CRYPTOCROME 1 (CRY1) IS CRITICAL IN MEDIATING
DEVELOPMENTAL PROCESS IN RESPONSE TO LIGHT INTENSITY

DISSERTATION

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Plants can perceive light signals through three major classes of photoreceptors: phytochromes (red/far-red light), cryptochromes (blue and UV-A light), and phototropins (blue and UV-A light). Genome-wide gene expression analysis revealed that CRYPTOCHROME1 (CRY1) is critical in the response of Arabidopsis to high irradiance. Database analysis also showed that expression of CRY1 was highly correlated to the expression of a number of anti-oxidant genes. Therefore, I hypothesized that CRY1 is involved in cellular communication responsible for the integration of irradiance-dependent responses in tobacco.

To understand the role of CRY1 in seed germination and early seedling development in response to light intensity, experiments were conducted using transgenic plants overexpressing CRY1. Germination kinetics were determined by measuring the time of radicle emergence in seeds subjected to light intensities of 6, 60, or 600 µmol m\(^{-2}\) s\(^{-1}\). In wild type plants, the final germination percentage was unaffected by different light intensities, but high light intensity caused a significant delay in germination. Plants overexpressing CRY1 showed reduced germination percentage and a delay in the timing of germination under both low and high light intensities. Surprisingly, the lower germination percentages in the CRY1 overexpressors was reversed by high light intensities in contrast to seeds of wild-type.
Under high light conditions, leaves of \textit{CRY1} overexpressing plants contained three-fold more total chlorophyll than wild-type plants, which was probably due to both increased synthesis and a reduction of photooxidative degradation. First, well-developed chloroplasts containing chlorophyll were observed in the cortex of roots of \textit{CRY1} overexpressing plants. Second, leaves of \textit{CRY1} overexpressing plants contained higher phenolics, flavonoids, and lignin content than wild-type plants. LC-MS results showed that leaves of \textit{CRY1} overexpressing plants contained two-fold higher level of chlorogenic acids and ten-fold higher level of rutin than wild-type plants. \textit{CRY1} overexpressing plants exhibited reduced ultraviolet-B injury. It may be due to the accumulation of secondary metabolites which have antioxidant activities in protecting cellular structures from oxidative damage.

\textit{CRY1} overexpression resulted in increased stomatal density. Wild-type plants followed a pattern known as the one cell spacing rule: two stomates are separated by at least one epidermal cell for proper stomatal physiology. In plants overpressing \textit{CRY1} there was elevated stomatal density and some of the stomates were clustered in violation of the one cell spacing rule, indicating that stomatal patterning was disrupted in \textit{CRY1} overexpression plants. I also observed a severe wilting phenotype in \textit{CRY1} overexpression plants when transferred to cold or high light conditions. This may be a result of a reduction in water transport (smaller vessel cells) and the increased transpiration.
Dedicated to my parents, sister, and brother.
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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ABSTRACT</td>
<td>iii</td>
</tr>
<tr>
<td>DEDICATION</td>
<td>v</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS</td>
<td>vi</td>
</tr>
<tr>
<td>VITA</td>
<td>vii</td>
</tr>
<tr>
<td>LIST OF TABLES</td>
<td>x</td>
</tr>
<tr>
<td>LIST OF FIGURES</td>
<td>xi</td>
</tr>
</tbody>
</table>

## CHAPTERS

1. LITERATURE REVIEW

   1.1 The role of photoreceptor in light perception                      | 1    |
   1.2 Phychromes                                                        | 2    |
   1.3 Cryptochromes                                                     | 3    |
   1.4 Phototropins                                                      | 4    |
   1.5 The photoreceptors in the model plant *Arabidopsis thaliana*       | 5    |
   1.6 Research objectives                                              | 10   |

2. THE ROLE OF *CRY1* IN MEDIATING SEED GERMINATION AND EARLY SEEDLING DEVELOPMENT IN RESPONSE TO LIGHT INTENSITY

   1.1 Introduction                                                      | 12   |
   1.2 Materials and methods                                            | 15   |
   1.3 Results                                                          | 19   |
   1.4 Discussion                                                       | 31   |

3. *CRY1* IS CRITICAL FOR SENSING CHANGES IN IRRADIANCE AND MAY BE INVOLVED IN PHOTOPROTECTION IN TOBACCO

   3.1 Introduction                                                      | 32   |
   3.2 Materials and methods                                            | 35   |
3.3 Results........................................................................................................42
3.4 Discussion....................................................................................................68

4. CRY1 MAY BE INVOLVED IN REGULATING SIZE AND SHAPE OF VESSEL ELEMENTS........................................................................................................70
   4.1 Introduction....................................................................................................70
   4.2 Materials and methods..................................................................................73
   4.3 Results..........................................................................................................76
   4.4 Discussion....................................................................................................90

LIST OF REFERENCES............................................................................................91

APPENDIX

GLZ1, A MEMBER OF FAMILY 8 GLYCOSYLTRANSFERASE, MAY PLAY A ROLE IN PROGRAMMED CELL DEATH.................................................................97
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1. Time of germination of Ns, S168, Nt, S135, and S144 under different light intensities</td>
<td>27</td>
</tr>
<tr>
<td>2.2. The marginal effects of control variables on germination time</td>
<td>28</td>
</tr>
<tr>
<td>3.1. Negative ions with corresponding retention time and suggested compound</td>
<td>57</td>
</tr>
<tr>
<td>3.2. Negative ions with corresponding retention time and suggested compound in root</td>
<td>65</td>
</tr>
<tr>
<td>4.1. Stomatal index of wild-type and CRY1 overexpressing plants grown under short and long day conditions</td>
<td>83</td>
</tr>
<tr>
<td>4.2. Mean length and width of vessels in Ns and S168</td>
<td>85</td>
</tr>
<tr>
<td>4.3. The results of ANOVA in mean length of Ns, mean width of Ns, mean length of S168, and mean width of S168</td>
<td>86</td>
</tr>
<tr>
<td>4.4. The results of paired-t test in mean length of Ns, mean width of Ns, mean length of S168, and mean width of S168</td>
<td>87</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1. Domain organization of phytochromes, cryptochromes, and phototropins</td>
<td>4</td>
</tr>
<tr>
<td>1.2. The data on light responsive expression were compiled by Genevestigator</td>
<td>7</td>
</tr>
<tr>
<td>1.3. The data on stimulus responsive expression were compiled by Genevestigator</td>
<td>8</td>
</tr>
<tr>
<td>1.4. Co-expressed genes of \textit{CRY1} in \textit{Arabidopsis thaliana}</td>
<td>9</td>
</tr>
<tr>
<td>2.1. Molecular characterization of \textit{N. sylvestris} and \textit{N. tabacum} cv. Maryland Mammoth transgenic lines</td>
<td>22</td>
</tr>
<tr>
<td>2.2. \textit{CRY1} affects seed size</td>
<td>23</td>
</tr>
<tr>
<td>2.3. \textit{CRY1} affects seed weight</td>
<td>24</td>
</tr>
<tr>
<td>2.4. \textit{CRY1} overexpression results in a reduced germination</td>
<td>25</td>
</tr>
<tr>
<td>2.5. The lower germination percentages in the \textit{CRY1} overexpressors were reversed by high light intensities</td>
<td>26</td>
</tr>
<tr>
<td>2.6. \textit{CRY1} affects the early seedling development</td>
<td>29</td>
</tr>
<tr>
<td>2.7. \textit{CRY1} overexpression results in an inhibition of early seedling development</td>
<td>30</td>
</tr>
<tr>
<td>3.1. \textit{CRY1} overexpression reduces photodamage in plants</td>
<td>49</td>
</tr>
<tr>
<td>3.2. \textit{CRY1} overexpression results in increased chlorophyll content</td>
<td>50</td>
</tr>
<tr>
<td>3.3. \textit{CRY1} overexpression results in root greening</td>
<td>51</td>
</tr>
<tr>
<td>3.4. Chloroplasts are located in cortex of the maturation zone</td>
<td>52</td>
</tr>
<tr>
<td>3.5. \textit{CRY1} overexpression results in increased chlorophyll content in root</td>
<td>53</td>
</tr>
</tbody>
</table>
3.6. CRY1 overexpression results in increased secondary metabolite content. ..........54
3.7. HPLC analysis shows increased secondary metabolite content in CRY1
overexpressing plants .................................................................................55
3.8. Total ion chromatogram of phenolic compounds in wild-type and CRY1
overexpressing plants ..................................................................................56
3.9. Total ion chromatogram of phenolic compounds in wild-type, CRY1 overexpressing,
PHYB1 overexpressing, PHYB1 underexpressing plants, and PHYA1 underexpressing
plants ...........................................................................................................58
3.10. Molecular characterization of wild-type, CRY1 overexpressing, PHYB1
overexpressing, PHYB1 underexpressing plants, and PHYA1 underexpressing
plants ............................................................................................................59
3.11. Quantification of chlorogenic acids, rutin, and kaempferol-3-rutinoside in wild-type
and CRY1 overexpressing plants ..................................................................60
3.12. The accumulation of phenolic compounds is correlated to reciprocity (intensity x
time). .............................................................................................................61
3.13 The accumulation of phenolic compounds is correlated to light intensity .........62
3.14. The accumulation of phenolic compounds is correlated to light intensity and
regulated by the expression of CRY1. ..........................................................63
3.15. CRY1 overexpression results in increased secondary metabolite content in root....64
3.16. CRY1 overexpressing plants exhibit reduced UV-B injury .........................66
3.17. CRY1 overexpressing plants exhibit reduced UV-B injury .........................67
4.1. \textit{CRY1} overexpression results in increased stomatal density and violation of the one cell spacing rule .........................................................81
4.2. The stomate on tobacco leaves of Nt and S135........................................82
4.3. \textit{CRY1} overexpression results in increased lignin content in the vascular tissue……84
4.4. \textit{CRY1} overexpressing plants show wilting phenotype under cold stress........88
4.5. \textit{CRY1} overexpressing plants show wilting phenotype under high light stress……89
CHAPTER 1

LITERATURE REVIEW

The role of photoreceptor in light perception

Light is the major energy source for the earth. It is generally referred as electromagnetic radiation, and behaves like a wave. Plants can convert light energy into chemical energy through photosynthesis. An increase the absorption of light energy can increase the photosynthesis rate and generate more chemical compounds. Therefore, agriculture seeks to optimize the capture of light energy in order to improve the crop productivity (Dixon 2005).

Plants can perceive light signals through at least four distinct families of photoreceptors: phytochromes, cryptochromes, phototropins, and unidentified UV-B photoreceptors (Gyula et al., 2003). For perception of the full range of the solar light spectrum, phytochromes predominately absorb the red and far-red wavelength, cryptochromes and phototropins perceive blue and UV-A wavelengths, and an unidentified photoreceptor absorbs UV-B wavelengths (Jiao et al., 2007). Photoreceptors
typically consist of a protein moiety and a non-protein pigment (chromophore) that can react to light signals (Matute 2008). After activation by light, photoreceptors trigger a signal transduction cascades and regulate light-modulate gene expression (Muleo et al., 2007).

**Phytochromes**

Phytochromes are the most studied photoreceptors by far, and the functions of phytochromes are involved in regulating plant growth and development throughout the entire plant life cycle including seed germination, photomorphogenesis, shade avoidance, circadian rhythms, and control of flowering (Smith 2000; Mathews et al., 2006). Phytochromes are red and far-red light photoreceptors which absorb wavelengths between 600 and 750 nm. Phytochromes are composed of two 125-kDa polypeptides which carry a covalently linked open-chain tetrapyrrole chromophore, and exist in two inter-convertible forms: a red light-absorbing form (Pr) and a far-red light-absorbing form (Pfr). The conformation change of phytochromes results in the effects of red light and is reversible by far-red light (Su et al., 2007).

Phytochromes are dimeric chromoproteins containing a photosensory part, core/quail-box, and a regulatory domain. The domain at the N-terminus, which determines the photosensory properties, contains a single bilin chromophore (PΦB) covalently bound to a bilin lyase domain and a phytochrome domain (PHY). The core/quail-box contains two PER-AMT-SIM domains (PAS) which is required for
dimerization of phytochrome. The histidine kinase related domain (HKRD), which displays homology to bacterial two-component sensor kinase, is the regulatory domain at C-terminus (Muller et al., 2009).

**Cryptochromes**

Cryptochromes are blue and UV-A (320-500 nm) light photoreceptors that function in both plant and animal kingdoms, and the sequence comparison indicates that the plant and animal cryptochrome families have distinct evolutionary histories. The sequences of eukaryotic cryptochromes are homologous to bacterial DNA photolyases which are involved in repairing UV radiance-induced DNA damage by removing pyrimidine dimers. However, plant cryptochromes lack DNA-repair activity (Balland et al., 2009). Cryptochromes are flavoproteins that contain a photolyase-related domain (PHR) at the N-terminus and DQXVP-acidic-STATES (DAS) motif at the C-terminus (Sang et al., 2005). There are two chromophores: a flavin adenine dinucleotide (FAD) and a pterin non-covalently bound to the PHR domain (Fig. 1.1). An intrinsically unstructured C-terminal DAS domain (CCT) directly interacts with an E3 ligase, CONSTITUTIVE PHOTOMORPHOGENIC 1 (COP1) (Liu et al., 2008). Cryptochrome inhibits COP1 mediated degradation of transcription factors that activate light dependent gene expression. This ubiquitin-dependent degradation of cryptochromes is critical in mediating blue light signaling (Busino et al., 2007).
The functions of cryptochromes are involved in mediating photomorphogenesis, stomatal opening, and circadian rhythms (Toth et al., 2001; Kang et al., 2008).

**Phototropins**

Phototropins are autophosphorylating protein kinases that are activated in response to UV-A and blue light (320-500 nm). Photopropins harbor two flavin mononucleotide (FMN) chromophore-binging LOV domains at N-terminus and a serine/threonine kinase at C-terminus (Fig. 1.1). When blue light interacts with a phototropin protein in the cell membrane, the phototropin protein unfolds and undergoes phosphorylation that cause a cascade of events inside of the cell (Katsura et al., 2009).

The functions of phototropins are involved in stomatal opening, chloroplast movement, and phototropism (Jiao et al., 2007).

Figure 1.1. Domain organization of phytochromes, cryptochromes, and phototropins.
The photoreceptors in the model plant *Arabidopsis thaliana*

In the *Arabidopsis* genome, there are five phytochrome (*PHYA, PHYB, PHYC, PHYD, PHYE*), three cryptochromes (*CRY1, CRY2, CRY3*) and two phototropins (*PHOT1, PHOT2*) have been identified.

To gain insight into the responses to light signals among these ten photoreceptors, I analyzed the recently available expression data from 22k *Arabidopsis* Affymetrix microarray chip experiments compiled by Genevestigator (http://www.genevestigator.ethz.ch) (Grennan 2006). Genevestigator is a web-based application providing Meta-Profile analysis which represents vectors of expression for a given type of condition. It can characterize the expression level of genes of interest across anatomy, development, stimuli (drug treatments, diseases, stress) and mutations. The expression level of ten photoreceptors in response to different light treatments are shown in Fig. 1.2, and the data were calculated as log2 fold change. A value of 0 means no change, less than 0 means that the gene’s expression is reduced, and greater than 0 means that the gene’s expression is increased. The result suggested that most photoreceptors did not show significant transcriptional changes in response to light stimuli. Moreover, the expression levels of photoreceptors were similar to control in response to environmental stimuli (Fig. 1.3)

Some Affymetrix microarray analyses have been performed in the photoreceptor null mutant background to characterize the regulatory network of light signals. The 8k-microarray data performed in phyA verify that *PHYA* is responsible for regulation of the
genes that respond to FRc signals (Ma et al., 2001). The experiment performed in phyB mutant suggests that one or more of the remaining phy family members is predominantly responsible for perception and transduction of FRc signals (Tepperman et al., 2004). Genome-wide gene expression analysis performed in cry1 reveals that CRY1 is critical in the response to high irradiance. The results of the microarray experiments showed that high irradiance dependent regulation of 77 genes was only altered in cry1 mutants and unaffected in cry2, phyA, and phyB mutants. This study also demonstrated a novel function of CRY1 as a mediator of plant response to changes in irradiance (Kleine et al., 2007).

I tried to estimate the function of CRY1 from the relationship with the coregulated genes by using ATTED-II which provides the publicly available microarray data from 58 experiments (1388 slides) collected by AtGenExpress (http://www.atted.bio.titech.ac.jp/). CRY1 expression was highly related to anti-oxidant genes such as VTE5 (a phytol kinase involved in Vitamin E biosynthesis), LYC (a lycopene β-cyclase involved in forming the bicyclic β-carotene), and AtGPX1 (a glutathione peroxidase involved in ROS degradation). It was also highly correlated with genes involved in phenylpropanoid biosynthesis such as CYP71B24, CYP71B4 (cytochrome P450s involved in biosynthesis of secondary metabolites), and SNG1 (a serine carboxypeptidases function as an acyltransferase in secondary metabolism) (Whitbred et al., 2000; Lehfeldt et al., 2000) (Fig. 1.4).
Figure 1.2. The data on light responsive expression were compiled by Genevestigator.
Figure 1.3. The data on stimulus responsive expression were compiled by Genevestigator.
Figure 1.4. Co-expressed genes of CRY1 in *Arabidopsis thaliana*
Research objectives

Light regulates the developmental processes throughout the plant life cycle including seed germination, seedling photomorphogenesis, shade avoidance, and the photoperiod response. It is well known that plants can monitor the light environment and perceive signals through three distinct families of photoreceptors: phytochromes, cryptochromes, and phototropins (Jiao et al., 2007). However, the molecular mechanism of how plants are able to measure the quantity of light is still unclear. Using a web-based database analysis, I have discovered that the expression level of CRY1 does not show changes in response to light stimuli. Therefore, CRY1 is likely involved in cellular communication responsible for the integration of information for plant response to light intensity.

This is very significant because crops typically lose 10-15% yield to photoinhibition (reduction in photosynthesis due to excessive light) (Nielsen et al., 2006), and CRY1 overexpression may reduce that. It would be a great tool for crop improvement through either traditional breeding or biotechnology. In addition, by manipulation of CRY1 level in plants, many full-sun plants can be grown indoors without expensive gardening equipment. Based on the floriculture and nursery crops yearbook released by USDA (http://www.ers.usda.gov/Publications/Flo/), in the last decade, the value of floriculture crops in wholesale value of sales and large operations in Ohio State is approximately $170,000,000 per year. It is conceivable that this research will have a significant contribution to Ohio floriculture business.
To determine the role of *CRY1* in light signal transduction, I performed a series of phenotypic analyses using overexpression mutants to determine the effects of *CRY1* gene in response to light signaling. **The central hypothesis is:** *CRYPTOCHROME 1* (*CRY1*), a blue light photoreceptor, is critical in mediating developmental process in response to light intensity. My goal is to test whether altered expression of *CRY1* can enhance or decrease the sensitivity to light. Three objectives are included in this proposed study:

1. To characterize the role of *CRY1* in seed germination and early seedling development in response to light intensity
2. To determine the role of *CRY1* involved in photoprotection against excess light
3. To characterize the role of *CRY1* in regulating stomatal distribution and size and shape of vessel elements
CHAPTER 2

THE ROLE OF CRY1 IN MEDIATING SEED GERMINATION AND EARLY SEEDLING DEVELOPMENT IN RESPONSE TO LIGHT INTENSITY

INTRODUCTION

Seed is a plant embryo packaged with a supply of nutrients within a protective seed coat. The process by which a seed embryo develops into a seedling is called seed germination. Seed germination requires certain external stimuli and physical conditions to prevent damage or death from inappropriate conditions. One of the most important environmental factors affecting seed germination is light. Light is required for seed germination in many plant species, and the quality of light is critical because it regulates the promotion and inhibition of seed germination (Penfield et al., 2005). The red/far-red light photoreceptors, the phytochromes, have been shown to directly mediate this process. Light acting through phytochromes regulates the expression of GA 3 beta-hydroxylases
that catalyze the final biosynthetic step to produce bioactive GAs and stimulates the germination in *Arabidopsis* (Oh et al., 2009). Seeds germinate well in white and red light, but far-red light, blue light and UV-A irradiations strongly reduce seed germination. Seeds usually germinate better in partial shade, and this inhibition is classified as a high irradiance response (Pezzani et al., 2006). However, little is known about the amount of light that individual seeds really need for germination. The quantity of light required for seeds to break dormancy and the cellular communication responsible for the integration of information between seed germination and light quantity is still unclear.

To understand the role of photoreceptors in seed germination and early seedling development in response to light quantity, the experiments were conducted by using the transgenic plants overexpressing *CRYPTOCHROME 1 (CRY1)* which is critical to sense the changes in irradiance in leaf development. *CRY1* is a photoreceptor perceiving blue and UV-A wavelengths. *CRY1* is a class of flavoproteins that contain photolyase-related (PHR) domain and DAS domain (Sang et al., 2005). The functions of *CRY1* are involved in mediating hypocotyl elongation and stomatal opening. Genome-wide gene expression analysis reveals that *CRY* is critical in the response of *Arabidopsis* to high irradiance. It also demonstrates a novel function of *CRY1* as a mediator of plant response to changes in irradiance (Kleine et al., 2007).

The aim of the present study was to analyze the light quantity on seed germination and early seedling development in tobacco by testing whether altered expression of *CRY1* can enhance or decrease the germination rate in response to different light intensities. A
series of phenotypic analyses were performed to determine the effects of CRY1 gene in response to light signaling.
MATERIALS AND METHODS

Plant material

The CRY1 full length sequence cloned from *Nicotiana sylvestris* was driven by a 2x 35S promoter and transferred into *Nicotiana sylvestris* (Ns) and *Nicotiana tabacum* cv Maryland Mammoth (Nt) (Yendrek, 2006). S168 is a transgenic line in *Nicotiana sylvestris* background. S135 and S144 were the transgenic lines in *Nicotiana tabacum* background. T₄ seeds were used as plant materials for all experiments.

Seeds were surface-sterilized and rinsed with sterile water several times. Seeds were water-imbibed in the dark for 1 d at 4°C, and transferred to 1x Murashige and Skoog basal salt mixture with B5 vitamins and 0.05% (w/v) MES (pH 5.7), and 1% (w/v) Phytagel (Sigma Chemical Co., St. Louis, MO, U.S.A.). Seeds were individually spotted using a 1-µL sterile loop. Seeds were incubated in the dark at 4°C for 1 d and then were transferred to light intensities of 6, 60, or 600 µmol m⁻² s⁻¹. Germination kinetics were determined by measuring the time of radicle emergence from repeated experiments with duplicate plates of approximately 25 seeds each.

Western analysis
Seven-day-old seedlings were harvested and homogenized in protein extraction buffer (0.125M Tris, 4% SDS, 20% glycerol, 2% 2- mercaptoethanol, pH 6.8). After 5 minutes centrifugation, the supernatant was mixed with four times the sample volume of 100% acetone at 4°C for 30 minutes. Protein was precipitated by high speed centrifugation for 5 minutes and pellet was resuspended in protein extraction buffer.

Protein samples were fractionated on 8% acrylamide gel and transferred to Immobilon-P PVDF membrane (Millipore, Bed-ford, MA, USA) via electrophoretic transfer. Membranes were blocked in blocking buffer (5% skim milk, 0.1% Tween-20, TBS solution) overnight, and followed by one-hour incubation with CRY1 specific primary antibodies (1: 500). After washing three times, the membranes were incubated with an alkaline phosphatase conjugated goat-anti-rabbit secondary antibody (1: 500; Promega, Madison, WI, USA) for 1 hour. Membranes were washed and developed in an alkaline phosphate buffer with NBT/BCIP for 20 min.

**The ordinary least square**

The germination time was hypothesized to be a linear function of its associated factors. As such, the model was formulated as

\[ y_i = \beta_0 + \beta_1 M_i + \beta_2 H_i + \beta_3 S_i + \beta_4 C_i + \varepsilon_i \text{ for } i=1,\ldots,30 \]

where
$y_i$: Germination time.

$M_i$: 1 indicated light intensity is $60 \mu \text{ mol quanta m}^{-2}\text{s}^{-1}$, otherwise 0.

$H_i$: 1 indicated light intensity is $600 \mu \text{ mol quanta m}^{-2}\text{s}^{-1}$, otherwise 0.

$S_i$: 1 indicated observation $i$ is associated with 1% sucrose, otherwise 0.

$C_i$: 1 indicated observation $i$ is CRY1 overexpressing, otherwise 0.

$\varepsilon_i$: indicating regression error which follows normal distribution with mean 0 and variance $\sigma^2$.

Since there were 30 observations in the model, the observations were stacked into a matrix form as follows.

$$Y = \begin{bmatrix} y_1 \\ y_2 \\ \vdots \\ y_{30} \end{bmatrix}, \quad X = \begin{bmatrix} 1 & M_1 & H_1 & S_1 & C_1 \\ 1 & M_2 & H_2 & S_2 & C_2 \\ \vdots \\ 1 & M_{30} & H_{30} & S_{30} & C_{30} \end{bmatrix}, \quad \beta = \begin{bmatrix} \beta_0 \\ \beta_1 \\ \beta_2 \\ \beta_3 \end{bmatrix}, \quad \varepsilon = \begin{bmatrix} \varepsilon_1 \\ \varepsilon_2 \\ \varepsilon_3 \end{bmatrix}$$

The model was then expressed in a compact form as follows

$$Y = X\beta + \varepsilon, \text{ where } \varepsilon_i \sim N(0, \sigma^2)$$

The model parameter estimates were derived through the following analytical forms,

$$\beta = (X'X)^{-1}(X'Y)$$
\[ \text{var}(\beta) = \sigma^2 (X' X)^{-1} \]

\[ \sigma^2 = \frac{\hat{e}' \hat{e}}{N - k}, \] where \( N \) was the number of observations (30) and \( k \) was the number of coefficients (5) in the regression model.

The model was solved by the statistical software Stata10. Stata10 was used to derive the model coefficients and their associated variances. In order to know the explanatory power of the variables included in the model, \( R^2 \) and \( F \) values were also derived. \( R^2 \) was an indicator showing the percentage of the total variation explained by the model. \( F \) was an indicator showing whether the variables included in the model were jointly different from zero.
RESULTS

Transgenic tobacco with increased expression levels of CRY1 protein

Western analysis revealed that one *N. sylvestris* transgenic line (S168) and two *N. tabacum* transgenic lines (S135 and S144) contained higher CRY1 protein level than wild-type plants (Fig. 2.1). Because of the position effect, the expression of CRY1 level in S144 was less than S135.

CRY1 affects seed weight and size

Seed size and weight can be a predictor of germination success and early seedling growth. To understand the effect of *CRY1* on seed quality, the size and weight of seeds were observed and measured in wild-type and *CRY1* overexpressors. The results showed that size and weight of seeds of S168 were similar to Ns, but the seed sizes and weight of S135 and S144 were 20% less than Nt (Fig. 2.2 and Fig. 2.3).

CRY1 overexpression results in a reduced germination

To understand the role of *CRY1* in seed germination in response to different light intensities, germination assays were conducted at low, medium, and high light intensities. Seeds were grown at 6, 60, and 600 µmol quanta m$^{-2}$ s$^{-1}$ for 24 h (Fig. 2.4). Increasing light intensity only slightly affected the final percentage of germination in wild-type.
Seeds overpressing \textit{CRY1} exhibited reduced germination percentage compared to WT. Both S135 and S144 showed 40% reduction at high light intensity. S168 exhibited 40% reduction and 25% reduction at low and high light intensity. Moreover, increasing light intensity resulted in a longer time interval between successive peaks. This suggests that \textit{CRY1} is involved in regulating seed germination in response to light intensity.

\textbf{\textit{CRY1} in seed germination and nutrient usage}

To understand the role of \textit{CRY1} in seed germination and nutrient usage, seeds were transferred to MS medium including 1% sucrose, and grown at 6, 60 and 600 µmol quanta m\(^{-2}\) s\(^{-1}\) for 24 h (Fig. 2.5). Seeds overexpressing of \textit{CRY1} had a reduced germination percentage under both low and high light intensities. Surprisingly, the lower germination percentages in the \textit{CRY1} overexpressors were reversed by high light intensities in contrast to seeds of WT.

\textbf{\textit{CRY1} overpression results in a significant delay of germination}

The mean of germination time of \textit{N. sylvestris}, S168, \textit{N. tabacum} cv. Maryland Mammoth, S135, and S144 under 6, 60, and 600 µmol quanta m\(^{-2}\) s\(^{-1}\) was listed in Table 2.1. To determine the marginal effects of control variables on germination time, the ordinary least squares was performed using software Stata. The germination time was the function of these treatment variables. The results showed that increasing light intensity...
led to prolong the timing of germination (Table 2.2). *CRY1* overexpression results in a significant delay in seed germination in both *N. sylvestris* and *N. tabacum*.

**CRY1 affects the seedling development**

To understand the role of *CRY1* in seedling development, seedlings were grown at 60 μmol quanta m\(^{-2}\) s\(^{-1}\) continuous light for five days (Fig. 2.6) and ten days (Fig. 2.7). It showed that *CRY1* overexpression results in an inhibition of early seedling development. The size of cotyledons of *CRY1* overpressior was smaller than WT.
Figure 2.1. Molecular characterization of *N. sylvestris* and *N. tabacum* cv. Maryland Mammoth transgenic lines. The band presented as CRY1 was corresponding to the *N. sylvestris* cryptochrome 1 (GenBank Protein ID: ABB36796.1). Coomassie-stained large subunit of RUBISCO was shown as a control of protein loading.
Figure 2.2. *CRY1* affects seed size. Mature seeds of Ns, S168, S135, and S144. Bars represent 1 mm.
Figure 2.3. *CRY1* affects seed weight. Mean weight per 100 seeds of Ns, S168, Nt, S135, and S144.
Figure 2.4. *CRY1* overexpression results in a reduced germination. Seed germination percentage of Ns, S168, Nt, S135, and S144 grown on MS medium without sucrose under different light intensities.
Figure 2.5. The lower germination percentages in the *CRY1* overexpressors were reversed by high light intensities. Seed germination percentage of Ns, S168, Nt, S135, and S144 grown on MS medium with 1% sucrose under different light intensities.
Table 2.1. Time of germination of Ns, S168, Nt, S135, and S144 under different light intensities.

<table>
<thead>
<tr>
<th>Light intensity (µmol quanta m⁻² s⁻¹)</th>
<th>Ns</th>
<th>S168</th>
<th>Nt</th>
<th>S135</th>
<th>S144</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>60.90±5.55</td>
<td>93.93±38.74</td>
<td>56.88±4.31</td>
<td>85.81±25.47</td>
<td>97.06±25.31</td>
</tr>
<tr>
<td>60</td>
<td>72.19±10.69</td>
<td>106.95±20.83</td>
<td>65.22±6.01</td>
<td>109.70±27.82</td>
<td>104.86±20.88</td>
</tr>
<tr>
<td>600</td>
<td>72.97±13.44</td>
<td>129.32±27.12</td>
<td>77.63±16.34</td>
<td>138.39±35.92</td>
<td>138.53±31.18</td>
</tr>
<tr>
<td>With1% sucrose</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>69.15±7.83</td>
<td>108.00±41.83</td>
<td>68.63±8.41</td>
<td>86.32±11.88</td>
<td>90.53±11.58</td>
</tr>
<tr>
<td>60</td>
<td>72</td>
<td>104.31±13.48</td>
<td>72</td>
<td>86.48±13.44</td>
<td>88.80±11.09</td>
</tr>
<tr>
<td>600</td>
<td>82.17±11.96</td>
<td>113.14±16.95</td>
<td>92.34±26.30</td>
<td>105.68±26.78</td>
<td>102.11±24.10</td>
</tr>
<tr>
<td>Variable</td>
<td>Estimates</td>
<td>Standard error</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------------------</td>
<td>-----------</td>
<td>----------------</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>64.117***</td>
<td>4.302</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light intensity (60 mol m(^{-2}) s(^{-1}))</td>
<td>6.530</td>
<td>4.493</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Light intensity (600 mol m(^{-2}) s(^{-1}))</td>
<td>23.507***</td>
<td>4.493</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>With 1% sucrose</td>
<td>-4.579</td>
<td>3.669</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRY1 overexpressing</td>
<td>33.156***</td>
<td>3.744</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

N 30, K-1 4, R\(^2\)(Adj- R\(^2\)) 0.8136(0.7838), F value 27.29***

*** denotes the variable is significant at 1% level of significance

Table 2.2. The marginal effects of control variables on germination time.
Figure 2.6. *CRY1* affects the early seedling development. Five-day-old seedlings of Ns, S168, Nt, S135, and S144
Figure 2.7. *CRY1* overpression results in an inhibition of early seedling development.

Ten-day-old seedlings of Nt, S135, and S144
DISCUSSION

Seed germination is affected by environmental factors including temperature, water, oxygen, and light. The seed begins to germinate when it is under favorable conditions, or it stays dormant to prevent from suffering damage or death from bad weather. Seeds may fail to germinate even under proper environmental condition due to the internal factors. These internal factors include the immature embryos, hard seed coats, abscisic acid, or presence of some chemicals which inhibit the development of the embryos. Phenolic compounds are implicated to be a class of inhibitors in the regulation of seed germination. For starting the embryo growing, seeds must active metabolism mechanisms to eliminate these chemicals before they can sprout.

In wild type plants, the final germination percentage was unaffected by different light intensity, but high light intensity caused a significant delay in germination. Interestingly, plants overexpressing CRY1 showed reduced germination percentage and a delay in the timing of germination under both low and high light intensities. Surprisingly, the lower germination percentages in the CRY1 overexpressors can be reversed by high light intensities in contrast to seeds of wild type. Therefore, CRY1 is involved in mediating seed germination in response to different light intensities.
CHAPTER 3

CRY1 IS CRITICAL FOR SENSING CHANGES IN IRRADIANCE AND MAY BE INVOLVED IN PHOTOPROTECTION IN TOBACCO

INTRODUCTION

Light provides energy required for plant growth and development. While lack of sufficient light results in poor plant growth, too much light can also be harmful. When light is more than plant needs for photosynthetic productivity, the accumulation of excess excitation energy leads to photooxidative damage to the photosynthetic apparatus (Mullineaux et al., 2002; Li et al., 2008). To mitigate photodamage, plants have evolved several protection mechanisms including chloroplast avoidance movement (Kasahara et al., 2002), repair processes for damaged photosystem II reaction center (Vass et al., 2007), and accumulation of antioxidant molecules (Penuelas et al., 2005). Flavonoid, phenolics,
anthocyanin, lignin and carotenoids, which have antioxidant activity, can protect the cellular anatomy from oxidative damage (Close et al., 2002). The leaf morphology and anatomy are altered in response to high light intensity for optimizing the photosynthetic efficiency (Kim et al., 2005). Leaves grown in high light intensity become thicker because they develop longer palisade cells or an additional layer of palisade cells. The greater mesophyll surface area per unit facilitates CO₂ uptake into the cells thereby increasing the rate of photosynthesis. To prevent excessive loss of water during transpiration, they also have thicker cuticle and contain higher stomatal density and small epidermal cells (Yano et al., 2001).

To understand the activation and protection mechanism against photodamage, I attempted to determine which photoreceptors critical sensing the changes in irradiance. There are three major classes of known photoreceptors: phytochromes, cryptochromes, and phototropins. For perception of the full range of the solar light spectrum, phytochromes predominately absorb the red and far-red wavelength, and both cryptochromes and phototropins perceive blue and ultraviolet A wavelengths (Jiao et al., 2007). Phototropins are not essential for the distinct leaf developmental and gene expression responses to light quantity in Arabidopsis (López-Juez et al., 2007). Genome-wide gene expression analysis reveals that CRYPTOCHROME1 (CRY1) is critical in the response of Arabidopsis to high irradiance. The results of the microarray experiments showed that high irradiance dependent regulation of 77 genes was only altered in cry1 mutants and unaffected in cry2, phyA, and phyB mutants. This study also demonstrated a
novel function of \textit{CRY1} as a mediator of plant response to changes in irradiance (Kleine et al., 2007).

Cryptochromes are blue-light signaling receptors found in plants, animals, and humans. They are a class of flavoproteins, which contain photolyase-related (PHR) domain and DQXVP-acidic-STATES (DAS) domain, control plant development and the entrainment of circadian rhythms (Li et al., 2007). In the \textit{Arabidopsis} genome, there are three cryptochromes: \textit{CRY1}, \textit{CRY2}, \textit{CRY3} have been identified. The sequence of \textit{CRY1} is homologous to DNA photolyases which are involved in repairing UV radiance-induced DNA damage by removing pyrimidine dimers, but \textit{CRY1} lacks DNA-repair activity (Balland et al., 2009). In \textit{Nicotiana sylvestris}, \textit{CRY1} full length fragments (GenBank: DQ231576.1) has been cloned and obtained by RT-PCR and RACE techniques (Yendrek 2006). The reverse genetics approach was performed by transferring \textit{CRY1} overexpression to the short day plant \textit{Nicotiana tabacum} cv Maryland Mammoth (Nt) and long day plant \textit{Nicotiana sylvestris} (Ns) to generate \textit{CRY1} overexpressors (Yendrek 2006). The results showed that \textit{CRY1} overexpression results in a short-hypocotyl phenotype and leads to floral initiation in non-inductive photoperiod. \textit{CRY1} also affects plant height, leaf area, dry weight, leaf chlorophyll content and photosynthetic rate (Yendrek 2006). These results suggest that \textit{CRY1} is not only critical for sensing changes in irradiance in \textit{Arabidopsis} but also in tobacco. \textit{CRY1} is likely involved in cellular communication responsible for the integration of information for excess light regulated gene expression and leaf development in response to light intensity.
MATERIALS AND METHODS

Plant material and growth conditions

Wild-type plants *Nicotiana sylvestris* (Ns) and *CRY1* overexpressor plants (S168) were germinated and planted in pots containing commercial soilless media (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH). For low light (LL) conditions, plants were grown under fluorescent lamps in growth chamber: light intensity 60 $\mu$mol quanta m$^{-2}$ s$^{-1}$, photoperiod 8 h, and temperature setpoint of 22°C. For high light (HL) condition, plants were grown under direct sunlight in greenhouse: light intensity 600-1500 $\mu$mol quanta m$^{-2}$ s$^{-1}$, photoperiod 8 h, and temperature setpoint of 22°C An 8 h photoperiod was achieved by covering the bench from 17:00 HR to 9:00 HR with blackout cloth. For different light intensities treatments, plants were grown under fluorescent lamps in a growth chamber with a temperature setpoint of 22°C, and a 16h photoperiod. The light intensities were 60, 300, or 600 $\mu$mol m$^{-2}$ s$^{-1}$.

For root assay, seeds were surface-sterilized and individually spotted on 1x Murashige and Skoog basal salt mixture with B5 vitamins, 1% (w/v) sucrose, 0.05% (w/v) MES (pH 5.7), and 1% (w/v) Phytage (Sigma Chemical Co., St. Louis, MO, U.S.A.). Seeds were germinated and grown on vertical-positioned plates under light intensities of 60 $\mu$mol m$^{-2}$ s$^{-1}$ 24 h for 4 weeks. Hand-cut sections of root were observed using light microscope.
Measurement of chlorophyll

The measurement of chlorophyll was as described by the method of Moran 1982. Leaf tissues were dried at 60°C for 48 h and ground into fine powder. A 0.01 g sample was taken and added 5 mL of N,N-dimethylformamide. The extract was stored in the dark at 4°C for 72 hr and then centrifuged at the highest speed for 5 minutes. The supernatant was collected and measured the absorbance readings at 603nm, 625nm, 647nm and 664nm was measured. Chlorophyll content was calculated using formulae:

Chlorophyll b: (647nm*26.01) - (664nm*4.93) + (625nm*3.74) – (603nm*15.55)
Chlorophyll a: (664nm*12.81) – (647nm*2.16) + (625nm*1.44) – (603nm*4.91)
Chlorophyll total: (664nm*8.24) + (647nm*23.97) – (603nm*16.64)

Total flavonoid and phenolics determination

Total soluble flavonoid and phenolics were determined by Pourmorad et al., 2006. Leaf tissue was dried at 60°C for 48 h, and ground into fine powder. A 0.1 g sample was taken and added with 5 mL of methanol. The plant extract was centrifuged at the highest speed for 5 minutes. For total flavonoid assay, the 0.5 mL supernatant was mixed with 1.5 mL of methanol, 0.1ml of 10% aluminum chloride, 0.1 mL 1M potassium acetate, and 2.8 mL of distilled H2O. The mixture was incubated at room temperature for 30 minutes and measured the absorbance readings at 415nm. The amount of total flavonoids was calculated from a linear calibration curve (0–100 µg) with commercial quercetin
(Sigma-Aldrich, Steinheim, Germany). For total soluble phenolic assay, the 0.5 mL supernatant was mixed with 5 mL of 10% Folin Ciocalteu reagent, and 4mL of 1M sodium carbonate (Na$_2$CO$_3$). The total phenolic concentration was determined at 765nm. The amount of phenolics was calculated from a linear calibration curve (0–200 µg) with commercial gallic acid (Sigma-Aldrich, Steinheim, Germany).

**Lignin determination**

Lignin was extracted and measured by the method of Müsel et al.,1997. A 0.5 g sample was homogenized in 10 mL of 99.5% (v/v) ethanol and the extract was centrifuged at maximum rpm for 15 minutes. The pellet was transferred to a glass Petri dish and air-dried, 50 mg of dried residue was placed in a screw-cap tube, and then 750 µL of 2N HCl and 250 µL of thioglycolic acid were added. The sealed tube was heated at 100°C for 4 h. After cooling, the contents were centrifuged at highest rpm for 30 minutes at 4°C. The pellet was washed once with 2.5 mL of water, then resuspended in 1 mL of 0.5N NaOH. The solution was agitated gently at 25°C for 18 h. After centrifugation at the highest rpm for 30 minutes, the supernatant was transferred to a test tube. Concentrated HCl (1 mL) was added to the test tube and the lignin thioglycolate was allowed to precipitate at 4°C for 4 h. After centrifugation at the highest rpm for 30 minutes, the pellets were dissolved in 10 mL of 0.5N NaOH. The absorbance was measured against a NaOH blank at 280nm. The amount of lignin was calculated from a linear calibration curve (0–20 µg) with commercial alkali lignin (Sigma-Aldrich, Steinheim, Germany).
Lignin staining of vascular tissues was using phloroglucinol method described by Liljegren et al., 2000. The hand-cut cross sections of fresh leaf petiole were stained for 2 minutes in 2% phloroglucinol solution in 95% ethanol and then photographed in 50% HCl. Lignin appeared red-violet.

**Determination of phenolics content using high performance liquid chromatography**

The method was described by Graham 1991. One hundred mg of fresh tobacco leaf was ground with 800 µL 80% ethanol. The extract was centrifuged at the highest speed for 5 minutes and the supernatant subjected to high performance liquid chromatography (HPLC). 40 µL were injected into a 4.6 mm i.d. x 250 mm Hibar Ec Cartridge containing Merck Lichrosorb RP-18 10 µm C18 reverse phase packing (Alltech Associates, Deerfield, IL). The extract was fractionated using a linear gradient of 5 to 30% acetonitrile in pH 3 water for 15 minutes, followed by a step increase to 60% acetonitrile in pH 3 water for 10 minutes, and then increased to 100% acetonitrile for 2 minutes. The gradient was held at 100% acetonitrile for 1 minute before returning to 5% acetonitrile. A flow rate of 1.5 mL min⁻¹ was used and the column eluates were detected at 240nm and 260nm.

**Liquid chromatography-mass spectrometry**
The liquid chromatography-mass spectrometry system comprised a Varian 212-LC binary gradient LC/MS pump (Varian Inc., USA), an autosampler, and a Varian 500-MS ion trap spectrometer. Samples were fractionated by reverse phase HPLC 4.6 mm i.d. x 250 mm Hibar Ec Cartridge containing Merck Lichrosorb RP-18 10 µm C18 reverse phase packing (Alltech Associates, Deerfield, IL) was used at a flow rate of 0.4 mL min⁻¹, and a linear gradient of 5 to 30% acetonitrile in water containing 1% formic acid for 37 minutes, followed by a step increase to 60% acetonitrile in water containing 1% formic acid for 25 minutes, and then increase to 100% acetonitrile for 5 minutes. The column was maintained at 100% acetonitrile was held for 3 min and then returned to 5% acetonitrile in water containing 1% formic acid. This system was operated with MS workstation software (version 6.9; Varian, Inc.). Mass spectra were acquired using an electrospray ionization (ESI) source in the negative ionization mode. Full-scan mass spectra were recorded over a range of 50 to 1000 m/z. Operating parameters for samples in the negative ion mode included a spray needle voltage of -5000 volts, capillary voltage of 80.0 volts, drying temperature of 350°C, drying gas pressure of 20.0 psi nitrogen, nebulizer gas pressure of 40.0 psi air, and helium as the neutral collision gas. Tandem mass spectrometry analysis was conducted with scan-type turbo data-dependent scanning (DDS). The collision-induced dissociation (CID) voltage was automatically set by DDS, and the mass width was 3 m/z.

Western analysis
Four-week-old seedlings were harvested and homogenized in protein extraction buffer (0.125M Tris, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8). After 5 minutes centrifugation, the supernatant was mixed with four times the sample volume of 100% acetone at 4°C for 30 minutes. Protein was precipitated by high speed centrifugation for 5 minutes and pellet was resuspended in protein extraction buffer.

Protein samples were fractionated on an 8% acrylamide gel and transferred to Immobilon-P PVDF membrane (Millipore, Bed-ford, MA, USA) via electrophoretic transfer. Membranes were blocked in blocking buffer (5% skim milk, 0.1% Tween-20, TBS solution) for overnight, and followed by one-hour incubation with CRY1 specific primary antibody (1: 500). After washing three times, the membranes were incubated with an alkaline phosphatase conjugated goat-anti-rabbit secondary antibody (1: 500; Promega, Madison, WI, USA) for 1 hour. Membranes were washed and developed in an alkaline phosphate buffer with NBT/BCIP for 20 min.

**UV-B treatment**

Visible and UV light were provided from Reptisun 10.0 UVB lamps (Zoo Med, San Luis Obispo, CA, USA). Four 18 W tubes in two double-lamp nonreflector luminaire were 20cm from plants. UV irradiances were measured with a radiometer/photometer (Model IL1350, International Light Inc., Newburyport, MA), and photosynthetic photon flux (PPF) was measured with a quantum/radiometer/photometer: Model LI-250 (LI-
COR Inc., Lincoln, Nebraska, USA). The lamps provided irradiances of UV-A and UV-B
44 kJ m\(^{-2}\) d\(^{-1}\) and 88 kJ m\(^{-2}\) d\(^{-1}\) respectively. The PPF was 60 \(\mu\)mol quanta m\(^{-2}\) s\(^{-1}\).
RESULTS

**CRY1 overexpression reduces photodamage in plants**

Plants cannot tolerate excessively high light levels. When light intensity exceeds what the plants need, reactive oxygen species are generated in the chloroplast and cause photodamage (Li et al., 2008). When wild-type *Nicotiana sylvestris* (Ns) and *CRY1* overexpressor (S168) were grown in high light (HL) conditions, leaves of S168 remained green but leaves of Ns displayed a dramatic photodamage phenotype: bleaching, chlorosis, and bronzing of leaves (Fig. 3.1). Total chlorophyll content of leaves in S168 was 3-fold higher than Ns in high light conditions (Fig. 3.2a), and no changes were observed in chlorophyll a/b ratio of Ns and S168 (Fig. 3.2b). The increase of chlorophyll content was probably due to increased synthesis of chloroplasts. When plants were grown in tissue culture condition, root greening was observed in *CRY1* overexpression plants (Fig. 3.3). Well-developed chloroplasts occurred in S168 root with exposure of seedlings to light. The region of maturation was green, but the regions of cell elongation and cell division, and root hair were not (Fig. 3.4). Well-developed chloroplasts were located in cortex of mature root segments. Total chlorophyll content of root in *CRY1* overexpressing plants was 100-fold more than wild-type plants (Fig. 3.5). It suggested that the function of *CRY1* may be involved in enhanced chloroplast development or increased synthesis of chloroplasts.
**CRY1 overexpression results in increased secondary metabolite content**

S168 contained higher total phenolic (Fig. 3.6 a) and flavonoid (Fig. 3.6 b) content than Ns under either HL or LL conditions. Although there is no significant change between Ns and S168 in total lignin content (Fig. 3.6 c), the result of lignin staining of leaf petiole showed much thicker secondary cell wall and darker purple color in S168 (Fig. 3.6 d). It suggested that relatives more lignin accumulated in the vascular bundles of S168 than Ns.

In order to evaluate the potential impact of CRY1 overexpression on different pathways of phenolic metabolism, the phenolic compounds of plants grown under high light conditions were separated by HPLC (Fig. 3.7). There were three major peaks detected in plant leaf extracts and were numbered consecutively according to their order of retention time. The peaks were quantified by the area of peaks. The compounds of peak IV and VI showed 3-fold more than peak I and III, and the compounds of peak V showed more than 10-fold more than peak II.

Identification of phenolic compounds was attempted using information derived from HPLC (Fig. 3.7), LC-MS (Fig 3.8), and LC-MS/MS data. Table 3.1 summarized the data of major compounds. Compounds that were eluted from 19 to 32 minutes exhibited UV spectral characteristics similar to chlorogenic acid isomers, and displayed an m/z of 353 [M-H] and a major fragmentation in at 191 [M-H] which is likely to correspond to the quinic acid moiety, suggesting that those compounds are chlorogenic acids. All these
compounds had the same mass spectra, and the daughter fragment ion mass spectra from parent ion \([M-H]^-\) was the same. The difference between each peak was the abundance of an m/z of 191. Compound 2 was confirmed as chlorogenic acid (1, 3, 4, 5-tetrahydroxycyclohexanecarboxylic acid 3-(3,4-dihydroxycinnamate)) by comparing of the retention time and mass spectra of standard.

Compound 3 exhibited an m/z of 609 \([M-H]^-\) was identified as quercetin-\(O\)-rutinoside (rutin) based on the identical retention time and mass spectrum of authentic rutin. It was also confirmed to correspond to rutin by spiking with a standard. Based on the retention time and mass spectra, compound 3 is the same compound as the peak VI shown in HPLC analysis. Rutin content of the leaves in the \(CRY1\) overexpressor was 10-fold higher than wild-type.

Compound 4 exhibited an m/z of 593 \([M-H]^-\) was identified as kaempferol-3-rutinoside, and an m/z of 623 was a fragment ions resulting from addition of formic acid \([M-H+HCOOH]^-\). Kaempferol-3-rutinoside is an isomerd rutin, but the hydroxide is in 3' position carbon.

Compound 5 exhibited an m/z of 623 \([M-H]^-\) and was identified as isorhamnetin-3-\(O\)-rutinoside.

There were no commercial standards of kaempferol-3-rutinoside and isorhamnetin-3-\(O\)-rutinoside available, so the results of MS/MS and MS/MS/MS of compound 3 and 4 were confirmed by analogy with information provided from MassBank (http://www.massbank.jp/).
The accumulation of secondary metabolite is independent of the function of PHYA and PHYB

To determine the role of photoreceptors in mediating the accumulation of phenolic compounds, the phenolic content of CRY1 overexpressing plants was compared with phytochrome overexpressing or underexpressing plants. SOB36 is the transgenic Nicotiana sylvestris overexpressing PHYB1, and SCB35 is cosuppressing the PHYB1 gene. SUA2 is the transgenic Nicotiana sylvestris underexpressing PHYA1 (Zheng et al., 2006). The phenolic compounds in Ns, S168, SOB36, SCB35, SUA2, Nt, S135, and S144 were identified by LC-MS (Fig. 3.9). The result showed that there were no changes in chlorogenic acids, rutin, and kaempferol-3-rutinoside content whether altered expression of PHYA or PHYB. Therefore, the accumulation of these compounds was independent of the function of PHYA and PHYB.

The accumulation of phenolic compounds is correlated to the expression level of CRY1

Western analysis revealed that CRY1 overpressing plants (S168, S135, and S144) contain higher CRY1 level than wild-type plants (Ns and Nt) (Fig. 3.10). Although both S135 and S144 were CRY1 transgenic in N. tabacum background, the expression of CRY1 level in S144 was less than S135. The expression level of CRY1 in SOB36,
SCB35, and SUA2 was similar to Ns. It suggested that the expression level of CRY1 was unaffected by the changes of expression level of either PHYB1 or PHYA1.

The phenolic compounds in Ns, S168, Nt, S135, and S144 were identified by LC-MS, and the data were confirmed in six replica experiments. The amount of chlorogenic acids, rutin, and kaempferol-3-rutinoside in fresh tissue were based on the area of each compound and calculated from a linear calibration curve with authentic standard (Fig. 3.11). Leaves of CRY1 overexpressing plants contained two-fold chlorogenic acids, ten-fold rutin, and six-fold kaempferol-3-rutinoside more than wild-type plants. Although both S135 and S144 were CRY1 transgenic in N. tabacum background, the expression of CRY1 level in S144 was less than S135. Interestingly, the amount of chlorogenic acids, rutin, and kaempferol-3-rutinoside in S144 were also less than S135, and the reduction of three compounds was all 20%. It suggested that the accumulation of phenolic compounds is correlated to the expression level of CRY1.

The accumulation of phenolic compounds is correlated to reciprocity and light intensity

In an attempt to separate the effects of light intensity and total fluence, plants were grown in the same reciprocity conditions: 60 µmol quanta m⁻² s⁻¹ for 24 h and 180 µmol quanta m⁻² s⁻¹ for 8 h (Fig. 3.12) for 4 weeks. The LC-MS results showed that there were no changes in the amount of chlorogenic acids, rutin, and kaempferol-3-rutinoside
between two conditions. Therefore, the accumulation of phenolic compounds was related to reciprocity.

To determine the role of light intensity in regulating the accumulation of phenolic compounds, plants were grown in the same photoperiod: 16 h but different light intensities: 60, 300, and 600 µmol quanta m\(^{-2}\) s\(^{-1}\). *CRY1* overexpression resulted in increased secondary metabolite content. The amount of chlorogenic acids, rutin, and kaempferol-3-rutinoside were increased with increasing light intensity and finally reached the saturation status in both wild-type and *CRY1* overexpressing plants (Fig. 3.13). S135, which contained higher *CRY1* expression level, reached saturation status under 300 µmol quanta m\(^{-2}\) s\(^{-1}\) condition. S144 reached saturation status under 600 µmol quanta m\(^{-2}\) s\(^{-1}\) condition (Fig. 3.14). It suggested that saturation kinetics was correlated to the expression level of *CRY1*.

**Phenolic compounds in root identified by LC-MS/MS**

Identification of phenolic compounds was attempted using LC-MS (Fig. 3.15), and LC-MS/MS data. Table 3.2 showed summarizes the data of major compounds.

Compounds 1 that were eluted on 29.1 displayed an m/z of 355 [M-H]\(^{-}\), a fragmentation at 733 [2M+Na-H]\(^{-}\) which is likely to correspond to the dimer, and a fragmentation at 1111 [3M+2Na-3H]\(^{-}\) which is likely to correspond to the trimer. Compound 1 was identified as ferulic acid hexoside. The results of MS/MS and MS/MS/MS of compound 1 were confirmed by analogy with information provided from literature (Rest et al., 2006).
Compound 2 exhibited an m/z of 609 [M-H]⁻ was identified as quercetin-\(O\)-rutinoside (rutin) based on the identical retention time and mass spectrum of authentic rutin. It was also confirmed to correspond to rutin by spiking with a standard.

Compound 3 exhibited an m/z of 623 [M-H]⁻ was identified as Isorhamnetin-3-\(O\)-rutinoside. The results of MS/MS and MS/MS/MS of compound 3 were confirmed by analogy with information provided from MassBank (http://www.massbank.jp/).

**CRY1 overexpressing plants exhibited reduced UV-B injury**

UV-B (280-320nm) causes oxidative damage in plants. Leaf cupping appeared on wild-type plants after 3 day UV-B exposure (Fig. 3.16), but CRY1 overexpressing plants did not. S135, which contained higher expression level of CRY1 and secondary metabolite content, exhibited reduced UV-B injury than S144 after 6 day UV-B exposure (Fig. 3.17). It may be due to the presence of phenolic compounds in the epidermal cells of CRY1 overexpressing leaves which protect mesophyll tissues from the harmful effects of UV-B radiation.
Figure 3.1. *CRY1* overexpression reduces photodamage in plants. Wild-type *Nicotiana sylvestris* (Ns) and *CRY1* overexpressor (S168) were grown under high light condition for three months. Leaves of Ns showed a dramatic photodamage phenotype, but leaves of S168 remained green.
**Figure 3.2.** *CRY1* overexpression results in increased chlorophyll content. Total chlorophyll content (a) and ratio of chlorophyll a to chlorophyll b (b) of Ns and S168 grown under low light (LL) and high light (HL) conditions for three months. Total chlorophyll content of leaves in S168 was 3-fold higher than Ns in HL conditions and 1.5-fold higher than Ns in LL conditions. There was no difference in chlorophyll a/b ratio between Ns and S168 in both HL and LL conditions.
Figure 3.3. *CRY1* overexpression results in root greening. Ns (a) (c) and S168 (b) (d) were grown on vertical agar plates under 60 μmol quanta m⁻² s⁻¹ 24 h for 4 weeks. Well-developed chloroplasts were observed in S168 root.
Figure 3.4. Chloroplasts are located in cortex of the maturation zone. S168 root (a).

The cross section of S168 root (b). The longitudinal section of S168 root (c).
Figure 3.5. *CRY1* overexpression results in increased chlorophyll content in root.

Total chlorophyll content in *CRY1* overexpressing root was 100-fold higher than wild-type root.
Figure 3.6. CRY1 overexpression results in increased secondary metabolite content.

Total phenolic content (a), total flavonoid content (b), and total lignin content (c) were obtained in leaves of three months old plants grown under LL and HL conditions. The vascular tissues were stained with phloroglucinol on hand-cut cross sections of leaf petiole of Ns and S168 grown under HL condition (d).
Figure 3.7. HPLC analysis shows increased secondary metabolite content in *CRY1* overexpressing plants. Ns (a) and S168 (b) were grown under high light condition for three months. Chromatograms were obtained with UV detector at 240nm.
Figure 3.8. Total ion chromatogram of phenolic compounds in wild-type and *CRY1* overexpressing plants. Ns (a) and S168 (b) were grown under high light condition for three months. Negative ion mass spectra of compound 1 (c), compound 2 (d), compound 3 (e), compound 4 (f), and compound 5(g).
<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time (min)</th>
<th>[M-H] (m/z)</th>
<th>Daughter ions MS/MS</th>
<th>Daughter ions MS/MS/MS</th>
<th>Suggested compound</th>
</tr>
</thead>
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<tr>
<td>1</td>
<td>22.2</td>
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<td>191</td>
<td>173, 111, 85, 151</td>
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</tr>
<tr>
<td>2</td>
<td>27.7</td>
<td>353</td>
<td>191</td>
<td>173, 111, 85, 151</td>
<td>Chlorogenic acid</td>
</tr>
<tr>
<td>3</td>
<td>37.5</td>
<td>609</td>
<td>301</td>
<td>271</td>
<td>Rutin</td>
</tr>
<tr>
<td>4</td>
<td>40.9</td>
<td>593</td>
<td>285</td>
<td>257</td>
<td>Kaempferol-3-rutinoside</td>
</tr>
<tr>
<td>5</td>
<td>41.5</td>
<td>623</td>
<td>315</td>
<td>300, 272</td>
<td>Isorhamnetin-3-O-rutinoside</td>
</tr>
</tbody>
</table>

Table 3.1 Negative ions with corresponding retention time and suggested compound.
Figure 3.9. Total ion chromatogram of phenolic compounds in wild-type, *CRY1* overexpressing, *PHYB1* overexpressing, *PHYB1* underexpressing plants, and *PHYA1* underexpressing plants. Ns (wild-type plant), S168 (*CRY1* overexpressing plant), SOB36 (*PHYB1* overexpressing plant), SCB35 (*PHYB1* cosuppressing plant), SUA2 (*PHYA1* underexpressing plant), Nt (wild-type plant), S135 (*CRY1* overexpressing plant), and S144 (*CRY1* overexpressing plant) were grown under 60 µmol quanta m⁻² s⁻¹ 24 h for 4 weeks.
Figure 3.10. Molecular characterization of wild-type, *CRY1* overexpressing, *PHYB1* overexpressing, *PHYB1* underexpressing plants, and *PHYA1* underexpressing plants. The band presented as CRY1 was corresponding to the *N. sylvestris* cryptochrome 1 (GenBank Protein ID: ABB36796.1). Ns (wild-type plant), S168 (*CRY1* overexpressing plant), SOB36 (*PHYB1* overexpressing plant), SCB35 (*PHYB1* cosuppressing plant), SUA2 (*PHYA1* underexpressing plant), Nt (wild-type plant), S135 (*CRY1* overexpressing plant), and S144 (*CRY1* overexpressing plant) were grown under 60 μmol quanta m⁻² s⁻¹ 24 h for 4 weeks.
Figure 3.11. Quantification of chlorogenic acids, rutin, and kaempferol-3-rutinoside in wild-type and CRY1 overexpressing plants. Ns, S168, Nt, S135, and S144 were grown under 60 µmol quanta m^{-2} s^{-1} 24 h for 4 weeks. Leaves of CRY1 overexpressing plants contained two-fold chlorogenic acids, ten-fold rutin, and six-fold kaempferol-3-rutinoside more than wild-type plants.
Figure 3.12. The accumulation of phenolic compounds is correlated to reciprocity (intensity x time). Total ion chromatogram of phenolic compounds of Ns, S168, SOB36, SCB35, SUA2, Nt, S135, and S144 grown under 60 µmol quanta m\(^{-2}\) s\(^{-1}\) 24 h and 180 µmol quanta m\(^{-2}\) s\(^{-1}\) 8h for 4 weeks.
Figure 3.13. The accumulation of phenolic compounds is correlated to light intensity.

Chromatogram of phenolic compounds in four-week-old Ns and S168 grown under 60, 300 and 600 µmol quanta m$^{-2}$ s$^{-1}$ light for 16 h.
Figure 3.14. The accumulation of phenolic compounds is correlated to light intensity and regulated by the expression of CRY1. Chromatogram of phenolic compounds in four-week-old Nt, S135, and S144 grown under 60, 300 and 600 μmol quanta m$^{-2}$ s$^{-1}$ light for 16 h.
Figure 3.15. *CRY1* overexpression results in increased secondary metabolite content in root. Chromatogram of phenolic compounds in Ns (a) and S168 (b) roots grown under high light condition for 4 weeks. Negative ion mass spectra of compound 1 (c), compound 2 (d), and compound 3 (e).
<table>
<thead>
<tr>
<th>Peak number</th>
<th>Retention time (min)</th>
<th>[M-H]- (m/z)</th>
<th>Daughter ions</th>
<th>MS/MS</th>
<th>MS/MS/MS</th>
<th>Suggested compound</th>
</tr>
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<td>29.1</td>
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<td>193</td>
<td></td>
<td>149, 134</td>
<td>Ferulic acid hexoside</td>
</tr>
<tr>
<td>2</td>
<td>37.7</td>
<td>609</td>
<td>301</td>
<td></td>
<td>271</td>
<td>Rutin</td>
</tr>
<tr>
<td>3</td>
<td>41.3</td>
<td>623</td>
<td>315</td>
<td></td>
<td>300, 272</td>
<td>Isorhamnetin-3-O-rutinoside</td>
</tr>
</tbody>
</table>

Table 3.2. Negative ions with corresponding retention time and suggested compound in root.
Figure 3.16. *CRY1* overexpressing plants exhibit reduced UV-B injury. The phenotypes of plants after three days UV-B exposure. Ns (a), S168 (b), Nt (c), S135 (d), and S144 (e).
Figure 3.17. *CRY1* overexpressing plants exhibit reduced UV-B injury. The phenotypes of plants after six days UV-B exposure. Ns (a), S168 (b), Nt (c), S135 (d), and S144 (e).
DISCUSSION

Light provides energy required for plant growth and development. While lack of sufficient light results in poor plant growth, too much light can also be harmful. When light levels exceed plants need for photosynthetic productivity, the accumulation of excess excitation energy leads to photooxidative damage to the photosynthetic apparatus. Under high light conditions, leaves of CRY1 overexpressing plants contained three-fold more total chlorophyll than wild-type plants, which was probably due to both increased synthesis and a reduction of photooxidative degradation. First, well-developed chloroplasts containing chlorophyll were observed in the cortex of roots of CRY1 overexpressing plants. Second, leaves of CRY1 overexpressing plants contained higher phenolics, flavonoids, and lignin content than wild-type plants. LC-MS results showed that leaves of CRY1 overexpressing plants contained two-fold chlorogenic acids, ten-fold rutin, and six-fold kaempferol-3-rutinoside more than wild-type plants. CRY1 overexpressing plants exhibited reduced Ultraviolet-B injury. It may be due to the accumulation of secondary metabolites which have antioxidant activities in protecting cellular structures from oxidative damage.

For decades, agriculture has sought to optimize the capture of light energy in order to improve the crop productivity. An increase in the sensitivity of light perception may increase the photosynthesis rate and generate more chemical compounds. The
research published in Nature Biotechnology (Richard 2005) provides the idea in tomato nutritional enhancement. They knock out a transcription factor called DET1 which is a negative regulator of the photomorphogenic responses in tomato. The significant of this study is DET1 is upstream of light signaling and have the ability to upregulate three classes of compounds such as carotenoids, chorogenic acids, and flavonoids. They claimed that this is the first time that plant antioxidant phytonutrients produced have been increased in parallel and create an extra nutritious tomato. Although I didn’t have a genetic evidences that can prove CRY1 is directly interact with DET1 in tobacco, the phenotypes showed in det1 such as accumulation of secondary metabolite content and root greening are similar to CRY1 overexpressor. Therefore, CRY1 may be upstream of DET1 in regulating secondary metabolism. Altered expression of CRY1 can enhance or decrease the sensitivity to light, and CRY1 overpression resulted in highly increased level of rutin and chlorogenic acids. It would be a great tool for crop improvement through either traditional breeding or biotechnology by manipulation of CRY1 level in plants.
Chapter 4

Cry1 overexpression leads to changes in leaf morphology
and may affect size and shape of vessel elements

Introduction

Water transport is important for the uptake of essential nutrients from soil and is crucial to the survival of plants. The main forces that move water upward in the xylem is the evaporation of water through stomata on the leaves, and the rate of transpiration is related to the degree of stomatal opening and the stomatal distribution. The stomatal distribution is variable between species, but in most species it is typically followed a pattern known as the one cell spacing rule: two stomates are separated at least one cell apart for proper stomatal physiology (Nadeau 2009). Cell communicates to cell and makes a fate decision to initiate a stomatal lineage base on the position of existing stomata. The genetic components of this cell-cell signal transduction that enforce one-cell
spacing rule have been identified in *Arabidopsis* (Hara et al., 2007). First, **STOMATAL DENSITY AND DISTRIBUTION 1 (SDD1)**, which encodes a subtilisin-like Ser protease, modifies unidentified extracellular ligands secreted from developing stomata (Von et al., 2002). After ligands binding to the putative receptors **TOO MANY MOUTHS (TMM)** (Nadeau et al., 2002), the signal transduction is activated and mediated by **YODA-MAPK** cascades (Gray et al., 2004; Nordberg 2005). Finally it suppresses the stomatal development and prevents clustered stomata. Stomatal development is also significantly affected by the environmental factors such as CO₂ concentrations, humidity, and light intensity. It is well documented that plants have fewer stomata than normal when grown at elevated CO₂ levels, higher air humidity, or lower light intensity (Casson et al., 2008). The environmental influence on stomatal development has been purposed to be the long-distance signaling mechanism regulated by hormones. The changes of stomatal density in response to both CO₂ concentration and humidity are correlated with the whole-plant transpiration and leaf abscisic acid (ABA) concentration (Lake et al., 2008). The potential integrating of environmental stimuli and stomatal development is the mitogen-activated protein kinase (MAPK) cascade (Wang et al., 2007). However, little is known about the mechanism by which plants perceive the light signals and initiates the stomatal lineage. To determine whether **CRY1** was involved in regulating stomatal development in response to light signals, the stomatal frequency and distribution were examined in transgenic tobacco overpressing **CRY1**. To understand stomatal density and water transport properties, three kinds of shape factors of xylem: cell arrangement, cell shape,
and cell wall thickness have also been analyzed. The goal is to address the coordination between xylem structure and water loss under stress condition.
MATERIALS AND METHODS

Plant material and growth conditions

Wild-type plants: *Nicotiana tabacum* and *Nicotiana sylvestris* and CRY1 overexpressing plants: S135, S144, and S168 were germinated and planted in pots containing a commercial soilless mix (Metro-Mix 360; Scotts-Sierra Horticultural Products Company, Marysville, OH). Plants were grown under fluorescent lamps in growth chamber: light intensity 300 µmol quanta m\(^{-2}\) s\(^{-1}\), photoperiod 16 h, and temperature setpoint of 22°C. For cold stress treatment, two-month-old plants were transfer from temperature setpoint of 22°C to 4°C for 10 minutes. For high light stress, three-month-old plants were transferred to from light intensity 300 µmol quanta m\(^{-2}\) s\(^{-1}\) to 1500 µmol quanta m\(^{-2}\) s\(^{-1}\) for 10 minutes.

Stomatal impression

Leaf impressions were as described by Gitz et al., 2000. Leaves were detached and coated with a thin layer of cyanoacrylate adhesive (The Original Super Glue® gel., CA, USA) on the adaxial surface. Gently and evenly pressed against glass microscope slides 10 minutes until super glue was completely dry. After peeling a leaf, the resulting epidermal impression remained in hardened adhesive attached to the slide and was viewed under a light microscope. The process was repeated for the abaxial surface on the
opposite side of the midrib. With this method, both total and relative adaxial and abaxial stomatal densities for a single leaf were determined.

**Statistical analysis**

ANOVA is a statistical method used to test whether the means of several groups (more than two) are equal. The following hypothesis were tested,

\[ H_0 : \bar{x}_1 = \bar{x}_2 = \cdots = \bar{x}_n \] (Means in several groups were equal)

\[ H_1 : \text{Not all population means were equal.} \]

When performing ANOVA, the observed variance for each observation could be partitioned into two components: within-treatment variance and between treatment variance. That is,

\[ x_{ij} - \bar{x} = (x_{ij} - \bar{x}_i) + (\bar{x}_i - \bar{x}) \]

where

\[ x_{ij} : \text{denoted } jth \text{ observation in } ith \text{ group} \]

\[ \bar{x}_i : \text{denoted the mean of } ith \text{ group} \]

\[ \bar{x} : \text{denoted the mean of all observations, that is, } \bar{x} = \frac{\sum\sum x_{ij}}{n}, \text{ where } n \text{ was the number of total observations.} \]

Taking the sum of squares for both sides of the above equation resulted in the following equation.

74
\[
\sum_{i} \sum_{j} (x_{ij} - \bar{x})^2 = \sum_{i} \sum_{j} (x_{ij} - \bar{x}_i)^2 + \sum_{i} \sum_{j} (\bar{x}_i - \bar{x})^2
\]

The total sum of squares (SST) was equal to sum of squares within treatments (SSE) plus sum of squares between treatments (SSF). If SSF was statistically significant greater than SSE, we could conclude that the population means in several groups were different.

A series of paired t-tests were performed to determine whether the means of paired samples are equal.

The test statistics can be derived by the following equation,

\[
t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{S_{\bar{x}_1 - \bar{x}_2}}, \text{ where } S_{\bar{x}_1 - \bar{x}_2} = \sqrt{\frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}}
\]

If the t statistic was greater than the critical value, it indicated that means of paired samples are not equal; otherwise, there was no difference between these paired samples means.
RESULTS

*CRY1* overexpression results in increased stomatal density and violation of the one cell spacing rule.

To determine the effect of *CRY1* in mediating the spacing of stomatal pores on leaves surface, an epidermal impressions was used to view and quantify the number of epidermal cells and stomates on wild type and *CRY1* overexpression plants. The results showed that the stomatal distribution on wild-type plants: (Fig. 4.1 (a) (c)) followed a pattern known as the one cell spacing rule: two stomates are separated at least one cell apart for proper stomatal physiology (Ngwenyama et al., 2007). In *CRY1* overexpression (Fig. 4.1 (b) and (d)), there were elevated numbers of stomates and some of the stomates were clustered. There were no significant difference in length and width of stomata between wild-type and *CRY1* overexpressing plants (Fig. 4.2). It suggested that the stomatal patterning was disrupted in *CRY1* overexpression plants, but the size and shape of stomata were not affected by the function of *CRY1*.

**CRY1 may play a role in photoperiod-depend modulation of stomatal development**

In plants, leaves grown on high light condition have higher stomatal densities than low light condition (Yendrek 2006). However, photoperiod-depend modulation of stomatal development has been less studied (Lepistö et al., 2008). To understand the role
of *CRY1* in photoperiod-depend regulation of stomatal development, Nt and S135 were grown at the same light intensity (300 μmol quanta m⁻² s⁻¹) but different photoperiod: 16 h for long-day condition and 8 h for short-day condition. The data were quantified by determining the stomatal index: the ratio of the number of stomata divided by the total number of cells in one area (Table 4.1). It showed that there was no difference in wild-type plants whether grown under short-day or long-day condition. There was a 1.5 fold increases when *CRY1* overexpressing plants grown under long day condition.

**CRY1 overexpression results in increased lignin content in the vascular tissue**

To understand the function of *CRY1* in affecting the vascular tissue structure of leaf petiole, the petioles were dissected and stained with phloroglucinol. The results showed that the vessel cells of wild type were four-fold larger than S168, but S168 had increased thickness of secondary cell wall (Fig. 4.3).

**The size and shape of vascular tissue in *CRY1* overexpressor**

Mean length and width of ten tracheids in *N. sylvestris* and S168 were measured (Table 4.2). A 95% confidence interval could be constructed to deliver a general idea about how the mean values distributed and enable us to make probability statements about how close the sample mean is to the population mean.

In order to compare the differences of size and shape in tracheids between wild-type and *CRY1* overexpressing plants, the paired t-test and analysis of variance (ANOVA)
were performed. Prior to any paired-t test, ANOVA was performed to exam whether mean length of NS, mean width of NS, mean length of S168, and mean width of S168 were equal by using the following hypothesis,

\[ H_0 : \text{NS}_{\text{mean length}} = \text{NS}_{\text{mean width}} = \text{S168}_{\text{mean length}} = \text{S168}_{\text{mean width}} \]

\[ H_1 : \text{Not all population means were equal.} \]

The results (Table 4.3) showed that the differences in mean values among the groups were greater than would be expected by chance; there was a statistically significant difference (P<0.001). Therefore, it suggested that mean length of NS, mean width of NS, mean length of S168, and mean width of S168 were not equal to each other.

A series of paired t-tests were performed to determine whether the means of paired samples are equal. When performing paired t-test, the hypothesis can be formulated as follows,

\[ H_0 : \mu_1 - \mu_2 = 0 \]

\[ H_1 : \mu_1 - \mu_2 \neq 0 \]

For the comparison of length and width of tracheids in wild-type and \textit{CRY1} overexpressing plants, (1) Ns mean length - S168 mean length=0 (2) Ns mean length - S168 mean width=0 (3) Ns mean length - Ns mean width=0 (4) Ns mean width - S168 mean length=0 (5) Ns mean width - S168 mean width=0 (6) S168 mean length - S168 mean width=0 were tested (Table 4.4). The results showed that Ns mean length and S168 mean length were not equal. Ns mean length and S168 mean width were not equal. Ns
mean length and Ns mean width were not equal. Ns mean width and S168 mean length were not equal. Ns mean width and S168 mean width were not equal. S168 mean length and S168 mean width were equal.

The ratio of length versus width in Ns was 1.611±0.123 and S168 was 0.0932±0.086. For the comparison of the ratio of length versus width in wild-type and CRY1 overexpressing plants, the test statistic was performed by

\[ t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{S_{\bar{x}_1 - \bar{x}_2}} = \frac{(0.932 - 1.611) - 0}{\sqrt{\frac{0.086^2}{10} + \frac{0.123^2}{10}}} = -15.474 \]

The test statistic is smaller than the critical value \( t_{9,0.025} = -2.262 \). As such, we can conclude that length vs. width ratio for Ns and s168 were not equal at 5% level of significance.

Since the shape of Ns and s168 were close to ellipse, the area of Ns and s168 could be calculated by \( \pi ab \), where \( \pi = 3.1416 \), a=length/2, and b=width/2. The area of Ns was 0.001591±0.000164, and the area of S168 was 0.000671±0.000141. For the comparison of the area of wild-type and CRY1 overexpressing plants, the test statistic was performed by

\[ t = \frac{(\bar{x}_1 - \bar{x}_2) - (\mu_1 - \mu_2)}{S_{\bar{x}_1 - \bar{x}_2}} = \frac{(0.000671 - 0.001591) - 0}{\sqrt{\frac{0.000141^2}{10} + \frac{0.000164^2}{10}}} = -22.203 \]

The test statistic was smaller than the critical value \( t_{9,0.025} = -2.262 \). It showed that area for Ns and s168 were not equal at 5% level of significance.
**CRY1 overexpressing plants show wilting phenotype under stress conditions**

Water is essential for the biological function in cell, and any environmental factor disrupt the water balance in cell results in a serious problem. There was a severely wilting phenotype showed in S168 when it is transferred to cold (Fig. 4.4) or high light conditions (Fig. 4.5). It may be a result of the loss of water transport (small vessel cells) and the increased water transpiration (higher stomatal densities).
Figure 4.1. *CRY1* overexpression results in increased stomatal density and violation of the one cell spacing rule. The stomatal distribution on tobacco leaves of wild-type plants: Nt (a); Ns (c) and *CRY1* overexpressing plants: S135 (b); S168 (d). Bars represent 1 mm.
Figure 4.2. The stomate on tobacco leaves of Nt and S135. There were no significant difference in length and width of stomata between wild-type and CRY1 overexpressing plants. Bars represent 1 mm.
<table>
<thead>
<tr>
<th></th>
<th>Nt</th>
<th>S135</th>
</tr>
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<tbody>
<tr>
<td>Long day</td>
<td>0.196 ± 0.031</td>
<td>0.365 ± 0.046</td>
</tr>
<tr>
<td>Short day</td>
<td>0.199 ± 0.022</td>
<td>0.244 ± 0.052</td>
</tr>
</tbody>
</table>

Table 4.1. Stomatal index of wild-type and *CRY1* overexpressing plants under long day and short day conditions.
Figure 4.3. *CRY1* overexpression results in increased lignin content in the vascular tissue. The vascular tissue on leaf petiole of Ns (a) (c) and S168 (b) (d). Bars represent 1 mm
<table>
<thead>
<tr>
<th></th>
<th>NS Length (µm)</th>
<th>Width (µm)</th>
<th>S168 Length (µm)</th>
<th>Width (µm)</th>
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<tr>
<td>Mean</td>
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<td>35.5</td>
<td>28</td>
<td>30.25</td>
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<tr>
<td>Stdev</td>
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<tr>
<td>Std Error</td>
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<td>0.82</td>
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<td>1.32</td>
</tr>
<tr>
<td>95% C.I.</td>
<td>57.00±1.90</td>
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<td>28.00±1.76</td>
<td>30.25±2.58</td>
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Table 4.2. Mean length and width of ten vessels in Ns and S168. Each confidence interval had 95% of probability to include its population mean.
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<th>Source of variation</th>
<th>DF</th>
<th>SS</th>
<th>MS</th>
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<th>P-value</th>
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<tr>
<td>Between Treatment</td>
<td>3</td>
<td>5269.219</td>
<td>1756.406</td>
<td>169.462</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Within Treatment</td>
<td>36</td>
<td>373.125</td>
<td>10.365</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>39</td>
<td>5642.344</td>
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Table 4.3. The results of ANOVA in mean length of Ns, mean width of Ns, mean length of S168, and mean width of S168.
<table>
<thead>
<tr>
<th>comparison</th>
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<th>q</th>
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<tbody>
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Table 4.4. The results of paired-t test in mean length of Ns, mean width of Ns, mean length of S168, and mean width of S168.
Figure 4.4. *CRY1* overexpressing plants show wilting phenotype under cold stress.

The phenotypes of plants after transferred to 4°C for 10 minutes.
Figure 4.5. *CRY1* overexpressing plants show wilting phenotype under high light stress. The phenotypes of plants after transferred to 1500 µmol quanta m$^{-2}$ s$^{-1}$ for 10 minutes.
DISCUSSION

Xylem is differentiated from vascular cambia and undergoes a process encountering radial and longitudinal elongation. The process is followed by thickening and lignification of cell wall, and the completely differentiated vessel element became oval to oblong. However, the mechanism by which the plant regulates the diameter of xylem vessels is still unclear. There was a significantly difference in the shape of vessel elements between Ns and S168. According to the Hagen-Poiseulle Law, the water conductance of each vessel is proportional to the fourth power of vessel radius. The length of vessels in S168 was nearly half that of Ns. Therefore, theoretical petiole hydraulic conductance of \textit{CRY1} overexpressing plants was greatly decreased compared to the wild-type plants. Insufficient movement of water may result in serious water stress and lead to leaf wilting under stress conditions.
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Yendrek, C. (2006). A reverse genetics approach to investigate the role of CRY1 and CRY2 in mediating floral initiation in the long day plant nicotiana sylvestries and the short day plant N. tabacum CV. Maryland Mammoth. In H&CS (Columbus: The Ohio State University).

Programmed cell death (PCD) is a regulated process that is critical for plant survival and development. Localized cell death can block the spread of the infection during pathogen attack. For certain pathogens, an infection can trigger oxidative burst and generate reactive oxygen intermediates (ROIs). The ROIs can in turn induce salicylic acid (SA) accumulation and induce the PCD. The *Arabidopsis* dwarf mutant *glz1* was initially identified as having defects in development as well as sugar accumulation and translocation. *GLZ1* encodes a glycosyltransferase with a sequence highly homologous to Avr9/Cf-9 Rapidly Elicited (ACRE231) protein in tobacco. The *glz1* mutants seem to be tolerant to spontaneous powdery mildew infections, where macroscopic cell death lesions are not apparent on infected plants. To understand the mechanisms underlying this abnormal response, we conducted artificial inoculations
using fungal pathogens under a controlled environment. Unlike the typical hypersensitive response seen in wild type plants, in which a few large lesions were found, hundreds of small lesions were formed on \textit{glz1} plants. However, these micro-lesions could neither expand nor effectively prevent the pathogen from spreading to surrounding healthy tissues. Interestingly, while SA application on wild-type leaves can cause high levels of ROIs accumulation and cell death, \textit{glz1} plants were unaffected by the SA treatment. However, \textit{glz1} plants were not completely insensitive to SA because SA-induced \textit{PR1} and \textit{PR5} expression appeared to be normal. We hypothesize that the lack of SA-induced cell death may be due to defects in ROI accumulation and signal transduction. This notion is supported by evidence indicating that low levels of H$_2$O$_2$ accumulation and compromised cell death (\textit{ACD6}) gene induction occur when \textit{glz1} plants are treated with INA or SA. The \textit{glz1} defect appears to have a profound effect on defense and cell death response, because microarray analyses revealed a trend of down regulation of \textit{ACD6, MEK1, RIN4, WAK1, and WRKY70} in the absence of pathogen infection.

**Unlike the WT, powdery mildew infection of \textit{glz1} does not cause large necrotic lesions**

Upon inoculation of leaves with powdery mildew for 1 d, trypan blue staining of wild ecotype Landsberg \textit{erecta} shows that infected leaves have large necrotic lesions relatively few in number (a, c). In contrast, similarly infected leaves of the \textit{glz1} mutant (b, d) do not have large lesions but instead have numerous microscopic lesions. There
were fewer necrotic cells were involved in each $glz1$ lesion (e). This suggests that cell death can occur in $glz1$, but the genetic defect prevents PCD from spreading into surrounding tissues. Bars represent 1 mm.

(a)      (b)      (c)       (d)      (e)
Small necrotic lesions in glz1 do not effectively contain the powdery mildew infection.

Leaves of glz1 (b) 9 days after inoculation with powdery mildew have significantly more conidiophores per mm2 (c) than similarly infected WT (a). Leaves were stained with trypan blue in (a) and (b). Later in the infection, many WT leaves become fully necrotic (d), while glz1 remains green despite abundant fungal growth (e). This suggests that glz1 is susceptible to powdery mildew and the abundance of the micro-lesions is not sufficient to prevent the fungus from spreading.
SA-induced programmed cell death is defective in the *glz1* mutant

Application of SA on leaves can generate high levels ROS accumulation and cause the onset of PCD. In (a), WT leaves were sprayed with either with H2O or 1mM SA; after 24 h, all plants sprayed with SA showed evidence of PCD (the proportion of plants that show the SA-induced cell death is listed below each picture). Surprisingly, similarly treated *glz1* plants were unaffected by the SA (b). In (b), The *glz1* plants were not completely insensitive to SA because SA-induced *PR1* and *PR5* expression appeared to be normal (c). Therefore, this suggests that the defective of SA-induced programmed cell death in *glz1* was not caused by the loss of SA sensitivity.
untreated  1mM SA 6h  1mM SA 24h

PR1

PR5

Ler  glz1  Ler  glz1  Ler  glz1
The production H$_2$O$_2$ may be defective in *glz1*

Application of H$_2$O$_2$, INA (SA analog), and SA on leaves can generate ROS and cause cell death. WT and *glz1* leaves were sprayed with H$_2$O, 10 mM H$_2$O$_2$, 1 mM INA, or 1 mM SA, and stained for peroxides *in situ* with DAB. While WT (Ler) leaves showed high levels of ROS accumulation, only small amounts of ROS accumulated in *glz1*. After 24h, whereas many large lesions (Trypan blue stain) can be seen in the WT leaves, fewer large lesions were been found only in H$_2$O$_2$ treated *glz1*. This suggests that exogenous H$_2$O$_2$ can cause the spread of cell death and partially rescue the defective runaway cell death mechanism in *glz1*. Quantification of cell death per leaf for different treatments.
Ler
DAB
Trypan blue

glzI
DAB
Trypan blue

$H_2O$   $10 \text{ mM } H_2O_2$   $1\text{mM INA}$   $1\text{mM SA}$
The response to H$_2$O$_2$ may be defective in glz1

WT and glz1 leaves were sprayed with H$_2$O, 10mM H$_2$O$_2$, 1mM INA, or 1mM SA. RNA was isolated from tissues 2 and 24 h after treatment. LSD1, a zinc-finger protein, is a negative regulator of runaway cell death. The expression of LSD1 in glz1 were similar to WT, suggesting that LSD1 may not be involved in GLZ1-dependent cell death. ACD6, a novel ankyrin protein, is involved in activating SA-dependent cell death. There was no induction of ACD6 in glz1. This raises the possibility that GLZ1 is upstream of ACD6 or that GLZ1 directly interacts with ACD6. WRKY70, a transcription factor, is a key regulator of JA-SA crosstalk. WAK1, wall associated kinase 1, is important for host-pathogen interactions. The responses of WAK1 and WRKY70 to SA and INA were normal in glz1. However, WAK1 and WRKY70 were not induced by H$_2$O$_2$. This suggests that a defect in ROS accumulation or signal transduction may occur in glz1.
Microarray Analysis of Ler versus *glz1* indicates that genes associated with defense and cell wall have significantly lower expression in *glz1*

Leaf pairs 4 and 5 from Ler were compared with leaf pairs 3 and 4 from *glz1* in 29 day-old plants using microarray analyses with the ATH1 GeneChip. Two replicates for each leaf pair were conducted, and log transformed normalized data were statistically analyzed using 3-way ANOVA (p=.003, measuring for genotype differences, leaf position differences, and genotype-leaf position interactions). Shown are functional categories associated with genotype effect for genes showing at least a 2-fold change in expression above background levels (n = 43 and 39 for genes with higher and lower *glz1* expression respectively).

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The sequence of GLZ1 is highly homologous to Avr9/Cf-9 Rapidly Elicited (ACRE231) protein

BLASTP analysis revealed that GLZ1 is highly related to ACRE231. The amino acid sequence of Arabidopsis GLZ1 (At1g19300), tobacco ACRE231 (AF211536), and the putative rice ACRE231 (BAD45664) were aligned using ClustalW (http://www.ebi.ac.uk/clustalw/). ACRE231 was rapidly induced when tobacco Cf-9 cell suspension cultures were challenged with Avr9. Its function is still unknown, but ACRE231 is hypothesized to be essential for activation of defense mechanisms (Plant Cell. 2005 17: 295-310). This suggests that GLZ1 may be induced by fungal elicitor to activate programmed cell death.
The role of *GLZ1* in regulating programmed cell death