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The Role of Ferrochelatase II (FC2) in Chloroplast Physiology & Signaling

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The Role of Ferrochelatase II (FC2) in Chloroplast Physiology & Signaling

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in Biology

by

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2013
The thesis of Andrew Beltran Sinson is approved and it is acceptable in quality and form for publication on microfilm and electronically:

Co-Chair

Chair

University of California, San Diego

2013
Dedications

I dedicate this thesis to my family. Thank you for watching over me,
your enduring support and understanding are unmatched.

To my mother and father, for providing a loving home and virtuous morals.
To my sisters, for filling my life with smiles and laughter.
Epigraph

“For the Snark was a Boojum, you see.”

The Hunting of the Snark, Lewis Carroll
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ABSTRACT OF THE THESIS

The Role of Ferrochelatase II (FC2) in Chloroplast Physiology & Signaling

by

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Master of Science in Biology

University of California, San Diego, 2013

Professor Joanne Chory, Chair

Two-way communication between the nucleus and DNA-containing organelles such as mitochondria and chloroplasts provide proper regulation of the separate genomes within each compartment. Communication is achieved through the use of anterograde and retrograde signaling. The nucleus genome dictates cell identity and encodes >95% of organellar proteins by anterograde regulation. In turn, mitochondria and chloroplasts are
sensors of stress and energy state – both organelles signal back to the nucleus in a process called retrograde signaling. Previous studies in multiple laboratories and organisms have implicated the tetrapyrrole biosynthetic pathway as a source of retrograde signals. In chloroplasts, the tetrapyrrole pathway is branched, leading to both chlorophyll and heme. In the plant model system *Arabidopsis*, the tetrapyrrole biosynthetic pathway in chloroplast utilizes Ferrochelatase II (FC2), one of two enzymes responsible for synthesizing heme. Here I detail work using an *fc2* mutant which has altered heme and tetrapyrrole synthesis resulting in a photodamaged phenotype. Using genetic and biochemical tools, we show a novel retrograde signal may be responsible. Through the use of transgenic complementation, we show that plant FC2 activity has a conserved role in the signaling which cannot be replaced by FC1 (the other, conserved plant ferrochelatase). By using an *fc2* suppressor screen, I have discovered genes associated with this putative retrograde signaling pathway.
I. INTRODUCTION
Intracellular signaling & the tetrapyrrole biosynthetic pathway

According to the endosymbiotic theory, DNA-containing organelles such as mitochondria and plastids (e.g. chloroplasts) are the result of symbiosis amongst microorganisms. In this model, proposed by Lynn Margulis (Sagan, 1967), microorganisms such as proteobacteria and cyanobacteria were internalized by other prokaryotes 1.5 to 1.8 billion years ago and became what are currently known as mitochondria and chloroplasts, respectively. Chloroplasts, for example, still have genomes, but over 95% of their genes were lost or transferred to the nucleus over time. The remaining plastid genes, encode protein subunits required for essential complexes involved in primary photosynthetic reactions and gene expression as well as rRNAs and a complete set of tRNAs.

Photosynthetic reaction centers can only be assembled when gene products from both the nucleus and the plastid are present, monomeric pigmented complexes must aggregate into trimers and attach to pre-existing core components (Dreyfuss and Thornber, 1994). The combined proteome for all plastid types ranges from 2,000 to 3,500 proteins in the plant model organism *Arabidopsis thaliana* (van Wijk and Baginsky, 2011). Functional chloroplasts cannot be sustained by only their own DNA which varies in length and codes for approximately 87-183 genes depending on the species (Shimada and Sugiura, 1991). The remaining 95-99% of proteins are nuclear-encoded (Nott et al., 2006). Thus, communication between the chloroplast and nucleus is a necessity in order to maintain homeostatic chloroplast function through gene regulation in response to the challenges of dynamic environments such as light intensity, drought, and chemical stress.
This communication is accomplished by anterograde and retrograde signaling. Anterograde regulation is the process in which the nucleus communicates with the chloroplast to regulate organellar gene expression and other essential and basic processes such as division and DNA replication. Retrograde signaling is the process in which chloroplasts communicate information concerning the state of organelle function to the nucleus. (Nott et al., 2006; Woodson and Chory, 2008) (Figure 1). This process is known to regulate transcription of hundreds of genes in the nucleus, many of which encode proteins involved in photosynthesis and chloroplast function as well as stress responses.

Methods of intracellular communication involved with plastid retrograde signaling and subsequent regulation of nucleus encoded genes in response to stress have been documented and characterized. For example, in the absence of SAL1 dephosphorylating enzymatic activity, the phosphonucleotide 39-phosphoadenosine 59-phosphate (PAP), cannot be converted to AMP and begins to accumulate. This accumulation has been shown to lead to alteration of nuclear gene expression during high light and drought stress (Estavillo et al., 2011). Additionally, high light stress has been documented to elevate methylerthritol cyclodiphosphate (MEcPP) levels. MEcPP is a precursor of isoprenoids produced by the plastidial methylerthritol phosphate (MEP) pathway and has been documented to elicit expression of stress-responsive nuclear-encoded plastid proteins (Xiao et al., 2012).

Perhaps the best understood retrograde signaling pathways are known from studies of chloroplast development – in these studies, herbicide was used to photobleach *Arabidopsis* and inhibit early chloroplast development. In conditions where plastid to
chloroplast development was blocked by antibiotics or such herbicides, expression of hundreds of Arabidopsis photosynthesis-associated nuclear genes (PhANGs) was strongly down regulated. Through genetic screens, mutants that continued to express PhANGs in spite of photobleaching were discovered and identified as gun mutants, or Genome UNcoupled mutants. All but one gun mutant effect enzymes in the chloroplast specifically at the branch-point of Protoporphyrin IX. This data has led to models where a tetrapyrrole product or intermediate is the source of a retrograde signal.

In plants, the tetrapyrrole biosynthetic pathway is localized to the chloroplast and is home to many compounds that have significant developmental, metabolic, and physiological roles in all organisms. Tetrapyrroles contain four pyrrole rings and have the distinct ability to bind metals. The tetrapyrrole pathway begins with a common intermediate, 5-Aminolevulinic acid (ALA), which can eventually be synthesized to siroheme or Protoporphyrin IX (Proto IX) (Figure 2). Proto IX, is at a critical branch point because it can be directed towards the synthesis of heme, bilins, and chlorophyll (Figure 2; Figure 3).

Siroheme serves as a prosthetic group in reductases responsible for converting sulfur and nitrogen compounds into absorbable derivatives (Murphy et al., 1974) and is an important co-factor for p450s. Heme serves as the crucial oxygen binding cofactor in hemoglobin responsible for respiration in mammals. Heme is also a primary co-factor for redox reactions in cytochromes responsible for generation of ATP. Moreover, heme also serves as an important cofactor for peroxidases and catalases that function as anti-oxidants for the purpose of free radical and reactive oxygen species (ROS) scavenging.
Heme levels are carefully monitored by feedback regulation and by metabolism to a less reactive, linear tetrapyrrole. In plants, this linear tetrapyrrole is phytochromobilin, the chromophore for the red/far-red light photo-receptor called phytochromes (Hanzawa et al., 2002; Kohchi et al., 2001). Chlorophyll serves as an essential pigment component for light absorption and subsequent transformation of energy in photosystems.

In order to regulate the levels of each compound and manage their roles in homeostasis, it is absolutely necessary for chloroplast-nucleus communication to regulate tetrapyrrole metabolism (Tanaka and Tanaka, 2007). In this context, retrograde signaling pathways could be mediated by tetrapyrrole compounds or their derivatives.

**Protochlorophyllide, an indicator of tetrapyrrole synthesis**

If misregulated, the tetrapyrrole pathway can lead to immense photo-oxidative damage as intermediates can accumulate and induce photo-oxidation. (Mock and Grimm, 1997). One such intermediate, protochlorophyllide (pchlide), is found on the chlorophyll branch of the tetrapyrrole pathway and serves as a precursor to chlorophyllide and chlorophyll. Notably in angiosperms, conversion of pchlide into chlorophyllide by Protochlorophyllide Oxidoreductase A (PORA) requires the presence of light (Fujita and Bauer, 2000) (Figure 2; Figure 3). Thus, pchlide accumulates in the dark. Researches have taken advantage of this by growing *Arabidopsis* in the dark and using pchlide levels as on output to quantify the level of tetrapyrrole synthesis in chloroplasts.

Due to its photo-oxidative nature, when high levels of pchlide accumulate and are subsequently exposed to light, reactive oxidative species (ROS) are generated in high
volume (particularly singlet oxygen) and cause cellular damage which can be observed as necrotic lesions and stunted growth. This process was characterized through the \textit{flu} mutant (Meskauskiene et al., 2001). The precise function of the FLU protein is not known. However, the FLU protein is located on the chloroplast membrane but not in the stroma of the chloroplast. It is unrelated to tetapyrrole enzyme activity but responsible for negative regulation of tetapyrrole synthesis in response to elevated pchlide levels by binding the Glu-tRNA reductase enzyme which is responsible for conversion of tRNA-glu to GSA (Figure 2; Figure 3). In the loss of function \textit{flu} mutant, negative tetapyrrole regulation is unable to occur and pchlide levels are elevated 9 fold that of dark grown wild type \textit{Arabidopsis}. As a result of elevated pchlide levels, necrotic lesions occur after light exposure (Figure).

However, the subsequent damage caused by ROS such as singlet oxygen is not completely responsible for the cell death and growth arrest phenotypes in the \textit{flu} mutant. Through a \textit{flu} mutant suppressor screen in which mutants with 9 fold-elevated pchlide levels continued to grow in the presence of high levels of singlet oxygen, the Apel lab also demonstrated the induction of singlet oxygen stress genes in \textit{Arabidopsis} required \textit{EXECUTER1 (EXE1)} (op den Camp et al., 2003). EXE1 is a thylakoid protein in the chloroplast with little similarity to other known proteins. While the biochemical characterization and mechanistic action of EXE1 have been elusive, the mutant screen revealed that \textit{Arabidopsis} seedlings have an EXE1-dependent apoptosis response to elevated pchlide and singlet oxygen levels.
This was supported by the fact that when EXE1 is inactivated in the presence of elevated pchlide, (flu/exe1 double mutant), plants continued to live in spite of photodioxidative damage associated with elevated pchlide (Wagner et al., 2004) (Figure 15). This finding revealed that genetically encoded retrograde signaling components respond to conditions in which tetrapyrroles and ROS are accumulating.

**Ferrochelatase II (FC2) and the fc2 mutant**

Another tetrapyrrole proposed to be involved in retrograde signaling is heme. Due to its metabolic and physiological roles as a cofactor for many types of enzymes, the electron accepting nature of the internalized iron atom for ROS scavenging, and utilization in photosynthetic cytochromes, there is significant potential for heme to effect chloroplast physiology and signaling. Heme has also been shown to be a signal in bacteria, yeast, *C. elegans*, algae, and humans (Mense and Zhang, 2006). By extension, we chose to analyze the two conserved plant ferrochelatases (FCs) responsible for synthesizing heme in *Arabidopsis*, FC1 and FC2. FC1 and FC2 both have an N-terminal sorting domain and a conserved catalytic domain (Figure 16). Structurally, the two enzymes differ in that FC1 has a 51 amino acid unknown C-terminal domain while FC2 has a 42 amino acid spacer region in addition to a hydrophobic Light Harvesting Complex (LHC) domain which may be involved in anchoring FC2 to a membrane in the chloroplast. *In vitro*, the enzymes have almost identical catalytic activity; however, *in vivo*, the two enzymes appear to have different physiological roles (Suzuki et al., 2002). Expression levels of FC1 increase in response to general stress conditions such as abiotic wounding and reactive oxygen species damage. In contrast, expression levels of FC2
decrease under the same stress conditions (Nagai et al., 2007). FC1 is expressed ubiquitously in all *Arabidopsis* tissue and the heme that it generates is presumably used for general housekeeping functions such as respiration and free radical scavenging. FC2 is expressed only in photosynthetic *Arabidopsis* tissue and the heme that it produces is presumably used specifically for cytochromes in photosynthesis.

Previous genetic studies by Jesse Woodson in the Chory lab have found that elevated FC1 activity increases the expression of photosynthesis-associated nuclear genes (PhANGs) in seedlings where chloroplast development has been blocked (Woodson et al., 2011). Elevated FC2 activity, however, was unable to produce the same effect – further demonstrating that the two enzymes have different roles. This data encouraged further research using FC mutants. However, *fc1* alleles have proven to be difficult to work with. When working with a weak mutant line of *fc1* in which the 5’ UTR had a T-DNA insertion and transcription levels were decreased (Figure 4), samples had a wild-type/*Columbia* phenotype. Alternatively, when attempting to work with a strong mutant line of *fc1* in which a T-DNA insertion was introduced to the third *FC1* exon (Figure 4), the phenotype was embryo lethal (Figure 5). The resulting phenotypes associated with these *fc1* mutant lines are understandable considering the role of FC1 as a general housekeeping ferrochelatase, and FC1 specific heme being used for processes such as respiration and ROS scavenging.

On the other hand, mutant *fc2* lines were viable and easier to work with. A strong mutant line of *fc2* (*fc2s*) in which a T-DNA insertion was introduced to the seventh *FC2* exon (Figure 4), resulted in loss of function and a strong phenotype: very pale in 24hr
light and seedling lethal in cycling 4hr light, 20hr dark conditions. The alternate weak mutant line of FC2 (fc2) in which the 5’ UTR had a T-DNA insertion and transcription levels were decreased by 50% (Figure 4), resulted in a distinct phenotype that grew pale in 24hr light and elongated in 4hr light but did not green or de- etiolate in the 4hr light conditions (Figure 5). Upon further investigation with the help of TEM through the Waitt Advanced Biophotonics Center at the Salk Institute, it was revealed that the fc2 mutant had undeveloped chloroplasts relative to wild type/Columbia after de- etiolation (Figure 6) and dead cells in 4hr light conditions.

The fc2 mutation has a profound effect on chloroplast and seedling development as a result of impaired heme synthesis due to decreased FC2 activity. This suggests a link between heme synthesis and retrograde signaling components involved in communicating misregulation of the tetrapyrrole pathway in the chloroplast to the nucleus. Because the fc2 phenotype is conditional, it is a useful tool to clarify the role of heme synthesis in chloroplast and seedling development. To understand what was responsible for the visual 4hr cell death fc2 phenotype on a molecular level, we conducted experiments using tetrapyrrole pathway inhibitors, additional tetrapyrrole biosynthetic mutant combinations, and transgenic lines expressing hybrid ferrochelatase genes.

A genetic screen for fc2 suppressors

Due to the fact that the Arabidopsis genome has been completely sequenced with high resolution and Arabidopsis itself has a relatively fast growing nature with established physiologically relevant growth conditions, the organism is an ideal model to use genetics for basic research. Due to these benefits, coupled with the nature of the fc2
phenotype in 4hr light conditions, and the available time I had to invest in the Chory lab over the course of my Master’s research, I performed a genetic screen to find fc2 suppressors under the tutelage of Jesse Woodson. Seedlings with fc2 mutant background were exposed to the chemical mutagen ethyl methanesulfonate (EMS) and suppressors of the 4hr light phenotype were screened and rescreened over three generations before subsequently being back-crossed into the parent line in addition to the Landsberg ecotype of Arabidopsis for the purpose of mapping the mutations. We have started a collaboration with the Weigel Lab, Max Planck Institute for Developmental Biology, Tübingen to sequence the fc2 suppressors which will identify the causative genes responsible for suppression (Manavella et al., 2012).

SOUL proteins, candidate tetrapyrrole-trafficking proteins

To assess our research topic from another angle and provide insight on heme trafficking and signaling, we have begun looking at heme binding proteins (HBPs) or SOUL proteins which are proposed to be involved in heme chaperoning and/or trafficking. Six SOUL genes were identified in Arabidopsis based on homology to the mammalian SOUL family. SOUL1 and SOUL4 have been shown to be capable of reversibly binding porphyrin tetrapyrrole intermediates in vitro, supporting their role as heme chaperones (Takahashi et al., 2008). SOUL6 has been shown to be capable of interacting with Heme Oxygenase I (HY1) and decreased SOUL6 correlates with increased H₂O₂ and oxidative stress (Lee et al., 2012).

Understanding the functions of the SOUL proteins may add to our understanding of tetrapyrrole pathway regulation and retrograde signals. To this end, we have localized
the SOUL proteins to their subcellular compartments to determine where they may function. We have also created *Arabidopsis* lines overexpressing individual SOUL proteins and identified loss of function, T-DNA insertion SOUL mutant. Together with the *fc2* mutant, these lines will be used to test the role of SOUL proteins in tetrapyrrrole trafficking and signaling through potential genetic and biochemical interactions.

Here I present evidence for the accumulation of tetrapyrrrole intermediates being responsible for the cell death phenotype in *fc2* mutants. Interestingly, this phenotype is independent of pchlde accumulation and the classic singlet oxygen EXE1 signaling pathway. Furthermore, we show that this pathway is specific to FC2, as FC1 activity cannot compensate for the *fc2* mutant phenotype in its entirety. To identify novel components involved in this signaling pathway, a suppressor screen identified 25 mutants affecting the cell death phenotype. Lastly, we have also begun characterization of the SOUL proteins involved in tetrapyrrrole trafficking. Together, these studies have elucidated a second retrograde pathway with signaling dependent on FC2 activity.
II. RESULTS
The *fc2* mutant has uncontrolled tetrapyrrole synthesis

The activity of the tetrapyrrole pathway is regulated by heme levels. When free heme is present the synthesis of ALA is negatively regulated and flux through the pathway decreases (Tanaka and Tanaka, 2007)(Figure 3). Due to the *fc2* mutant having impaired heme synthesis capabilities, we believed that flux through the tetrapyrrole pathway and synthesis of other tetrapyrrole intermediates would increase. We checked if this was the case by looking at pchlide levels in the *fc2* mutant and found that they were 2.5 to 3 fold increased relative to wild type (Figure 13). Thus, we determined two types of stress in the *fc2* mutant: decreased heme and increased tetrapyrrole intermediates, particularly pchlide. Because heme produced by FC2 is associated with cytochromes, stress in the form of decreased heme in the *fc2* mutant could manifest as impaired photosynthetic activity. Additionally, as a result of increased tetrapyrrole intermediates (particularly pchlide), stress in the form of ROS in the *fc2* mutant could manifest as light sensitive damage. Thus, we needed to determine which of the two stresses caused *fc2* seedlings to be stressed under light cycling conditions – decreased heme or increased tetrapyrrole intermediates.

If increased tetrapyrrole intermediate levels were responsible for the 4hr light cell death *fc2* phenotype, we expected the 4hr light cell death phenotype to be mildly suppressed when the tetrapyrrole pathway is downregulated and less tetrapyrroles are being photo-oxidized. Alternatively, if decreased heme levels were responsible for the 4hr light cell death *fc2* phenotype, we expected the 4hr light cell death *fc2* phenotype to be amplified in the event that the tetrapyrrole pathway is downregulated and even less heme is available. As a preliminary experiment, we decreased flux through the
tetrapyrrole biosynthetic pathway using the inhibitory drug Gabaculine. Gabaculine blocks conversion of Glutamate-1-Semialdehyde (GSA) to ALA by irreversibly inhibiting GABA transaminase, the enzyme responsible for this step in the pathway (Demko et al., 2010; Rando, 1977) (Figure 3).

Wild type/Columbia and fc2 seeds were grown on individual plates of increasing Gabaculine concentration under 4hr light and 24hr light conditions. In 4hr light, at concentrations of [25μM] Gabaculine and higher, the 4hr light cell death fc2 phenotype became partially suppressed; cotyledons opened but did not green (Figure 7). In 24hr light, both wild type and fc2 seedling were unable to turn green in the presence of [25μM] Gabaculine (Figure 8). The suppression of the 4hr light cell death fc2 phenotype correlated with a decrease in pchlide levels (Figure 9). This supports the hypothesis that the 4hr light cell death fc2 phenotype is caused by high tetrapyrrole intermediate levels. However, suppression of the 4hr light cell death fc2 phenotype may have been due to incomplete chloroplast development as the results also correlated with dramatically low chlorophyll levels under increasing gabaculine concentrations (Figure 10).

We next took a genetic approach in order to determine if increased tetrapyrrole flux in the fc2 mutant was contributing to the 4hr light cell death phenotype. To decrease flux through the tetrapyrrole biosynthetic pathway, we used T-DNA insertions to cause genetic mutations that targeted HEMA1 and HEMA2, genes coding for the Glu-tRNA reductase enzyme which is responsible for conversion of Glu-tRNA to GSA (Figure 3). The following mutants were generated and tested for their ability to synthesize pchlide and survive 4hr light conditions: fc2/hema1/hema2, fc2/hema1, and fc2/hema2.
These mutants were then plated with wild type/Columbia and fc2 under 24hr light and 4hr light conditions. In 24hr light conditions, fc2/hema1, fc2/hema2, and fc2/hema1/hema2 genotypes had decreased chlorophyll levels relative to wild type (Figure 14). These measurements in conjunction the 4hr light phenotype observations (Figure 11; Figure 12) indicated that there was no correlation between chlorophyll levels and the 4hr light cell death fc2 phenotype.

In 4hr light conditions, the fc2/hema2 and fc2/hema1/hema2 genotypes were capable of suppressing the 4hr light cell death fc2 phenotype. Suppression was demonstrated by the ability to green and de-etiolate (Figure 11). Pchlide measurements were taken for all genotypes and the results depicted a correlation between decreasing pchlide levels and the suppression of the 4hr light fc2 phenotype (Figure 13). Thus, we determined the 4hr light cell death fc2 phenotype was the result of increased tetrapyrrole biosynthesis.

**The fc2 phenotype is distinct from the EXE1/singlet oxygen pathway**

While high pchlide levels indicate high flux through the tetrapyrrole biosynthetic pathway, pchlide can be photo-activated and create reactive oxidative species (ROS), particularly singlet oxygen. In excess, this process results in cellular damage that manifests as necrotic lesions visual to the naked eye - a phenotypical trait characteristic of the flu mutant (Meskauskiene et al., 2001)(Figure 15). In the flu mutant, pchlide levels are nine times that of wild type and senescence occurs shortly after light exposure (Meskauskiene et al., 2001). However, photo-oxidative species and the subsequent damage they cause are not the only components responsible for Arabidopsis cell death.
Arabidopsis responds to chloroplast localized singlet oxygen from elevated pchlide levels by inducing genes in stress-response pathways responsible for apoptosis (op den Camp et al., 2003), through the EXE1 signaling pathway. This genetic interaction is illustrated by the flu/exe1 double mutant in which suppression of the necrotic flu mutant phenotype occurs in spite of elevated pchlide and singlet oxygen levels (Wagner et al., 2004).

Next, we crossed both of the fc2 mutants with the exe1 mutant to check if EXE1 was required for the 4hr light cell death phenotype of either fc2 mutation in a similar manner to that of the flu mutant. Under these 4hr light conditions, the exe1 mutation was incapable of suppressing the 4hr light cell death phenotype of either fc2 mutant or the flu mutant (images omitted). As EXE1 may not be active in seedlings, we simulated the conditions in which the flu/exe1 double mutant was analyzed (Wagner et al., 2004); genotypes were grown to adulthood in 24hr light conditions and subsequently moved to cycling 16hr light/8hr dark conditions in order to induce pchlide intermediate accumulation and subsequent singlet oxygen production. Unlike the flu/exe1 double mutant, the fc2/exe1 double mutants did not suppress the cell death phenotype (Figure 15). This indicates that suppression of the 4hr light cell death fc2 phenotype is not only distinct from the flu mutant, but it is also EXE1 independent.

FC1 cannot fully replace FC2 function

To further characterize the fc2 mutant and determine what protein domains of FC2 are necessary for the 4hr light cell death fc2 phenotype, we generated transgenic lines expressing various domains of FC2 in the fc2 background (Figure 16; Table 1). Additionally, we analyzed transgenic lines expressing FC1 in the fc2 background to test
what FC1 domain(s) (if any) could compliment the *fc2* phenotype. We also manipulated
gene expression via FC1/FC2 promoter swaps and spacial manipulation of localization
via FC1/FC2 N-terminal signal swaps to test if FC1 could suppress the *fc2* phenotype.
The following lines were used as controls in all transgenic experiments were tested in
unison: wild type/ *Columbia*, *fc2*, empty vector, complete *fc2*, and catalytically dead FC2
generated by a point mutation, amino acid swap in which histadine (his, H) 295 was
converted to alanine (ala, A) and iron became incapable of binding to the FC2 catalytic
site. To test the ability of a transgene to complement the *fc2* mutant, we tested for its
ability to suppress the following phenotypes associated with the *fc2* mutant: 4hr light cell
death, chlorophyll levels, and pchlide levels. In both the *fc2* and *fc2s* mutant
backgrounds, the full length FC2 transgene always complimented all phenotypes and the
catalytically dead transgene did enhanced the phenotypes (Figure 17; Figure 18; Figure
19; Figure 20).

In all construct combinations in the *fc2* mutant background, the 24hr light
phenotypes did not provide insight as the 24hr light chlorophyll measurements did not
increase or decrease in a significant manner (Figure 17; Figure 20). On the other hand, in
the more severe *fc2s* mutant background chlorophyll levels in 24light were complimented
by transgenes encoding active FC2 or FC1. This complementation was not promoter
specific as using FC1, FC2, or 35S promoters had a similar effect (Figure 18; Figure 20).

By using the transgenic lines expressing different FC2 domains, we determined
that the FC2 LHC domain is not necessary for complementation of the 4hr light cell death
*fc2* phenotype, but the linker region is. We also determined that the N-terminal sorting
sequence of FC2 is important for complementation of the 4hr light cell death *fc2* phenotype (Figure 17).

Strikingly, even though we swapped the N-terminal sorting sequences and sent the FC2 protein to the location of FC1, the 4hr light cell death *fc2* phenotype was capable of being suppressed. In contrast, when we sent the FC1 catalytic domain to the location of FC2, the 4hr light cell death *fc2* phenotype was not suppressed. In all transgene conditions, the catalytic domain of FC1 was unable to suppress the 4hr light-cell death *fc2* phenotype when expressed with either a 35S promoter or an FC2 promoter. However, regardless of promoter, in all transgenic lines with the catalytic FC1 domain present, pchlide was decreased to wild type or comparable levels (Figure 19).

As supported by our transgenic lines, it is clear that FC1 and FC2 are catalytically similar enzymes but with functional differences (Singh et al., 2002; Tanaka and Tanaka, 2007). Through these experiments we determined that any full length active FC2 protein complements the 4hr light cell death *fc2* phenotype and even when looking at monocots, maize FC2 was capable of complimenting the 4hour light cell death *fc2* phenotype. We also learned that the FC2 Linker domain is required to compliment the 4hr light cell death *fc2* phenotype, the FC2 LHC domain is not required to compliment the 4hr light cell death *fc2* phenotype, and the FC2 N-terminal sorting sequence is not specific for complementation of the 4hr light cell death *fc2* phenotype. Notably, we were capable of uncoupling the 4hr light-cell death *fc2* phenotype from elevated pchlide levels and eliminated the possibility of excess pchlide being the main tetrapyrrole molecule responsible for the *fc2* mutant’s inability to survive 4hr light cycling conditions.
At the beginning of my time in the Chory lab, we also began work on an \textit{fc2} mutant suppressor screen. Jesse D. Woodson, Ph.D., had already soaked \textit{fc2} mutant seeds in EMS which I then began growing in pools for a total number of 3,500 M1 individuals. Individual M2 seedlings were hand selected and moved to new plates if they could suppress the 4hr light cell death \textit{fc2} phenotype. In the M2 generation, 200,000 seedlings were screened in this manner and 600 suppressor candidates were obtained. The M3 generation was rescreened in the same manner and 41 \textit{fc2} suppressors with robust phenotypes from 21 distinct M1 pools were obtained. These \textit{fc2} mutant suppressors, now denoted as \textit{fts} mutants, were categorized according to pchlide levels.

Four pchlide classifications were developed for the 21 distinct \textit{fts} mutants to be categorized into: decreased pchlide relative to wild type, wild type levels of pchlide, increased pchlide relative to wild type, and \textit{fc2} levels of pchlide. Visual phenotypes and corresponding pchlide measurements are depicted for each classification (Figure 21; Figure 22; Figure 23)

Total genomic DNA samples for each \textit{fts} mutant were isolated and sent to Patrice Salome, Ph.D. at the Weigel Lab, Max Planck Institute for Developmental Biology, Tübingen for SHOREmap deep sequencing analysis of causative mutations responsible for suppression of the \textit{fc2} phenotype.
SOUL proteins, potential tetrapyrrole trafficking proteins, are localized throughout the cell

Due to their capacity to reversibly bind cyclic tetrapyrrole molecules, we also began looking at SOUL proteins in order to determine their possible role in trafficking or signaling. Analysis of the SOUL proteins will also help us understand FC1/FC2 tetrapyrrole regulatory relationships in addition to the results of the fc2 mutant suppressor screen. If tetrapyrrole intermediates are complexed with SOUL proteins for the purpose of sequestration, being aware of these interactions will help to establish a clarified tetrapyrrole biosynthetic pathway and signaling model.

Knowing that FC1 and FC2 are localized to the chloroplast, (Tanaka et al., 2011), we cloned all 6 SOUL proteins with YFP fluorescent tags attached to each into Arabidopsis to check their respective localization sites using confocal microscopy. If a SOUL protein were to be co-localized to the chloroplast, there could be a possible interaction between two proteins affecting catalytic activity. Knowing the location of each SOUL protein and how they interact with tetrapyrroles could potentially influence measurements of tetrapyrrole levels. The 6 SOUL proteins were localized accordingly – SOUL1 localized to the cytoplasm and/or endoplasmic reticulum, SOUL2 localized potentially to the cytoplasm, SOUL3 localized to the cytoplasm, SOUL4 localized to the plasma membrane, and SOUL5 and SOUL6 both localized to the chloroplast (Table 2; Figure 24).

Additionally, we generated lines overexpressing all 6 SOUL proteins in the fc2 mutant background; none of them were able to suppress the fc2 phenotype (images
omitted). T-DNA insertion line mutants for all SOUL proteins have also been acquired for future work and possible crossing with the fc2 mutant but homozygous lines have not yet been isolated.
Figure 1. Illustration of Nucleus-Chloroplast and Chloroplast-Nucleus signaling resulting in change of gene expression. Retrograde signals affect the expression of nuclear genes such as LHCB (light harvesting complex chlorophyll binding protein) and RBCS (RuBisCo small subunit).
Figure 2. A detailed model of The Tetrapyrrole Biosynthetic Pathway (Tanaka and Tanaka, 2007) denoting tetrapyrrole intermediates by name, structure, and position.
**Figure 3.** A simplified model of The Tetrpyrrole Biosynthetic Pathway with significant tetrapyrroles and regulatory feedback loops derived from (Meskauskiene et al., 2001; Woodson et al., 2011)

**Figure 4.** Visual representation of T-DNA insertion locations in FC1 and FC2 mutants
Figure 5. *Columbia*, Wild Type (wt), the *fc2s* mutant, and the *fc2* mutant phenotypes in 24-hour light conditions and cycling 4-hour light/20-hour dark conditions; the *fc2s* mutant is very pale in 24-hour light and dead in 4-hour light conditions; the *fc2* mutant is somewhat pale in 24-hour light conditions and unable to green or de-etiolute in 4-hour light conditions.
Figure 6. TEM Imaging of etioplasts in Columbia, Wild Type and the fc2 mutant (Matt Joens, Waitt Advanced Biophotonics Core, Salk Institute for Biological Research, La Jolla, CA)
Figure 7. *Columbia*, Wild Type and the *fc2* mutant grown in 4hour light/20hour dark cycling conditions under increasing gabaculine concentrations on individual plates; the 4hour light cell death *fc2* phenotype is suppressed at [25µM] Gabaculine and higher.
Figure 8. *Columbia*, Wild Type and the *fc2* mutant grown in 24-hour light conditions under increasing gabaculine concentrations on individual plates; the 24-hour light phenotypes for both *Columbia*, Wild Type and the *fc2* mutant begin to dramatically photobleach at [25\(\mu\text{M}\)] Gabaculine and higher.
**Figure 9.** Protochlorophyllide levels of five-day-old, 24 hour dark grown *Columbia*, Wild Type and *fc2* mutant seedlings under increasing gabaculine concentrations on individual plates. Data shown is the mean of 2 samples of 10 seedlings each as a function of pchlide with respect to *Columbia*, Wild Type; pchlide levels for both *Columbia*, Wild Type and the *fc2* mutant begin to drop significantly at [25µM] Gabaculine.
Chlorophyll levels of five-day-old, 24hour light grown *Columbia*, Wild Type and *fc2* mutant seedlings under increasing gabaculine concentrations on individual plates. Data shown is normalized to total protein levels in each sample; chlorophyll levels for both *Columbia*, Wild Type and the *fc2* mutant are insignificant at [25µM] Gabaculine.
Figure 11. Columbia, Wild Type and the following mutants: fc2, hema1, hema2, fc2/hema1, fc2/hema2, and fc2/hema1/hema2 grown in 4hour light/20hour dark cycling conditions on individual plates; the 4hour light cell death fc2 phenotype is suppressed for the fc2/hema2 and fc2/hema1/hema2 mutants
Figure 12. *Columbia*, Wild Type and the following mutants: fc2, hema1, hema2, fc2/hema1, fc2/hema2, and fc2/hema1/hema2 grown in 24-hour light conditions on individual plates.
Figure 13. Protochlorophyllide levels of five-day-old, 24-hour dark grown seedlings for the following genotype conditions: Columbia, Wild Type and the following mutants: fc2, hema1, hema2, hema1/hema2, fc2/hema1, fc2/hema2, and fc2/hema1/hema2. Data shown is the mean of 2 samples of 10 seedlings each as a function of pchlide with respect to Columbia, Wild Type; decreasing pchlide correlates with suppression of the 4-hour light cell death fc2 phenotype.
Figure 14. Chlorophyll levels of five-day-old, 24hour dark grown seedlings for the following genotypes: Columbia, Wild Type and the following mutants: fc2, hema1, hema2, hema1/hema2, fc2/hema1, fc2/hema2, and fc2/hema1/hema2. Data shown is normalized according to total protein levels.
Figure 15. On the left, leaves of *Columbia*, Wild Type and the following mutants: *flu*, *flu/exe1*, *fc2*, *fc2/exe1*, *fc2s*, and *fc2s/exe1* grown to adulthood in 24hour light conditions. On the right, leaves of the same genotypes after being grown to adulthood in 24hour light conditions and moved to 16hour light/8hour dark cycling conditions for 1 week (Wagner et al., 2004); the *exe1* mutant is able to suppress the *flu1* mutant phenotype but not the *fc2* and *fc2s* mutant necrotic lesion phenotypes.
Figure 16. FC1 and FC2 protein domains denoted by amino acid number composition; FC1 415-466 domain has unknown function.
Table 1. List of transgene constructs inserted into the \( fc2 \) and \( fc2s \) mutant backgrounds; \emph{Columbia}/Wild Type, the \( fc2 \) mutant, and \emph{the fc2s mutant} with no transgene construct, empty vector, and catalytically dead \( fc2 \) serve as negative controls.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Background</th>
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<tbody>
<tr>
<td>n/a</td>
<td>( Col )</td>
</tr>
<tr>
<td>n/a</td>
<td>( fc2 )</td>
</tr>
<tr>
<td>empty</td>
<td>( fc2 )</td>
</tr>
<tr>
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<td>( fc2 )</td>
</tr>
<tr>
<td>35S::H295A</td>
<td>( fc2 )</td>
</tr>
<tr>
<td>35S::FC1</td>
<td>( fc2 )</td>
</tr>
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<td>( fc2 )</td>
</tr>
<tr>
<td>35S::FC2ΔLinker, ΔLHC</td>
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</tr>
<tr>
<td>35S::FC2 Catalytic, FC1</td>
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<tr>
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<tr>
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<td>( fc2 )</td>
</tr>
<tr>
<td>Linker, FC2 LHC</td>
<td></td>
</tr>
<tr>
<td>FC2::FC2</td>
<td>( fc2 )</td>
</tr>
<tr>
<td>35S::maize FC1-a</td>
<td>( fc2 )</td>
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Table 1. Continued

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<tr>
<td>35S::FC2ΔN-terminal, Sorting Signal</td>
<td>fc2</td>
</tr>
<tr>
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</tr>
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<td>fc2s</td>
</tr>
<tr>
<td>FC1::FC2</td>
<td>fc2s</td>
</tr>
<tr>
<td>FC2::FC1</td>
<td>fc2s</td>
</tr>
</tbody>
</table>
Figure 17. Columbia/Wild Type, the fc2 mutant, and transgenic lines from Table 1 grown in 24hour light and 4hour light/20hour dark cycling conditions checking for complementation of the 4hour light cell death phenotype.
Figure 18. Columbia/Wild Type, the fc2s mutant, and transgenic lines from Table 1 grown in 24hour light and 4hour light/20hour dark cycling conditions checking for complementation of the 4hour light cell death phenotype.
Figure 19. Protochlorophyllide levels of five-day-old, 24hour dark grown seedling genotypes described in Table 1; Data shown is the mean of 2 samples of 10 seedlings each as a function of pchlide with respect to Columbia, Wild Type. Error bars derived from 2 independently generated lines.
Figure 20. Chlorophyll levels of five-day-old, 24 hour light grown seedlings. Data shown is normalized according to total protein.
Figure 21. *Columbia/Wild Type, the fc2 mutant, and suppressors of the 4hour light cell death fc2 phenotype (fts mutants) grown in 24hour light and 4hour light/20hour dark cycling conditions*
Figure 22. Protochlorophyllide levels of five-day-old, 24 hour dark grown Columbia/Wild Type, fc2 mutant seedlings, and suppressors of the 4 hour light cell death fc2 phenotype (fts mutants) categorized by pchlide levels; Data shown is the mean of 2 samples of 10 seedlings each as a function of pchlide with respect to Columbia, Wild Type
Figure 23. *Columbia/Wild Type, the fc2 mutant, and representative robust suppressors of the 4hour light cell death fc2 phenotype (fts38 and fts39 mutants) grown in 24hour light and 4hour light/20hour dark cycling conditions*
Table 2. List of SOUL proteins and their respective localization in Arabidopsis using confocal microscopy

<table>
<thead>
<tr>
<th>SOUL protein</th>
<th>Location</th>
</tr>
</thead>
<tbody>
<tr>
<td>SOUL 1</td>
<td>Cytoplasm/ER</td>
</tr>
<tr>
<td>SOUL 2</td>
<td>Cytoplasm (?)</td>
</tr>
<tr>
<td>SOUL 3</td>
<td>Cytoplasm</td>
</tr>
<tr>
<td>SOUL 4</td>
<td>Plasma Membrane</td>
</tr>
<tr>
<td>SOUL 5</td>
<td>Plastid/Chloroplast</td>
</tr>
<tr>
<td>SOUL 6</td>
<td>Plastid/Chloroplast</td>
</tr>
</tbody>
</table>

Figure 24. Confocal Imaging of SOUL-YFP proteins, Chloroplast auto-fluorescence, and overlapped-merge image in Columbia/Wild Type for localization purposes.
Figure 24. continued
III. DISCUSSION
In this work, I have used the *fc2* mutant to detail the importance and role of FC2 in tetrapyrrole regulation and retrograde signaling. I have shown that the 4hr light cell death *fc2* phenotype is the result of tetrapyrrole intermediate accumulation. I have also shown that the 4hr light cell death *fc2* phenotype is independent of the EXE1 singlet oxygen signaling pathway. Moreover, the *fc2* mutant phenotype is not solely the result of pchlide accumulation, indicating that the *fc2* mutant is not a mild *flu* mutant. By isolating *fc2* suppressor mutants, I have shown that it is possible to genetically alter the *fc2* mutant cell death signal. In the following discussion, I will describe the significance of these findings.

The tetrapyrrole pathway is a source of retrograde signals

Due to the negative feedback nature of heme on the tetrapyrrole biosynthetic pathway (Tanaka et al., 2011)(Figure 3), we know that the *fc2* mutant has increased ALA levels relative to wild type (Papenbrock et al., 2001)(Figure 3). These measurements have been confirmed by Jesse D. Woodson, Ph.D., also at the Chory Lab, Salk Institute for Biological Research, La Jolla, CA (data not yet published). Increased ALA levels imply an increase in subsequent tetrapyrrole intermediates which could be retrograde signaling molecules involved in the *fc2* mutant phenotype.

This idea is supported by experiments detailed in this thesis. Using Gabaculine and *HEMA1* and *HEMA2* mutations together with the *fc2* mutant to decrease tetrapyrrole synthesis, we learned that the molecular stress on the *fc2* mutant was the accumulation of a tetrapyrrole intermediate and not the result of decreased heme levels. Using the *flu* and *exe1* mutants together with the *fc2* mutant, we learned that the molecular stress on the *fc2*
mutant was not the result of pchlide accumulation or singlet oxygen stress. Through this experiment we also learned that EXE1 was not a part of the fc2 retrograde signal pathway. Using our transgenic lines, we learned that increased pchlide levels do not correlate with the 4hr light cell death fc2 phenotype, leading us to conclude that pchlide is not the primary cause of the fc2 cell death phenotype in 4hour light.

To reconcile the uncoupling of the fc2 cell death phenotype with elevated pchlide levels and determine which tetrapyrrole intermediate is responsible for the fc2 phenotype and involved in retrograde signaling, one of our highest research priorities has been the measurement of other tetrapyrrole intermediates by using HPLC assays. One such intermediate, Protoporphyrin IX (Proto IX), has been reported to increase in tobacco when FC2 activity is impaired (Papenbrock et al., 2001). If we were to find elevated Proto IX in the fc2 mutant, it would suggest that Proto IX accumulation is responsible for the cell death phenotype.

Preliminary HPLC results depict elevated Proto IX levels in the fc2 mutant relative to wild type/Columbia (data not yet published). These results, in tandem with our knowledge of tetrapyrrole feedback regulation in response to decreased heme as a result of the fc2 mutation, help validate the hypothesis that Proto IX could be acting as a retrograde signal or is causing damage that triggers a signal. Multiple HPLC measurements of Proto IX must still be taken for the fc2 mutant at different time points with respect to cycling light conditions to confirm that it truly is the tetrapyrrole molecule involved with retrograde signaling. Further work will be done to develop an
understanding of what molecules are being created as a result of Proto IX photo-oxidation and the effects they have on complex structures in the chloroplast and cell.

**FC1 & FC2 appear to be physiologically distinct**

Because FC1 and FC2 have similar, if not identical, biochemical activity and both enzymes are localized to plastids in the cotyledon (Woodson et al., 2011), we decided to use both enzymes for our research on the fc2 mutant. Further characterization of the fc2 mutant was accomplished by trying to complement the fc2 phenotypes with transgenic constructs containing FC1, FC2, or various truncations and hybrids. In doing so, we were capable of identifying which FC2 domains were important for the 4hr light fc2 phenotype and elevated. We also tested which FC1 domain(s) (if any) could compliment the fc2 phenotype.

Through these experiments we also learned that ferrochelatase conserved in monocot maize FC2, but not FC1, is capable of complementing the 4hour light cell death fc2 phenotype. We also learned that the LHC domain does not contribute significantly to FC2 function. Most importantly, we learned that when the FC1 catalytic domain is expressed in the fc2 mutant background, we could uncouple the 4hr light phenotype and elevated pchlide levels. FC1 was able to synthesize heme and reduce pchlide accumulation, but could not suppress the 4hr light cell death phenotype. Although the transgenic experiments still need to be supplemented with Western blot data to ensure that all the protein domains in question are being expressed, the results clearly depict functional differences between the two FCs. These experiments are supported by the fact that swapping the promoters between FC1 and FC2 did not change their functions. FC1
driven by the FC2 promoter was able to decrease pchlide levels, but had no effect on the cell death phenotype (Figure 19).

In spite of the fact that both FCs have been localized to the thylakoids and envelope membranes of the chloroplast, the sides in which FC1 and FC2 are have not yet been established. Conducting experiments to determine which side of the membrane either FC is facing would help us clarify and understand what could be happening on a sub-compartment level in response to elevated tetrapyrrole levels (presumably Proto IX). This could possibly be accomplished through gold plated/high resolution chloroplast-thylakoid imaging or chloroplast-thylakoid detergent emulsifying experiments.

Knowledge of specific FC locations would provide appropriate physiological context for exactly what compartment tetrapyrroles such as Proto IX may be accumulating and retrograde signaling may be beginning. As preliminary HPLC results depict elevated Proto IX levels in the fc2 mutant relative to wild type/Columbia (data not yet published), it is possible that there is a pool of tetrapyrrole substrates dedicated to FC2. Presumably, FC1 and the Mg-Chelatase are unable to utilize this pool, implicating that separate tetrapyrrole pools exist in the chloroplast, an idea suggested by previous research (Woodson et al., 2011). Experiments calculating $K_m$ and $V_{max}$ for FC1 and FC2 must also be conducted, as their functional differences may be due to a difference in enzymatic kinetics. For instance, FC2 may have a higher catalysis rate and may be able to detoxify accumulated Proto IX more quickly.
A suppressor screen may identify signaling components

The SHOREmap analysis of fts mutants from the genetic screen is paramount in determining the direction of future projects. Results will yield insight into what pathways are activated in response to tetrapyrrole accumulation, subsequent photo-oxidation, and retrograde signaling. Due to the flexibility of Arabidopsis, identification of these genes provides many different avenues of research for future pursuits. Complementation of each gene must still be performed to validate SHOREmap analysis.

I have shown that the fc2 phenotype is EXE1 independent and this is supported by a recent microarray study by Jesse D.Woodson, Ph.D., also at the Chory Lab, Salk Institute for Biological Research, La Jolla, CA. He demonstrated that the fc2 mutant has a distinct subset of stress response and heat shock protein (HSP) genes that are upregulated upon light exposure (data not yet published). Altogether, this suggest that the fts mutant genes encode components of a previously uncharacterized retrograde signaling pathway.

SOUL proteins as potential tetrapyrrole trafficking proteins

In addition to potentially interacting with fts gene products from the suppressor screen, SOUL pose interesting research questions due to their ability to reversibly bind tetrapyrroles such as heme (Lee et al., 2012; Takahashi et al., 2008). Understanding these interactions may prove paramount to interpreting future tetrapyrrole research. In order to begin characterization of the SOUL proteins, we attached fluorescent tags to each one and transformed them into Arabidopsis to check their respective localization sites using confocal microscopy. Localization of SOUL proteins should identify physiological
compartments potentially associated with tetrapyrroles. Through this experiment we also
learned the following – SOUL1 is localized to the cytoplasm and/or endoplasmic
reticulum, SOUL2 could potentially be localized to the cytoplasm, SOUL3 is localized to
the cytoplasm, SOUL4 is localized to the plasma membrane, and SOUL5 and SOUL6 are
both localized to the chloroplast. Because tetrapyrroles may be accumulating both inside
and outside the chloroplast, their interactions with SOUL proteins may be dynamic and
complex. Pull downs can be designed and performed to check if SOUL proteins are
binding tetrapyrroles and altering tetrapyrrole levels and/or gene regulation.

In addition, we made combinations of over expression lines for all six SOUL
proteins with the fc2 mutant background but none of them suppressed the 4hour light cell
death fc2 phenotype (images omitted). We have also been working on isolating
homozygous T-DNA insertion line mutants for the SOUL genes and crossing them into
the fc2 mutant background – fts mutants may also be crossed into the SOUL lines to
check for interesting genetic interactions.
IV. MATERIALS AND METHODS
Biological Material, Growth Conditions, and Treatments

The Arabidopsis transgenic lines pOCA107-2 (Col-0/107) (Susek et al., 1993) and 6-3 (Col-0/6-3) (Koussevitzky et al., 2007) in the Columbia background were used parental lines for transgenic constructs except where indicated. The Col-0/107 line contains a hygromycin-resistance cassette and the uidA (GUS) histochemical marker, each under the control of the LHCBI.2 promoter. The Col-0/6-3 line contains the LUC (Luciferase) marker under the control of a LHCBI.2 promoter. The T-DNA insertion lines (Alonso et al., 2003) FC2W (GABI_766H08), FC2S (SAIL_20_C06), EXE1, HEMA1 (SALK_0566220, SALK_053036), HEMA2 (SALK_052000), SOUL1 (GK_041H09), SOUL2 (SALK_012200C, SALK_019423C), SOUL3 (SAIL_120_D09, GK_686D04), SOUL4 (SALK_018386, GK_497H12), SOUL5 (SALK_000557, SAIL_12520_E10, SALK_018155C), and SOUL6 (SAIL_215_C02) were obtained from the Arabidopsis Biological Resource Center (http://abrc.osu.edu/) (SALK lines) or the Nottingham Arabidopsis Stock Centre (Sutton Boning Campus, UK) (Gabi-Kat, lines). Double and triple mutants were obtained by crossing mutant lines and all genotypes were confirmed by PCR-based markers.

For plate assays, seeds were surface sterilized using chlorine gas for 16h and plated on Linsmaier and Skoog medium pH 5.7 (Caisson Laboratories, North Logan, UT, USA) with 0.6% micropropagation type-1 agar power (Caisson Laboratories). After 3-7 day stratification in the dark at 4°C, plates were moved to one of three conditions using white light at 75 µmol m⁻² s⁻¹: 24hr light, cycling conditions of 4hr light/20hr dark (Short Day), or 24hr dark. Dark-grown seedlings were harvested under dim green light. To
minimize differences in seed quality for a given experiment, all seeds from all genotypes were harvested from plants grown concurrently in the same growth chambers.

For cloning purposes, *E. coli* and *Agrobacterium tumefaciens* strains were grown in liquid Miller nutrient broth or solid medium containing 1.5% agar (w/v). Cells were grown at 37°C (*E. coli*) or 28°C (*A. tumefaciens*) with appropriate antibiotics. Liquid medium was shaken at 225 rpm.

**Construction of plasmids for SOUL transformants**

DNA fragments were amplified by using Phusion enzyme (Finnzymes Espoo, Finland) and *Columbia/Wild type Arabidopsis* genomic DNA as a template. DNA fragments were gel-purified using the QIAquick Gel Extraction Kit (Qiagen) and cloned into the Gateway compatible vector pENTR-D/TOPO (Invitrogen) according to manufacturer instructions. The cloned DNA fragments were then transferred to appropriate vectors using LR clonase (Invitrogen) according to manufacturer instructions. Plasmids were transformed into the *A. tumefaciens* strain GV301 and these cells were used to transform *Arabidopsis*.

Plasmids were transformed into *A. tumefaciens* and colonies were selected for kanamycin resistance. *Arabidopsis* plants were transformed with *A. tumefaciens* cultures through the floral dip method. Basta-resistant T1 plants were selected and propagated. Lines with T2 progeny segregating 3:1 for Basta resistance:sensitivity were further propagated and T3 seeds homozygous for the construct were selected for further study (Woodson et al., 2012).
Confocal imaging, Fluorescent microscopy

A Leica SP/2 inverted microscope was used for confocal imaging.

Protochlorophyllide (pchlide) quantification

All pchlide extractions were quantified according to (Shin et al., 2009). Ten, five-day-old, dark-grown seedlings were collected under a green safe light and immediately frozen in liquid N\textsubscript{2}. After homogenization, pchlide was extracted once in 1ml of ice-cold 80% acetone by shaking at 4°C for 1 hour. Cellular debris was removed by centrifugation at 14,000g for 10 minutes at 4°C. By exciting samples at wavelength of 440nm and measuring emission at wavelength of 636nm, we were capable of measuring fluorescence of 100μl of extract with a Tecan Safire\textsuperscript{2} fluorometer. Using this absorbance value, pchlide levels were calculated and averaged for each sample in biological duplicates as a function of percent weight relative to Columbia/wt.

Chlorophyll quantification

Five-day-old seedlings were frozen in liquid N\textsubscript{2} and homogenized. Chlorophyll was extracted twice in ice-cold 80% acetone and cellular debris was removed by centrifugation at 14,000g for 10 minutes at 4°C. Protein levels were determined by Bradford assay after solubilizing the dried pellet in 0.1M NaOH. Chlorophyll was measured spectrophotometrically with a Tecan Safire\textsuperscript{2} fluorometer by checking absorbance at wavelengths 663nm and 645nm. Chlorophyll A and Chlorophyll B levels were calculated according to (Hendry and Price, 1993).
Suppressor Screen

*fc2* mutant seeds with T-DNA insertion in the 5’ UTR were soaked in ethyl methanesulfonate (EMS), rinsed thoroughly, and dried. EMS-treated-*fc2* seeds were then plated in pools and 3,500 M1 seedlings were grown and M2 seeds were collected. 200,000 M2 seedlings were screened in 4hour light, 20hour dark cycling conditions (in which *fc2* cell death occurs) and the candidates that survived were rescued. M3 progeny from self-fertilized candidates was rescreened for robust phenotypes which yielded 41 *fc2* suppressors from 21 distinct M1 pools.

Approximately 250 F2 individuals with the *fts* suppressor phenotype from *fc2* parental backcrosses for each of the 41 *fc2* suppressors were grown for a month on soil. Leaves were collected and DNA was extracted and pooled via Qiagen Maxiprep Kit. Libraries were sent to Patrice Salome, Ph.D. in Professor Detlef Weigel’s Lab at the Max Planck Institute for Developmental Biology in Tübingen for SHOREmap (simultaneous mapping and mutation by deep sequencing) analysis. Causative mutations for each suppressor are being determined (Hartwig et al., 2012; Schneeberger et al., 2009).


