IN VIVO CHARACTERIZATION OF PGR5 HOMOLOGS FROM ARABIDOPSIS AND PINE: THE ROLE OF CYCLIC ELECTRON TRANSPORT IN STRESS TOLERANCE

by

TERRI ANITA LONG

(Under the Direction of Sarah Covert)

ABSTRACT

During photosynthesis light energy is converted to chemical energy primarily by the transfer of electrons through photosysems I and II. However, electrons can also cycle around photosysems I, generating ATP. Recent studies suggest that along with ATP synthesis, CET plays a role in photoprotection by mediating nonphotochemical quenching of excess light and maintaining the proper stromal redox status. There are at least two partially compensatory CET pathways in most C_3 plants, the NAD(P)H dehydrogenase (NDH)-dependent pathway, and the Proton Gradient Regulation 5 (PGR5)-dependent pathway. We analyzed *A. thaliana PGR5 (AtPGR5)* RNAi suppression lines and over-expressed both the native *AtPGR5* and the *Pinus taeda PGR5 (PtPGR5)* genes in *A. thaliana*. Compared to wild-type, RNAi plants displayed decreased NPQ, decreased growth under high light, and increased susceptibility to photodamage. Constitutive over-expression of *AtPGR5* or *PtPGR5* caused alterations in linear and cyclic electron transport, which led to decreased growth under low to moderate light, but increased tolerance of high light and drought compared to wild-type. *AtPGR5:GUS* fusions and *PtPGR5* tissue-specific northerns indicate that *PGR5* is

predominantly expressed in young, rapidly expanding leaves and phloem. Wounding, exposure to excess light and low temperature induce *AtPGR5* transcription. Previous research indicates that *PGR5* expression is suppressed in *A. thaliana* and *P. taeda* tissues infected biotrophic pathogens. We conclude that PGR5 has an essential, conserved role in protecting plants from excess light energy that may accumulate under stress conditions. It is likely that the chloroplast redox status, which is directly affected by physiological stress, regulates the expression of *PGR5*. Furthermore, we propose that NDH-dependent CET cannot compensate for the function of PGR5-dependent CET under stress conditions.

INDEX WORDS: photosynthesis, cyclic electron transport, nonphotochemical quenching, photoinhibition, PGR5, NDH

IN VIVO CHARACTERIZATION OF PGR5 HOMOLOGS FROM ARABIDOPSIS AND PINE: THE ROLE OF CYCLIC ELECTRON TRANSPORT IN STRESS TOLERANCE

by

TERRI ANITA LONG

B.S., Biology, University of North Carolina at Chapel Hill, 1998

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2005

© 2005

Terri Anita Long

All Rights Reserved

IN VIVO CHARACTERIZATION OF PGR5 HOMOLOGS FROM ARABIDOPSIS AND PINE: THE ROLE OF CYCLIC ELECTRON TRANSPORT IN STRESS TOLERANCE

by

TERRI ANITA LONG

Major Professor: Sarah F. Covert

Committee: Richard Meagher
Susan Wessler
Zheng-Hua Ye
Gregory Schmidt

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
May 2005

DEDICATION

I dedicate this body of work in loving memory of my father, Oliver Earl Long, whose love of nature inspired me to begin this journey, and to my mother, Geraldine L. Long, and brother, Travis Earl Long, whose faith and encouragement inspired me to complete it.

ACKNOWLEDGEMENTS

First and foremost, I would like to thank Dr. Sarah F. Covert for her dedication to me as a scientist and friend, and for her tenacity while completing this project. I would like to thank the members of my committee: Dr. Richard Meagher, Dr. Susan Wessler, Dr. Zheng-Hua Ye, and Dr. Gregory Schmidt for their assistance and support during my tenure here at UGA. I also thank the entire Covert lab, the Meagher lab, Dr. Jeff Dean and lab, Dr. Joe Nairn and lab, and Dr. Scott Merkle and lab for provision of numerous supplies, support and advice.

Thanks to my friends and family near and far for their support and encouragement

…and, thank you, Lord, for your continuous blessings….

TABLE OF CONTENTS

Page

ACKNOWLEDGEMENTS...V

CHAPTER

I Introduction and Literature Review.. 1

II *In vivo* characterization of PGR5 homologs from Arabidopsis and Pine: The role of cyclic electron transport in stress tolerance.. 21

III Conclusions... 86

APPENDIX.. 97

A PGR5 and Plant Defense... 97

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Photosynthesis is arguably the most important biological process on earth. During photosynthesis O_2 is generated while light energy is used to convert CO_2 to chemical energy in the form of sugars and starches. Therefore, photosynthesis serves directly or indirectly to meet all plant and animal nutritional needs, and to maintain the proper balance of atmospheric gases essential for life on earth.

Why study photosynthesis?

While traditional farming practices currently meet the world's nutritional needs, in the next 50 years the global population is projected to be over 9 billion - over 76 million more people to feed per year (UNFPA, 2004). Farmers will need to double crop yield to meet global nutritional demands in the next three decades (UNFPA, 2001). By better understanding photosynthetic mechanisms, scientists can develop new cultivation techniques and new genetically engineered plant lines with increased photosynthetic yield.

It is also important that we understand photosynthesis so that we may learn how to avoid altering our atmosphere in a manner that will lead to large-scale ecological problems. In the last 10,000 years photosynthetic organisms such as plants, algae, and cyanobacteria have helped maintain the proper balance of atmospheric gases by

cycling carbon between the atmosphere and earth (Raven et al., 1992). However, within the last century, especially since the 1970's, global atmospheric CO_2 has increased by 50% due to deforestation, and the increased use of fossil fuels (Hansen and Sato, 2004). This excess CO_2 contributes to the Greenhouse Effect, and is the cause of a 1°C increase in global temperature within the last century, and numerous subsequent global climatic alterations (EPA, 2000). With a greater understanding of the genetic mechanisms involved in photosynthetic intake and output, scientists might be able to generate plants with enhanced CO_2 consumption capabilities.

Photosynthesis – linear electron transport

When plants are exposed to light, the light energy is first utilized by the linear electron transfer (LET) chain (Figure 1-1). LET consist of a series of redox reactions mediated by photosystems II (PSII) and I (PSI), large protein complexes embedded within the chloroplast thylakoid membrane (Raven et al., 1992). PSII first catalyses photolysis or the light-induced transfer of electrons from water to the plastoquinone pool, which also results in the synthesis of H^+ and O_2. Electrons are transferred from reduced plastoquinone to the cytochrome b_6/f complex, and then on the plastocyanin pool. Plastocyanin can then transfer electrons to PSI, which catalyses the light-induced transfer of electrons to ferredoxin. Ferredoxin $NADP^+$ reductase catalyses the transfer of electrons from ferredoxin to $NADP^+$, producing NADPH. During this process, electron transfer between plastoquinone and cytochrome b_6/f is mediated by the Q cycle, which pumps H^+ from the chloroplast stroma into the thylakoid lumen. The accumulation of H^+ in the lumen by H_2O oxidation and the Q cycle creates a large pH gradient (ΔpH) across

the thylakoid membrane. Protons move down the gradient from the lumen to the stroma via ATP synthase, which uses the energy to drive ATP synthesis. Therefore, LET generates both NADPH and ATP. These molecules are used in a wide variety of important processes, such as the Calvin Cycle, protein synthesis, and nitrogen metabolism (Raven et al., 1992).

Photosynthesis – cyclic electron transport

In the late 1950's researchers discovered that electrons can also cycle around PSI and generate ATP in a light-dependent manner, without reducing $NADP^+$ (Arnon et al., 1954). Since then, analyses of chlorophyll fluorescence, photoacoustic signals, P700 oxidation, and the use of chemical inhibitors specific to certain photosynthetic processes have demonstrated that there are at least two cyclic electron transport (CET) pathways; NAD(P)H dehydrogenase-dependent CET and ferredoxin-dependent CET (Bendall and Manasse, 1995). For our purposes I will refer to NAD(P)H dehydrogenase-dependent CET as NDH-dependent CET, and ferredoxin-dependent CET as Proton Gradient Regulation 5 (PGR5)-dependent CET.

NDH-dependent and PGR5-dependent cyclic electron transfer

Reduction of the plastoquinone pool by NADPH and NADH in the absence of light was initially observed in isolated chloroplasts (Mills et al., 1979). NAD(P)H dehydrogenase (NDH) subunit genes homologous to subunits of the mitochondrial complex I (NADH-ubiquinone reductase) were later found in the plastid genomes of higher plants (Shinozaki et al., 1986). This discovery, along with the observation that

ndh mutants do not demonstrate post-illumination reduction of the plastoquinone pool, prompted researchers to believe that the chloroplast-encoded NDH acts as an NAD(P)H:plastoquinone oxidoreductase (Burrows et al., 1998; Endo et al., 1998; Sazanov et al., 1998b; Shikanai et al., 1998).

Since the 1980's NDH activity has been found in the leaves of a number of plant species, yellow algae and cyanobacteria (Bukhov and Carpentier, 2004). The genomes of cyanobacteria and higher plant plastids encode 11 genes (ndhA-K) that are homologous to those found in the mitochondrial NADH-ubiquinone reductase. However, three subunits essential for NADH-ubiquinone reductase have not been found in the chloroplast genomes of higher plants (Grohmann et al., 1996). To date, all functional NDH genes are missing from the plastid genomes of all tested *Pinus* species, as well as green and red algae, and certain protists (Wakasugi et al., 1994; Neyland and Urbatsch, 1996; Odintsova and Yurina, 2003). NDH is an approximately 550 kDa thylakoid membrane protein complex. It only constitutes 0.2% of total thylakoid membrane protein (Sazanov et al., 1998a). *ndh* mutants do not have an obvious phenotype under normal growth conditions, although they do display impaired growth and CET under water stress, and anaerobic conditions (Horvath et al., 2000). These results indicate that although NDH is not essential for normal plant growth, it may have a role in stress tolerance that is not conserved in some photosynthetic species, *Pinus* species in particular.

A decade after discovering CET, Arnon and co-workers reported that electrons can also transfer from ferredoxin or ferredoxin:NAD(P)H reductase to the plastoquinone pool (Tagawa et al., 1963). For years, a ferredoxin plastoquinone oxidoreductase was

postulated to mediate the transfer of electrons from ferredoxin to plastoquinone (Bendall and Manasse, 1995). However, researchers have not been able to identify or purify such an enzyme (Bukhov and Carpentier, 2004; Johnson, 2005). The recent discovery that ferredoxin:NAD(P)H reductase forms a complex with ferredoxin and cytochrome b_6/f strongly indicates that electrons likely flow from ferredoxin to plastoquinone via this complex (Zhang et al., 2001).

Recently, Munekage et al. (2002) discovered that a mutation in *Proton Gradient Regulation 5 (PGR5)* inhibits ferredoxin-dependent CET. PGR5 is a nuclear-encoded, thylakoid membrane-associated protein that is only 10 kD once its transit peptide is cleaved (Munekage et al., 2002). It does not have a metal binding motif or a redox reactive prosthetic group, and its absence does not affect the stability of other major photosynthetic complexes such as PSII and PSI (Munekage et al., 2002). Therefore, the biochemical function of *PGR5* is still an open question. PGR5 homologs can be found throughout the plant kingdom, including angiosperms and gymnosperms such as pine, as well as green algae and cyanobacteria (Munekage et al., 2002). Although *pgr5* loss-of-function mutants display enhanced PSII photoinhibtion, decreased CET, and reduced nonphotochemical quenching (NPQ) in response to excess light, they are reported to lack a conspicuous physical phenotype (Munekage et al., 2002; Munekage et al., 2004).

While interruption of neither the PGR5-dependent nor the NDH-dependent pathway causes obvious changes in growth or development, double mutants display very slow growth and low chlorophyll content (Munekage et al., 2004). These results indicate that the two CET pathways are somewhat redundant and that some form of CET is essential for normal growth (Munekage et al., 2004).

The role of CET in photoprotection

When plants are exposed to high light, excess energy, unused by the Calvin Cycle, can accumulate within the PSII light harvesting complex and the linear electron transport chain, causing over-reduction of the LET chain and the stromal redox carriers, ferredoxin and NADPH (Heber and Walker, 1992). This in turn can cause O_2 reduction, which leads to the formation of reactive oxygen species (ROS) (Heber and Walker, 1992). If not scavenged or quenched, ROS damage the thylakoid membranes, PSII and PSI proteins and light harvesting centers, as well as metabolic enzymes involved in CO_2 fixation (Niyogi, 1999). If this damage is not repaired, it leads to permanent photoinhibition and death (Niyogi, 1999).

Excess chemical energy can also accumulate within the electron transport chain when plants are exposed to physiologically stressful conditions, such as cold, drought, or wounding. Under these conditions, CO_2 assimilation is limited, causing an imbalance of energy acquisition and energy utilization (Savitch et al., 2001; Golding and Johnson, 2003; Chang et al., 2004). This imbalance can also lead to the formation of ROS, photodamage, and death (Niyogi, 1999). Therefore, photoprotection, or the shielding of the photosynthetic machinery from excess energy, is a key component of almost all plant stress responses.

Plants have evolved numerous methods to prevent damage due to excess light exposure, such as alterations in leaf orientation and leaf size, movement of chloroplasts and increased production of anthocyanins, which absorb excess light and shield the photosynthetic apparatus (Steyn et al., 2002). Plants also utilize several biochemical mechanisms, such as the Mehler reaction and water-water cycle, and NPQ to absorb

and dissipate excess energy (for review see Niyogi, 1999). During the water-water cycle ferredoxin donates electrons to O_2 instead of NADP+, creating O_2^- (a superoxide radical anion), super oxide dismutase catalyses the conversion of O_2^- to H_2O_2, then ascorbate peroxidase converts H_2O_2 and ascorbate into H_2O. Nonphotochemical quenching (NPQ) or pH-dependent thermal dissipation, occurs when increased lumenal pH triggers a series of responses in the PSII antenna pigments that cause excess light energy to be dissipated as heat (Muller et al., 2001). These responses include activation of the xanthophyll cycle (a process in which violaxanthin de-epoxidase converts violaxanthin to zeaxanthin), which may aid in chlorophyll de-excitation. During NPQ there are also conformational changes of the thylakoid membrane, and possibly protonation and structural changes of light harvesting complex polypeptides (Muller et al., 2001).

There is growing evidence that CET plays a key role in photoprotection by mediating NPQ of excess light energy. Research suggests that CET is elevated under high light stress, anaerobic conditions, and high heat stress (Thomas et al., 2001; Joet et al., 2002; Munekage et al., 2004). Also, *ndh* mutants display decreased tolerance to drought stress and salt stress, while *pgr5* mutants display decreased NPQ of excess light (Tanaka, 1997; Horvath et al., 2000; Munekage et al., 2002). It is likely that under conditions causing over-reduction of the LET pathway and the stroma, CET increases the transfer of electrons from the stromal redox carriers and from the LET to the plastoquinone pool. Increased plastoquinone reduction subsequently increases Q cycle activity, which in turn increases the intake of H^+ into the lumen. This process causes increased ΔpH, which triggers NPQ of excess light energy, and a corresponding increase in ATP synthesis (Heber and Walker, 1992).

The role of CET in preventing stroma over-reduction

Generally, in C_3 plants, the Calvin Cycle requires ATP and NADPH in a ratio of 3:2. While there is still some discussion about whether LET produces sufficient ATP and NADPH for the Calvin Cycle, ATP made in the chloroplasts is also required for numerous other cellular activities, such as starch and protein synthesis (Bendall and Manasse, 1995; Allen, 2002). Therefore the proper ATP:NADPH ratio must be maintained for normal growth. When insufficient ATP is produced, NADPH unused by the Calvin Cycle accumulates in the stroma. This causes over-reduction of the LET and stromal redox carriers, and subsequently photoinhibition (Munekage et al., 2004). The phenotype of double mutants lacking PGR5-dependent CET and NDH-dependent CET suggests that both pathways contribute ATP needed for CO_2 fixation, as well as for other cellular activities (Bendall and Manasse, 1995; Munekage et al., 2004). However, it is unclear why many plants have evolved two distinct CET pathways.

Different plants – different photosynthetic mechanisms

Cyanobacteria, algae and C_4 plants exhibit a significant amount of CET, while CET is negligible in C_3 plants under normal growing conditions (Bukhov and Carpentier, 2004). This difference may be due to differences in carbon storage and CO_2 fixation.

In C_3 plants, the light and dark reactions both occur in the mesophyll cells (Raven et al., 1992). Because RUBISCO can fix both O_2 and CO_2, oxygen competes with CO_2 for fixation and causes photorespiration if bound to rubisco. When rubisco fixes O_2 instead of CO_2, one molecule of PGA is produced and eventually converted to sucrose, and one molecule of phosphoglycolate is produced, which is broken down into CO_2.

Therefore, during photorespiration, oxygen is consumed and CO_2 is released. Photorespiration can cause up to 50% of fixed carbon to be lost under normal atmospheric conditions in C_3 plants (Foyer and Noctor, 2000). C_3 plants are most energy efficient in a high CO_2:O_2 ratio environment because there is more CO_2 to compete for rubisco and, therefore, less fixed carbon is lost in photorespiration.

In C_4 plants and crassulacean acid metabolism (CAM) plants, the light reactions occur in the mesophyll cells and CO_2 fixation occurs in the rubisco-rich bundle sheath cells or subcellular compartments, which lack PSII. In C_4 plants, CO_2 is first converted to oxaloacetate by PEP carboxylase, then often to malate, which is transported to the bundle sheath cells and decarboxylated to yield CO_2 and pyruvate. This transfer helps maintain a high CO_2:O_2 ratio close to rubisco activity (Moroney and Somanchi, 1999). C_4 plants therefore require less CO_2, less stomatal opening, and suffer less under drought conditions than C_3 plants (Proctor and Tuba, 2002). C_4 plants also fare better then C_3 plants in high light conditions, which cause increased photosynthesis and thus increased O_2 generation during photolysis (Raven et al., 1992). However, although C_4 fixation uses CO_2 more efficiently, it requires five ATP molecules to fix one molecule of CO_2, while C_3 fixation requires only three ATP molecules. Due to the increased CET activity found in C_4 plants, this extra ATP is believed to be provided by CET (Bendall and Manasse, 1995).

Cyanobacteria and unicellular green algae also collect carbon internally because they live in an aquatic environment in which there is low CO_2 availability (Moroney and Somanchi, 1999; Ghoshal and Goyal, 2001). In both cases, carbon is absorbed as HCO_3^- by either active transporters or diffusion. Cyanobacteria, which do not have

organelles such as mitochondria or chloroplasts, collect HCO_3^- in carboxysomes, electron-dense polyhedreal particles surrounded by a protein shell. Unicellular green algae store CO_2 in pyrenoids, proteinaceous structures located in the chloroplasts. Both carboxysomes and pyrenoids are rubisco-rich particles, which contain enzymes that convert HCO_3^- back to CO_2. Therefore, similar to C_4 photosynthesis, CO_2 is localized close to rubisco, and can out-compete O_2 for fixation (Moroney and Somanchi, 1999; Ghoshal and Goyal, 2001). Though cyanobacteria and unicellular green algae utilize a more CO_2 efficient photosynthetic mechanism, they too require more ATP than C_3 plants for HCO_3^- transport. Again, CET is proposed to provide the extra ATP required for this activity (Moroney and Somanchi, 1999).

PGR5 – from tree pathogens to photosynthesis

My study of PGR5 began several years ago in an effort to elucidate the molecular genetic mechanisms underlying the interaction between *Cronartium quercuum f. sp. fusiforme* and its host, *Pinus taeda* (loblolly pine). *C. q. fusiforme* is the causative agent of fusiform rust, a destructive disease of *P. taeda* and *Pinus elliottii* (slash pine) in the southeastern Unites States. Estimated losses due to this disease are 25-135 million dollars per year (Cubbage and Wagner, 2000). Fusiform rust leads to the development of spindle-shaped galls on the trunks and limbs of pine trees. These galls form due to increases in the number and size of xylem, phloem, and cortical cells, which form the secondary vascular tissue, or wood. Fusiform rust galls also display a higher than normal proportion of ray parenchyma cells, decreased cell wall synthesis, and increased soluble carbohydrate content. It is apparent that the vascular cambium,

the ring of meristematic cells from which secondary xylem and phloem cells develop, is affected by *C. q. fusiforme* infection, and that cell fate is altered in galled tissues (Jackson and Parker, 1958; Jewell et al., 1962; Miller and Cowling, 1977). Because relatively little is known about how wood forms in trees, I expected that by studying the molecular genetic mechanisms of gall formation I would learn more about what genes are important for normal wood development.

In previous work, the Covert lab used a genomics approach to isolate a set of partial cDNA transcripts differentially regulated by *C. q. fusiforme* infection (Warren and Covert, 2004). The then uncharacterized *PGR5* loblolly pine ortholog (*PtPGR5*) was found to be suppressed three-fold in pine tissue infected with *C. q. fusiforme* (Warren and Covert, 2004). Using PCR, I isolated the full-length sequence of *PtPGR5* and determined that it had high homology to a single uncharacterized protein in *A. thaliana*, cyanobacteria and green-algae, and that it was over 80% similar to numerous expressed sequence tags from species throughout the plant kingdom (Figure 1-2). Because *PtPGR5* was suppressed in galled tissues and its structure was so highly conserved, I hypothesized that this protein could be involved in wood development, plant defense, or basic plant metabolism.

At the outset, my primary objective was to gain an in-depth understanding of the function of PtPGR5 in healthy trees and thus infer how its suppression in fusiform rust galls contributed to abnormal wood formation, disease development, or basic plant metabolism. Due to the difficulty of performing transgenic procedures in pine and the novel nature of PtPGR5, I also decided to characterize the function of the PtPGR5 ortholog from *A. thaliana* (AtPGR5). To meet these objectives I suppressed *AtPGR5*

expression in *A. thaliana* by RNA interference, and over-expressed *AtPGR5* and *PtPGR5* in *A. thaliana* to look for changes in growth and development, or changes in pathogen resistance*.* I also planned to determine the expression pattern of *AtPGR5* and *PtPGR5* in *A. thaliana* and *P. taeda*, respectively, and to determine if *AtPGR5* is influenced by the hormone signal transduction pathways that regulate plant responses to pathogens. However, while I was performing these experiments, AtPGR5 was isolated and named in a search for *A. thaliana* mutants that displayed decreased NPQ of excess light (Munekage et al., 2002). A subsequent study by Munekage et al. (2004) demonstrated that the PGR5-dependent and NDH-dependent CET pathways may operate together to maintain the stromal ATP:NAPDH ratio, thus preventing stromal over-reduction.

Since the description of AtPGR5, my objectives have remained largely the same; namely, to further elucidate the function of *PGR5* in plants with RNA interference and over-expression mutants, and to determine the expression pattern of *PGR5*. However, based on the information about AtPGR5's role in photosynthesis, I expanded the objectives of the study to further characterize the role of PGR5-dependent CET in photosynthesis across distantly related plant species.

In contrast to previous reports, this study describes whole-plant RNAi phenotypes that verify the role of *PGR5* in photoprotection, and demonstrate that NDH-dependent CET cannot compensate for this function. I have also determined that AtPGR5 over-expression confers increased tolerance to high light and drought. Finally, I describe the temporal and spatial expression patterns of *PGR5* in *A. thaliana* and pine, and in response to physiological stresses that disturb photosynthesis. *AtPGR5:GUS*

and *PtPGR5* are predominantly expressed in young, rapidly expanding leaves and phloem. Wounding, exposure to excess light and low temperature induce *AtPGR5* transcription. Because I have worked on two distantly related plant species, one of which lacks NDH activity, I also address the possible evolutionary significance of the two distinct CET pathways in C_3 plants.

LITERATURE CITED

Allen, J. (2002). Photosynthesis of ATP-electrons, proton pumps, rotors, and poise. Cell **110,** 273-276.

Arnon, D.I., Allen, M.B., and Whatley, F.R. (1954). Photosynthesis by isolated chloroplasts. Nature **174,** 394-396.

Bendall, D.S., and Manasse, R.S. (1995). Cyclic photophosphorylation and electron transport. Biochimica Et Biophysica Acta-Bioenergetics **1229,** 23-38.

Bukhov, N., and Carpentier, R. (2004). Alternative Photosystem I-driven electron transport routes: mechanisms and functions. Photosynthesis Research **82,** 17-33.

Burrows, P.A., Sazanov, L.A., Svab, Z., Maliga, P., and Nixon, P.J. (1998). Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants

containing disrupted plastid ndhgenes. The EMBO Journal **17,** 868-876.

Chang, C.C.C., Ball, L., Fryer, M.J., Baker, N.R., Karpinski, S., and Mullineaux, P.M. (2004). Induction of ASCORBATE PEROXIDASE 2 expression in wounded

Arabidopsis leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis. Plant Journal **38,** 499-511.

Cubbage, F.W., and Wagner, J.E. (2000). An economic evaluation of fusiform rust protection research. Southern Journal of Applied Forestry **24,** 77-85.

Endo, T., Shikanai, T., Sato, F., and Asada, K. (1998). NAD(P)H dehydrogenase-dependent, antimycin A-sensitive electron donation to plastoquinone in tobacco chloroplasts. Plant and Cell Physiology **39,** 1226-1231.

EPA, U. (2000). Global Warming - Climate (http://yosemite.epa.gov/oar/globalwarming.nsf/content/climate.html) (US Environmental Protection Agency).

Foyer, C.H., and Noctor, G. (2000). Tansley Review No. 112 Oxygen processing in photosynthesis: regulation and signalling. New Phytologist **146,** 359-388.

Ghoshal, D., and Goyal, A. (2001). Cyanobacteria collect CO_2 in rubisco-rich carboxysomes, while green alga store them in rubisco-rich pyrenoids. Journal of Plant Biochemistry and Biotechnology **10,** 83-90.

Golding, A.J., and Johnson, G.N. (2003). Down-regulation of linear and activation of cyclic electron transport during drought. Planta **218,** 107-114.

Grohmann, L., Rasmusson, A.G., Heiser, V., Thieck, O., and Brennicke, A. (1996). The NADH-binding subunit of respiratory chain complex I is nuclear-encoded in plants and identified only in mitochondria. Plant Journal **10,** 793-803.

Hansen, J., and Sato, M. (2004). Greenhouse gas growth rates. Proc Natl Acad Sci U S A **101,** 16109–16114.

Heber, U., and Walker, D. (1992). Concerning a dual function of coupled cyclic electron transport in leaves. Plant Physiology **100,** 1621-1626.

Horvath, E.M., Peter, S.O., Joet, T., Rumeau, D., Cournac, L., Horvath, G.V., Kavanagh, T.A., Schafer, C., Peltier, G., and Medgyesy, P. (2000). Targeted inactivation of the plastid *ndhB* Gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. Plant Physiology **123,** 13378-11349.

Jackson, L.W.R.e.a., and Parker, N.L. (1958). Anatomy of fusiform rust galls and loblolly pine. Phytopathology **46,** 637-640.

Jewell, F.F., True, R.P., and Mallett, S.L. (1962). Histology of Cronartium in slash pine seedlings. Phytopathology **52,** 850-888.

Joet, T., Cournac, L., Peltier, G., and Havaux, M. (2002). Cyclic electron flow around photosystem I in C-3 plants. In vivo control by the redox state of chloroplasts and involvement of the NADH-dehydrogenase complex. Plant Physiology **128,** 760-769.

Johnson, G.N. (2005). Cyclic Electron Transport in C_3 plants: fact or artefect. Journal of Experimental Botany **56,** 407-416.

Miller, T., and Cowling, E.B. (1977). Infection and colonization of different organs of slash pine seedlings by *Cronartium fusiforme*. Phytopatholgy **67,** 179-186.

Mills, J.D., Crowther, D., Slovacek, R.E., Hind, G., and McCarthy, R.E. (1979). Electron transport pathways in spinach chloroplasts. Reduction of the primary accpetor of Photosystem II by reduced nicotinamide adenine nucleotide phosphate in dark. Biochim Biophys Acta **547,** 127-138.

Moroney, J.V., and Somanchi, A. (1999). How do algae concentrate CO_2 to increase the efficiency of photosynthetic carbon fixation? Plant Physiol. **119,** 9-16.

Muller, P., Li, X.P., and Niyogi, K.K. (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiology **125,** 1558-1566.

Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., and Shikanai, T. (2002). PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell **110,** 361-371.

Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K., Endo, T., Tasaka, M., and Shikanai, T. (2004). Cyclic electron flow around photosystem I is essential for photosynthesis. Nature **429,** 579-582.

Neyland, R., and Urbatsch, L.E. (1996). The *ndhf* chloroplast gene detected in all vasular plant divisions. Planta **200,** 273-277.

Niyogi, K.K. (1999). Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 333-359.

Odintsova, M.S., and Yurina, N.P. (2003). Plastid genomes of higher plants and algae: structure and functions. Molecular Biology **37,** 649-662.

Proctor, M.C.F., and Tuba, Z. (2002). Tansley review no. 141 Poikilohydry and homoihydry: antithesis or spectrum of possibilities? New Phytologist **156,** 327-349.

Raven, P.H., F., E.R., and Eichhorn, S.E. (1992). Biology of plants. (New York: Worth Publishers).

Savitch, L.V., Barker-Astrom, J., Ivanov, A.G., Hurry, V., Oquist, G., Huner, N.P., and Gardestrom, P. (2001). Cold acclimation of *Arabidopsis thaliana* results in

incomplete recovery of photosynthetic capacity, associated with an increased reduction of the chloroplast stroma. Planta **214,** 295-303.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998a). The plastid *ndh* genes code for an NADH-specific dehydrogenase: Isolation of a complex I analogue from pea†thylakoid†membranes. PNAS **95,** 1319-1324.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998b). The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. FEBS Lett **429,** 115-118.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998). Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proceedings of the National Academy of Sciences of the United States of America **95,** 9705-9709.

Shinozaki, K., Ohme, M., Tanaka, M., Wakasugi, T., Hayashida, N., Matsubayashi, T., Zaita, N., Chunwongse, J., Obokata, J., Yamaguchi- Shinozaki, K., Ohto, C., Torazawa, K., Meng, B.Y., Sugita, M., Deno, H., Kamogashira, T., Yamada, K., Kusuda, J., Takaiwa, F., Kato, A., Tohdoh, N., Shimada, H., and Sugiura, M. (1986). The complete nucleotide sequence of the tobacco chloroplast genome: its gene organization and expression. The EMBO Journal **5,** 2043-2049.

Steyn, W.J., Wand, S.J.E., Holcroft, D.M., and Jacobs, G. (2002). Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist **155,** 349-361.

Tagawa, K., Tsuminto, H.Y., and Arnon, D.I. (1963). Role of chloroplast ferredoxin in the energy conservation process of photosynthesis. Proc Natl Acad Sci U S A **49,** 567-572.

Tanaka, Y., Katada, S., Ishikawa, H., Ogawa, T. and Takabe, T ... . (1997). Electron flow from NAD(P)H dehydrogenase to photosystem I is required for adaptation to salt shock in cyanobacterium Synechocystis sp. PCC 6803. Plant Cell Physiology **38,** 1311-1319.

Thomas, D.J., Thomas, J., YOuderin, P.P., and Herbert, S.K. (2001). Photoinhibition and light-induced cyclic transport in *ndhB*- and psaE- mutants of *Synechocystic* sp. PCC6803. Plant Cell Physiology **42,** 803-812.

UNFPA. (2001). State of the World Population 2001. (United Nations Population Fund).

UNFPA. (2004). State of the World Population 2004. (United Nations Population Fund).

Wakasugi, T., Tsudzuki, J., Ito, S., Nakashima, K., Tsudzuki, T., and Sugiura, M. (1994). Loss of all *ndh* genes as determined by sequencing the entire chloroplats genome of the black pine *Pinus thunbergii*. Proc Natl Acad Sci U S A **91,** 9794-9798.

Warren, J.A., and Covert, S.F. (2004). Differential expression of pine and *Cronartium quercuum f. sp fusiforme* genes in fusiform rust galls. Applied and Environmental Microbiology **70,** 441-451.

Zhang, H., Whitelegge, J.P., and Cramer, W.A. (2001). Ferredoxin:NADP+ Oxidoreductase Is a subunit of the chloroplast cytochrome b_6f Complex. The Journal of Biological Chenistry **276,** 38159–38165.

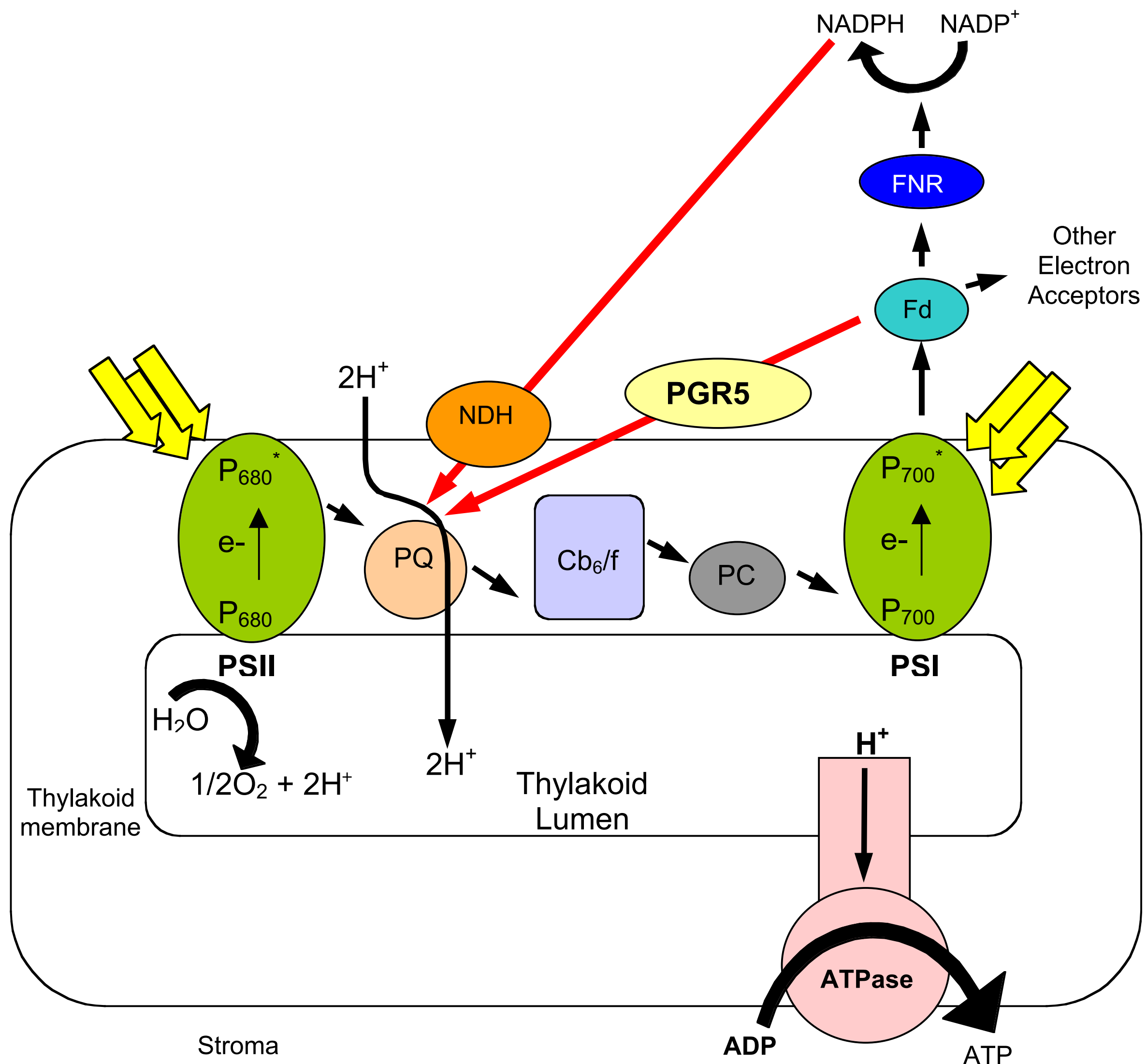


Figure 1-1. Photosynthetic linear and cyclic electron transfer. Light causes electrons (e-) associated with pigments (P_{680}) in Photosystem II (PSII) to rise to a higher energy state and transfer to the cytochrome b_6/f complex (Cb_6/f) via plastoquinone (PQ). The PSII electron deficit is filled by H_2O oxidation, leaving H^+ and O_2. The movement of electrons from PQ to Cb_6/f is induced by the Q cycle, which pumps protons into the thylakoid lumen, thus driving ATP synthesis. Plastocyanin (PC) transfers electrons from Cb_6/f to Photosystem I (PSI). There, light induces the excitation of e- associated with pigments (P_{700}) in PSI, and causes them to transfer to ferredoxin (Fd). Fd can then reduce a variety of electron acceptors, including ferredoxin NADP reductase (FNR), which reduces $NADP^+$ to NADPH. Electrons can also cycle around PSI by transferring from Fd, FNR, or NADPH back to the PQ pool. NAD(P)H dehydrogenase (NDH) mediates electron transfer between NADPH. Electron transfer from Fd to PQ requires Proton Gradient Regulation 5 (PGR5).

```
Arabidopsis thaliana     M A - - - - A - A S I S A I G C N Q - T L I G T S F Y G - G  23
Zea mays                 M A - - - - A A V S L S R V - - R A L P - - - - - - - - - T  15
Pinus taeda              M A - - - - T I S S I P A A G N N V L R R G M T I N D G S G  26
Ceratopteris richardii   M A - - - - S - S A L - - I G S S Q L S L Q H N P L H S I S  23
Physcomitrella patens    M A - - - - T A R S T G S - - - - - - P M S S A E L V R L N  20
Volvox carteri           M L V A K R N A V Q V R A S G S A A L S M S R S A A R S V A  30
Synecocystis sp.         M - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1

Arabidopsis thaliana     W G S S I S G E D Y Q T - - M L S K T V A - P P Q Q A R V S  50
Zea mays                 W S S S V S G D D H H A Y S V L A T S S V - S A R P - R S G  43
Pinus taeda              W K K S S M A G D G S A - - Q L N A R L W - S - - - Y R T S  50
Ceratopteris richardii   H S Q S V C G D G V Y L - - L K S G A F R - P P R - - - - -  45
Physcomitrella patens    W S S S S I N G N G A D R F L H T S N F T - - - - - - R S S  44
Volvox carteri           V S S R I A L S S W D D C R Q T A S S L A Q S A P K L Q N T  60
Synecocystis sp.         - - - - - - - - - - - - - - - - - - - - - - - - - - - - - -  1

Arabidopsis thaliana     R K A I R - A V P - M M K N V N E G K G L F A P L V V V T R  78
Zea mays                 A R P L R - S P A Q M M G N V N D G K G L F A P L V V V A R  72
Pinus taeda              G K A V R - A Q P - V M G N K N E G K G L F A P L V V L A R  78
Ceratopteris richardii   R T S L R - A V P - R M G N V N E G K G I F A P L V V L V R  73
Physcomitrella patens    V S C P R V A Q P R A M G N K N E G K G I F A P L V V V T R  74
Volvox carteri           S N A P R R K P V T M M G N K A T T - G P F A P L V V V V R  89
Synecocystis sp.         - - - - - - - - - - - - - - - - - - - - - F A P I V I L V R  10

Arabidopsis thaliana     N L V G K K R F N Q L R G K A I A L H S Q V I T E F C K S I  108
Zea mays                 N I I G R K R F N Q L R G K A I A L H S Q V I N E F C K T I  102
Pinus taeda              N I I G K K P F N Q L R G K A I A L H S Q V I T E F C K S I  108
Ceratopteris richardii   N A V G K K R F N Q L R G K A I A L H S Q V I T E F C R S I  103
Physcomitrella patens    N V M G K K E F N Q L R G K A I A L H S Q V I G E F C K T I  104
Volvox carteri           G A I G E K E F N Q F R G K A I S L H S Q V I K D F C K L L  119
Synecocystis sp.         Q Q L G K A K F N Q I R G K A I A L H C Q T I T N F C N R V  40

Arabidopsis thaliana     G A D A K Q R Q G L I R L A K K N G E R L G F L A .          134
Zea mays                 G A D Y K Q R Q G L I R L A K K N G E K L G F L A .          128
Pinus taeda              G A D A K Q R Q G L I R L A K K N G E K L G F L A .          134
Ceratopteris richardii   G A D A K Q R Q G L I R L A K K N G E K L G F L A .          129
Physcomitrella patens    G A D S K Q K Q G L I R L A K K N G E K L G F L A .          130
Volvox carteri           G V D N K Q V Q G V I R L A K K N G E K L G F L A .          145
Synecocystis sp.         G I D A K Q R Q N L I R L A K S N G K T L G L L A .          66
```

Figure 1-2. Alignment of PGR5 proteins from diverse photosynthetic species

CHAPTER II

IN VIVO CHARACTERIZATION OF PGR5 HOMOLOGS FROM ARABIDOPSIS AND PINE: THE ROLE OF CYCLIC ELECTRON TRANSPORT IN STRESS TOLERANCE[1]

[1] Long, T.A., G. Schmidt, S.F. Covert. To be modified for submission to Plant Cell

ABSTRACT

Although the importance of cyclic electron transport (CET) in C_3 plants has long been controversial, recent studies suggest that CET plays a role in photoprotection by mediating NPQ and maintaining the proper stromal redox status. There are at least two partially compensatory CET pathways in most C_3 plants, the NAD(P)H dehydrogenase (NDH)-dependent pathway, and the Proton Gradient Regulation 5 (PGR5)-dependent pathway. We analyzed *A. thaliana PGR5 (AtPGR5)* RNAi suppression lines and over-expressed both the native *AtPGR5* and the *Pinus taeda PGR5 (PtPGR5)* genes in *A. thaliana*. Compared to wild-type, RNAi plants displayed decreased NPQ, decreased growth under high light, and increased susceptibility to photodamage. Constitutive over-expression of *AtPGR5* or *PtPGR5* caused alterations in linear and cyclic electron transport, which led to decreased growth under low to moderate light, but increased tolerance of high light and drought compared to wild-type. *AtPGR5:GUS* fusions and *PtPGR5* tissue-specific northerns indicate that *PGR5* is predominantly expressed in young, rapidly expanding leaves and phloem. Wounding, exposure to excess light and low temperature induce *AtPGR5* transcription. We conclude that PGR5 has an essential, conserved role in protecting plants from excess light energy that may accumulate under stress conditions. Furthermore, we suggest that NDH-dependent CET cannot compensate for the function of PGR5-dependent CET under these conditions.

INTRODUCTION

In most photosynthetic organisms, the conversion of light energy to chemical energy occurs largely through linear electron transport. Electrons are transferred through photosystem II (PSII), the cytochrome b_6/f complex (Cb_6/f), and photosystem I (PSI) to oxidized ferredoxin$^+$ (Fd^+) and $NADP^+$ to generate energy in the form of reduced NADPH. Protons released into the thylakoid lumen by PSII-catalyzed H_2O oxidation and the Q cycle increase the concentration of H^+ in the thylakoid lumen and generate a pH gradient (ΔpH) used for ATP synthesis. Thus, linear electron transport (LET) generates both the NADPH and the ATP required for CO_2 assimilation.

Electrons can also transfer cyclically around PSI (Arnon et al., 1954; Bukhov and Carpentier, 2004). During cyclic electron transport (CET), electrons from NADPH or Fd flow back to the plastoquinone pool (PQ), generating ATP (for review see Bukhov and Carpentier, 2004; Johnson, 2005). In cyanobacteria, green algae, and C_4 plants, CET can be the exclusive source of photosynthetically-derived ATP, or act to provide extra ATP for different cellular processes (Leegood et al., 1981; Jeanjean et al., 1993; Ravenel et al., 1994; Mi et al., 1995). In C_3 plants, the importance of CET has long been controversial (Herbert et al., 1990; Bendall and Manasse, 1995; Allen, 2002; Joet et al., 2002; Bukhov and Carpentier, 2004; Johnson, 2005). In these plants CET has been proposed to generate ATP for the Calvin Cycle, and other metabolic processes such as starch and protein synthesis. CET has also been proposed to play a role in photoprotection in C_3 plants (Herbert et al., 1990; Bendall and Manasse, 1995; Allen, 2002; Joet et al., 2002; Johnson, 2005). When plants are exposed to high light or conditions that limit CO_2 fixation, excess energy unused by the Calvin Cycle can

accumulate and cause over-reduction of the linear electron transport chain and the stromal redox components (Niyogi, 1999). This over-reduction leads to the generation of reactive oxygen species (ROS), which, in turn, can cause photoinhibition of PSI and PSII, and eventually death (Long et al., 1994; Niyogi, 1999; Savitch et al., 2001; Golding and Johnson, 2003; Chang et al., 2004). Among other photoprotective measures, plants utilize energy dissipation mechanisms such as nonphotochemical quenching (NPQ) to absorb and dissipate excess energy (Demmig-Adams and Adams, 1992; Muller et al., 2001). NPQ occurs when increased lumen pH (ΔpH) triggers a series of responses in the PSII antenna bed that lead to chlorophyll de-excitation and dissipation of excess light energy as heat (Muller et al., 2001). It has been proposed that CET enhances the ΔpH which stimulates NPQ (for review see Bendall and Manasse, 1995; Bukhov and Carpentier, 2004).

Electron transfer between NADPH and the PQ pool is mediated by NAD(P)H dehydrogenase (NDH). NDH is a large, chloroplast encoded, thylakoid membrane-bound, multi-subunit complex, which facilitates the movement of electrons from NADPH to the PQ pool (Burrows et al., 1998; Endo et al., 1998; Sazanov et al., 1998b; Shikanai et al., 1998). *ndh* mutants do not have an obvious phenotype under normal growth conditions, though they do display decreased CET. Under water stress and anaerobic conditions they also display decreased CO_2 fixation and decreased growth (Burrows et al., 1998; Shikanai et al., 1998; Horvath et al., 2000; Joet et al., 2001; Bukhov and Carpentier, 2004; Munekage et al., 2004). Electron transfer between Fd and PQ requires Proton Gradient Regulation 5 (PGR5) (Munekage et al., 2002). PGR5 is a small, nuclear encoded, thylakoid membrane-associated protein (Munekage et al.,

2002). *pgr5* loss-of-function mutants display impaired electron flow through PSII and from Fd to PQ, as well as decreased P700 oxidation, yet no visible whole plant phenotype (Munekage et al., 2002; Munekage et al., 2004). *pgr5* mutants also display decreased light-dependent NPQ, and damage to photosystem I under high light (Munekage et al., 2002). These studies provide biochemical evidence, which suggests that PGR5-dependent CET acts to induce ΔpH under photoinhibitory conditions by facilitating the movement of electrons from NADPH or Fd to the PQ pool (Munekage et al., 2002). Analysis of *pgr5 crr* double mutants indicate that the two CET pathways act in an partially redundant manner to generate the ATP needed for proper growth (Munekage et al., 2004). However, is it still uncertain whether CET is important for photoprotection in whole plants, when and where CET is utilized and whether the function of CET is conserved in photosynthetic species.

In previous research, a *Pinus taeda* (loblolly pine) partial cDNA (*DD55*) was identified whose expression was suppressed over three-fold in *P. taeda* infected with the obligate phytopathogen, *Cronartium quercuum* f. sp. *fusiforme* (Warren and Covert, 2004). Cloning of the *DD55* full-length cDNA revealed that it encoded a highly conserved plant protein whose apparent homolog was encoded by a single gene in *A. thaliana* (this study). Based on this data we initiated a series of transgenic experiments and expression studies to determine the function of DD55 in pine and that of its homolog in *A. thaliana.* While we were in the midst of this work, Munekage et al. (2002) identified the *A. thaliana* homolog of DD55 as essential for NPQ and Fd-dependent CET, and they named the gene *PGR5*. The goals of our research, therefore, were expanded to further investigate the roles of the *PGR5* genes in *A. thaliana* (*AtPGR5*) and *Pinus taeda*

(*PtPGR5*) in CET. In contrast to earlier reports, we found that a reduction in AtPGR5 protein levels caused visible phenotypes in whole plants indicating a role in ATP synthesis or photoprotection. Constitutive over-expression of *AtPGR5* caused changes in electron transport, which led to decreased growth, but increased tolerance of excess light and drought. Consistent with these effects, *AtPGR5* transcription was induced in developing leaves, and regulated to a small degree by physiological stresses that reduce CO_2 fixation. By over-expressing *PtPGR5* in *A. thaliana* and studying its expression in different pine tissues, we determined that the function and developmental regulation of *PGR5* are likely to be conserved throughout the plant kingdom. We suggest that PGR5-dependent CET plays a more significant role than NDH-dependent CET in preventing over-reduction of the electron transport pathway and the stromal redox components under stress conditions in C_3 plants.

RESULTS

PGR5 is important for normal growth and development under high light

RNA interference (RNAi) efficiently silenced *AtPGR5* expression in independent, transgenic lines. In three of these lines, Ri5, Ri9, and Ri19, only trace amounts of *AtPGR5* transcript and protein were produced. All subsequent experiments focused on these lines, and similar results were obtained for all three lines. The two most suppressed lines, Ri5 and Ri19 are shown in Figure 2-1A,B and described here in detail. Under moderate to high light (100-700 $\mu E/m^2s$) *AtPGR5* RNAi plants displayed decreased availability of PSI electron acceptors, increased reduction of PQ, decreased PSII yield, decreased electron transport through PSII (ETR), and decreased NPQ when

compared to wild-type (Figure 2-2). These findings are consistent with the results of Munekage et al. (2002, 2004) for the *atpgr5* EMS-generated point mutants. However, in contrast to *atpgr5*, the RNAi plants did not display a discernable decrease in steady state P700 oxidation (Figure 2-2A), perhaps due to incomplete suppression of *AtPGR5* by RNAi.

RNAi-mediated suppression of *AtPGR5* also did not effect growth or development when the plants were grown in media containing excess or deficient amounts of sucrose, low MS salts, or when exposed to sudden increases in light intensity (data not shown). Also, RNAi suppression of *AtPGR5* caused no changes in growth or development when the plants were grown under moderate (100-200 $\mu E/m^2s$) or low light (50 $\mu E/m^2s$; data not shown). This low light level replicated the conditions used by Munekage et al. (2002, 2004) in their studies of *AtPGR5*. They reported that loss of AtPGR5 function resulted in no whole plant phenotype.

However, since both types of *AtPGR5* loss-of-function mutants exhibited decreased NPQ (Figure 2-2E) (Munekage et al., 2002; Munekage et al., 2004), we predicted that they should be reduced in their ability to tolerate highly intense light. Indeed, when *AtPGR5* RNAi plants were germinated and grown in high light (2000 $\mu E/m^2s$), they exhibited slow growth (Figure 2-3A) during the seedling and early adult phases of the life cycle (Figure 2-4).

Suppression of *PGR5* causes photodamage in a sealed environment

A lack of atmospheric CO_2 causes decreased Calvin Cycle activity and the over-reduction of the stromal redox carriers ferredoxin and $NADP^+$. This in turn leads to the formation of reactive oxygen species (ROS) that can damage the photosynthetic machinery and cause chlorosis (Niyogi, 1999; Savitch et al., 2001; Golding and Johnson,

2003; Chang et al., 2004). AtPGR5's apparent role in mediating NPQ (Figure 2-2E; Munekage et al. 2002) suggested that PGR5-dependent CET may act to protect plants from ROS-induced damage. Consistent with this idea, the leaves of *AtPGR5* RNAi plants grown in airtight chambers for two weeks displayed chlorotic patches under moderate light (Figure 2-3B). This chlorosis occurred whether plants were grown in media with or without 1% sucrose, but it faded when the RNAi plants were shifted to open air, and did not appear if the plants were germinated and grown in an open environment (data not shown). To determine if the RNAi leaf chlorosis could be due to ROS accumulation, we applied nitro blue tetrazolium salt (NBT), which preferentially stains O_2, to the leaves of RNAi and wild-type plants grown in airtight chambers. The *AtPGR5* RNAi plants displayed a dramatic increase in ROS under these conditions compared to wild-type (Figure 2-3C).

Constitutive over-expression of *AtPGR5* causes changes in electron transfer

We generated multiple *A. thaliana* over-expression lines (AtOE) with increased *AtPGR5* expression levels. The results presented below are derived form the most highly expressed line: AtOE20, but is representative of other highly expressed lines. Line AtOE20 displayed dramatically increased *AtPGR5* transcript levels and a two-fold increase in AtPGR5 protein (Figure 2-1C,D). We monitored the PSI redox state, and changes in chlorophyll fluorescence parameters to determine how over-expression of *AtPGR5* affected the movement of electrons from PSI to Fd to PQ. Oxidation of the PSI reaction center, $P700^+$, can be determined by measuring absorbance changes at 830nm (Schreiber et al., 1988). We measured $P700^+$ oxidation under actinic light (ΔA),

relative to P700 oxidation under far red light (ΔAmax). The P700 oxidation ratio ($\Delta A/\Delta$Amax) was increased in AtOE plants (Figure 2-2A) indicating that steady-state PSI was more oxidized in the over-expression lines than in wild-type. By measuring ΔAmax due to a saturating flash under actinic light relative to far red light ($\Delta Amax_{AL}$ / $\Delta Amax_{FR}$), it is also possible to determine the availability of PSI electron acceptors (Munekage et al., 2002). Over-expression of *AtPGR5* caused an increase in $\Delta Amax_{AL}$ / $\Delta Amax_{FR}$ (Figure 2-2A), indicating that elevated AtPGR5 acted to increase the level of Fd^+.

AtPGR5 over-expression plants also displayed increased PQ reduction, decreased PSII photosynthetic yield, and a corresponding decrease in PSII ETR compared to wild-type plants (Figure 2-2). It is likely that elevated PQ reduction in the over-expression lines caused electrons to accumulate in PSII, and thus reduced PSII yield. In addition, NPQ was elevated in over-expression lines in a light-dependent manner (Figure 2-2E), suggesting that elevated AtPGR5 increased the movement of electrons from PSI to PQ, and thus increased ΔpH.

Constitutive over-expression of *AtPGR5* causes changes in growth and development

Over-expression of *AtPGR5* caused numerous physical effects when the plants were germinated and grown under either moderate or high light. AtOE seedlings had white cotyledons when grown on soil (Figure 2-5A). When AtOE seeds were germinated on solid medium with or without sucrose, the cotyledons were paler than wild-type, but were not completely white (data not shown). The true leaves from AtOE plants were green (Figure 2-5B), but AtOE plants grew more slowly and were smaller

than wild-type plants at maturity (Figure 2-5B, G, 6A). They also had decreased chlorophyll content (Figure 2-6B). The rosette and inflorescence leaves of AtOE plants were dimpled and crinkled (Figure 2-5C). After bolting, they senesced and dried more slowly than wild-type plants (Figure 2-5D).

AtOE plants retained white cotyledons and many died without producing true leaves if they were germinated and grown in low light (Figure 2-5E). This may have been due to impairment of the protochlorophyllide (Pchlide):protochlorophyllide oxidoreductase POR:NADPH complex in developing cotyledons. The dark-stable Pchlide:POR:NADPH complex is essential for proper greening of cotyledons (Armstrong et al., 1995). Upon exposure to light, the Pchlide is reduced and transformed into chlorophyll, however, when NADPH is unavailable, POR is degraded (Griffiths, 1991; Ryberg and Sundqvist, 1991). To test the hypothesis that increased PGR5-dependent CET sequestered electrons away from NADPH and therefore destabilizes the Pchlide:POR:NADPH complex, wild-type and AtOE plants were grown in complete darkness for four days and the levels of the Pchlide:POR:NADPH complex were measured with an antibody that recognizes POR. However, POR was not degraded in the AtOE plants as compared to wild-type plants (Figure 2-1E).

Constitutive over-expression of *AtPGR5* increases high light tolerance

Because a reduction in AtPGR5 reduced high light tolerance, we predicted that over-expression of *AtPGR5* would increase high light tolerance. To test this idea we grew *AtPGR5* over-expression lines in moderate light (200 $\mu E/m^2s$) for three weeks, and then shifted them to high light (2000 $\mu E/m^2s$). Within four days, the wild-type

leaves displayed dramatically increased levels of anthocyanin, while the over-expression lines retained their bright green color (Figure 2-5F), producing visible anthocyanin only in the underside of leaves (data not shown). Measurement of the foliar anthocyanin content confirmed that over-expression lines produced significantly less anthocyanin than wild-type plants (Figure 2-5F). After three weeks in high light, AtOE plants were still growing and primarily green while wild-type plants were shriveled and dead (Figure 2-5G). Wild-type plants maintained under moderate light during the three week incubation period remained lush (Figure 2-5G, center), indicating that the death of wild-type plants exposed to long-term high light is due to high light and not age-induced senescence.

AtPGR5:GUS expression is developmentally regulated in leaves

AtPGR5 is present in the chloroplasts of 4-5 week old wild-type plants grown in low intensity light (Munekage 2002), but little is known about the regulation of *AtPGR5* expression. In an effort to expand our understanding of AtPGR5's importance at different developmental stages, we examined the AtPGR5-driven expression of the beta-glucuronidase (GUS) reporter gene. *AtPGR5:GUS* was expressed in cotyledons during the seedling stage, as well as in all true leaves until at least two weeks after germination (Figure 2-7A, B). By four weeks after germination *AtPGR5:GUS* was only moderately expressed in emerging leaves and older leaves but its expression peaked in rapidly expanding and young, fully expanded rosette leaves (Figure 2-7C). In the emerging leaves, expression was initially concentrated at the apex and then it spread throughout the leaves as they expanded (Figure 2-7C). By the fifth or sixth week after

germination, *AtPGR5:GUS* expression had rapidly decreased in the oldest rosette leaves with the remaining expression concentrated at the base of the oldest leaves, and in the youngest leaves (Figure 2-7D). After the seventh week there was little or no *AtPGR5:GUS* expression in any rosette leaves (data not shown).

AtPGR5:GUS was also expressed in inflorescence stems. Visual inspection of whole plants stained for GUS revealed that *AtPGR5:GUS* expression was highest at the top of the stem (data not shown). GUS staining of transverse sections from the top two inches of inflorescence stems from six week old plants revealed expression in primary cortical cells and phloem cells in the vascular bundles (Figure 2-7E). After the induction of secondary vascular tissue in the hypocotyls by continually decapitating the floral stem (Lev-Yadun, 1994; Zhao et al., 2000), *AtPGR5:GUS* was expressed primarily in the secondary phloem (Figure 2-7F). Immunoblots of total protein from adult plants verified that *AtPGR5* is present in young emerging leaves, fully expanded leaves, older leaves, stems, and flowers, but not roots (data not shown).

AtPGR5:GUS **is expressed in etiolated seedlings and reproductive tissues**

Analysis of the *AtPGR5* regulatory region (-1100 to +1) with PLACE Signal Scan (Higo et al., 1999) indicates that it includes several putative regulatory motifs, such as pollen-, endosperm-, and etiolation-specific elements (Figure 2-8), which suggested that *AtPGR5* is expressed in non-photosynthetic tissues. Consistent with this, GUS staining indicated that *AtPGR5:GUS* was highly expressed in the cotyledons of dark-grown seedlings, as well as mature stigmas, anthers, and pollen (Figure 2-7A, I). Although there was slight expression in the siliques and ovary funicules, developing embryos did

not display *AtPGR5:GUS* expression (data not shown). However, *AtPGR5:GUS* was expressed in the hypocotyl and cotyledons as the embryos emerged from the seed coat (Figure 2-7G). It was also expressed in the aleurone cells associated with the seed coat after embryo emergence (Figure 2-7H).

***AtPGR5* is up-regulated by high light and other stresses that inhibit Calvin Cycle activity**

Under normal CO_2 concentrations, photoinhibition can occur upon exposure to high light intensities (Niyogi, 1999). Based on AtPGR5's proposed role in photoprotection, and the presence of numerous putative light regulatory elements in the *AtPGR5* promoter (Figure 2-8), we suspected that *AtPGR5* might be induced by high light. To test this theory, we exposed three week old wild-type plants to 2000 $\mu E/m^2s$ for up to 12 hours and performed northern analysis to visualize changes in *AtPGR5* expression. After one hour of high light *AtPGR5* expression increased over 2-fold and it retained this approximate level for at least 12 hours (Figure 2-9A).

Photoinhibition can also occur under moderate light if CO_2 assimilation is limited. Physiological stresses, such as wounding, cold, drought and excess exogenous sucrose decrease CO_2 fixation and lead to elevated NPQ to prevent photoinhibition (Niyogi, 1999; Savitch et al., 2001; Golding and Johnson, 2003; Chang et al., 2004). Because of AtPGR5's apparent role in photoprotection, it seemed likely that transcription of *AtPGR5* would rise in response to conditions that decrease CO_2 fixation. The fact that the *AtPGR5* promoter has wound-, cold-, drought-, and sucrose-responsive elements further supported this theory (Figure 2-8). Thus, we exposed wild-

type plants to these four conditions and analyzed changes in gene expression by GUS or northern analysis. GUS analysis of wounded leaves indicated that *AtPGR5:GUS* was up-regulated between one and 24 hours after wounding (Figure 2-7J). This up-regulation was not systemic; it was only seen at the wound site and in the surrounding veins. Northern analysis indicated that *AtPGR5* was also up-regulated four-fold after three days of chilling at $4^{o}C$ (Figure 2-9B). However, dehydration only caused a 23% increase in *AtPGR5* expression, and exogenous sucrose or glucose only decreased *AtPGR5* expression by approximately 24% (data not shown). The latter effects were reproducible, but due to their small size, it is difficult to gauge their biological significance.

Is PGR5 function conserved in a distantly related plant species?

PGR5 and NDH protein sequence (Wakasugi et al., 1994; Neyland and Urbatsch, 1996; Munekage et al., 2002) are conserved throughout the plant kingdom. A notable exception to this rule, however, is the apparent absence of *NDH* genes in all *Pinus* species that have been examined to date (Wakasugi et al., 1994; Neyland and Urbatsch, 1996). Although this data does not prove unequivocally that there is no chloroplast NDH activity in pine, it does suggest that this ancient and widely distributed genus has been an evolutionary success despite its probable lack of NDH-dependent CET. In this context, we were interested in determining if PGR5-dependent CET was likely to function in *Pinus*, and in particular if the putative *AtPGR5* loblolly pine homolog (*PtPGR5*) could be its functional equivalent. This possibility was supported by the degree of sequence conservation between the two proteins encoded by these genes;

outside of their chloroplast targeting sequences PtPGR5 (GenBank: BE656689) and AtPGR5 (AGI: At2g05620) are 76% identical to each other (Figure 1-2).

Constitutive over-expression of *PtPGR5* in *A. thaliana* (Figure 2-1F) caused changes in electron transport, growth, and development similar to those seen in the *AtPGR5* over-expression plants. When compared to wild-type plants, plants over-expressing *PtPGR5* displayed an increased P700 oxidation ratio and increased availability of PSI electron acceptors (Figure 2-2). *PtPGR5* over-expression lines (PtOE) also displayed increased plastoquinone reduction, decreased PSII quantum yield, and a corresponding decrease in PSII ETR (Figure 2-2). PtOE lines exhibited the same physical phenotypes as AtOE plants compared to wild-type plants: slower growth, lower chlorophyll content (no effect on POR content (Figure 2-1E)), dimpled leaves, poor tolerance to low light, and elevated tolerance of drought and high light (Figure 5). Northern analysis of RNA from *P. taeda* shoot apical meristems, primary vascular tissue, mature needles, phloem, xylem, and roots indicated that *PtPGR5* was most strongly expressed in mature needles and phloem, in a manner similar to that of *AtPGR5* in *A. thaliana* (Figure 2-7, Figure 2-10). *PtPGR5* was also expressed at a low level in the shoot apical meristem and in young stems, compared to needles and phloem (Figure 2-10). However, unlike *AtPGR5*, *PtPGR5* expression was not detected in pollen (data not shown). This disparity may reflect differences in angiosperm and pine pollen development; i.e. angiosperm pollen is mature at its release, while pine pollen is not mature until 12-15 months after pollination (Raven et al., 1992).

DISCUSSION

PGR5 mediates an alternative electron transport pathway with an essential role in photoprotection

Plants with decreased amounts of *AtPGR5* due to RNAi suppression exhibited decreased P700 oxidation, PQ reduction, PSII yield, and ETR, as well as decreased NPQ (Figure 2-2). This data supports previous studies, which indicate that AtPGR5 plays a role in CET and that PGR5-dependent CET is important for preventing damage to PSII (Munekage et al., 2002; Munekage et al., 2004). This phenotype also demonstrates that under high light conditions NDH cannot compensate for PGR5, as previously suggested (Munekage et al., 2004).

When we germinated *AtPGR5* RNAi plants under high light they grew more slowly than wild-type plants. This phenotype is the first line of physical evidence that PGR5-dependent CET is essential for normal growth in young plants, and may substantiate the proposed role of PGR5-dependent CET in ATP synthesis, independent of NDH. Previous studies have indicated that plants acclimated to high light (600-2000 $\mu E/m^2s$) have increased amounts of Calvin Cycle, PSII, and Cb_6/f complex proteins, and ATP synthase compared to those grown under low to moderate light (35-200 $\mu E/m^2s$) (for review see Walker, 2005). Synthesis of these and other proteins requires ATP, and, therefore, may explain the elevated ATP synthase activity (Anderson and Osmond, 1987) observed in plants grown under high light. Plants acclimated high light also exhibit increased oxygen evolution and CO_2 fixation activity compared to those grown under low to moderate light (Walker, 2005). Decreased CO_2 fixation in the presence of the PGR5-dependent CET inhibitor antimycin A (Furbank and Horton, 1987; Cornic et

al., 2000) indicate that CET is an important energy source for CO_2 fixation under high light. These data suggests that plants require more ATP for proper growth when acclimated to high light, and that RNAi plants may grow slower than wild-type plants because PGR5-dependent CET is an important source of ATP under high light.

The decreased growth of the RNAi plants could also be a photoprotective mechanism. Plants acclimated to high light produce thicker leaves with a smaller surface area (Walters and Horton, 1994; Pearcy, 1998; Bailey et al., 2004; Muller-Moule et al., 2004). These changes enable plants to minimize light exposure. It is possible that because PGR5-dependent CET mediates NPQ, RNAi plants grew slower then wild-type to minimize absorption of excess light, thereby preventing photoinhibition.

The timing of the *AtPGR5* RNAi phenotype under high light suggests that PGR5-dependent CET is most important when plants are young. This idea is supported by the fact that *AtPGR5* transcription is highest at this stage, and in young, expanding leaves. During *A. thaliana* leaf development, photosynthetic mechanisms decline early, even before leaves fully expand (Wingler et al., 2004). The decline in CO_2 assimilation along with a high chlorophyll content causes an imbalance between energy intake and utilization, which can lead to photodamage and photoinhibition (Badger, 1985; Wingler et al., 2004). To prevent photoinhibition, NPQ gradually increases during the life of the plants, and peaks during the beginning of senescence (Wingler et al., 2004). This process correlates with the expression patterns of *AtPGR5:GUS* and *PtPGR5*, and with the *AtPGR5* RNAi phenotype in young plants. Therefore, it appears that PGR5-dependent CET may be more important in younger leaf tissues because there is a significant need for photoprotection.

Decreased *AtPGR5* also led to visible photodamage and to the accumulation of ROS when *AtPGR5* RNAi plants were grown in an airtight environment under moderate light. Under these conditions, the atmospheric CO_2 concentration decreases over time and the Calvin Cycle is inhibited. In the absence of AtPGR5, this apparently led to over-reduction of the LET pathway and stromal redox carriers, and subsequently to the formation of ROS. Our data suggests, therefore, that PGR5-dependent CET prevents over-reduction of the stroma when the Calvin Cycle is suppressed by sequestering electrons from NADPH and reduced Fd, and inducing NPQ. Furthermore, these results, along with the observation that suppression of *AtPGR5* causes slowed growth when plants are grown under high light, suggest that PGR5-dependent CET provides an alternative electron transport pathway for excess light energy that is not compensated by NDH-dependent CET. The biochemical differences between *pgr5, ndh* and *crr* mutants support this theory. While disruption of NDH activity causes no change in LET or CET under normal growth conditions, reduced PGR5 activity causes alterations in CET, LET, and decreased NPQ (Munekage et al., 2002; Munekage et al., 2004). It is likely, therefore, that PGR5-dependent CET serves a role in preventing stroma over-reduction, and subsequent photoinhibition, independent of NDH-dependent CET.

Elevated PGR5 supports the role of PGR5-dependent CET in photoprotection

Constitutive over-expression of *PGR5* caused a domino effect in which there was an increase in electrons moving from PSI to Fd to PQ. We theorize that this increased CET activity led to an increase in Δph, which in turn elevated NPQ. Increased NPQ and prevention of stromal over-reduction enabled *PGR5* over-expression plants to survive

longer than wild-type under high light. It also allowed over-expression lines to tolerant excess light without the need for excessive anthocyanin, a pigment that protects mesophyll cells by absorbing excess light (Smillie and Hetherington, 1999; Feild et al., 2001; Steyn et al., 2002).

PGR5 over-expression plants also consistently survived longer than wild-type plants under drought conditions. Because the over-expression plants grew more slowly than wild-type plants they could have withstood dry conditions due to their relatively low transpiration rate. Therefore, we manipulated the plant growth conditions to produce wild-type and over-expression plants of equal size by shifting the plants to high, constant light before ceasing water application. The over-expression plants had a similar aerial mass to wild-type plants, but they still exhibited increased drought tolerance. Given PGR5's role in photoprotection under CO_2 limiting conditions, this result raises the possibility that drought-induced plant death is accelerated by stromal over-reduction and the consequent damage caused by ROS. Regardless of the under lying mechanism, both the drought and high light tolerance phenotypes of the over-expression plants warrant further study due to their potential agricultural utility.

Interestingly, although decreases and increases in AtPGR5 protein caused contrasting effects on P700+ oxidation and NPQ, they both caused decreased PSII yield, and ETR. This may have occurred because both a decrease and an increase in PGR5-dependent CET elevates PQ reduction, resulting in decreased PSII oxidation, and subsequently decreased PSII yield and ETR. However, elevated PGR5 caused direct reduction of PQ by Fd, resulting in increased P700 oxidation, and elevated ΔpH. In contrast, decreased AtPGR5 resulted in increased PQ reduction by inhibiting the

transfer of electrons from stromal reductants to PQ. This process prevented NPQ, and led to reduced P700 oxidation, which caused excess energy to accumulate within the LET.

Elevated PGR5-dependent CET competes with other metabolic activities essential for proper growth

The slow growth of the *AtPGR5* over-expression plants indicates that when PGR5-dependent CET was increased, it successfully diverted electrons from NADPH and Fd, and donated them to the PQ pool. Presumably, this diversion left little energy for CO_2 fixation, and accounts for their poor growth under moderate light. This may also explain why the over-expression plants produced dimpled, discolored leaves. Elevated PGR5-dependent CET may have caused localized alterations in energy acquisition and utilization such that patches of cells grew more then others, causing the appearance of dimple leaves.

PGR5 over-expression plants also displayed decreased chlorophyll content, raising the possibility that elevated PGR5-dependent CET also sequestered electrons away from enzymatic reactions involved in chlorophyll synthesis. Although NADPH-dependent protochlorophylide reductase does not appear to be effected by increased PGR5-dependent CET (Figure 2-1E) there are numerous other redox reactions required for chlorophyll production (von Wettstein et al., 1995) that may have been disrupted in these plants.

Under low light, the cotyledons of over-expression plants did not generate chlorophyll and many seedlings died without producing true leaves. It is likely,

therefore, that elevated PGR5-dependent CET strongly competed with other metabolic processes for the limited energized electrons, and that this caused seedlings to die prematurely. The complete lack of chlorophyll in the cotyledons of over-expression plants grown under moderate and low light may be due to the fact that the chloroplasts in cotyledons have less extensive thylakoid membranes than true leaves (Deng and Gruissem, 1987). Thus, the cotyledons may be particularly susceptible to the damage caused by elevated PGR5-dependent CET.

Specific developmental processes regulate PGR5-dependent CET activity

In addition to its expression in cotyledons and young leaves, *AtPGR5:GUS* was expressed in phloem, certain reproductive tissues, aleurone cells, and etiolated seedlings. The expression of a CET protein in several of these tissues is not wholly unexpected. For example, *AtPGR5* expression in the phloem is likely due to the presence of chloroplasts in sieve companion cells. Also, angiosperm stigma, filaments and anthers, especially, contain a variety of plastids, some of which are photosynthetically active (Clement et al., 1997; Clement and Pacini, 2001).

However, *AtPGR5:GUS* expression in etiolated seedlings mature pollen and aleurone cells is unexpected, as these tissues are not photosynthetically active (Jones, 1969; Griffiths, 1991; Ryberg and Sundqvist, 1991; Clement et al., 1997; Bethke et al., 2000; Clement and Pacini, 2001). Etiolated seedlings contain etioplasts, which can differentiate into chloroplasts upon light exposure. *AtPGR5* therefore, may be expressed in etioplasts in preparation for differentiation into chloroplasts, as suggested for PC, Lhcb (Cab) and ribulose-1,5-bisphosphate carboxylase (Brusslan and Tobin,

1992; Dijkwel et al., 1996). During pollen development, chloroplasts develop, de-differentiate into starch-producing amyloplasts, and then regenerate lamellar structures (Clement et al., 1997; Clement and Pacini, 2001). Therefore, the presence of photosynthetic proteins in pollen is not entirely unlikely. However, we are uncertain why the *AtPGR5* promoter is activated in aleurone cells, which contain only starch-bearing plastids (Jones, 1969).

AtPGR5 expression is induced by exposure to physiological stress

Abiotic stress can lead to photoinhibition by causing an imbalance in energy acquisition and utilization (Long et al., 1994; Niyogi, 1999; Savitch et al., 2001; Golding and Johnson, 2003; Chang et al., 2004). Studies have indicated that alterations in the chloroplast redox status caused by physioloigical stress regulate expression of both chloroplast and nuclear encoded genes whose products are involved in photoprotection, as well as other processes (Karpinski et al., 1997; Karpinski et al., 1999; Oswald et al., 2001; Rossel et al., 2002).

We found that *AtPGR5* expression is also regulated by abiotic stress. *AtPGR5* transcription was increased by exposure to high light. Under high light plants can accumulate excess light energy beyond that which is utilized by the Calvin Cycle, causing photoinhibition (Niyogi, 1999). It is likely that high light elevates PGR5-dependent CET, which triggers an increase in NPQ to prevent photodamage. We propose that high light-induced alterations in the chloroplast redox status may activate the transcription of *AtPGR5* to further increase PGR5-dependent CET.

There was a noticeable increase in *AtPGR5* expression when plants were wounded. This expression was concentrated around the wound site, especially in the veins (Figure 2-7J). When plants are wounded, numerous physiological and biochemical changes occur that lead to both decreased LET and CO_2 assimilation, as well as increased production of ROS (Karpinski et al., 1999; Fryer et al., 2003; Chang et al., 2004). Similar to high light, oxidative damage or alterations in the chloroplast redox status caused by wounding increases the expression of *AtPGR5*. These results indicate that AtPGR5 is part of a general stress response mechanism important for preventing photodamage.

AtPGR5 expression was also significantly affected by low temperature. When plants are exposed to low temperature, CO_2 fixation and photorespiration are decreased, (Hirotsu et al., 2004). Under these conditions plants are more susceptible to photodamage, and demonstrate increased xanthophyll cycle activity and NPQ (Krause et al., 1999; Tsonev and Hikosaka, 2003). Savitch et al (2001) observed that cold exposure of *A. thaliana* corresponds to decreased LET, but also to unaccountable increases in PQ reduction, the intersystem electron-donor pool, and the ATP:NADPH ratio. Although CET is a likely candidate for these effects, the authors observed an appreciable decrease in CET (Savitch et al., 2001). It should be noted, however, that in this study, only NDH-dependent CET was analyzed by measuring the $P700^+$ dark-re-reduction rate, which is typical of NDH activity. Cornic et al (2000) also observed that Fd-dependent CET induces ΔpH, and subsequently NPQ, at chilling temperatures in pea leaves (Cornic et al., 2000). Our observation that *AtPGR5* expression was

increased four-fold after exposure to low temperature, supports the idea that PGR5-dependent CET is the key mediator of NPQ under low temperature.

PGR5-dependent CET may compensate for NDH deficiency in *Pinus* species

AtPGR5 and *PtPGR5* have similar expression patterns in *A. thaliana* and *P. taeda* tissues. In addition, over-expression of *PtPGR5,* like over-expression of *AtPGR5*, caused elevated CET and NPQ, along with decreased growth, and alterations in development, chlorophyll content, and light tolerance. These data suggest that PGR5 is functionally conserved between these distantly related plant species. Although *PGR5* DNA sequences are present in almost all photosynthetic plants, including bryophytes, photosynthetic protists, green algae, and even cyanobacteria (Munekage et al., 2002), DNA sequences for all the NDH subunits have not been found in green and red alga, and certain protists (Odintsova and Yurina, 2003). Even more surprising, the plastid NDH subunits are all either completely lost or are truncated psuedogenes in *Pinus thunbergii,* (Wakasugi et al., 1994). Furthermore, using a well-conserved DNA sequence as a probe, researchers found NHDf sequences in all vascular divisions, including conifers, however, they found no evidence of the NDHf sequence in the three *Pinus* species analyzed; *Pinus glabra, Pinus taeda, and Pinus elliottii* (Neyland and Urbatsch, 1996). Assuming NDH is not present in pine, it is possible that PGR5-dependent CET alone can provide photoprotection and modulate the stromal redox state under stress conditions.

It appears that NDH may be more dispensable than PGR5 in photosynthetic organisms. This idea is consistent with our conclusions with the RNAi lines; namely that

PGR5 alone is important for photoprotection and redox control. This theory is also supported by the discovery that disruption of NDH activity has no effect on PSII electron transport, P700 oxidation or NPQ under normal growth conditions (Munekage et al., 2002; Munekage et al., 2004). In contrast, *pgr5* mutants exhibit decreases in all three aspects of photosynthesis.

Based upon the phenotypes of *PGR5* RNAi and over-expression plants and the analysis of *PGR5* expression in wild-type plants, we conclude that PGR5-dependent CET is essential not only to maintain the proper redox status when C_3 plants are exposed to high light, but also when plants are subjected to stress conditions. Although it appears that NDH-dependent CET can compensate for PGR5-dependent CET in maintaining the ATP:NADPH ratio for the Calvin Cycle (Munekage et al., 2004) under low light, we conclude that it cannot compensate for PGR5's role in photoprotection under stress conditions.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

For experiments performed on media, *Arabidopsis thaliana* (Columbia ecotype) seeds were surface sterilized (McKinney et al., 2001) and germinated on MS (Gibco/BRL) agar plates, pH adjusted to 5.7 with potassium hydroxide, containing 0.8% (wt/vol) Phytagar (Gibco/BRL), 1% sucrose, and 30μg/ml kanamycin, except where indicated. Seedlings germinated on media, as well as those germinated on soil, were grown under 150-200 $\mu E/m^2s$ with a 16 hour photoperiod at 23^oC, except where indicated.

Production of *AtPGR5:GUS*, over-expression and RNAi lines

AtPGR5:GUS Construct: Genomic DNA was isolated from *A. thaliana* using the EZDNA Plant DNA Miniprep Kit (OmegaBio-tek). 1.1kb of the *AtPGR5* regulatory region and the first 30bp of coding region (-1100 to +30) was PCR-amplified from *A. thaliana* genomic DNA using the following primers, which contained restriction enzyme sites for subcloning into binary vectors: 5' - ATTAGCAAGCTTATCTGTTAC AGATTATTACTGTACAATCCAGCA - 3' and 5' - TCGGGATCCTATTGCAGAAATCG AAGCA GCAG - 3'. The product was cloned into the pCRII-TOPO cloning vector using the TOPO TA Cloning Kit (Invitrogen), then subcloned into pBI101 (Clontech) upstream of the GUS reading frame to generate an *AtPGR5:GUS* translational fusion.

AtPGR5 RNA interference (Ri) construct: 200bp from the 5’ end of the *AtPGR5* cDNA (-100 to +100) were cloned into pCRII-TOPO in the sense orientation (primers: 5' - TACGGGTACCAAACTCA AAATCCAACCACACACAC - 3' and 5' - AACGACTAGT CATGGTTTGGTAATCTTCTCCGGAG - 3'). This sequence was also cloned into pCRII-TOPO in the antisense orientation (primers 5' - TAGTGGATCCCATGGTTTGGTAAT CTTCTCCGGAG - 3' and 5' - AACGTCTAGAGAGCTCAAACTCAAAATCCAACC ACACAC - 3'). The antisense and sense sequences were then ligated sequentially on either side of 1kb of GUS coding sequence via restriction enzyme sites in the PCRII-TOPO vector. The result was subcloned into pCAMBIA (CAMBIA) behind the constitutive cauliflower mosaic flower (CMV) 35S promoter.

35S:AtPGR5 (AtOE) and *35S:PtPGR5* (PtOE) constructs: Full-length *AtPGR5* cDNA was amplified from an *A. thaliana* leaf cDNA library using the following primers: 5' - GTACGGATCCAT GGCTGCTGCTTCGATTTCTGCAA - 3' and 5' - CACTGGATC

CGAGCTCCTAAGCAAGGAAACCAAGCCTCTCTCCAT - 3'. Using *DD55* (Warren and Covert, 2004) as the starting point, sequential, over-lapping PCR was performed with vector- and gene- specific primers on a *P. taeda* vascular cambium cDNA library. The full-length cDNA sequence of *PtPGR5* was then PCR-amplified using the following primers: 5' - TCGGGATCCATGGCTACAATTTC CAGCATA CCAGC - 3' and 5' - CGAGAGCTCCTACGCCAAGAACCCCAACTTTTC - 3'. Both sets of primers annealed to the 5' UTR and the 3' UTR of each gene, respectively, and contained restriction enzyme sites for subcloning. Full-length *AtPGR5* and *PtPGR5* was subcloned into pBIN19 (Bevan, 1984) behind the CMV 35S constitutive promoter to generate the over-expression constructs.

All transformation constructs, as well as corresponding control empty vectors, were transformed into *Esherichia coli* strain DH5α, sequenced, and transferred into *Agrobacterium tumefaciens* strain C58C1. Wild-type plants were transformed by *Agrobacterium*-mediated vacuum infiltration (An et al., 1996; Bechtold and Pelletier, 1998). For the empty vector controls, the *AtPGR5:GUS* fusion and all three over-expression and suppression lines displaying the expected 3:1 Kan^R:Kan^S ratio for a single insertion event, T3 or T4 transgenic plants were verified by GUS, northern, or western analyses. Five independent *AtPGR5* promoter:GUS fusion lines were analyzed, and expression patterns common to all lines were described. The three RNAi and over-expression lines with the lowest and highest *PGR5* expression levels, respectively, were self-crossed for selection of T4 homozygous plants. Both T3 and T4 plants from these lines were used to analyze plant phenotypes. Plant containing empty

vector controls were indistinguishable transcriptionally and physically from wild-type plants.

GUS Analyses

Young seedlings transformed with the *AtPGR5:GUS* gene construct were germinated on kanamycin selection medium, and when necessary, transferred to soil after two weeks. Plants were fixed and stained for GUS as previously described (An et al., 1996). To determine the *AtPGR5* expression pattern in etiolated seedlings, seeds were sown on MS medium with 1% sucrose, wrapped in aluminum foil and placed in a light-sealed container at 22^0 C in the dark for four days. The seedlings were then collected in a darkened room under a green safe light and placed directly in GUS staining solution (An et al., 1996). For wound analyses of *AtPGR5:GUS* expression, mature rosette leaves of five week old plants were gently crushed between the midvein and the leaf edge with padded forceps, then fixed and stained for GUS expression as described previously (An et al., 1996).

Stress Treatments

For light intensity treatments, seeds were germinated directly on soil and grown at 150 $\mu E/m^2s$ for three weeks, then shifted to 2000 $\mu E/m^2s$ for three weeks. For cold treatments, seeds were sown on soil for three weeks, then transferred to a 4° growth chamber for three days. Drought treatments for northern analysis were performed on wild-type plants according to a previous protocol (Yamaguchi-Shinozaki and Shinozaki, 1994). Briefly, seeds were sown on sterile SpectraMesh Nylon Filters (Spectrum) on

MS media containing 0 mM sucrose. After 10-14 days growth the filters were removed an allowed to dry for up to three hours. For the sucrose treatments, seeds were sown on sterile SpectraMesh Nylon Filters on MS medium containing 0 mM soluble carbohydrates, 100 mM sucrose, 100 mM glucose, or 100 mM mannitol. The plates were germinated and grown under constant light (150 $\mu E/m^2s$) for five days.

All plant materials were collected at the indicated intervals, plunged in liquid nitrogen, and stored at -80° until used in northern analyses. All treatments and RNA extractions were repeated at least twice.

Polyclonal Antibody Preparation and Western Analyses

The *AtPGR5* cDNA was subcloned into the pMALc2 vector (New England Biolabs) to generate the maltose-binding protein (MBP):PGR5 fusion protein construct pMBP:PGR5. pMBP:PGR5 was transformed into the RosettaBlue strain of *E. coli* (Novagen, Madison, WI) by electroporation, and induced with IPTG. After sonication and purification on an amylose resin column (New England Biolabs) the MBP-PGR5 fusion protein was concentrated with a YM-10 Centricon Centrifugal Filter (Millipore), and injected into rabbits to raise the AtPGR5 antibody. Western analysis of pre-immune and immune serum verified production of the AtPGR5 antibody in response to the fusion protein (data not shown).

For western analyses, *A. thaliana* total proteins were extracted in 1X running buffer (50 mM Tris, pH6.8, 1% SDS, 2.5% β mercaptoethanol, 7.5% glycerol, and bromophenol blue), boiled for five minutes, and centrifuged at 13,000 g for 10 minutes. The concentration of the supernatant was determined by the RC/DC Protein

Concentration Kit (Biorad). Total plant proteins were run on a 15% SDS PAGE gel and stained with coomassie blue. Equal loading of stained gels was verified by densitometry analyses using the ChemiImager Low Light Imaging system (Alpha Innotech CO). Protein was blotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech) using the Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Biorad). Blots were probed with the AtPGR5 rabbit serum using the ECL Plus Western Blotting Detection system (Amersham Pharmacia Biotech). POR western analyses were performed in a similar manner with the PORB antibody. Alterations in protein levels were quantified with ImageQuant software (Amersham Pharmacia Biotech).

RNA Preparation and Northern Analyses

A. thaliana and *P. taeda* RNA was extracted as previously described (Chang, 1993; Weigal and Glazebrook, 2002; Warren and Covert, 2004). Total RNA was run on a debaturing gel, blotted onto nylon membranes and probed with either an *AtPGR5*- or *PtPGR5*-specific DNA probe according to a previous protocol (Ausubel et al., 1998). To quantify alterations in transcript levels the membranes were exposed to a phosphor screen. The signal was analyzed with a STORM phosphoimager and quantified with ImageQuant software (Amersham Pharmacia Biotech).

Fluorescence and P700 Absorbance Measurements

Chlorophyll fluorescence parameters were measured with a Diving PAM Underwater Fluorometer (Walz) by placing the probe on top of attached leaves. F_o (minimum fluorescence yield at the open PSII center) was determined at a light intensity

of 5 $\mu E/m^2 s$. F_m (maximum fluorescence yield of the closed PSII center in the dark) and $F_{m'}$ (maximum fluorescence yield of the closed PSII center under actinic light) were determined under saturating pulses (100ms) of white light. F_s (steady state fluorescence yield) was determined under actinic light intensities of 1-1000 $\mu E/m^2 s$. The efficiency of PSII photochemistry (ϕ) was calculated as $(F_m - F_o)/F_m$. NPQ was calculated as $(F_m - F_{m'})/F_{m'}$. ϕ_{PSII} was calculated as (Fm'-Fs)/Fm'. ETR was calculated as ϕ_{PSII} x light intensity X 0.5 X 0.85. qP was calculated as $(F_{m'} - F_s)/(F_{m'} - F_o)$. PQ reduction was determined as 1-qP. P700 absorbance change was measured in the reflective mode using a PAM101/102 unit with a dual-wavelength emitter-detector unit ED-P700/DW (Walz) as previously described (Munekage et al., 2002). n=2-5 for each set of measurements, and measurements were taken on three different sets of plants with consistent results.

Measurement of Chlorophyll and Anthocyanin Content

Mature leaves of three week old plants grown at 150 $\mu E/m^2 s$ were collected and ground to powder in liquid nitrogen under low light. The powder was resuspended in either 80% acetone (for chlorophyll content), or 1% HCl in methanol (for anthocyanin content) on ice, and centrifuged at 10,000g at 4°C for five minutes. The chlorophyll concentrations were determined from spectroscopy absorbance measurements at 663.2 nm, 646.8 nm, and 470 nm according to calculations from a previous protocol (Lichtenthaler, 1987). Anthocyanin content was determine from spectroscopy measurements taken at 530 nm and 653 nm as previously described (Gould et al., 2000).

Detection of reactive oxygen species

To measure O_2^- production, plants were germinated and grown for two to three weeks on MS medium in sealed GA7 vessels (Magenta Corp.). As wounding also induces production of ROS, whole plants were gently removed from the medium, and immersed in a solution containing 0.025% nitro blue tetrazolium salt (NBT (Sigma)), and 0.05% Tween 20 in potassium phosphate buffer, pH 7.0. (Cuevas et al., 2004). The plants were vacuum infiltrated for five minutes, and illuminated until a dark blue formazan precipitate was visible in the leaves. The plants were bleached in 95% ethanol overnight to enhance visualization of formazan precipitate.

ACKNOWLEDGEMENTS

We thank Dr. Richard Meagher and Elizabeth McKinney for provision of plant binary vectors, the *A. thaliana* cDNA library, and helpful suggestions, Yolanda Lay for technical assistance, Dr. Jeff Dean and John Barnes for provision of *P. taeda* vascular cambium cDNA library, Dr. Greg Armstrong for provision of PORB antibody, and Dr. Joseph Nairn for helpful suggestions. This work was supported by a University of Georgia Graduate School Fellowship, The State of Georgia's Traditional Industries Program for Pulp and Paper (TIP3), NIH training grant GM07103, NSF Graduate Research Fellowship DGE-9616054, and NSF grant NSFOCE-013-7007.

LITERATURE CITED

Allen, J. (2002). Photosynthesis of ATP-electrons, proton pumps, rotors, and poise. Cell **110,** 273-276.

An, Y.-Q., Huang, S., McDowell, J.M., McKinney, E.C., and Meagher, R.B. (1996). Conserved expression of the Arabidopsis *ACT1* and *ACT3* actin subclass in organ primordia and mature Pollen. Plant Cell **8,** 15-30.

Anderson, J.M., and Osmond, C.B. (1987). Shade-sub responses: compromises between acclimation and photoinhibition. In Photoinhibition, D.J. Kyle, C.B. Osmond, and C.J. Arntzen, eds (Amsterdam: Elsevier), pp. 1-38.

Armstrong, G.A., Runge, S., Frick, G., Sperling, U., and Apel, K. (1995). Identification of NADPH:protochlorophyllide oxidoreductases A and B: a branched pathway for light-dependent chlorophyll biosynthesis in Arabidopsis thaliana. Plant Physiol **108,** 1505-1517.

Arnon, D.I., Allen, M.B., and Whatley, F.R. (1954). Photosynthesis by isolated chloroplasts. Nature **174,** 394-396.

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (1998). Current protocols in molecular biology. (New York, N.Y: John Wiley and Sons Inc.).

Badger, M.R. (1985). Photosynthetic oxygen exchange. Annual Review of Plant Physiology **36,** 27-53.

Bailey, S., Horton, P., and Walters, R. (2004). Acclimation of *Arabidopsis thaliana* to the light environment: the relationship between photosynthetic function and chloroplast composition. Planta **218,** 793-802.

Baker, S.S., Wilhelm, K.S., and Thomashow, M.F. (1994). The 5'-region of Arabidopsis thaliana cor15a has cis-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol **24,** 701-713.

Bate, N., and Twell, D. (1998). Functional architecture of a plant pollen promoter: pollen-specific transription is developmentally regulated by multiple stage-specific and co-dependent activator elements. Plant Mol Biol **37,** 859-869.

Bechtold, N., and Pelletier, G. (1998). In planta Agrobacterium-mediated transformation of adult *Arabidopsis thaliana* plants by vacuum infiltration. Methods Mol Biol **82,** 259-266.

Bendall, D.S., and Manasse, R.S. (1995). Cyclic photophosphorylation and electron transport. Biochimica Et Biophysica Acta-Bioenergetics **1229,** 23-38.

Bethke, P.C., Jacobsen, J.V., and Jones, R.L. (2000). Barley biotechnology. In: Seed Biotechnology,. (Sheffield Press).

Bevan, M. (1984). Binary Agrobacterium vectors for plant transformation. Nucleic Acids Research **12,** 8711-8721.

Brusslan, J.A., and Tobin, E.M. (1992). Light-independent developmental regulation of *cab* gene epxression in *Arabidopsis thaliana*. Proc Natl Acad Sci U S A **89,** 7791-7795.

Bukhov, N., and Carpentier, R. (2004). Alternative Photosystem I-driven electron transport routes: mechanisms and functions. Photosynthesis Research **82,** 17-33.

Burrows, P.A., Sazanov, L.A., Svab, Z., Maliga, P., and Nixon, P.J. (1998). Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndhgenes. The EMBO Journal **17,** 868-876.

Busk, P.K., Jensen, A.B., and Pages, M. (1997). Regulatory elements in vivo in the promoter of the abscisic acid responsive gene *rab17* from maize. Plant J **11,** 1285-1295.

Chang, C.C.C., Ball, L., Fryer, M.J., Baker, N.R., Karpinski, S., and Mullineaux, P.M. (2004). Induction of ASCORBATE PEROXIDASE 2 expression in wounded Arabidopsis leaves does not involve known wound-signalling pathways but is associated with changes in photosynthesis. Plant Journal **38,** 499-511.

Chang, S., Puryear, J., Cairney, J. (1993). A simple and effective method for isolating RNA from pine trees. Plant Molecular Biology **11,** 113-116.

Clement, C., and Pacini, E. (2001). Anther plastids in angiosperms. Botanical Review **67,** 54-73.

Clement, C., Mischler, P., Burrus, M., and Audran, J.C. (1997). Characteristics of the photosynthetic apparatus and CO_2 fixation in the flower bud of Lilium. II. Anther. International Journal of Plant Sciences **158,** 801-810.

Cornic, G., Bukhov, N.G., Wiese, C., Bligny, R., and Heber, U. (2000). Flexible coupling between light-dependent electron and vectorial proton transport in illuminated leaves of C_3 plants. Role of photosystem I-dependent proton pumping. Planta **210,** 468-477.

Cuevas, J.C., Diego, S.H., Marina, M., and Ruiz, O. (2004). Do polyamines modulate the *Lotus glaber* NADPH oxidation activiy induced by the herbicide methyl viologen. Functional Plant Biology **31,** 921-928.

Degenhardt, J., and Tobin, E.M. (1996). A DNA binding activity for one of two closely defined phytochrome regulatory elements in an *Lhcb* promoter is more abundant in etiolated than in green plants. Plant Cell **8,** 31-41.

Demmig-Adams, B., and Adams, W.W. (1992). Photoprotection and other responses of plants to high light stress. Annual Review of Plant Physiology and Plant Molecular Biology **43,** 599-626.

Deng, X.-W., and Gruissem, W. (1987). Control of plastid gene expression during development: the limited role of transcriptional regulation. Cell **49,** 379-387.

Dijkwel, P.P., Kock, P.A.M., Bezemer, R., Weisbeek, P.J., and Smeekens, S.C.M. (1996). Sucrose represses the developmentally controlled transient activation of the plastocyanin gene in *Arabidopsis thaliana* seedlings. Plant Physiology **110,** 455-463.

Dubouzet, J.G., Sakuma, Y., Ito, Y., Kasuga, M., Dubouzet, E.G., Miura, S., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). *OsDREB* genes in rice, *Oryza sativa* L., encode transcription activators that function in drought-, high-salt- and cold-responsive gene expression. Plant Journal **23,** 751-763.

Dunn, M.A., White, A.J., Vural, S., and Hughes, M.A. (1998). Identification of promoter elements in a low-temperature-responsive gene (*blt4.9*) from barley (*Hordeum vulgare* L.). Plant Mol Biol **38,** 551-564.

Endo, T., Shikanai, T., Sato, F., and Asada, K. (1998). NAD(P)H dehydrogenase-dependent, antimycin A-sensitive electron donation to plastoquinone in tobacco chloroplasts. Plant and Cell Physiology **39,** 1226-1231.

Feild, T.S., Lee, D.W., and Holbrook, N.M. (2001). Why leaves turn red in autumn. The role of anthocyanins in senescing leaves of red-osier dogwood. Plant Physiol **127,** 566-574.

Fryer, M.J., Ball, L., Oxborough, K., Karpinski, S., Mullineaux, P.M., and Baker, N.R. (2003). Control of Ascorbate Peroxidase 2 expression by hydrogen peroxide and leaf water status during excess light stress reveals a functional organisation of Arabidopsis leaves. Plant Journal **33,** 691-705.

Furbank, R.T., and Horton, P. (1987). Regulation of photosynthesis in isolated barley protoplasts: the contribution of cyclic photophosphorylation. Biochem Biophys Acta **894**.

Gilmartin, P.M., Saroki, L., Memelink, J., and Chua, N.-H. (1990). Molecular light switches for plant genes. Plant Cell **2,** 369-378.

Golding, A.J., and Johnson, G.N. (2003). Down-regulation of linear and activation of cyclic electron transport during drought. Planta **218,** 107-114.

Gould, K.S., Markham, K.R., Smith, R.H., and Goris, J.J. (2000). Functional role of anthocyanins in the leaves of *Quintinia serrata* A. Cunn. J Exp Bot **51,** 1107-1115.

Griffiths, W. (1991). Protochlorophyllide photoreduction. In Chlorophylls, H. Sheer, ed (Boca Raton: CRC Press), pp. 433-449.

Herbert, S.K., Fork, D.C., and Malkin, S. (1990). Photoacoustic measurements *In vivo* of energy-storage by cyclic electron flow in algae and higher plants. Plant Physiology **94,** 926-934.

Higo, K., Ugawa, Y., Iwamoto, M., and Korenaga, T. (1999). Plant cis-acting regulatory DNA elements (PLACE) database: 1999. Nucl. Acids Res. **27,** 297-300.

Hirotsu, N., Makino, A., Ushio, A., and Mae, T. (2004). Changes in the thermal Dissipation and electron flow in the water-water cycle in rice grown under conditions of physiologically low temperature. Planta **45,** 635-644.

Horvath, E.M., Peter, S.O., Joet, T., Rumeau, D., Cournac, L., Horvath, G.V., Kavanagh, T.A., Schafer, C., Peltier, G., and Medgyesy, P. (2000). Targeted inactivation of the plastid *ndhB* Gene in tobacco results in an enhanced sensitivity of photosynthesis to moderate stomatal closure. Plant Physiology **123,** 13378-11349.

Jeanjean, R., Matthijs, H.C.P., Onana, B., Havaux, M., and Joset, F. (1993). Exposure of the cyanobacterium Synechocystis Pcc6803 to salt stress induces concerted changes in respiration and photosynthesis. Plant and Cell Physiology **34,** 1073-1079.

Joet, T., Cournac, L., Peltier, G., and Havaux, M. (2002). Cyclic electron flow around photosystem I in C-3 plants. In vivo control by the redox state of chloroplasts and involvement of the NADH-dehydrogenase complex. Plant Physiology **128,** 760-769.

Joet, T., Cournac, L., Horvath, E.M., Medgyesy, P., and Peltier, G. (2001). Increased sensitivity of photosynthesis to antimycin A induced by inactivation of the chloroplast *ndhB* gene. Evidence for a participation of the NADH-dehydrogenase

complex to cyclic electron flow around photosystem I. Plant Physiology **125,** 1919-1929.

Johnson, G.N. (2005). Cyclic Electron Transport in C_3 plants: fact or artefect. Journal of Experimental Botany **56,** 407-416.

Jones, R.L. (1969). The fine structure of barley aleurone cells. Planta **85,** 359–374.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G., and Mullineaux, P.M. (1997). Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9,** 627-640.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., and Mullineaux, P. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science **284,** 654-657.

Krause, G.H., Carouge, N., and Garden, H. (1999). Long-term effects of temperature shifts on xanthophyll cycle and photoinhibition in spinach (*Spinacia oleracea*). Australian Journal of Plant Physiology **26,** 125-134.

Leegood, R.C., Crowther, D., Walker, D.A., and Hind, G. (1981). Photosynthetic electron transport in the bundle sheath of maize. Febs Letters **126,** 89-92.

Lev-Yadun, S. (1994). Induction of sclereid differentiation in the pith of *Arabidopsis thaliana* (L) Heynh. Journal of Experimental Botany **45,** 1845-1849.

Lichtenthaler. (1987). Chlorophylls and caratenoids: pigments of photosynthetic biomembranes. In Methods in Enzymology, S.P. Colowick and N.O. Kaplan, eds (Harcourt Brace Janovich), pp. 350-382.

Long, S.P., Humphries, S., and Falkowski, P.G. (1994). Photoinhibition of photosynthesis in nature. Annual Review of Plant Physiology and Plant Molecular Biology **45,** 633-662.

McKinney, E.C., Kandasamy, M.K., and Meagher, R.B. (2001). Small changes in the regulation of one Arabidopsis profilin isovariant, PRF1, alter seedling development. Plant Cell **13,** 1179-1191.

Mi, H.L., Endo, T., Ogawa, T., and Asada, K. (1995). Thylakoid membrane-bound, NADPH-specific pyridine-nucleotide dehydrogenase complex mediates cyclic electron transport in the cyanobacterium Synechocystis Sp Pcc-68038. Plant and Cell Physiology **36,** 661-668.

Morita, A., Umemura, T., Kuroyanagi, M., Futsuhara, Y., Perata, P., and Yamaguchi, J. (1998). Functional dissection of a sugar-repressed alpha-amylase gene. FEBS Lett **423,** 81-85.

Muller, P., Li, X.P., and Niyogi, K.K. (2001). Non-photochemical quenching. A response to excess light energy. Plant Physiology **125,** 1558-1566.

Muller-Moule, P., Golan, T., and Niyogi, K.K. (2004). Ascorbate-deficient mutants of Arabidopsis grow in high light despite chronic photooxidative stress. Plant Physiol. **134,** 1163-1172.

Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., and Shikanai, T. (2002). PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell **110,** 361-371.

Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K., Endo, T., Tasaka, M., and Shikanai, T. (2004). Cyclic electron flow around photosystem I is essential for photosynthesis. Nature **429,** 579-582.

Neyland, R., and Urbatsch, L.E. (1996). The *ndhf* chloroplast gene detected in all vasular plant divisions. Planta **200,** 273-277.

Niyogi, K.K. (1999). Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 333-359.

Odintsova, M.S., and Yurina, N.P. (2003). Plastid genomes of higher plants and algae: structure and functions. Molecular Biology **37,** 649-662.

Oswald, O., Martin, T., Dominy, P.J., and Graham, I.A. (2001). Plastid redox state and sugars: Interactive regulators of nuclear-encoded photosynthetic gene expression. Proceedings of the National Academy of Sciences of the United States of America **98,** 2047-2052.

Pearcy, R.W. (1998). Acclimation to sun and shade. In Photosynthesis: A Comprehensice Treatise, A. Raghavendra, ed (Cambridge: Cambride University Press), pp. 250-263.

Raven, P.H., F., E.R., and Eichhorn, S.E. (1992). Biology of plants. (New York: Worth Publishers).

Ravenel, J., Peltier, G., and Havaux, M. (1994). The cyclic electron pathways around Photosystem-I in *Chlamydomonas-Reinhardtii* as determined *in-vivo* by photoacoustic measurements of energy storage. Planta **193,** 251-259.

Rogers, H.J., Bate, N., Combe, J., Sullivan, J., Sweetman, J., Swan, C., Lonsdale, D.M., and Twell, D. (2001). Functional analysis of cis-regulatory elements within the promoter of the tobacco pollen gene *g10*. Plant Mol Biol **45,** 577-585.

Rossel, J.B., Wilson, I.W., and Pogson, B.J. (2002). Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol. **130,** 1109-1120.

Rushton, P., Torres, J.T., Parniske, M., Wernert, P., Hahlbrock, K., and Somssich, I.E. (1996). Interaction of elicitor-induced DNA-binding proteins with elicitor response elements in the promoters of parsley PR1 genes. Embo Journal **15,** 5690-5700.

Ryberg, M., and Sundqvist, C. (1991). Structural and functional significance of pigment-protein complexes of chlorophyll precursors. In Chlorophylls, H. Shees, ed (Boca Raton: CRC PRess), pp. 587-612.

Savitch, L.V., Barker-Astrom, J., Ivanov, A.G., Hurry, V., Oquist, G., Huner, N.P., and Gardestrom, P. (2001). Cold acclimation of *Arabidopsis thaliana* results in incomplete recovery of photosynthetic capacity, associated with an increased reduction of the chloroplast stroma. Planta **214,** 295-303.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998a). The plastid *ndh* genes code for an NADH-specific dehydrogenase: Isolation of a complex I analogue from pea†thylakoid†membranes. PNAS **95,** 1319-1324.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998b). The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. FEBS Lett **429,** 115-118.

Schreiber, U., Klughammer, C., and Neubauer, C. (1988). Measuring P700 absorbance changes around 830 nm with a new tyoe of pulse modulation system. Z. Naturforsch **43C,** 686-698.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998). Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proceedings of the National Academy of Sciences of the United States of America **95,** 9705-9709.

Simpson, S.D., Nakashima, K., Narusaka, Y., Seki, M., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2003). Two different novel cis-acting elements of erd1, a clpA homologous Arabidopsis gene function in induction by dehydration stress and dark-induced senescence. Plant Journal **33,** 259-270.

Smillie, R.M., and Hetherington, S.E. (1999). Photoabatement by anthocyanin shields photosynthetic systems from light stress. Photosynthetica **36,** 451-463.

Steyn, W.J., Wand, S.J.E., Holcroft, D.M., and Jacobs, G. (2002). Anthocyanins in vegetative tissues: a proposed unified function in photoprotection. New Phytologist **155,** 349-361.

Terzaghi, W.B., and Cashmore, A.R. (1995). Light-regulated transcription. Annu rev Plant Physiol Plant Mol Biol **46**.

Tsonev, T.D., and Hikosaka, K. (2003). Contribution of photosynthetic electron transport, heat dissipation, and recovery of photoinactivated photosystem II to photoprotection at different temperatures in Chenopodium album leaves. Plant and Cell Physiology **44,** 828-835.

von Wettstein, D., Gough, S., and Kannangara, C.G. (1995). Chlorophyll Biosynthesis. Plant Cell **7,** 1039-1057.

Wakasugi, T., Tsudzuki, J., Ito, S., Nakashima, K., Tsudzuki, T., and Sugiura, M. (1994). Loss of all *ndh* genes as determined by sequencing the entire chloroplats genome of the black pine *Pinus thunbergii*. Proc Natl Acad Sci U S A **91,** 9794-9798.

Walker, R.G. (2005). Towards an understanding of photosynthetic acclimation. Journal of Experimental Botany **56,** 435-447.

Walters, R.G., and Horton, P. (1994). Acclimation of *Arabidopsis thaliana* to the light environment: changes in composition of the photosynthetic apparatus. Planta **195,** 248-256.

Warren, J.A., and Covert, S.F. (2004). Differential expression of pine and *Cronartium quercuum f. sp fusiforme* genes in fusiform rust galls. Applied and Environmental Microbiology **70,** 441-451.

Weigal, B., and Glazebrook, J. (2002). Arabidopsis, a laboratory manual. (Cold Spring Harbor: Cold Spring Harbor Press).

Wingler, A., Mares, M., and Pourtau, N. (2004). Spatial patterns and metabolic regulation of photosynthetic parameters during leaf senescence. New Phytologist **161,** 781-789.

Wu, C., Washida, H., Onodera, Y., Harada, K., and Takaiwa, F. (2000). Quantitative nature of the Prolamin-box, ACGT and AACA motifs in a rice glutelin gene promoter: minimal cis-element requirements for endosperm-specific gene expression. Plant Journal **23,** 415-421.

Yamaguchi-Shinozaki, K., and Shinozaki, K. (1994). A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell **6,** 251-264.

Zhao, C., Johnson, B.J., Kositsup, B., and Beers, E.P. (2000). Exploiting secondary growth in Arabidopsis. construction of xylem and bark cDNA libraries and cloning of three xylem endopeptidases. Plant Physiol. **123,** 1185-1196.

Figure 2-1. *PGR5* expression in wild-type (WT) and mutant *A. thaliana* leaves. A. Northern blot of WT and two *AtPGR5* RNAi mutants, Ri5 and Ri9. B. Immunodetection of AtPGR5 in WT, Ri5 and Ri9 plants. C. Northern blot of WT and *AtPGR5* over-expression mutant, AtOE20 plants. D. Immunodetection of AtPGR5 in WT, and AtOE20. E. Immunodetection of POR in four day old etiolated WT, AtOE20, and PtOE17 seedlings. F. Northern blot of WT and *PtPGR5* over-expression mutant, PtOE17. Northern blots were probed with *AtPGR5* (A and C) or *PtPGR5* (F). The bottom panels in A, C, and F show ethidium bromide staining of the corresponding RNA gel. The bottom panels in B, D, and E show coomassie blue staining of a corresponding protein gel. Except for E, which was probed with a POR antibody, immunoblots were probed with an *AtPGR5* antibody.

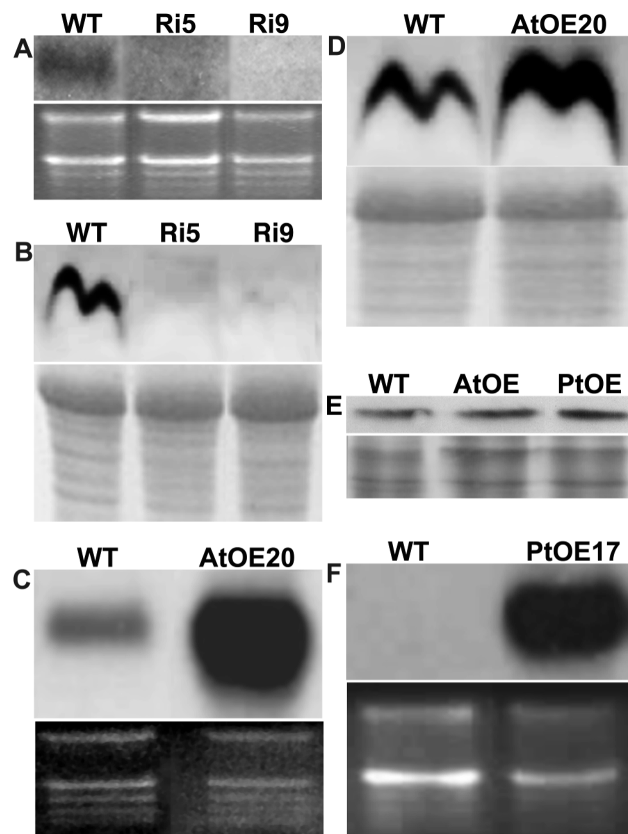

A
WT
Ri5
Ri9
B
WT
Ri5
Ri9
C
WT
AtOE20
D
WT
AtOE20
E
WT
AtOE
PtOE
F
WT
PtOE17

Figure 2-2. Electron transport analysis in 4 week old *A. thaliana* wild-type (WT), RNAi (Ri5), and over-expression mutants (AtOE20, and PtOE17). A. P700 oxidation ratio at 270 $\mu E/m^2 s$ (A/Amax), and limitation of PSI electron acceptors ($Amax_{AL}/Amax_{FR}$). B. Reduction of plastoquinone pool. C. PSII yield (ϕ_{PSII}). D. Electron transport rate (ETR) through PSII. E. Nonphotochemical quenching (NPQ) of chlorophyll fluorescence. Means $\pm$ SE (n = 2-5).

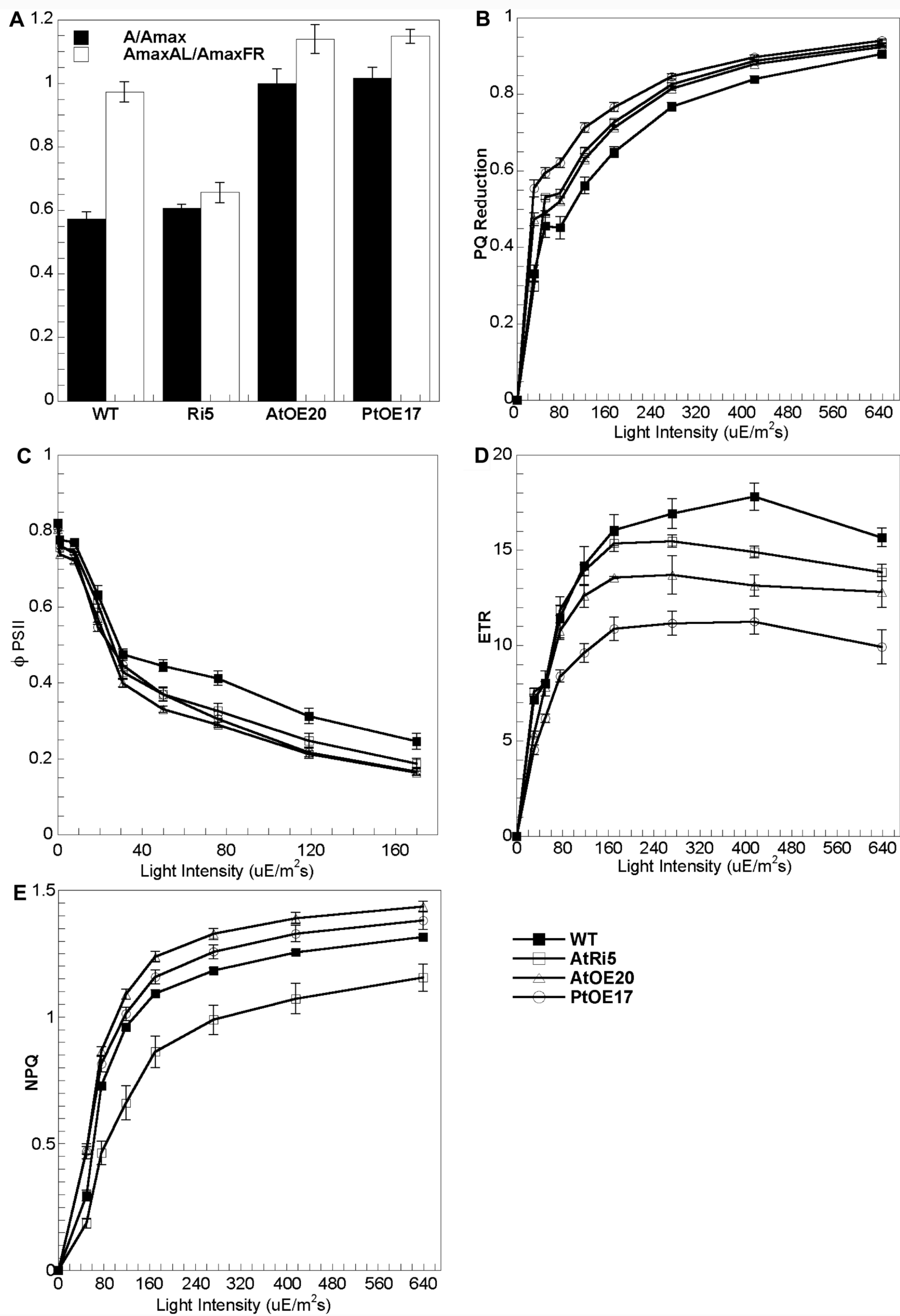

A
A/Amax
AmaxAL/AmaxFR
WT
Ri5
AtOE20
PtOE17
B
PQ Reduction
Light Intensity (uE/m2s)
C
ϕ PSII
Light Intensity (uE/m2s)
D
ETR
Light Intensity (uE/m2s)
E
NPQ
Light Intensity (uE/m2s)
WT
AtRi5
AtOE20
PtOE17

Figure 2-3. Visible phenotypes of *AtPGR5* RNAi mutants. A. Two week old wild-type (WT) and *AtPGR5* RNAi (Ri5) plants germinated and grown under high intensity light (2000 $\mu E/m^2s$). B. Leaves from 2 week old WT and Ri5 plants grown in sealed GA7 containers under moderate intensity light (150 $\mu E/m^2s$). C. Leaves from B treated with NBT to detect O_2^-.

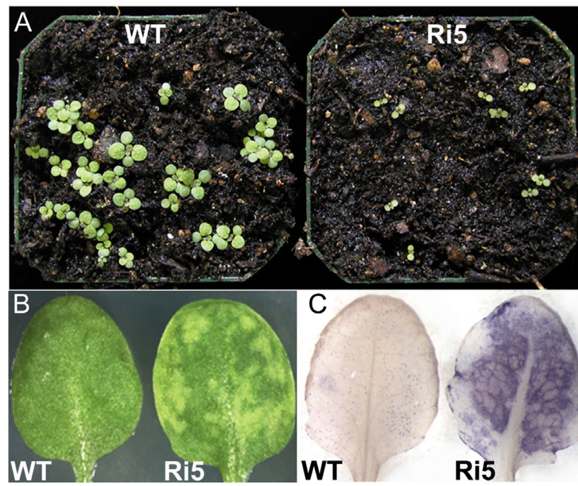

A
WT
Ri5
B
WT
Ri5
C
WT
Ri5

Figure 2-4. Average weight of wild-type (WT) plants, and *AtPGR5* RNAi mutants, Ri5 and Ri9, grown under high intensity light (2000 $\mu E/m^2 s$). Means $\pm$ SE (n=10).

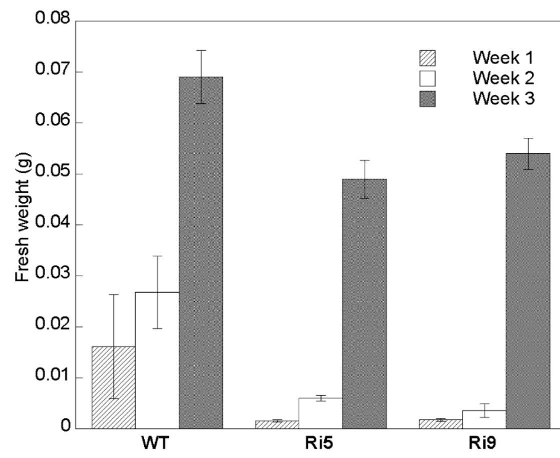

0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
Fresh weight (g)
Week 1
Week 2
Week 3
WT
Ri5
Ri9

Figure 2-5. Visible phenotypes of *AtPGR5* and *PtPGR5* over-expression mutants in *A. thaliana*. A. One week old, and B. 2 week old wild-type (WT) and OE plants grown on soil under moderate light (150 $\mu E/m^2 s$). C. Leaf surfaces of mature leaves from 4 week old plants. D. Six week old plants three weeks after cessation of water application and growth at constant high light (2000 light $\mu E/m^2 s$) to induce bolting and equal growth of WT and OE plants. E. Two week old plants germinated and grown in low light (50 $\mu E/m^2 s$). F. Four week old plants grown in moderate light, then shifted to high light (2000 $\mu E/m^2 s$) for four days. G. Left, three week old plants grown in moderate light. Center, seven week old WT plants grown in moderate light. Right, seven week old plants from left after a shift to very high light for 3 weeks. In all panels the over-expression plants (OE) are representative of both *AtPGR5* and *PtPGR5* over-expression mutants.

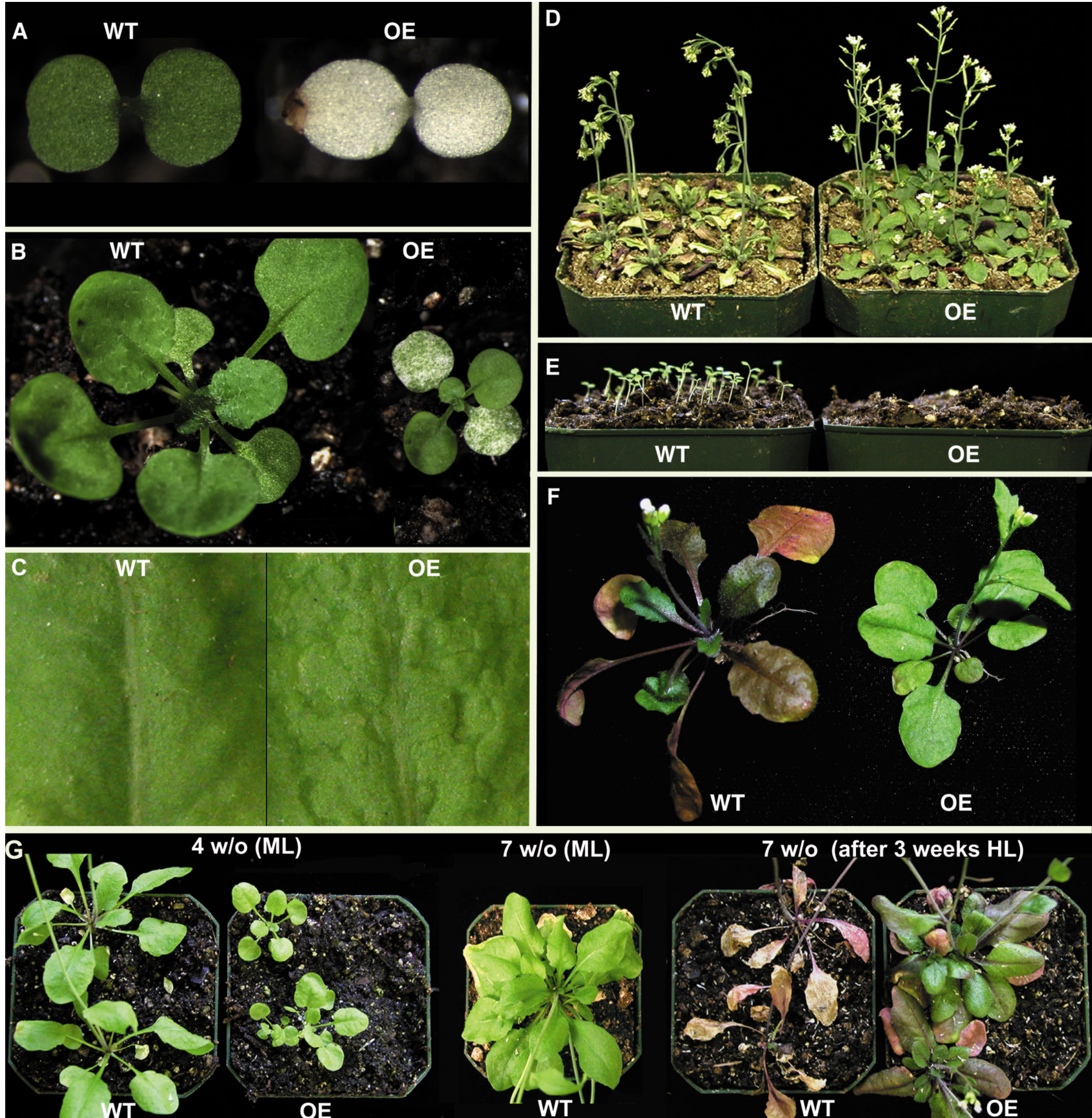

A
WT
OE
B
WT
OE
C
WT
OE
D
WT
OE
E
WT
OE
F
WT
OE
G
4 w/o (ML)
WT
OE
7 w/o (ML)
WT
7 w/o (after 3 weeks HL)
WT
OE

Figure 2-6. Growth and pigment content of 3 – 4 week old wild-type (WT) and *AtPGR5* (AtOE) and *PtPGR5* (PtOE) over-expression mutants. A. Leaf area and chlorophyll (chlor) content of plants grown under moderate light (150 $\mu E/m^2 s$). Means $\pm$ SE (n=4-20). B. Anthocyanin (Antho.) content under moderate light (-) and after four days exposure to high light (2000 $\mu E/m^2 s$) (+). Means $\pm$ SE (n=4).

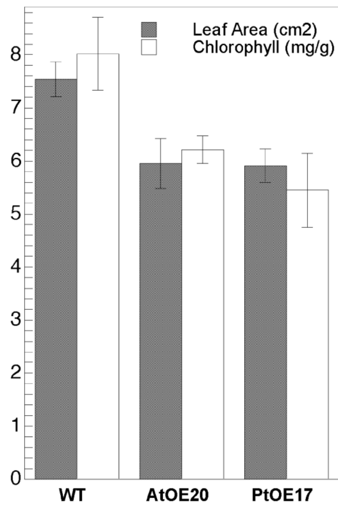
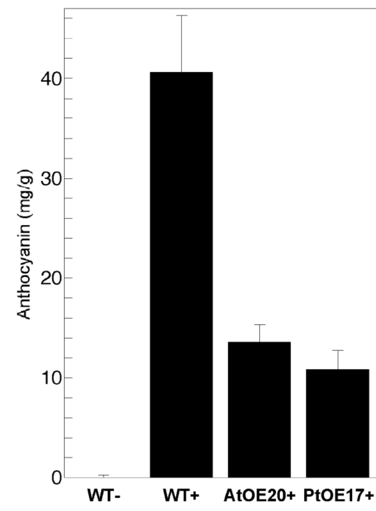

Leaf Area (cm2)
Chlorophyll (mg/g)
8
7
6
5
4
3
2
1
0
WT
AtOE20
PtOE17

Anthocyanin (mg/g)
40
30
20
10
0
WT-
WT+
AtOE20+
PtOE17+

Figure 2-7. *AtPGR5:GUS* expression pattern. A. Four day old seedlings grown under no light, and constant light. B. Two week old whole plant. C. Four week old whole plant. D. Five week old whole plant. E. Cross-section of stem primary vascular tissue. F. Cross-section of stem secondary vascular tissue. G. Germinating seed. H. Seed coat remaining after embryo emergence. I. Floral tissues. J. Fully expanded leaves from five week old plants at three time points post wounding (PW).

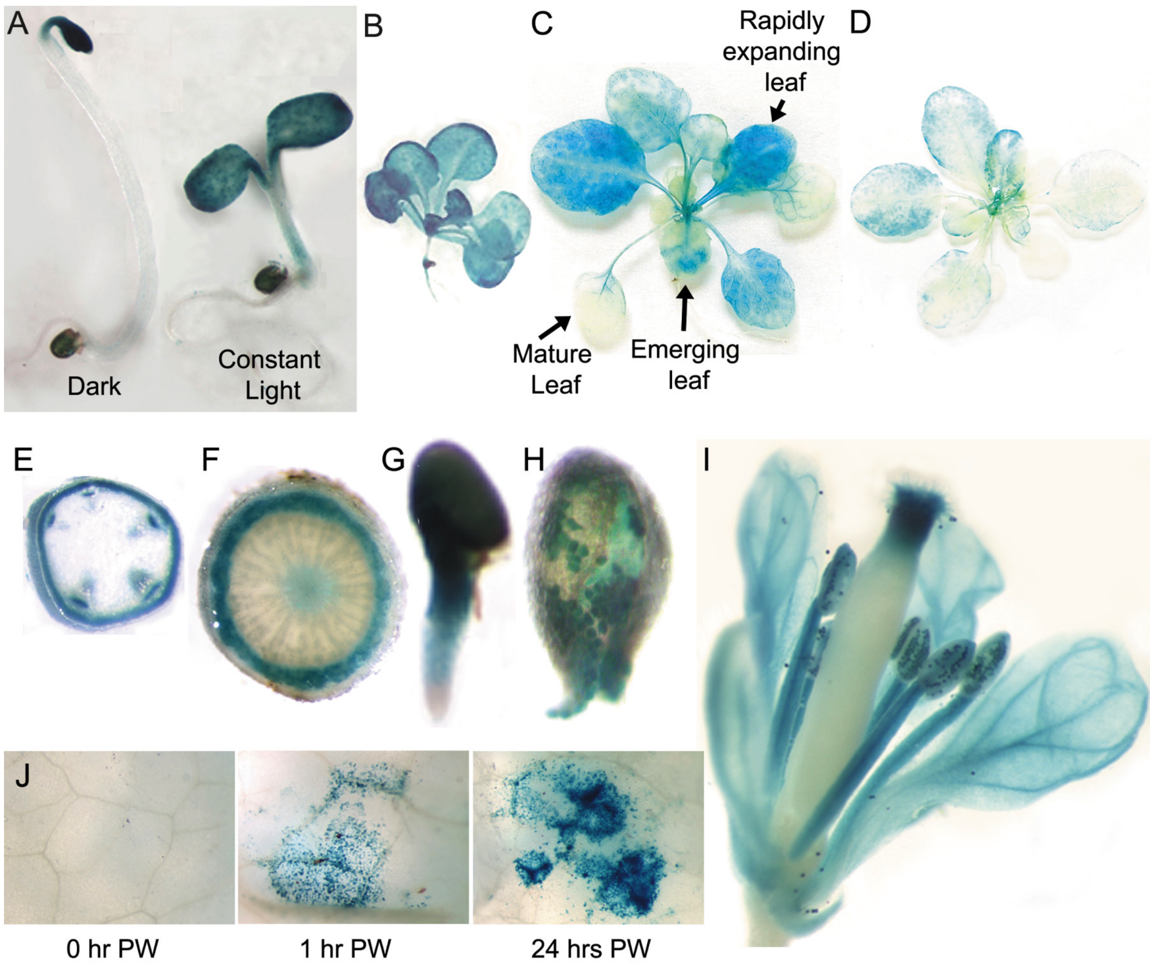

A
Dark
Constant Light
B
C
Rapidly expanding leaf
D
Mature Leaf
Emerging leaf
E
F
G
H
I
J
0 hr PW
1 hr PW
24 hrs PW

Figure 2-8. Putative cis regulatory elements (underlined or over-lined) in the *AtPGR5* regulatory region (1100 kb to +1). Pollen-specific elements: POLLEN1LELAT52 (Bate and Twell, 1998), and GTGANTG10 (Rogers et al., 2001); etiolation-specific elements: ABRELATERD1 (Simpson et al., 2003), ACGTATERD1 (Simpson et al., 2003), and REALPHALGLHCB21 (Degenhardt and Tobin, 1996); endosperm-specific element: CACOREOSGLUB1 (Wu et al., 2000); wound-specific element: ELRECOREPCRP1 (Rushton et al., 1996); light-specific elements (double underlined): GTICORE (Gilmartin et al., 1990), GATABOX (Gilmartin et al., 1990), IBOXCORE (Terzaghi and Cashmore, 1995); cold-specific elements: LTRECOREATCOR15 (Baker et al., 1994), and LTRE1HVBLT49 (Dunn et al., 1998); drought regulatory elements: DRE1COREZMRAB17 (Busk et al., 1997), and DRECRTCOREAT (Dubouzet et al., 2003) and a sucrose repression element: PYRIMIDINEBOXOSRAMY1A (Morita et al., 1998).

ATCTGTTACAGATTATTACTGTACAATCCAGCAAAAACTAGGCCTGGGATTTCGGATTAACCCAATGACCGAACTG
DRECRTCOREAT
AACAAACCGTTTTTTTGGTTCTGATAACCGACTATGAAGGGAAGGCTAAACCGATCGGCGAAATTATAATTGAAGT
AACACOREOSGLUB1 GATABOX
TCGGTTCTCGGTTCGGCAGACATAACCGATCAGTTTTCTCGGTTAATAGATACTATAAATATAAAGATAAAAACAT
GTICORE GATABOX GATABOX
TAGATCTAATCTTTTTAGCTTCTTCTCCGTTTCCTTTTGATCCTCATCCATGGCTATTTAGTCAATTCTCTCTATCTGC

ATGTGGTTTTCACTATGGTAGCACGTCGGGGCATATTTCGTTACGGAGATATCGTCATCCCTCGCTATCACCGTCAC
ACGTATERD1 GATABOX
CGTCACAGACGTAGATCTGCTGATTATGAAGAGGGTAGCGGCACCAAGATACCATCAACCCTAATTTTTAATAGGT
ACGTATERD1 GATABOX
TTGGATTTTTAGTTATTTTGGGTTTAAGTTTGTTTTTCGTTTTGGCCCAATTATTGGTTTGTAATTATAAAATTTTGTT

TATCTTCGCCCATTCTATAATTTTTGTTTTGACCCATTATCTTGATTTTGGACTTATTCGGTATAAACTAACCGAACT
ELRECOREPCRP1 DRECRTCOREAT
AAAAAAAAAATCGGTTTAGTTCAGAGTTGATTTTGAATCCTATAACCAAACCGACCCGAACCAAATCTTTATTCGG
REALPHALGLHCB12 REALPHALGLHCB12
TTTAATTTGACAAAATTTTAAAGAACCGAAAAAACCGATTAAACCAAACCGAACCGATTACCCGAATAAACCGAA
LTRE1HVBLT49 DRE1COREZMRAB17
TGCCCAGGCCAAAAACAACTGAAAATAAAAAATAAAAAATGAGATAGGAGAATATTTAATCCGACGGTGGAGAA
GTGANTG10 GATABOX POLLEN1LELAT52
ATGTTGATATAATAAAACGTGATACAGAGGATCATAAACCCGCAACATGAGAAACGTAATAAGTTAAGTCAAAAA
GATABOX ACGTATERD1 GATABOX ACGTATERD1
AACGAAAAAGCCAGAATCATATTTGTGGCTCTGGTTTCTCCATCCAAACAAAAACAACACCCAAACCTTGTCCACA

CCAAAATGTTAAACTCAAAATCCAACCACACACACACAATTTCTCTTCCTCTCTACCATTAACATCGATCAGAAAG
POLLEN1LELAT52

ACCGAGAGAGAGAGGGAGAAGCTGATTGATT
DRE1COREZMRAB17

Figure 2-9. Northern hybridizations showing the effects of abiotic stress on *AtPGR5* expression. *AtPGR5* transcript levels after (A) 0, 1, 3, 6 , or 12 hrs exposure to high light (2000 $\mu E/m^2s$), (B) 0, 1 or 3 days of chilling at 4^oC. The bottom row in each panel shows the corresponding RNA gel stained with ethidium bromide.

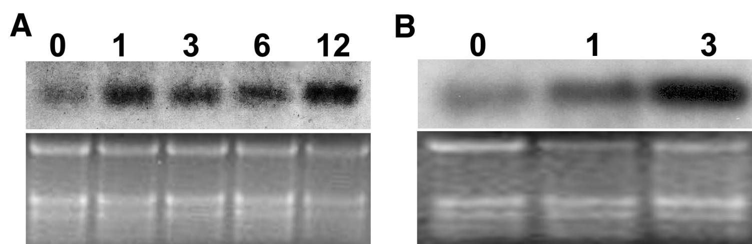
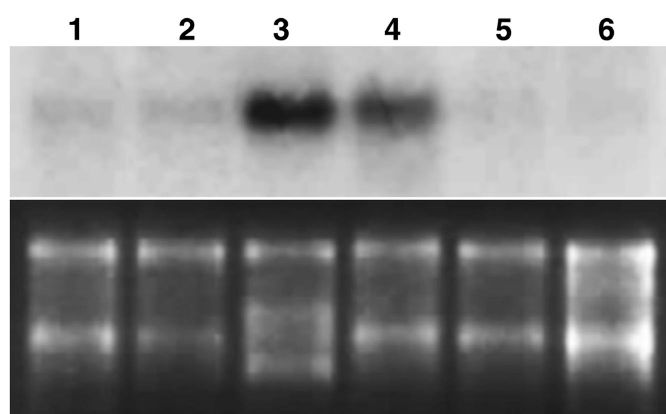

A
0
1
3
6
12
B
0
1
3

Figure 2-10. Northern analysis of *PtPGR5* expression in different *P. taeda* tissues. RNA from shoot apical meristems (1), young succulent stems (2), mature needles (3), phloem (4), xylem (5), or roots (6) was hybridized to a *PtPGR5* specific DNA probe. Corresponding RNA gel stained with ethidium bromide is below.


1
2
3
4
5
6

CHAPTER III

CONCLUSIONS

This project initially began as an effort to characterize the function of PtPGR5, a then unnamed *P. taeda* protein whose gene expression was known to be suppressed in tissues infected by *C. q. fusiforme*, a biotrophic pathogen (Warren and Covert, 2004). I initially predicted that this protein has a role in development, defense, or basic plant metabolism based on its suppression in woody galls formed by *C. q. fusiforme* infection, and because of its high conservation in the plant kingdom. Because *A. thaliana* is a more tractable model system than *P. taeda*, I planned to infer the function of PtPGR5 by characterizing the effects of suppressing *AtPGR5* expression in *A. thaliana*, and over-expressing the *A. thaliana* and *P. taeda* orthologs in *A. thaliana.*

Initially, I found that RNAi-mediated suppression of *AtPGR5* caused no phenotype under normal growth conditions. However, over-expression of both *PGR5* orthologs caused decreased growth and leaf abnormalities. I also found that *PGR5* was expressed in certain aerial tissues in *A. thaliana* and *P. taeda*. Based on these results I suspected the protein might be involved in regulating development. To test the hypothesis that the protein played a role in plant defense, I also assessed how *AtPGR5* transcription was affected by salicylic acid and ethylene, hormones commonly involved in plant defense responses (Penninckx et al., 1998; Thomma et al., 1999). These hormones did not effect *AtPGR5* expression (Appendix A). RNAi and over-expression

mutants were also tested for alterations in resistance to compatible and incompatible strains of *Peronospora parasitica*, a biotrophic pathogen of *A. thaliana* (Appendix A). I theorized that suppressing *AtPGR5* would cause decreased resistance, and over-expression would lead to increased disease resistance. However, I only found that over-expression mutants displayed increased susceptibility to the avirulent strain of *P. parasitica* (Appendix A). I suspected that this increase was most likely a secondary effect of the poor growth exhibited by plants over-expressing *AtPGR5*. At this point in the study, AtPGR5's role in photoprotection was discovered (Munekage et al., 2002) and the third of the three initial hypotheses was substantiated.

The multiple functions of CET

Cyclic electron transport (CET) is a controversial process with a proposed role in both NPQ and ATP synthesis (Bendall and Manasse, 1995; Bukhov and Carpentier, 2004; Johnson, 2005). During CET the reduction of the stroma is reduced as electrons are transferred from Fd or NADPH to the PQ pool and back to PSI via the Cb_6/f complex. As electrons are transferred from PQ to the Cb_6/f complex, and the Q cycle pumps protons into the chloroplast and elevates ΔpH (Niyogi, 1999). Elevated ΔpH triggers NPQ and ATP synthesis (Bendall and Manasse, 1995; Bukhov and Carpentier, 2004; Johnson, 2005). ATP generated by CET is proposed to be used to induce the Calvin Cycle during state transition from dark to light, and to supplement the ATP made by the LET for use in the Calvin Cycle and in other metabolic activities (Bendall and Manasse, 1995; Joliot and Joliot, 2002; Bukhov and Carpentier, 2004).

To further characterize how CET helps maintain the ATP:NADPH ratio, and prevent stromal over-reduction, in the future one could monitor ATP and NADPH synthesis in RNAi and over-expression mutants, and in plants exposed to moderate and high light. Future experiments could also include measuring and monitoring O_2 evolution and CO_2 assimilation in RNAi and over-expression mutants, determining how the O_2:CO_2 ratio is effected when RNAi and over-expression plants are grown in an air-tight chamber, measuring chlorophyll fluorescence parameters of the mutants in a completely anaerobic environment, and determining the exact ROS species produced in RNAi plants under these conditions.

There are also other alternative electron transport pathways that may coordinate or compete with CET for reduced Fd and NADPH (Backhausen et al., 2000). In the future, it would be interesting to determine how other electron acceptors and dissipation mechanisms, such as photorespiration, the water-water cycle, and nitrogen and sulfur assimilation are effected by alterations in PGR5-dependent CET.

When and where PGR5-dependent CET is utilized

The temporal and spatial expression pattern of *PGR5* also supports the idea of its role in photoprotection and stress tolerance. Our study has revealed that PGR5-dependent CET may be most important in young expanding leaves, which are most susceptible to photodamage. Surprisingly, we also found that *PGR5:GUS* is expressed in nonphotosynthetically active tissues. This raises the possibility that PGR5 may be expressed in plastids in anticipation of differentiation into chloroplasts. Obviously,

further studies are needed to measure CET activity in nonphotosynthetically active tissues such as pollen.

AtPGR5 expression is also moderately regulated by physiological stresses that inhibit Calvin Cycle activity. Presumably, CET is induced under these conditions to prevent photoinhibition. It would be interesting to perform more detailed analyses of how *PGR5* transcription and PGR5-dependent CET are affected by various stresses such as drought and abscisic acid, salt and high temperature; conditions which have been shown to elevate CET (Bukhov and Carpentier, 2004). Analyses of the effect of salt would be particularly interesting because salt has been shown to increase the expression of the PGR5 homolog in the cyanobacterium *Synechocystis Pcc6803* (Kanesakib et al., 2002). For practical purposes one could also subject the PGR5 over-expression mutants to these stress conditions to determine if elevated CET might increase stress tolerance.

The two CET pathways

Why do plants have two CET pathways? Do they serve different functions? Does one pathway serve a biological function that plants require more than the other? Maybe we can begin to answer these questions by looking at the differences between the two CET pathways. NDH genes have been reported in cyanobacteria and most higher plants, including seed-bearing and seedless plant divisions, and byrophytes (Wakasugi et al., 1994; Neyland and Urbatsch, 1996; Odintsova and Yurina, 2003). However, NDH genes have not been found in any *Pinus* species, green algae, red algae or certain protists (Wakasugi et al., 1994; Neyland and Urbatsch, 1996; Odintsova

and Yurina, 2003). This is particularly interesting in light of the fact that PGR5 shows high homology to ESTs and predicted proteins in seed-bearing and seedless plant divisions, byrophytes, as well as pine, cyanobacteria and green algae. Though loss of NDH genes in certain species might be due to differences in their physiology, I postulate that PGR5-dependent CET is the major CET pathway, essential for maintaining the chloroplast redox status in most photosynthetic organisms. Perhaps NDH-dependent CET is a secondary CET pathway that is dispensable for normal growth and development, provided that PGR5-dependent CET is present. This theory is supported by the fact that the concentration of chloroplast NDH is very low: only 0.2% of total thylakoid protein (Sazanov et al., 1998a). Also, disruption of NDH activity has no effect on PSII electron transport, P700 oxidation or, in particular NPQ under normal growth conditions, while *pgr5* mutants exhibit decreases in all three aspects of photosynthesis (this study; (Munekage et al., 2004).

Loss of NDH genes in pine is particularly interesting because pine, unlike green algae, is a higher plant species. It is important that there be continued analyses of *PGR5* and, possible NDH function in *Pinus* species. Obviously, additional examination of the genomes of *Pinus* species and other photosynthetic organisms will be necessary to further elucidate the evolution of CET proteins.

The two CET pathways also require different types of stromal redox carriers. NDH requires NADPH, a stromal reductant which is stable in light and darkness, while PGR5-dependent CET utilizes reduced Fd, a reductant stable only in light (Bukhov and Carpentier, 2004). Perhaps because it is only light stable, and because reduced Fd is the first mobile electron carrier after PSI, it is the preferential electron transport pathway

under light. This concept may explain why PGR5-dependent CET is more important for NPQ of excess light than NDH. It is likely that NDH-dependent CET is a secondary CET pathway, which plays a more important role in dark reduction of the PQ pool. Dark electron transport, or chlororespiration, occurs, transiently, after cessation of light exposure (Burrows et al., 1998; Sazanov et al., 1998b; Shikanai et al., 1998). This process is mediated only by NDH-dependent CET, and is caused by reduction of the PQ by stromal reductants which accumulate upon inhibition of the Calvin Cycle (Bukhov and Carpentier, 2004). Chlororespiration is proposed to maintain the thylakoid ATP synthase in a catalytically active state, and (or) aid in carotenoid biosynthesis. Because NDH-dependent CET also occurs in the light, it is also proposed to help maintain the ATP:NADPH ratio (Bukhov and Carpentier, 2004; Munekage et al., 2004).

This study has shown that NDH-dependent CET cannot compensate for PGR5-dependent CET when plants are grown under high light or when exposed to a low CO_2 environment. However, previous research has indicated that the functions of the two CET pathways are compensatory under low light (Munekage et al., 2004). It would be interesting to determine whether one CET pathway becomes elevated when the other is unavailable, in order to maintain the chloroplast redox state. Characterization of possible increases in PGR5- and NDH-dependent CET in *ndh* and *pgr5* mutants, respectively, could verify this idea.

What does PGR5 do?

Unfortunately, the biochemical function of PGR5 is still unknown, though I can use the information this study and others have provided to speculate about the function

of this protein. One of the most striking, and informative, characteristics of PGR5 is that it is a small membrane-associated protein with no indication of being an electron carrier or an enzyme involved in electron transfer (Munekage et al., 2002; Allen, 2003). In contrast, NDH is a large, multisubunit enzyme complex that mediates the transfer of electrons from NADPH to PQ (Bendall and Manasse, 1995). It is also intriguing that up-regulation of this small, apparently nonenzymatic protein alone can have a major effect on cyclic and linear electron transfer, and cause changes in overall growth and development. To better understand how PGR5 might do this we should consider what is currently known about the structure of other proteins involved in CET. It has recently been shown that Cb_6/f, Fd, and Fd:NADPH reductase form a complex which might complete the CET circuit around PSI (Zhang et al., 2001). CET may also occur during the transition from dark to light in a proposed supercomplex composed of Cb_6/f, plastocyanin, PSI, Fd, and Fd:NADPH reductase (Joliot and Joliot, 2002). Though PGR5 does not act to stabilize PSII, PSI, or the Cb_6/f complex (Munekage et al., 2002), perhaps PGR5 interacts with and helps stabilize the Cb_6/f , Fd, and Fd:NADPH reductase complex, or the proposed CET supercomplex, and, thereby helps facilitate the movement of electrons from PSI back to the LET chain. This may explain why over-expression of *PGR5* causes decreased growth, but increased NPQ. Perhaps ectopic PGR5 increases the formation of the CET supercomplex, inhibiting LET and growth, and increasing NPQ. Other studies describe the crystal structure of the Cb_6/f complex in detail (Kurisu et al., 2003, 2004). In Kurisu et al (2003) the authors describe a new heme molecule, Heme X. They speculate that Heme X is the long-sought Fd:plastoquinone reductase that facilitates the transfer of electrons from Fd to PQ. I

propose that PGR5 may interact with this molecule to aid electron transfer. Currently, studies are underway to determine how PGR5 and other proteins form complexes that mediate both LET and CET (J Meurer, personal communication).

LITERATURE CITED

Allen, J.F. (2003). Cyclic, pseudocyclic and noncyclic photophosphorylation: new links in the chain. Trends Plant Sci **8,** 15-19.

Backhausen, J.E., Kitzmann, C., Horton, P., and Scheibe, R. (2000). Electron acceptors in isolated intact spinach chloroplasts act hierarchically to prevent over-reduction and competition for electrons. Photosynthesis Research **64,** 1-13.

Bendall, D.S., and Manasse, R.S. (1995). Cyclic photophosphorylation and electron transport. Biochimica Et Biophysica Acta-Bioenergetics **1229,** 23-38.

Bukhov, N., and Carpentier, R. (2004). Alternative Photosystem I-driven electron transport routes: mechanisms and functions. Photosynthesis Research **82,** 17-33.

Burrows, P.A., Sazanov, L.A., Svab, Z., Maliga, P., and Nixon, P.J. (1998). Identification of a functional respiratory complex in chloroplasts through analysis of tobacco mutants containing disrupted plastid ndhgenes. The EMBO Journal **17,** 868-876.

Johnson, G.N. (2005). Cyclic Electron Transport in C_3 plants: fact or artefect. Journal of Experimental Botany **56,** 407-416.

Joliot, P., and Joliot, A. (2002). Cyclic electron transfer in plant leaf. Proc Natl Acad Sci U S A **99,** 10209-10214.

Kanesakib, Y., Suzukib, I., Allakhverdieva, S., Mikamia, K., and Muratab, N. (2002). Salt Stress and Hyperosmotic Stress Regulate the Expression of Different Sets of Genes in *Synechocystis* sp. PCC 6803. Biochemical and Biophysical Research Communications **290,** 339-348.

Kurisu, G., Zhang, H.M., Smith, J.L., and Cramer, W.A. (2003). Structure of the cytochrome b_6f complex of oxygenic photosynthesis: Tuning the cavity. Science **302,** 1009-1014.

Kurisu, G., Zhang, H.M., Smith, J.L., and Cramer, W.A. (2004). Crystal structure of the cytochrome b_6f complex of oxygenic photosynthesis. Plant and Cell Physiology **45,** S44-S44.

Munekage, Y., Hojo, M., Meurer, J., Endo, T., Tasaka, M., and Shikanai, T. (2002). PGR5 is involved in cyclic electron flow around photosystem I and is essential for photoprotection in Arabidopsis. Cell **110,** 361-371.

Munekage, Y., Hashimoto, M., Miyake, C., Tomizawa, K., Endo, T., Tasaka, M., and Shikanai, T. (2004). Cyclic electron flow around photosystem I is essential for photosynthesis. Nature **429,** 579-582.

Neyland, R., and Urbatsch, L.E. (1996). The *ndhf* chloroplast gene detected in all vasular plant divisions. Planta **200,** 273-277.

Niyogi, K.K. (1999). Photoprotection revisited: Genetic and molecular approaches. Annual Review of Plant Physiology and Plant Molecular Biology **50,** 333-359.

Odintsova, M.S., and Yurina, N.P. (2003). Plastid genomes of higher plants and algae: structure and functions. Molecular Biology **37,** 649-662.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998a). The plastid *ndh* genes code for an NADH-specific dehydrogenase: Isolation of a complex I analogue from pea†thylakoid†membranes. PNAS **95,** 1319-1324.

Sazanov, L.A., Burrows, P.A., and Nixon, P.J. (1998b). The chloroplast Ndh complex mediates the dark reduction of the plastoquinone pool in response to heat stress in tobacco leaves. FEBS Lett **429,** 115-118.

Shikanai, T., Endo, T., Hashimoto, T., Yamada, Y., Asada, K., and Yokota, A. (1998). Directed disruption of the tobacco *ndhB* gene impairs cyclic electron flow around photosystem I. Proceedings of the National Academy of Sciences of the United States of America **95,** 9705-9709.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional EHTYLENE-INSENSITIVE 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol **121,** 1093-1102.

Wakasugi, T., Tsudzuki, J., Ito, S., Nakashima, K., Tsudzuki, T., and Sugiura, M. (1994). Loss of all *ndh* genes as determined by sequencing the entire chloroplats genome of the black pine *Pinus thunbergii*. Proc Natl Acad Sci U S A **91,** 9794-9798.

Warren, J.A., and Covert, S.F. (2004). Differential expression of pine and *Cronartium quercuum f. sp fusiforme* genes in fusiform rust galls. Applied and Environmental Microbiology **70,** 441-451.

Zhang, H., Whitelegge, J.P., and Cramer, W.A. (2001). Ferredoxin:NADP+ Oxidoreductase Is a subunit of the chloroplast cytochrome b_6f Complex. The Journal of Biological Chenistry **276,** 38159–38165.

APPENDIX A

PGR5 AND PLANT DEFENSE

INTRODUCTION

Hormonal regulation of gene expression

To defend themselves against pathogens, plants employ a variety of defense responses. These responses include the generation of secondary signal molecules or stress hormones (Heath, 2000). Stress hormones such as salicylic acid (SA), jasmonic acid (JA), and ethylene trigger different signal transduction cascades that lead to the expression of specific pathogen response genes (Penninckx et al., 1998; Thomma et al., 1999). Generally, the SA pathway is induced by biotrophs, while the ethylene/JA pathway is induced by necrotrophic pathogens as well as other stresses such as wounding (for review see Thomma et al, 2001). Recent studies indicate, however, that there is substantial interplay between these and other signal transduction pathways during stress responses (Durrant et al., 2000; Schenk et al., 2000).

Biotrophic pathogens and disease resistance

In order for biotrophic pathogens to live within their host, they must suppress or prevent the elicitation of defense responses in plants (Heath, 1997). During fungal infection the growth of the hyphae through the intercellular spaces and the cell wall may play a role in this suppression. Haustorial growth within host cells invaginates the

plasma membrane and causes the plasma membrane to detach from the cell wall (Gray, 1983; Heath, 1997). During infection by incompatible fungal pathogens this detachment elicits nonspecific defense responses. However, compatible biotrophs are able to infect their host by negating or not triggering these adverse responses (Heath, 2000). A study on the rust fungus *Uromyces vignae* indicates that compatible rust fungi induce a decrease in plasma membrane-cell wall adhesion, which disrupts nonspecific plant defense responses in their host (Mellersh, 2001).

PGR5 suppression may indicate a role in defense

PGR5 is suppressed in *A. thaliana* and *P. taeda* tissues infected by biotrophic pathogens (Ramonell, 2004; Warren and Covert, 2004). In the initial phase of this project I hypothesized that PGR5 was suppressed by the infecting pathogen because it was important for plant defense. If so, then *PGR5* expression might be induced by one (or more) of the hormones regulating defense responses. I also proposed that plants with decreased *PGR5* expression might be more susceptible to avirulent strains of biotrophic pathogens, and over-expression mutants might be more resistant to virulent strains of biotrophic pathogens.

MATERIALS AND METHODS

To determine if *AtPGR5* expression is regulated by defense-related hormones, salicylic acid, jasmonic acid and ethylene were applied to three to four week old *A. thaliana* plants according to a published protocol (Schenk et al., 2000). For salicylic acid treatment, plants were either sprayed with a solution of 4 mM salicylic acid in water

or just water until run off. For jasmonic acid treatment, plants were placed in a 10 L clear plastic bag, along with a cotton ball soaked in either 200ul of 0.5% jasmonic acid, or water. For ethylene treatment, plants were placed in a sealed airtight container with 50 ppm, 100 ppm, or 200 ppm ethylene gas or just air. After 24 hours, fully expanded and mature leaves were collected in liquid nitrogen for use in northern or western analysis as described in Chapter II Materials and Methods.

To determine if *AtPGR5* over-expression influences pathogen response, we inoculated three to four week old wild-type, RNA, and over-expression mutants with spore suspensions of virulent (EMCO) and avirulent (CALA) isolates of *Peronospora parasitica* (kindly provided by Dr. Jeff Dangl) according to a previous protocol (McDowell et al., 1998). After five to seven days we observed differences in localized cell death and hyphal growth by staining inoculated leaves with lactophenol-trypan blue (data not shown) which stains for fungal hyphal growth (Koch, 1990). We also determined the percentage of disease infection by measuring spore formation according to a standard protocol (Weigal and Glazebrook, 2002).

RESULTS AND CONCLUSION

Salicylic acid, and ethylene did not regulate AtPGR5 expression (Figure A-1A, B) . The results for jasmonic acid were inconclusive (data not shown). RNAi suppression mutants did not exhibit noticeably altered disease resistance to virulent or avirulent strains of *P. parasitica* (Figure A-1C). Over-expression mutants did display a 50% increase in susceptibility to the avirulent strain of *P. parasitica*, though I think this is a secondary effect caused by the poor growth of these mutants (Figure A-1C).

Based on these results as well as PGR5's reported role in photoprotection I conclude that PGR5 is probably not directly involved in disease resistance. When plants are infected by pathogens or exposed to any type of stress they initialize numerous mechanisms to protect themselves. These mechanisms include alterations in the chloroplast redox status which regulate gene expression (Karpinski et al., 1997; Karpinski et al., 1999; Oswald et al., 2001; Rossel et al., 2002). In light of PGR5's role in photoprotection, I propose that biotroph infection causes alterations in the chloroplast redox status which repress the expression of PGR5.

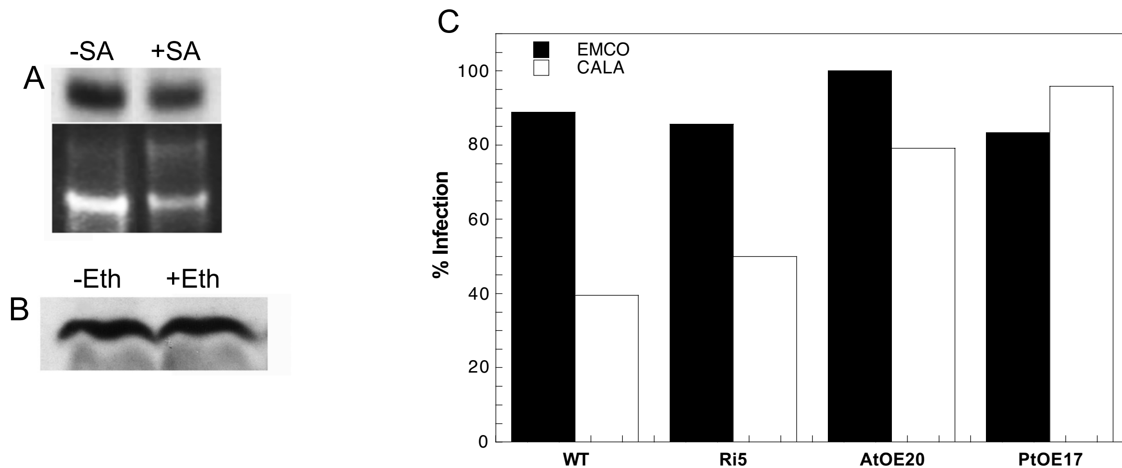


Figure A-1. The effect of hormones on *AtPGR* expression. A. Northern hybridizations showing the effects of salicylic acid (SA) on *AtPGR5* transcription. The bottom row shows the corresponding RNA gel stained with ethidium bromide. B. Immunoblot showing the effect of 50 ppm ethylene (Eth) on AtPGR5. C. The effect of alteration of *AtPGR5* expression on disease resistance 5 days after inoculation. Three leaves from seven to eight leaves were scored for formation of fungal spores. n= 21-24

LITERATURE CITED

Durrant, W.E., Rowland, O., Piedras, P., Hammond-Kosack, K.E., and Jones, J.D. (2000). cDNA-AFLP reveals a striking overlap in race-specific resistance and wound response gene expression profiles. Plant Cell **12,** 963-977.

Gray, D.J., Amerson, H.V., Van Dyke, C.G. (1983). Ultrastructure of the infection and early colonization of *Pinus taeda* by *Cronartium quercuum fromae speciales fusiforme*. Mycologia **75,** 117-130.

Heath, M.C. (1997). Signalling between pathogenic rust fungi and resistant or susceptible host plants. Annals of Botany **80,** 713-720.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. Curr Opin Plant Biol **3,** 315-319.

Karpinski, S., Escobar, C., Karpinska, B., Creissen, G., and Mullineaux, P.M. (1997). Photosynthetic electron transport regulates the expression of cytosolic ascorbate peroxidase genes in Arabidopsis during excess light stress. Plant Cell **9,** 627-640.

Karpinski, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., and Mullineaux, P. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science **284,** 654-657.

Koch, E., Slusarenko, A. (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell **7,** 1195-1206.

McDowell, J.M., Dhandaydham, M., Long, T.A., Aarts, M.G., Goff, S., Holub, E.B., and Dangl, J.L. (1998). Intragenic recombination and diversifying selection

contribute to the evolution of downy mildew resistance at the RPP8 locus of Arabidopsis. Plant Cell **10,** 1861-1874.

Mellersh, D.G., Heath M.C. (2001). Plasma membrane-cell wall adhesion is required for expression of plant defense responses during fungal penetration. The Plant Cell **13,** 413-424.

Oswald, O., Martin, T., Dominy, P.J., and Graham, I.A. (2001). Plastid redox state and sugars: Interactive regulators of nuclear-encoded photosynthetic gene expression. Proceedings of the National Academy of Sciences of the United States of America **98,** 2047-2052.

Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F. (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. Plant Cell **10,** 2103-2113.

Ramonell, K. (2004). Powdery mildew infection www.arabidopsis.org/servlets/TairObject?type=expression_set_full&id=10058239. The Arabidopsis Information Resource (TAIR) Accessed Februrary 11, 2005.

Rossel, J.B., Wilson, I.W., and Pogson, B.J. (2002). Global changes in gene expression in response to high light in Arabidopsis. Plant Physiol. **130,** 1109-1120.

Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M. (2000). Coordinated plant defense responses in Arabidopsis revealed by microarray analysis. Proc Natl Acad Sci U S A **97,** 11655-11660.

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999). Requirement of functional EHTYLENE-INSENSITIVE 2 gene for efficient resistance of Arabidopsis to infection by *Botrytis cinerea*. Plant Physiol **121,** 1093-1102.

Warren, J.A., and Covert, S.F. (2004). Differential expression of pine and *Cronartium quercuum f. sp fusiforme* genes in fusiform rust galls. Applied and Environmental Microbiology **70,** 441-451.

Weigal, B., and Glazebrook, J. (2002). Arabidopsis, a laboratory manual. (Cold Spring Harbor: Cold Spring Harbor Press).