NH_4^+) (Refer to Figure 2.5). Ammonia is used to synthesize the first organic forms of nitrogen, Gln and Glu. Glu is the starting point for the synthesis of other N-containing compounds in plants (Miflin and Lea 1977).

Nitrogen has a positive effect on photosynthesis and photosynthetic gene expression. For example, increasing N availability to *Arabidopsis* plants grown under N stress resulted in upregulation of the photosynthetic genes encoding a PSII family protein and a PSII reaction centre W PsbW family protein (Bi et al. 2007). Exogenous application of Glu to hawthorn (*Crataegus pinnatifida*) leaves has been found to increase chlorophyll content, net photosynthetic rate, carboxylation efficiency of Rubisco and several other photosynthetic parameters, indicating an overall improvement in photosynthetic capacity (Yu et al. 2010). In contrast, nitrogen limitation or deprivation resulted in repression of photosynthetic genes, genes involved in chlorophyll biosynthesis, and plastid protein synthesis (Bi et al. 2007; Scheible et al. 2004; Peng et al. 2007).

As mentioned in the Introduction, *GLK1* and *GLK2* have been found to be involved in organic nitrogen signalling (Gutiérrez et al. 2008). Nitrogen availability may generate regulatory signals affecting expression of nuclear photosynthesis-related genes and transcription factors such as *GLK1* and *GLK2*, as Glu is the precursor for the biosynthesis of

tetrapyrroles, which have been implicated in retrograde chloroplast-to-nucleus signalling (Foyer et al. 2011; Refer to Figure 2.5). Conversely, photosynthetic processes may affect nitrogen signalling, as Fd donates electrons to NiR and GOGAT (Foyer et al. 2011; Refer to Figure 2.5).

6.2 Effects of nitrogen signalling on *GLK* and *GLK2* expression

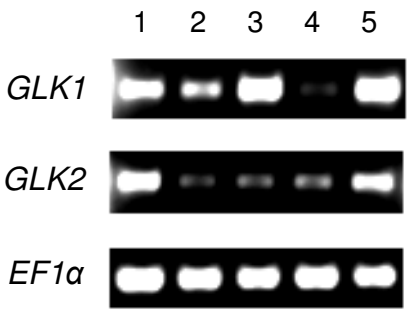
Incubation of *Arabidopsis* leaves in various solutions following vacuum infiltration was performed for 4h. Although a variety of different treatment methods and experimental conditions have been used by different researchers, MSX and Gln have been found to have a physiological effect within several hours of application (Rawat et al. 1999; Gutiérrez et al. 2008). MSX has been shown to inhibit the light activation of phospho*enol*pyruvate carboxylase kinase (PEPC-PK) in tobacco leaves after 3h of incubation. The inhibitory effect was partially reversed by simultaneous treatment of leaves with Gln (Li et al. 1996).

GLK1 transcript levels decreased upon treatment with MSX, which was reversed by the addition of GSH (MSX + GSH) or Gln (MSX + Gln), but not by the addition of GSSG (MSX + GSSG). *GLK2* responded to the experimental treatments in a similar manner, except that its downregulation by MSX was not reversed by GSH (Figure 6.1). Suppression of *GLK1* and *GLK2* by MSX and reversal of suppression by Gln indicate that organic nitrogen or its derivatives play a role in the regulation of these two genes.

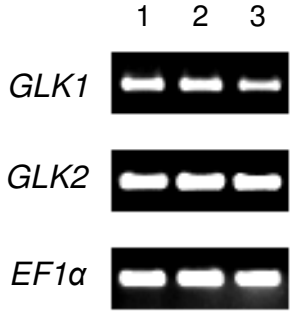
Treatment of *Arabidopsis* leaves with Gln had no effect on *GLK1* expression and slightly upregulated *GLK2* (Figure 6.1). These results may indicate that organic nitrogen signalling plays a role in *GLK* regulation but that the effect depends on the levels of organic nitrogen in the plant. This conclusion is supported by the complexity of organic nitrogen

Figure 6.1 Effects of the nitrogen assimilation inhibitor MSX and several nitrogen derivatives on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR (A and B) and qPCR analyses (C). Following vacuum infiltration, leaves were incubated for 4h from ZT4 to ZT8 in the following chemical solutions: **A.** (1) water (control), (2) 1 mM MSX, (3) 1 mM MSX + 5 mM GSH, (4) 1 mM MSX + 5 mM GSSG, (5) 1 mM MSX + 5 mM Gln; **B.** (1) water control, (2) 5 mM Gln, (3) 5 mM Glu. Two biological replicates were performed. For RT-PCR analysis, three replicates of RT-PCR were performed for each of the two biological replicates. Representative results are shown. Elongation factor 1α (*EF1α*) was used as housekeeping gene control. For qPCR analysis, three replicates of qPCR were performed for each of the two biological replicates. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE). Expression level of *GLK1* and *GLK2* in the empty vector control was set to 100.

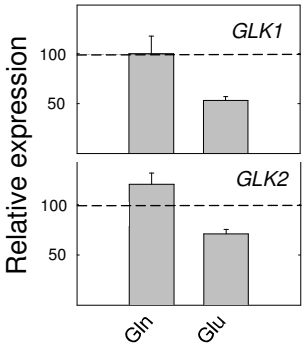
A.


1 2 3 4 5
GLK1
GLK2
EF1α

B.


1 2 3
GLK1
GLK2
EF1α

C.


Relative expression
GLK1
100
50
GLK2
100
50
Gln
Glu

signalling in plants, such that differences in plant growth conditions, pre-treatment of plants, and concentrations of solutions used for experimental treatments may play a role in the outcome of the experiment (Coruzzi and Zhou 2001). For example, the transcription factor *ANR1* was induced by nitrate in nitrate-starved plants (Zhang and Forde 1998), but not affected by nitrate in plants grown on ammonium (Wang et al. 2000). Another study found upregulation of two nitrate-regulated genes as a fast response to nitrate treatments, and repression after prolonged nitrate treatments, which was attributed to feedback inhibition (Rawat et al. 1999).

Treatment with Glu caused a two-fold reduction in *GLK1* and a small reduction in *GLK2* transcript levels (Figure 6.1). This is in agreement with previous findings of suppression of *GLK1* by internal Glu, and of *GLK2* by internal and external Glu (Gutiérrez et al. 2008). The inhibitory effect of excess Glu may be due to perturbations in tetrapyrrole levels, as Glu is the precursor for chlorophyll biosynthesis (Foyer et al. 2011; Refer to Figure 2.5). The tetrapyrrole biosynthesis inhibitor norflurazon has been shown to suppress both *GLK1* and *GLK2* (Waters et al. 2009).

Thus, at physiological concentrations organic nitrogen appears to have a positive effect on *GLK1* and *GLK2* transcription, possibly mediating induction of photosynthetic gene expression seen upon re-supplying nitrogen to nitrogen starved plants (Bi et al. 2007). However, at higher concentrations, the effect of organic nitrogen on *GLK* expression was not as pronounced or inhibitory, possibly due to feedback inhibition of organic nitrogen-responsive genes or perturbations in tetrapyrrole biosynthesis.

Exogenous GSH and GSSG were found to have a similar negative effect on expression of *GLK1* and *GLK2* (Figure 4.1), which, as suggested in Section 4.5, may be due

to inhibition of carbon fixation. However, when applied together with MSX, GSH and not GSSG reversed suppression of *GLK1* (Figure 6.1). Chlorophyll fluorescence analysis revealed that the MSX+GSH and MSX+GSSG treatments were similar in their effect on photosynthetic performance (Refer to Sections 3.19 and 3.20; Table 3.1). Although MSX+GSH caused a slightly more pronounced decrease in F_v/F_m and Φ_{PSII}, and a greater increase in $\Phi_{f,D}$, similar values of these photosynthetic parameters were observed in response to a number of other treatments (Table 3.1). Thus, it seems that the observed changes in photosynthetic performance were not responsible for mediating the effect of MSX+GSH/GSSG on *GLK1* expression.

The different effects of the two treatments on *GLK1* suggest that glutathione homeostasis was disrupted by the MSX treatment. MSX inhibits not only GS, but also γ-glutamylcysteine synthetase, the first enzyme in the glutathione biosynthesis pathway (Noctor et al. 2011). Perhaps disruption of glutathione biosynthesis resulted in changes in the GSH/GSSG ratio, which explains the different effects of MSX+GSH and MSX+GSSG treatments on *GLK1*. Although *GLK1* and *GLK2* have been suggested to be at least partially redundant in function (Fitter et al. 2002), their response to MSX+GSH treatment highlights differences in their regulation by environmental signals.

CHAPTER 7 PATHOGEN SIGNALLING

7.1 Plant defence against pathogens

Disease resistance in plants is divided into non-host resistance, in which all members of a plant species are resistant to all members of a pathogen species, and host, or race-cultivar specific resistance, in which a specific cultivar of a plant species is resistant to a certain race of a pathogen species (Thordal-Christensen 2003). Both types of plant-pathogen interactions involve basal defence mechanisms, which are induced when the plant recognizes pathogen-associated molecular patterns (PAMPS), such as lipopolysaccharides and flagellin (Nurnberger et al. 2004). Host resistance additionally involves effector-triggered immunity (ETI), also known as gene-for-gene resistance (Jones and Dangl 2006). Gene-for-gene resistance is induced when the plant detects pathogenic proteins, or effectors, that contribute to disease development in susceptible hosts. Those effectors that are recognized by the plant are called avirulence (Avr) proteins, and they confer resistance to the host upon being detected by the plant Resistance, or R, proteins (Jones and Dangl 2006).

Gene-for-gene resistance is associated with the SA-dependent signalling pathway, which induces expression of PR genes that have important functions in disease resistance. In addition, gene-for-gene resistance involves oxidative burst and induction of HR, a rapid death of cells at the site of pathogen infection (Ingle et al. 2006; Jones and Dangl 2006). H_2O_2 is produced as part of the oxidative burst and serves as an important signalling molecule (Apostol et al. 1989; Levine et al. 1994; Jabs et al. 1997).

The second major signalling pathway induced in response to pathogenesis involves JA (Kunkel and Brooks 2002). The SA- and JA-dependent pathways of defence against pathogens are mutually antagonistic, with the SA pathway generally being induced in

response to biotrophic pathogens and JA pathway in response to necrotrophic pathogens, insect herbivory and wounding (Kunkel and Brooks 2002).

To investigate the role of pathogenesis in *GLK* gene regulation, transcript levels of *GLK1* and *GLK2* were analyzed after infecting *Arabidopsis* plants with *P. syringae* carrying an empty vector or expressing one of two *avr* genes, *avrRpm1* or *avrRpt2*. The strains of *P. syringae* used were *Psm* and *Pst*, which are the two virulent strains most commonly employed in studies on *Arabidopsis-P. syringae* interactions (Katagiri et al. 2002). The *avr* genes *avrRpm1* and *avrRpt2* are known to convert *Psm* and *Pst* into avirulent strains in *Arabidopsis* Col-0 and to induce HR at different times after infection (4-5h and 12 h, respectively) (Ritter and Dangl 1996; Boyes et al. 1998). Empty vector was used as a control and is not expected to cause HR.

In addition to investigating the role of pathogenesis in *GLK* regulation by performing infection experiments, we tested *GLK* expression in response to two phytohormones which have important signalling functions in plant-pathogen interactions. As mentioned above, SA and JA are involved in signalling in response to biotrophic and necrotrophic pathogens, respectively (Kunkel and Brooks 2002). *P. syringae* is a hemi-biotroph, behaving as a biotroph during the phase of most aggressive intercellular growth, and as a necrotroph at later stages of the life cycle (Nomura et al. 2005). In addition, *P. syringae* releases coronatine, a toxin that mimics MeJA, thereby inducing plant defence against necrotrophic pathogens and diverting plant resources from defence against biotrophs (Nomura et al. 2005). Thus, both SA and JA signalling pathways are involved in plant response to *P. syringae*.

One additional important player in plant-pathogen interactions is H_2O_2, which is produced in plants in a localized oxidative burst in response to pathogen attack (Apostol et al. 1989; Levine et al. 1994; Jabs et al. 1997). H_2O_2 and other ROS may directly damage invading pathogens and stiffen the cell wall (Nimchuk et al. 2003). Together with another messenger molecule, nitric oxide (NO), SA and H_2O_2 induce HR as part of the gene-for-gene resistance response (Shirasu et al. 1997; McDowell and Dangl 2000). The three molecules appear to act synergistically in a positive feedback loop, such that SA acts both upstream and downstream of H_2O_2.

Previously, constitutive *GLK1* overexpression in *Arabidopsis* was found to result in enhanced resistance to the necrotrophic plant pathogen *Fusarium graminearum* (Refer to Introduction). *GLK* regulation by signalling related to infection with both necrotrophic and biotrophic pathogens was investigated through the use of *P. syringae* infection and SA, JA and H_2O_2 treatments. *Arabidopsis* leaves infected with *P. syringae* were collected 6h after inoculation with *Pst* EV and *Pst avrRpm1*, and 24h after inoculation with *Pst avrRpt2* and all *Psm* strains (*Psm* EV, *Psm avrRpm1* and *Psm avrRpt2*). The duration of incubation was chosen according to known speed of development of HR in response to infection with *Psm* and *Pst* expressing *avrRpm1* or *avrRpt2* (Ritter and Dangl 1996; Boyes et al. 1998).

Following spraying of plants with SA, JA, and H_2O_2 leaves were collected after 4h. Previously, the defence-related gene marker *PR1* was found to be upregulated 6h after infection of *Arabidopsis* with *Pst avrRpm1* and 3h after spraying of leaves with 1 mM SA (Jagadeeswaran et al. 2007). Thus, HR-related processes are expected to be induced in both the pathogen-signalling experiment and the 4h SA signalling experiment. Although exogenously applied H_2O_2 is known to be degraded fast, within minutes in *Arabidopsis* cell

cultures (Neill et al. 2002), it acts as part of positive feedback loop, activating SA-signalling and other defence responses. The 4h treatment was thus performed in order to investigate whether H_2O_2 is involved in regulation of *GLK* genes by pathogen defence signalling.

7.2 Effects of pathogen signalling on *GLK1* and *GLK2* expression

As seen in Figure 7.1A, infection of *Arabidopsis* with *P. syringae* expressing *avrRpm1* resulted in suppression of both *GLK* genes. Infection with *P. syringae* expressing *avrRpt2*, on the other hand, caused a slight suppression of *GLK1* and no suppression of *GLK2*, compared to the empty vector control. Effects of *P. syringae* infection on *GLK1* and *GLK2* expression were the same regardless of whether *Psm* or *Pst* was used. This result indicates that the observed effect on *GLK* expression is determined by gene-for-gene resistance arising from detection of AvrRpm1 or AvrRpt2 by the plant host, and not from differences in resistance of *Arabidopsis* to the two strains of *P. syringae*.

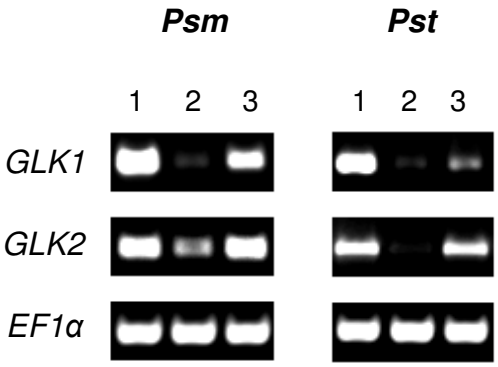
AvrRpm1 and AvrRpt2 interact with the same host plant protein, RPM1-INTERACTING PROTEIN4 (RIN4), triggering HR in resistant plants(Axtell and Staskawicz 2003; Mackey et al 2003; Chisholm et al. 2005). However, AvrRpt2 has multiple targets in the host, and may affect plant disease resistance through additional mechanisms (Belkhadir et al. 2004; Lim and Kunkel 2004). This difference in the mode of action of the two Avr proteins may explain the different effects of AvrRpm1 and AvrRpt2 on *GLK* gene expression.

Repression of *GLK* genes by the two avirulent *P. syringae* strains may be explained by the effect of pathogen infection on plant primary metabolism. Pathogen infection is

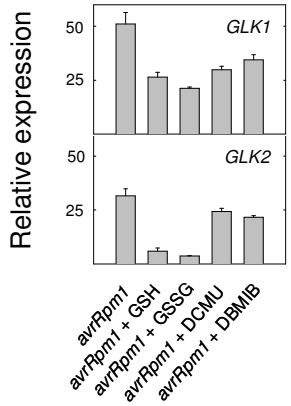
Figure 7.1 Effects of *P. syringae* avirulence factors and SA, JA, and H_2O_2 on *GLK1* and *GLK2* expression in *Arabidopsis*. **A.** Effects of *avrRpm1* and *avrRpt2* expressed in *Pst* or *Psm* strains of *P. syringae* on *GLK1* and *GLK2* expression, determined by RT-PCR analysis. Rosette leaves of *Arabidopsis* were inoculated with bacterial suspensions of $5x10^7$ cfu/mL *Psm* or *Pst* carrying empty vector pVSP61 (1), or expressing *avrRpm1* (2) or *avrRpt2* (3). Inoculation of leaves was performed at ZT6. Infected leaves were collected 6h later, at ZT12, after inoculation with *Pst* EV and *Pst avrRpm1*, and 24h later, at ZT6 the next day, after inoculation with *Pst avrRpt2*, *Psm* EV, *Psm avrRpm1* and *Psm avrRpt2*. **B.** Effects of *Pst* infection in combination with selected redox signalling compounds, determined by qPCR analysis. *Arabidopsis* leaves were inoculated with *Pst avrRpm1* and kept in the light for 1h from ZT6 to ZT7; the leaves were collected, vacuum infiltrated with 10 μM DCMU, 0.24 μM DBMIB, 5 mM GSH and 5 mM GSSG from ZT7 to ZT8, and kept in respective solutions in the light for additional 4h from ZT8 to ZT12. **C.** Effects of SA, JA and H_2O_2 on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR analysis. Incubation was performed for 4h from ZT4 to ZT8. (1) water (control), (2) SA, (3) JA, (4) H_2O_2.

Two biological replicates were performed. For RT-PCR analysis, two to five replicates of RT-PCR were performed for each of the two biological replicates. Representative results are shown. Elongation factor 1α (*EF1α*) was used as a housekeeping gene control. For qPCR analysis, three replicates of qPCR were performed for each of the two biological replicates. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE). Expression level of *GLK1* and *GLK2* in the empty vector control was set to 100.

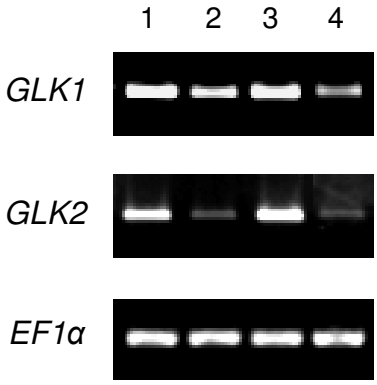
A.


Psm
Pst
1 2 3
1 2 3
GLK1
GLK2
EF1α

B.


Relative expression
50
25
GLK1
50
25
GLK2
avrRpm1
avrRpm1 + GSH
avrRpm1 + GSSG
avrRpm1 + DCMU
avrRpm1 + DBMIB

C.


1 2 3 4
GLK1
GLK2
EF1α

known to affect photosynthesis, assimilate partitioning and sink metabolism (Berger et al. 2007). Specifically, a decline in photosynthesis was observed in *Arabidopsis* as a result of interaction with both virulent and avirulent strains of *P. syringae* (Tao et al. 2003). Chlorophyll fluorescence analysis confirmed suppression of photosynthesis by *Pst avrRpm1* revealing a decline in the maximum and effective quantum yield of PSII and an increase in nonphotochemical energy quenching (Section 3.21; Table 3.1). Given their role in photosynthetic development, *GLK* genes are highly probable targets for repression of photosynthesis (Fitter et al. 2002; Waters et al. 2009).

In many plant-pathogen interactions changes in primary metabolism include an increase in soluble sugar levels (Berger et al. 2007). However, levels of glucose, fructose and sucrose did not change in *Arabidopsis* as a result of *P. syringae* infection (Bonfig et al. 2006). In addition, glucose treatment was found to upregulate *GLK1* (Figure 5.2), while both strains of avirulent *P. syringae* suppressed it (Figure 7.1A). Thus, it seems that the observed suppression of *GLK1* and *GLK2* by *P. syringae* is independent of sugar signalling.

7.3 Effects of SA, JA and H_2O_2 on *GLK* and *GLK2* expression

JA treatment did not affect *GLK1* or *GLK2* expression compared to the water-treated control, while both SA and H_2O_2 treatments downregulated both genes (Figure 7.1C). The negative effect of SA on *GLK1* and *GLK2* expression after 4h treatment suggests that the suppression of *GLK* genes by *avrRpm1* and *avrRpt2* may proceed through HR and the associated oxidative burst. Although chloroplast-derived ROS do not seem to affect *GLK* expression (Refer to Chapter 4), exogenous H_2O_2 suppressed both genes. Since both SA and H_2O_2 are involved in HR, which is known to be triggered by each of the two avirulence

genes, it appears that the effect of gene-for-gene interactions on *GLK1* and *GLK2* expression involves the activation of the SA-dependent signalling pathway.

7.4 Redox state of the cell and the chloroplast

Infection of *Arabidopsis* with *P. syringae* expressing *avrRpm1* and simultaneous treatments with GSH or GSSG revealed no difference in the effect of reduced and oxidized glutathione (Figure 7.1C). This finding may indicate that the plant maintains glutathione homeostasis (Noctor et al. 2011) and that the absolute level of glutathione rather than GSH/GSSG ratio plays a role in *GLK* gene regulation.

Treatments with GSH or GSSG on the background of infection with *P. syringae* expressing *avrRpm1* resulted in further suppression of *GLK1* and *GLK2* (Figures 7.1C). This may be due to the known involvement of glutathione in plant-pathogen interactions. For example, increased glutathione synthesis in transgenic tobacco (*Nicotiana tabacum*) caused lesion formation and induced expression of *PR* genes (Creissen et al. 1999). Conversely, depletion of cytosolic GSH in *Arabidopsis* was found to result in reduced ability to activate SA-dependent PR gene expression (Maughan et al. 2010).

Gene-for-gene resistance is known to result in HR, which involves an oxidative burst (Hammond-Kosack and Jones 1996). Production of ROS as a result of *Pst avrRpm1* infection is consistent with the observed decrease in the maximum quantum yield of PSII, F_v/F_m, indicating oxidative damage to PSII reaction centres (Table 3.1; Section 3.21). In addition, the observed increase in reaction centre quenching, $\Phi_{f,D}$, in the absence of changes in antenna quenching, Φ_{NPQ}, suggests extensive tissue damage, which resulted in altered energy distribution between the two photoprotective processes (Table 3.1; Section 3.21).

Perhaps glutathione exacerbated the oxidative burst caused by HR, thus causing greater suppression of *GLK* genes through increased production of ROS. As mentioned in Chapter 3, although glutathione participates in ROS detoxification, exogenous glutathione paradoxically results in increased oxidative stress (Creissen et al. 1999; Karpinska et al. 2000; Xu et al. 2000).

Alternatively, the enhanced downregulation of *GLK1* and *GLK2* by exogenous glutathione on the background of avirulent *P. syringae* infection may be explained by its inhibitory effect on photosynthesis. As discussed in Chapter 4, exogenous glutathione may suppress photosynthesis through interaction with the thioredoxin system, and alterations in carbon metabolism seem to affect *GLK1* and *GLK2* expression.

Compared to the *avrRpm1* infection, *avrRpm1*+GSH/GSSG treatments caused a greater suppression of *GLK2* than *GLK1* (Figure 7.1C). If glutathione affects *GLK* transcript levels through production of ROS, the observed differences in response of the two genes to the *avrRpm1*+GSH/GSSG treatments may be explained by differential regulation of *GLK1* and *GLK2* by ROS. Previously *GLK1* and *GLK2* were found to be differentially responsive to norflurazon, a carotenoid biosynthesis inhibitor which results in oxidative damage (Waters et al. 2009).

DCMU and DBMIB treatments on the background of infection with *P. syringae* expressing *avrRpm1* resulted in further reduction in *GLK* transcript levels compared to infection with the pathogen without any additional treatments. Although the suppression was less than two-fold, it was observed for both DCMU and DBMIB, as well as for both *GLK1* and *GLK2*. DCMU and DBMIB block the flow of electrons through the photosynthetic electron transport chain, resulting in increased oxidation or reduction of the PQ pool,

respectively (Trebst 1974; Kirilovsky et al. 1994; Ralph et al. 2011). Changes in the redox state of the PQ pool are a known source or retrograde chloroplast-to-nucleus signalling (Kleine et al. 2009). If PQ redox state plays a role in gene regulation, the effects seen after DCMU and DBMIB applications would be opposite. Since DCMU and DBMIB resulted in similar transcript levels of *GLK1* and *GLK2*, changes in redox state of the PQ pool do not seem to play a role in mediating the pathogen-induced suppression of *GLK1* and *GLK2*. Reduction in *GLK* transcript levels by DCMU and DBMIB on the background of infection with *P. syringae* expressing *avrRpm1* may be a result of blockage of the electron transport chain and subsequent suppression of photosynthesis.

CHAPTER 8 HORMONE SIGNALLING

8.1 Hormone signalling

Hormones are involved in regulation of numerous growth and development processes throughout the plant life cycle. Briefly, auxin regulates cell division, elongation and differentiation; cytokinin is involved in cell division, shoot initiation and light responses; GA_3 plays important roles in cell growth in vegetative tissues and in flower and fruit development; ABA inhibits transition between plant developmental stages and mediates responses to abiotic stresses; and, finally, BR is necessary for photomorphogenesis and many growth and developmental processes (Liscum and Reed 2002).

As mentioned in the Introduction, *GLK1* and *GLK2* have been found to be repressed by BR-derived signalling. Given the role of hormones and *GLK* genes in photosynthetic development, it seems likely that other hormones may also target *GLK1* and *GLK2*.

8.2 Cytokinin

Consistently with the roles of cytokinin and *GLK* genes in photosynthetic development, *GLK1* and *GLK2* were both found to be positively regulated by cytokinin signalling, which was seen in all three experiments (Figure 8.1). Cytokinin is known to activate photosynthetic processes, as indicated by increased oxygen evolution and CO_2 fixation rates in response to exogenous treatment with the hormone (Caers and Vendrig 1986; Nyitrai 2005).

Cytokinin is also involved in chloroplast development (Chory et al. 1994) and chlorophyll biosynthesis (Dei 1982; Lew and Tsuji 1982; Parthier 1989), and promotes formation of light harvesting complexes of PSI and PSII (Nyitrai 2005). Interestingly, *GLK1*

and *GLK2* have also been implicated in these processes (Rossini et al. 2001; Fitter et al. 2002; Waters et al. 2009). Since *GLK* genes were found to be upregulated by cytokinin and are involved in many of the same processes as cytokinin, it is possible that they mediate photosynthesis-related effects of this phytohormone. However, some of the effects of cytokinin on photosynthetic performance are not associated with expression of *GLK* genes. For example, unlike cytokinin, *GLK1* and *GLK2* have no regulatory role in plastid division or expression of Calvin cycle enzymes (Fitter et al. 2002; Nyitrai 2005; Okazaki et al. 2009; Waters et al. 2009).

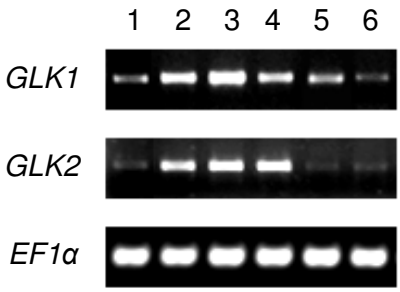
Additional evidence implicating *GLK* transcription factors in mediation of cytokinin responses comes from their relatedness to type B ARRs, which are transcription factors involved in cytokinin signalling (Mizuno 2004; Argyros et al. 2008). *GLK* and type B ARR genes belong to the GARP family of transcription factors and share high sequence similarity in their DNA binding domain (Imamura et al. 1999; Rossini et al. 2001). Taken together, these data suggest that *GLK1* and *GLK2* mediate signalling affecting a subset of the photosynthetic processes associated with cytokinin signalling in *Arabidopsis*.

8.3 Auxin

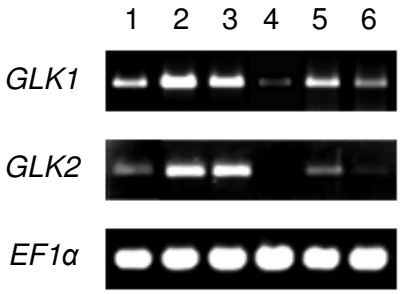
Another phytohormone that affected both *GLK1* and *GLK2* expression was auxin. *GLK1* was upregulated by auxin in all three experiments. *GLK2* upregulation by auxin was evident after 24h and not after 4h of treatment, suggesting that the positive effect of auxin on *GLK2* expression was due to secondary signalling mechanisms, as opposed to activation of early auxin-responsive genes.

Figure 8.1 Effects of phytohormone signalling on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by RT-PCR analysis. Following spraying of *Arabidopsis* plants with phytohormone solutions, incubations were performed for 24h from ZT4 to ZT4 the next day, for 24h from ZT12 to ZT12 the next day, and for 4h from ZT4 to ZT8. Time of collection is indicated. (1) water control, (2) 10 μM IAA, (3) 20 μM cytokinin, (4) 100 μM GA_3, (5) 100 μM ABA, (6) 100 μM BR. Two biological replicates, each with five replicates of PCR, were performed. Representative results are shown. Elongation factor 1α (*EF1α*) was used as a housekeeping gene control. Abbreviations: ZT, Zeitgeber time.

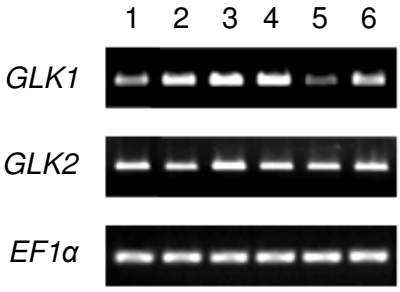
24h – ZT4



24h – ZT12



4h – ZT8



Auxin is known to play a role in many core growth processes in plants, such as tropic growth and apical dominance. Auxin-induced genes include MYB-like transcription factors, which are related to the GARP family of transcription factors to which *GLK1* and *GLK2* belong (Chapman and Estelle 2009). With respect to photosynthesis, auxin is believed to play a protective role, promoting remodelling of photosynthetic apparatus to minimize photoinhibition induced by various environmental stresses (Tognetti et al. 2012). For example, auxin deficiency in mutant tomato plants was associated with an increased number of plastoglobuli and lack of starch granules, as well as a strong reduction in levels of PSI and stromal thylakoids (Ehlert et al. 2008; Tognetti et al. 2012).

Auxin and cytokinin are known to be involved in a signalling crosstalk, which involves both agonistic and antagonistic interactions (Coenen and Lomax 1997; Moubayidin et al. 2009). Exogenous cytokinin treatment was found to upregulate members of all three major auxin response gene families, Aux/IAA, SAUR and GH3 (Brenner et al. 2005). Thus, *GLK1* and *GLK2* induction may be a common response to auxin and cytokinin signalling, consistently with the role of *GLK* genes and both phytohormones in growth and development processes.

8.4 Gibberellic acid

GA_3 was found to upregulate *GLK1* following 4h treatment. Longer treatments of 24h caused upregulation or downregulation of *GLK1*, depending on whether leaves were incubated from ZT4 to ZT4 or from ZT12 to ZT12, respectively (Figure 8.1). *GLK2* transcript levels did not change as a result of 4h treatment with GA_3. However, 24h treatments affected *GLK2* the same way as *GLK1*, causing upregulation in leaves collected

at ZT4, and downregulation in leaves collected at ZT12. These results suggest that, similarly to auxin, GA_3 signalling influences *GLK1* and *GLK2* expression through different mechanisms. The effect of the phytohormone on *GLK1* seems to be more direct, while its effect on *GLK2* is more likely to involve secondary signalling mechanisms. Results of the 24h treatments reveal that long-term effect of GA_3 on *GLK1* and *GLK2* is qualitatively the same.

The diurnal dependence of the effect of GA_3 on *GLK* expression may be explained by the finding that hormones modulate circadian clock parameters (Hanano et al. 2006). Exogenous application of various hormones has been found to cause changes in period length, amplitude or phase, as determined by monitoring circadian rhythms of selected marker genes. In particular, GA_3 was found to promote periodicity. Thus, effects of 24h treatments with GA_3 may be at least in part due to the disruption of circadian rhythmicity of *GLK1* and *GLK2* (Hanano et al. 2006).

Effect of GA_3 and other gibberellins on photosynthesis is unclear, as both stimulation and inhibition of photosynthetic capacity and rate have been previously observed (Cramer et al. 1995; Ashraf et al. 2002; Yuan and Xu 2001; Nagel and Lambers 2002; Biemelt et al. 2004). The contradictory results were proposed to arise from differences in the experimental systems, such as use of GA, GA inhibitors, or mutants, as well as differences in the methods of measuring photosynthesis (Nagel and Lambers 2002). Despite lack of conclusive evidence on the changes in photosynthesis caused by altered GA levels, exogenous GA_3 application has been found to enhance morphogenesis of plastids in greening cereal seedlings (Wellburn et al. 1973; Younis et al. 1995).

The positive effect of GA_3 on plastid morphogenesis is in agreement with the known role of *GLK* genes in photosynthetic development (Fitter et al. 2002; Waters et al. 2009). GA, cytokinin and auxin signalling are known to interact (Fu and Harberd 2003; Greenboim-Wainberg et al. 2005), which may explain the positive effect of all three hormones on *GLK1* expression following 4h treatment.

8.5 Abscisic acid

As can be seen in Figure 8.1, ABA caused a slight upregulation of *GLK1* following 24h treatments, and had no effect on *GLK2* expression. Exogenously applied ABA is known to inhibit greening of etiolated cotyledons and to interfere with plastid morphogenesis, including the assembly of photosynthetic complexes in the thylakoid membrane (Kusnetsov et al. 1994; Kusnetsov et al. 1998). In addition, ABA has been shown to decrease the rate of photosynthesis in a number of plants, and to suppress nuclear photosynthesis-related genes such as *CAB1* and *RbcS* (Raschke and Hedrich 1985; Bartholomew et al. 1991; Milla et al. 2003).

In contrast to ABA, *GLK* genes are positive regulators of chloroplast development (Fitter et al. 2002; Waters et al. 2009). While ABA was found to downregulate *CAB1* and *RbcS*, *GLK1* or *GLK2* overexpression upregulated *CAB1* and had no effect on *RbcS* transcript levels (Bartholomew et al. 1991; Waters et al. 2009). Thus, it seems that the photosynthesis-related effects of ABA are not related to the observed upregulation of *GLK1* in response to the 24h treatments of *Arabidopsis* with the hormone. Suppression of nuclear photosynthesis-related genes by ABA may instead be mediated by the transcription factor *ABI4*, which has been suggested to be involved in retrograde chloroplast-to-nucleus

signalling independently of *GLK1* (Koussevitzky et al. 2007; Kakizaki et al. 2009).

8.6 Brassinosteroid

BR caused upregulation of *GLK1* in response to 4h treatment and downregulation in response 24h treatments (Figure 8.1). Effects of phytohormones on gene expression may vary depending on duration of incubation. For example, expression of the senescence-specific gene *SAG12* decreased in response to 2h and 4h treatment with auxin, but increased in response to 24h and 48h treatments (Noh and Amasino 1999).

The downregulation of *GLK1* as a result of 24h treatments with BR is in agreement with a previous report of negative regulation of *GLK* genes by BR (Yu et al. 2011). It was suggested that BR-induced suppression of chloroplast development proceeds through downregulation of *GLK* genes by BES1, a transcription factor involved in BR signalling. Gain-of-function *Arabidopsis bes1-D* mutants exhibited reduced *GLK1* and *GLK2* transcript levels, reduced chlorophyll content and enlarged plastoglobules in their chloroplasts. Thus, while 4h treatment with BR increased transcript levels of *GLK1* (Figure 8.1), it seems that long-term BR signalling has a negative effect on *GLK1* expression.

Effect of BR on *GLK2* expression was evident only after 24h treatment in leaves collected at ZT12, suggesting that similarly to auxin and GA_3, the hormone affects *GLK2* indirectly. Diurnal dependence of *GLK2* regulation by BR may be related to the role of hormones in modulation of circadian clock parameters, as was suggested in Chapter 8 (Hanano et al. 2006).

CHAPTER 9 *pgp1* MUTATION AND COLD TEMPERATURE SIGNALLING

9.1 *GLK* expression in chloroplast mutants

Genetic studies can serve as a useful complement to those employing exogenous chemical treatments and may provide further insight into gene regulatory pathways. One advantage of using mutants is that the need to use combinatorial treatments is avoided, thus overcoming the problem of possible interactions between two different chemicals.

GLK1 and *GLK2* have been found to be suppressed in several albino and variegated *Arabidopsis* mutants with impaired chloroplast development. For example, transcript levels of both genes were reduced in the *ppi2* mutant of *Arabidopsis*, which lacks the chloroplast atToc159 protein import receptor (Kakizaki et al. 2009). The mutant exhibits a severe albino phenotype, and undifferentiated plastids lacking thylakoid membranes and starch granules (Bauer et al. 2000). In addition, several photosynthesis-related nuclear genes are suppressed in *ppi2* (Kakizaki et al. 2009). Genetic studies have indicated that this phenotype is at least partially caused by decreased expression of *GLK1* (Kakizaki et al. 2009).

GLK1 was also found to be suppressed in two other plastid protein import mutants, *attoc132 attoc120+/-*, which is defective in plastid import of nonphotosynthetic proteins, and *attic20-I*, which is defective in plastid import of both nonphotosynthetic and photosynthesis-related proteins (Kakizaki et al. 2009). Like *ppi2*, these mutants have disrupted chloroplast development, and are particularly impaired in thylakoid membrane and granal stack formation (Ivanova et al. 2004; Duy et al. 2007). In addition, *GLK1* and *GLK2* were suppressed in the *Arabidopsis* variegation mutants *msh1*, *var2*, and *immutans*, all of which have disrupted thylakoid membrane formation (Aluru et al. 2009; Miura et al. 2010; Xu et al. 2011).

Another *Arabidopsis* mutant with impaired chloroplast development is *pgp1*, characterized by reduced PG content as a result of mutation of the phosphatidylglycerolphosphate synthase isoform encoded by a gene on chromosome 2 (Krol et al. 2010). PG is a ubiquitous phospholipid found in almost all organisms (Dowhan 1997; Wada and Murata 2007). In plants, it is present mainly in the thylakoid membranes of chloroplasts, and has been shown to be necessary for chloroplast development, photosynthetic electron transport, and resistance to chilling (Hagio et al. 2002; Wada and Murata 2007).

The *pgp1* mutant of *Arabidopsis* with a 30% decrease in PG content was visually indistinguishable from wild type plants when grown at 20℃ (Refer to Introduction and Materials and Methods; Krol et al. 2010). However, growth of the mutant at 5°C resulted in a yellow-white phenotype characterized by decreased chlorophyll content and disrupted chloroplasts with reduced number of thylakoid membranes which failed to form granal stacks. PSII and PSI associated polypeptides such as Lhcb1, Lhcb2, PsaA, PsaB and PsaD, and a number of nuclear-encoded photosynthetic genes were suppressed in these conditions. Placement of the cold-acclimated mutant at 20°C and continuous light for 48h reversed the phenotype of plants from yellow-white to green (N.P.A Huner laboratory, unpublished).

Chlorophyll fluorescence analysis of the 5°C-grown *pgp1* mutant revealed decreases in the maximum and effective quantum yield of PSII, F_v/F_m and $\Phi_{PSII,}$ respectively. A pronounced increase in nonphotochemical reaction centre quenching, $\Phi_{f,D,}$ was also detected (Krol et al. 2010). These changes are qualitatively similar to those observed in cold-stressed *Arabidopsis* plants (Refer to Table 3.1 and Section 3.23). Additionally, chlorophyll fluorescence analysis of the cold-acclimated mutant indicated an increase in PSI-dependent

cyclic electron transport compared to cold acclimated wild type plants (Krol et al. 2010), which may serve as a photoprotective mechanism and reflects the inability of *pgp1* to acclimate to cold temperature conditions.

Interestingly, the phenotype of the *pgp1* mutant grown at 5℃ closely resembles that of the *glk1 glk2* double mutant, with respect to overall appearance, chlorophyll content, chloroplast ultrastructure, and nuclear photosynthesis-related gene expression (Yasumura et al. 2005; Waters et al. 2008, 2009; Krol et al. 2010). Given the similarities between the two mutants in their photosynthetic development, we hypothesized that the phenotype of *pgp1* grown at 5℃ is associated with reduced *GLK1* and *GLK2* expression. If this is the case, the mutant may serve as a useful system to understand *GLK* regulation in response to perturbed chloroplast development. Perhaps, as was suggested to occur in the *ppi2* mutant (Kakizaki et al. 2009), dysfunctional chloroplasts generate retrograde chloroplast-to-nucleus signals that target *GLK* genes, which in turn results in suppression of nuclear photosynthesis-related gene expression.

9.2 Effects of cold on *GLK1* and *GLK2* expression in wild type and *pgp1 Arabidopsis*

The *pgp1* mutation resulted in suppression of both *GLK1* and *GLK2* in plants grown at 20ºC (Figure 9.1). Suppression of *GLK1* was more pronounced than that of *GLK2*, with observed decreases in transcript levels of 2.9-fold and 1.7-fold compared to wild type, respectively. Although when grown at 20ºC, the mutant is visually indistinguishable from wild type, it exhibits a reduction in the number of thylakoids per granal stack and a redistribution of excitation energy in favour of PSI (Krol et al. 2010). Thus, *GLK1* and

GLK2 downregulation in *pgp1* grown at 20ºC correlates with negative changes in chloroplast development and photosynthetic performance.

In wild type plants, cold acclimation resulted in a slight but insignificant decrease in expression of *GLK1* and a pronounced decrease in expression of *GLK2*. The 6-fold suppression of *GLK2* in response to cold acclimation was partially reversed in plants placed at 20ºC in continuous light for 48h. The downregulation of *GLK2* by cold acclimation is consistent with the known effect of cold temperature on photosynthetic performance. Short-term (hours to days) exposure of *Arabidopsis* to cold temperatures results in suppression of photosynthesis, followed by a recovery over a period of days to weeks (Huner et al. 1993; Stitt and Hurry 2002). Chlorophyll fluorescence analysis revealed a recovery of photosynthesis in *Arabidopsis* plants exposed to 5ºC for 5 weeks as compared to those exposed to the cold (Section 3.23; Table 3.1). However, the recovery was not complete, which may explain why *GLK2* levels were lower following cold acclimation than in control plants grown at 20ºC (Figure 9.1).

While cold acclimation of wild type plants resulted in a significant suppression of *GLK2* and not *GLK1*, cold acclimation of *pgp1* suppressed both *GLK1* and *GLK2* (Figure 9.1). Exposure of the cold acclimated *pgp1* plants to 20ºC and continuous light for 48h restored expression of both genes to that seen in 20ºC-grown mutants. Chilling resistance in plants is known to be associated with unsaturation of fatty acids in the thylakoid membranes (Nishida and Murata 1996; Wada and Murata 2007). In particular, the unsaturation of fatty acids of PG may protect the PSII against low temperature-induced photoinhibition (Murata et al. 1992). Given the important role of PG in plant cold tolerance, it seems that the

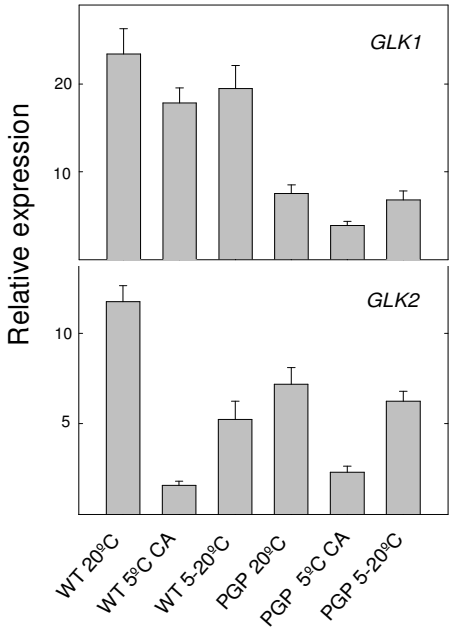


Figure 9.1 Effects of cold temperature and *pgp1* mutation on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by qPCR analysis. Plants were grown for 5 weeks at 20ºC or 5ºC, as indicated. In addition, 5ºC-grown plants were placed in continuous light for 48h (5-20ºC). Two biological replicates, each with three replicates of qPCR, were performed. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE).

downregulation of *GLK1* and *GLK2* in *pgp1* grown at 5ºC for 5 weeks is related to PG deficiency in the mutant and the resultant cold-induced damage to the photosynthetic machinery. Perhaps PG deficiency generates retrograde chloroplast-to-nucleus signals, which downregulate *GLK* genes, which in turn causes suppression of nuclear photosynthesis-related genes. Such signalling cascade targeting *LHCB* genes was suggested to occur in the *ppi2* mutant in response to defects in plastid protein import (Kakizaki et al. 2009).

Interestingly, some of the nuclear photosynthesis-related genes which were suppressed in the 5°C-grown *pgp1* mutant have been found to be affected by changes in *GLK* gene expression (Waters et al. 2009; Krol et al. 2010). For example, *Lhcb1* and *Lhcb2*, which were both suppressed in the cold-acclimated *pgp1*, were also suppressed in the *glk1 glk2* mutant, while overexpression of *GLK* genes lead to *Lhcb1* and *Lhcb2* upregulation compared to wild type (Waters et al. 2009). This suggests that the downregulation of photosynthesis-related genes in cold-acclimated *pgp1* proceeds through a decrease in *GLK1* and *GLK2* expression.

Taken together, results of *GLK* expression analyses in cold acclimated-wild type and *pgp1 Arabidopsis* reveal differences in the regulation of *GLK1* and *GLK2*. When the two conditions of cold temperature and PG deficiency were combined by growing *pgp1* mutants at 5ºC, both *GLK1* and *GLK2* were downregulated. Decrease in transcript levels of both genes at the same time may explain the appearance of the yellow-white phenotype in cold-acclimated *pgp1* and its similarity to the *glk1 glk2* double mutant phenotype.

9.3 Effects of cold on retrograde signalling in *pgp1*

DCMU, DBMIB, GSH and GSSG treatments all caused suppression of *GLK1* and *GLK2* in wild type plants and in the *pgp1* mutant irrespective of growth conditions (Figure 9.2). Since effects of DCMU and DBMIB on *GLK* expression were similar, it appears that redox state of the PQ pool does not play a role in *GLK1* and *GLK2* regulation under any of the conditions tested. GSH and GSSG also had the same effect on transcript levels of the two genes, suggesting that glutathione regulates their expression irrespective of the GSH/GSSG ratio. Effects of DCMU, DBMIB, GSH and GSSG on wild type *Arabidopsis* plants at 20°C are in agreement with those presented in Chapter 4.

The suppression of *GLK1* and *GLK2* by these chemicals was not reversed in the *pgp1* mutant (Figure 9.2). This result indicates that the action of these chemicals with respect to *GLK* regulation is not affected by PG deficiency. Previously, suppression of *GLK1* and *GLK2* by the plastid inhibitors norflurazon and lincomycin was reversed in the *gun1 gun5* mutant (Waters et al. 2009). Genetic analyses have confirmed that *GLK1* regulation by retrograde chloroplast-to-nucleus signalling seems to involve the *GUN1* signalling pathway (Kakizaki et al. 2009). Analogously, if suppression of *GLK* genes by DCMU, DBMIB, GSH or GSSG was reversed in the *pgp1* mutant, it would indicate that *GLK* regulation by redox signalling from the chloroplast to the nucleus requires functional chloroplasts with intact thylakoid membranes.

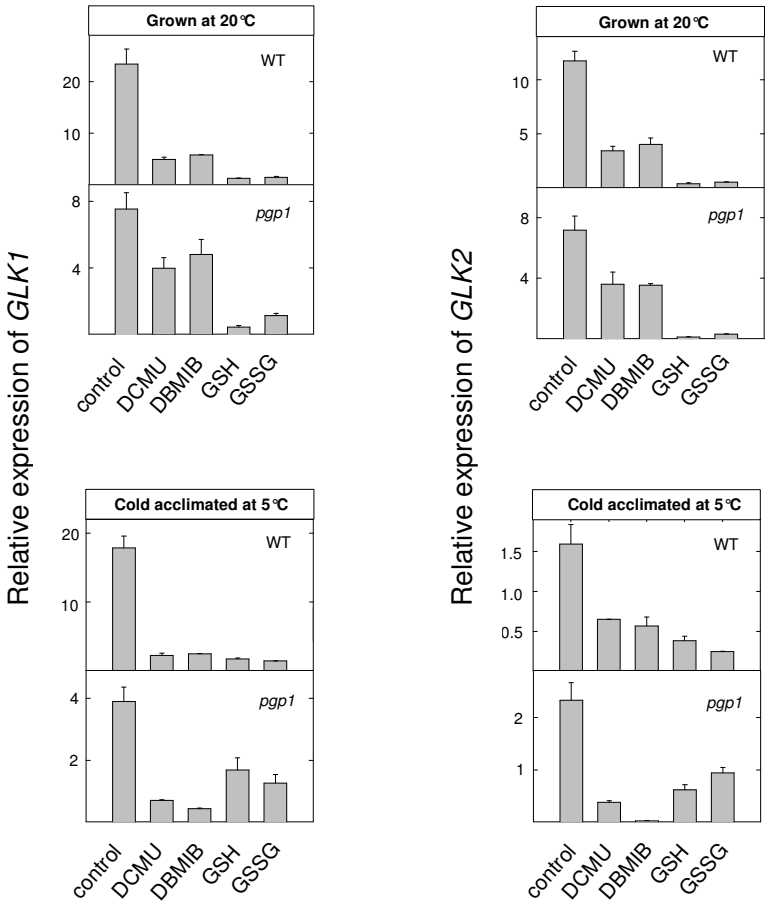


Figure 9.2 Effects of cold temperature and *pgp1* mutation in combination with redox signalling compounds on *GLK1* and *GLK2* expression in *Arabidopsis*, determined by qPCR analysis. Plants were grown for 5 weeks at 20ºC or 5ºC and leaves were treated with water (control), 10 μM DCMU, 0.24 μM DBMIB, 5 mM GSH and 5 mM GSSG. Incubations in respective chemical solutions were performed for 4h from ZT4 to ZT8. Two biological replicates, each with three replicates of qPCR, were performed. Mean transcript levels of *GLK1* and *GLK2* were normalized to the mean transcript levels of *EF1α*, and are represented by bars. Error bars represent standard error (SE).

As found previously (Chapter 4), glutathione had the same effect on *GLK* expression regardless of whether its reduced or oxidized form was used (Figure 9.2). Glutathione regulation of *GLK1* and *GLK2* was proposed to override other signalling pathways, possibly through glutathionylation and the resultant suppression of photosynthesis. In agreement with this finding, glutathione also had the same negative effect on both genes in wild type and *pgp1* plants, whether grown at 20°C or 5°C.

CHAPTER 10 CONCLUSIONS AND FUTURE DIRECTIONS

10.1 Conclusions

Arabidopsis transcription factors *GLK1* and *GLK2* play an important role in chloroplast development, and have also been implicated in organic nitrogen signalling and defence against pathogens (Waters et al. 2009; Gutiérrez et al. 2008; Savitch et al. 2007). Although a number of stimuli, such as retrograde chloroplast-to-nucleus signalling, organic nitrogen and pathogenesis, have been shown to affect transcription of the two genes (Kakizaki et al. 2009; Waters et al. 2009; Gutiérrez et al. 2008; Bozso et al. 2009), regulation of *GLK1* and *GLK2* has not received much attention. The objective of this study was to determine the signalling pathways regulating expression of *GLK1* and *GLK2* transcription factors in *Arabidopsis*. The hypotheses were that *GLK* genes are regulated differentially by endogenous and environmental stimuli, and that the different factors affecting *GLK* expression regulate the two genes by producing chloroplast-to-nucleus retrograde signals.

Pleiotropy

As mentioned in the Introduction, pleiotropic effects of the experimental treatments of *Arabidopsis* were taken into account by considering the different known signalling pathways activated by each treatment, as well as through the use of combinations of chemicals and the mutant *pgp1*. Pleiotropic effects of chemicals may also be reduced by performing treatments for short periods of time, during which only primary signalling mechanisms would presumably be activated without triggering downstream secondary responses. Time course experiments with selected factors identified in the present study may be performed in order to detect early changes in *GLK* transcript levels. In addition, an

inhibitor of protein translation such as cycloheximide may be used to identify signalling pathways affecting *GLK* expression in the absence of *de novo* protein synthesis.

GLK1 and *GLK2* regulation in *Arabidopsis*

Results of the chloroplast-to-nucleus retrograde signalling experiment suggest that under the experimental conditions used, *GLK1* and *GLK2* expression was not regulated by redox state of the PQ pool, Fd, or the stroma, or by levels of chloroplast-generated ROS. In most cases, glutathione was found to affect *GLK* expression independently of the GSH/GSSG ratio. Retrograde signals affecting *GLK* expression appear to arise from changes in metabolic state of the cell (Refer to Chapter 4).

The sugar signalling experiment confirmed regulation of *GLK2* by changes in photosynthetic metabolism, as its expression was downregulated by exogenous sucrose and glucose. The upregulation of *GLK1* by sucrose and glucose, however, may be related to the signalling cross-talk between sugars and nitrogen and the known role of *GLK1* in nitrogen signalling (Refer to Chapter 5).

As found previously, *GLK1* and *GLK2* were found to be affected positively by organic nitrogen signalling (Refer to Chapter 6; Gutiérrez et al. 2008). Results of the nitrogen signalling experiment suggest that when glutathione homeostasis is disrupted, changes in redox state of the glutathione pool may regulate *GLK1*. In addition, perturbations in tetrapyrrole biosynthesis may negatively affect both *GLK1* and *GLK2*.

Infection of *Arabidopsis* with *P. syringae* revealed a possible regulation of both genes by HR-induced increase in ROS levels and the associated SA signalling pathway. The downregulation of *GLK1* and *GLK2* by *P. syringae* infection may again be related to suppression of photosynthesis (Refer to Chapter 7). Conversely, phytohormones which are

known to have positive effects on photosynthetic development caused increased expression of *GLK1* and *GLK2* (Refer to Chapter 8).

Analysis of *GLK* expression in *Arabidopsis* grown for 5 weeks at 5°C revealed that cold temperatures negatively affect transcription of *GLK2*, and to a lesser extent that of *GLK1*. The mutation *pgp1* was found to downregulate both genes in normal and cold acclimation temperature conditions. The negative effects of cold and *pgp1* on *GLK* expression appear to be unrelated to changes in redox state of the PQ or glutathione pools, and may instead be a result of suppression of photosynthetic metabolism (Refer to Chapter 9).

Overall, *GLK1* and *GLK2* appear to be responsive to changes in photosynthetic metabolism, ROS levels, and organic nitrogen signalling. In addition, *GLK1* seems to be affected by changes in redox state of the glutathione pool. The proposed model of *GLK1* and *GLK2* regulation by the various signalling pathways is illustrated in Figure 10.1.

The hypothesis that *GLK1* and *GLK2* are affected by a number of different stimuli that produce chloroplast-to-nucleus retrograde signals was supported in the present study. Many of the treatments that caused changes in *GLK* expression are known to directly or indirectly affect carbon fixation. Plastidial metabolites have been proposed to be involved in retrograde chloroplast-to-nucleus signalling (Pfannschmidt 2010). Moreover, single metabolites may not be sufficient to act as signalling molecules, and rather metabolic states or metabolic signatures of the cell are sensed by the nucleus (Pfannschmidt 2010). Although the detailed mechanisms of these signalling processes are currently unknown, results of the experimental treatments suggest that *GLK1* and *GLK2* may be controlled by changes in metabolic state of the cell arising from altered carbon fixation.

GLK1 and *GLK2* also appear to be suppressed by increased levels of ROS generated outside of the chloroplast (Refer to Chapters 4 and 5). This regulation may be related either to ROS-induced oxidative damage or their signalling functions. Together with other redox-reactive molecules such as thioredoxin and glutathione, ROS contribute to the cellular redox environment, which is known to regulate gene expression (Foyer and Noctor 2009). Many genes that are redox regulated are involved in photosynthesis (Pfannschmidt et al. 2003b). Thus, *GLK1* and *GLK2* expression may be affected by increased ROS levels through changes in cellular redox state and subsequent changes in photosynthetic processes.

Organic nitrogen may also affect *GLK* expression through changes in photosynthetic metabolism, as a result of cross-talk between nitrogen and carbon metabolism (Coruzzi and Zhou 2001). As discussed in Chapter 5, sugars suppress photosynthesis through feedback inhibition. The interplay between nitrogen and sugar metabolism is exemplified by the finding that mutations in sugar signalling alter nitrogen responses (Hellmann et al. 2000; Coruzzi and Zhou 2001). Thus, it is possible that treatments of *Arabidopsis* with nitrogen inhibitors or derivatives affected sugar signalling, which in turn resulted in changes in photosynthesis and *GLK* expression.

Therefore, although several factors may independently affect *GLK1* and *GLK2* expression, the ROS and nitrogen signalling pathways may converge and regulate *GLK* genes through changes in photosynthesis and cellular metabolic state. In addition, according to the proposed model, suppression of photosynthetic metabolism is expected to override other signalling pathways with respect to *GLK* regulation. This was seen, for example, in the results of the sugar signalling experiment. While glucose upregulated *GLK1*,

glucose+GSH/GSSG downregulated it, likely as a result of suppression of photosynthesis by GSH/GSSG.

GLK1* vs. *GLK2

GLK1 and *GLK2* expression was affected similarly by most of the experimental treatments. However, some of the treatments had different effects on the two genes, supporting the hypothesis that the two genes are differentially regulated at the transcriptional level. For example, glucose and sucrose upregulated *GLK1* and downregulated *GLK2* (Figures 5.2 and 5.3). Suppression by MSX was reversed by GSH for *GLK1* but not for *GLK2* (Figure 6.1). Four-hour treatments with hormones affected *GLK1* but not *GLK2* (Figure 8.1), and, finally, growth of *Arabidopsis* for 5 weeks at 5°C suppressed *GLK2* to a greater extent than *GLK1* (Figure 9.1). Such differences in transcriptional regulation of the two genes are in agreement with previous finding of differential regulation of *GLK1* and *GLK2* by plastid retrograde signals generated by lincomycin and norflurazon applications (Waters et al. 2009).

Although both genes have been implicated in organic nitrogen signalling, *GLK1* has been called a “master regulator”, having more predicted regulatory interactions with targets than *GLK2* (Gutiérrez et al. 2008). Thus, as discussed in Chapter 5, the upregulation of *GLK1* by sucrose and glucose may be explained by its involvement in organic nitrogen signalling. Sucrose- and glucose-induced suppression of *GLK2*, on the other hand, may be related to its photosynthesis-related functions.

Based on *GLK1* and *GLK2* transcript accumulation profiles in *Arabidopsis*, it has been suggested that while *GLK2* regulates photosynthesis throughout the plant, *GLK1* boosts photosynthetic capacity in tissues with high photosynthetic workload (Fitter et al. 2002).

This difference in the function of the two genes seems to also be reflected by the greater downregulation of *GLK2* than *GLK1* in *Arabidopsis* plants acclimated to 5ºC, as cold temperatures are known to suppress photosynthesis (Huner et al. 1993; Stitt and Hurry 2002). Thus, it appears that *GLK2* has a more important role in photosynthesis, while *GLK1* is a more important player in organic nitrogen signalling, indicating specialization of the two genes.

10.2 Future Directions

Possible future directions related to investigating *GLK1* and *GLK2* regulation in *Arabidopsis* include further dissecting the retrograde chloroplast-to-nucleus signalling pathways affecting *GLK* transcript levels and verifying the proposed mechanisms of *GLK* regulation.

For example, expression of *GLK1* and *GLK2* in *pgp1 Arabidopsis* may be explored further to determine whether any of the known retrograde signalling pathways are responsible for the downregulation of the two genes in this mutant. One approach is to use double *pgp1 gun1* mutants to test whether retrograde signalling in *pgp1* involves GUN1. As discussed in the Introduction, *GUN1* is known to integrate several chloroplast-to-nucleus stimuli, including changes in redox state of the electron transport chain, plastid protein synthesis and chlorophyll biosynthesis (Koussevitzky et al. 2007). In addition, double mutants of *pgp1 gun2-5* can be used to test whether the negative effect of *pgp1* on *GLK* expression is mediated by perturbations in tetrapyrrole biosynthesis.

The effect of changes in redox state of the PQ pool on *GLK* expression also requires further investigation, as PQ pool was not found to have a regulatory role under the

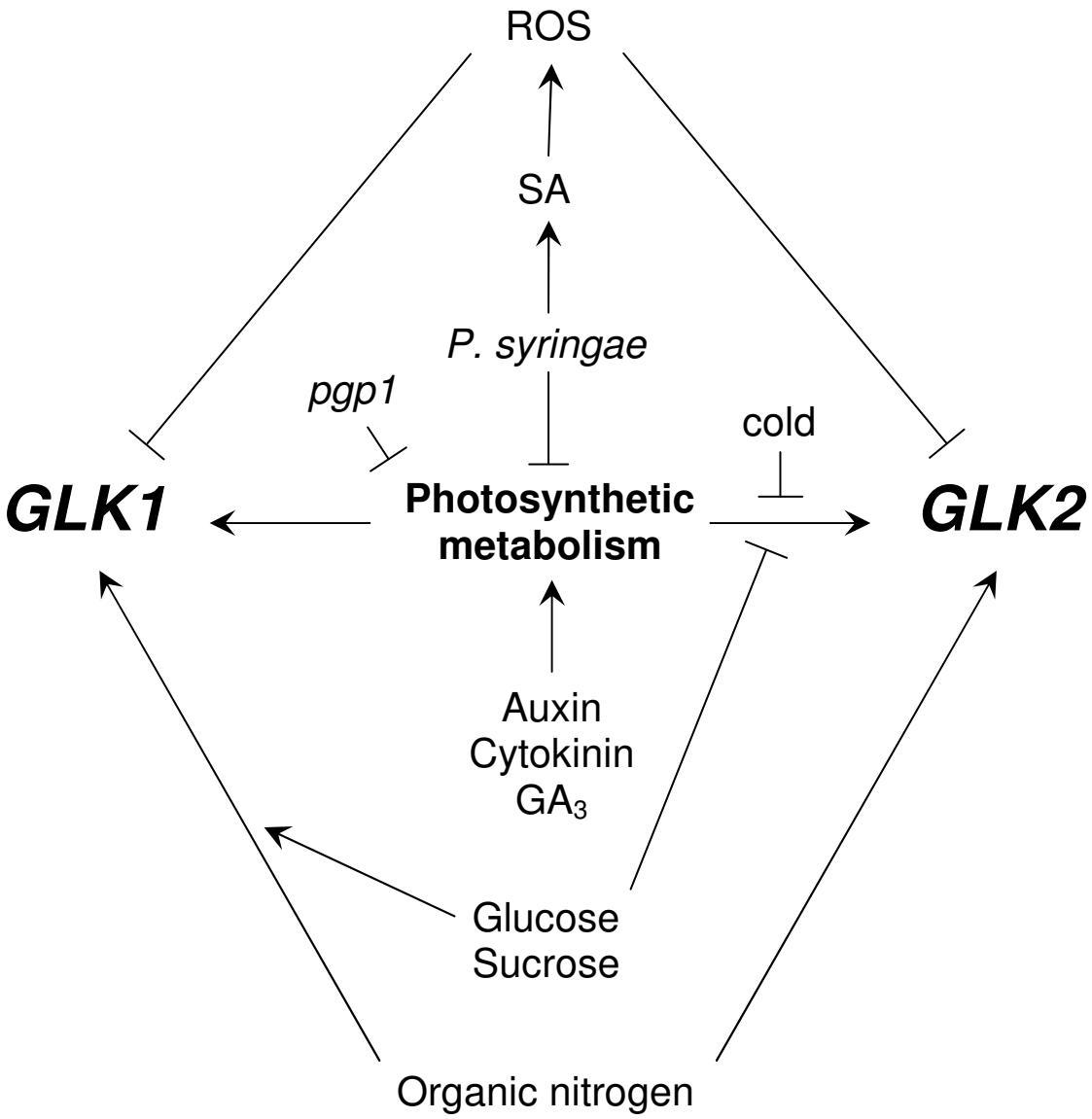


Figure 10.1 Proposed mode of regulation of *GLK1* and *GLK2* transcription in *Arabidopsis* by various stimuli and signalling pathways. Refer to text for more details on the signalling cross-talk as it relates to regulation of *GLK* genes. → indicates positive regulation and ⊣ indicates negative regulation.

experimental conditions, but may be involved when it is overreduced (Refer to Chapter 4; Figure 4.1A and B). To clarify the role of the PQ pool in *GLK1* and *GLK2* regulation, transcript levels of the two genes may be determined following DCMU and DBMIB application and illumination with light of a range of different intensities.

The proposal that *P. syringae* infection affects *GLK* expression through the SA signalling pathway can be investigated in more detail by using plants with decreased SA levels. For example, transgenic *Arabidopsis* plants expressing the bacterial *nahG* gene, encoding the SA-metabolizing enzyme salicylate hydroxilate, fail to accumulate high SA levels and to express PR genes and are hypersensitive to virulent and avirulent pathogens (Delaney et al. 1994; Vernooij et al. 1994; Vlot et al. 2009). Mutants deficient in SA biosynthesis and overexpressors of enzymes involved in SA metabolism display similar phenotypes (Wildermuth et al. 2001; Koo et al. 2007; Song et al. 2008). Such plants can be used to analyze *GLK* transcript levels following *P. syringae* infection, as was done in this study. Based on the proposed model, it is expected that *GLK1* and *GLK2* would not be suppressed in the absence of SA signalling pathway induction.

Finally, while based on the experimental evidence we proposed that *GLK* expression is affected by perturbations in photosynthetic metabolism, the detailed mechanisms of this retrograde signalling pathway are unknown. One approach that can be used to understand which metabolites or metabolite signatures are associated with different *GLK* expression levels is metabolic profiling. Metabolic profiling in conjunction with data-mining has been used to characterize plant genotypes, as well as phenotypes in response to different environmental conditions (Roessner et al. 2001). This technique can be used to compare metabolite changes in plants treated with chloroplast inhibitors such as DCMU or DBMIB to

those in plants treated with GA. The three chemicals all suppress photosynthesis but were found to affect *GLK* expression differently, with DCMU and DBMIB downregulating *GLK* genes and GA upregulating them (Refer to Chapter 4). Thus, the experiment is expected to give insight into the retrograde chloroplast-to-nucleus signalling pathway affecting *GLK* expression through changes in metabolic state of the cell.

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