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# Optimizing a Luciferase-Based Tool for Studying the Effects of Fatty Acid Desaturase 7 on Singlet Oxygen Accumulation in *Arabidopsis thaliana*

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Optimizing a Luciferase-Based Tool for Studying the Effects of *Fatty Acid Desaturase 7* on  
Singlet Oxygen Accumulation in *Arabidopsis thaliana*

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science in Cell and Molecular Biology

by

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This thesis is approved for recommendation to the Graduate Council.

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## Abstract

In plants, reactive oxygen species (ROS) are generated as a byproduct of normal metabolism, as well as in response to adverse conditions such as light stress, extreme temperatures, and exposure to pests and pathogens. Singlet oxygen ( $^1\text{O}_2$ ) is a ROS that is formed during photosynthesis in photosystem II (PSII) of the chloroplasts. Levels of  $^1\text{O}_2$  and other ROS are tightly controlled in healthy plants, but some studies suggest that levels of fatty acid desaturase (FAD) activity in *Arabidopsis thaliana* can influence constitutive and/or stress-responsive ROS accumulation. In this study, a luciferase-based reporter gene that is selectively stimulated by  $^1\text{O}_2$  (*AAA-ATPase: Luc*) was used to compare  $^1\text{O}_2$  levels in wild-type *Arabidopsis* plants (Col-0) and a mutant line with impaired fatty acid desaturation (*fad7-1*). The goals of this study were to establish a suitable method for measuring luminescence generated by the  $^1\text{O}_2$ -responsive reporter gene, and to use this method to see if alterations in fatty acid desaturation have an impact on ROS accumulation in the chloroplast. The *AAA-ATPase: Luc* reporter gene was successfully introduced into the two genetic backgrounds, Col-0 and *fad7-1*, and reporter gene activity was measured using a luminometer-based assay of leaf extracts, a plate reader-based assay of intact leaf discs or extracts, and a camera-based system for imaging intact rosettes (a Lumina XR). Our results suggested that the luminometer-based assay had greater sensitivity than the other two approaches. We also found that, in both genetic backgrounds, activity of the reporter gene increased in response to high light and rose bengal (a chemical elicitor of  $^1\text{O}_2$ ), but not in response to aphid infestation. Although *fad7-Luc* and Col-*Luc* were similar in their response to these three treatments, our data suggested that constitutive levels of reporter gene activity were higher in the *fad7-1* background than in Col-*Luc*. Potentially, decreased fatty acid desaturation in *fad7-1* may promote higher constitutive levels of  $^1\text{O}_2$ .

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## **Dedication**

This work is dedicated to my country, my parents, my husband, and my daughter.

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## Introduction

Plants are exposed to many different environmental stresses such as drought, extreme temperatures, and insect attacks. To respond to these adverse conditions, plants have developed many physiological mechanisms to adapt to stress (Arimura et al., 2005). Previous research has shown that plants accumulate reactive oxygen species (ROS) in response to stress, and that tight regulation of stress-responsive ROS levels can contribute to stress adaptation (Laloi et al., 2007; Dat et al., 2000). In addition, different stresses, including drought, cold, and salt stress, alter expression levels of fatty acid desaturases, and this too can influence levels of stress resistance in plants (Upchurch, 2008). Here, we will discuss ROS generation and fatty acid desaturation, the impacts of these processes on stress adaptation, and the potential linkages between fatty acid desaturation and ROS accumulation in plants.

## Reactive Oxygen Species

ROS are highly reactive molecules that are generated during the cellular metabolism of oxygen, and include such molecules as singlet oxygen ( $^1\text{O}_2$ ), the superoxide anion ( $\text{O}_2^-$ ), hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), and the hydroxyl radical ( $\text{HO}^\bullet$ ) (D'Autréaux & Toledano, 2007). ROS, which are also called reactive oxygen intermediates (ROI) or active oxygen species (AOS), can be actively generated in response to stress by enzymes such as NADPH oxidases and cell wall peroxidases (O'Brien et al. 2012); they can also be formed in chloroplasts and peroxisomes as toxic byproducts of photosynthesis or photorespiration (Foyer & Noctor, 2003; Laloi, Przybyla, & Apel, 2006). During photorespiration, the enzyme Rubisco uses  $\text{O}_2$  to oxygenate ribulose-1,5 biphosphate and form glycolate; then, glycolate moves to peroxisomes from the chloroplast and is oxidized by glycolate oxidase, generating  $\text{H}_2\text{O}_2$  (Wingler, Lea, Quick, & Leegood, 2000).

Although ROS are highly toxic, they also play many roles in cellular signaling in a diverse array of organisms including plants, animals and insects (Mittler et al., 2011). The chloroplast redox status and chloroplast electron transport are crucial sources for redox signals (Baier & Dietz 2005). ROS work as biological signals in stress adaptation by affecting the expression of several genes (Laloi, Apel, & Danon, 2004). When the concentration of the ROS is increased, and ROS specifically interact with the target molecules, this information will be translated via changes in the gene expression. These changes in the gene expression may be due to the oxidation of transcription factors that are redox-sensitive, or to the oxidation of other components that are involved in signaling pathways, resulting in the activation of transcription factors (Laloi et al., 2004). It has been demonstrated that ROS have effects on nuclear gene expression through discrete signaling pathways in a few photosynthetic organisms, and these pathways may cooperate with each other and do not always function independently (Kim, Meskauskiene, Apel, & Laloi, 2008). The interaction between ROS can be seen in Arabidopsis mutant plants that overexpress ascorbate peroxidase, an antioxidant enzyme that scavenges H<sub>2</sub>O<sub>2</sub> (Murgia et al, 2004). The overexpression of ascorbate peroxidase improved the intensity of singlet oxygen -mediated responses in the florescent (*flu*) mutant in Arabidopsis plants, which gives an indication that singlet oxygen -mediated signaling is influenced by H<sub>2</sub>O<sub>2</sub> either directly or indirectly (Laloi et al., 2007).

Under standard physiological circumstances, the creation and degradation of ROS is tightly regulated. Cells have a sturdy scavenging system to maintain the balance of ROS, which are constantly produced. This scavenging system consists of both enzymatic and non-enzymatic antioxidants that detoxify ROS. The controlled balance of ROS can be disturbed by a number of conditions such as light stress, high temperature, pathogen invasion, drought, wounding and



other mechanical stresses, which can lead to oxidative stress by increasing ROS production and/or decreasing ROS scavenging (Hernandez-Barrera et al., 2013).

## **ROS and Plant Stress**

ROS play a crucial role in improving plant resistance against environmental stresses. According to many studies, the production of ROS such as  $\text{H}_2\text{O}_2$  and  $\text{O}_2^-$  is one of the primary actions in plant defense responses to pathogen attack (Bolwell & Wojtaszek, 1997; Davies, Bindschedler, Strickland, & Bolwell, 2006; Lamb & Dixon, 1997).

Under abiotic stresses, the production of ROS increases rapidly. However, the type of ROS produced differs depending on the type of environmental stress and on the species of the plants (Laloi et al., 2007). For example, plants under moderate light stresses produce  $^1\text{O}_2$ ,  $\text{O}_2^-$ , and  $\text{H}_2\text{O}_2$  (Fryer, Oxborough, Mullineaux, & Baker, 2002; Laloi et al., 2007). However, high light intensities favor  $^1\text{O}_2$  production (Hideg, Kálai, Hideg, & Vass, 1998; E. Hideg, Spetea, & Vass, 1994; Laloi et al., 2007). Knowing the type of ROS is very important because ROS differ from each other in the specificity of their signaling; for example, different ROS trigger differing transcriptional responses in plants (Laloi et al. 204; Kim et al., 2008). Additionally, ROS have different biological properties such as half-life, chemical reactivity, and lipid solubility. For instance,  $\text{HO}^\bullet$  is not selective in its reactivity towards biological molecules (D'Autréaux & Toledano, 2007). However, singlet oxygen is likely to trigger a stress-response program modified to lessen the negative impact of environmental conditions (Fryer et al., 2002; Laloi et al., 2007).

ROS accretion in plant cells is involved in a number of processes such as hypersensitive response to pathogen invasion, growth and development, stress response and hormonal recognition (Mittler & Berkowitz, 2001). During pathogenesis, ROS play several essential roles.

First, they have the ability to limit the growth of the pathogen infection by reinforcement plant cell walls or by killing directly the pathogens. Second, in local and systemic acquired resistance, they act as signaling molecules. Finally, they are involved in the hypersensitive response in the incompatible interactions between plant and pathogen (Dat, et al., 2000). Kariola and coauthors have shown that Chlorophyllase1 is rapidly induced in *Arabidopsis thaliana* after tissue damage that caused by necrotrophic bacteria *Erwinia carotovora* or the necrotrophic fungus *Alternaria brassicicola* (2005). This will lead to increasing the production of ROS from thylakoid membranes of chloroplasts by the photosynthetic electron transport chain (Foyer, Lelandais, & Kunert, 1994).

### **Singlet Oxygen**

Singlet oxygen ( $^1\text{O}_2$ ) is an inevitable side-product of photosynthesis.  $^1\text{O}_2$  is very reactive and react rapidly with wide range of molecules, particularly molecules that have double bonds (Laloi & Havaux, 2015; Triantaphylidès & Havaux, 2009). The high levels of  $^1\text{O}_2$  can cause damage to proteins and lipids in the surrounding areas of PSII, causing an inhibition in photosynthetic productivity and a reduction of plant growth. Also, it has been reported that the overproduction of  $^1\text{O}_2$  can damage the  $\beta$ -carotene particles in the PSII reaction center. The  $\beta$ -carotene oxidation products act as stress signals that arbitrate the responses of the genes to  $^1\text{O}_2$  (Gutierrez et al., 2014; Ramel, Sulmon, Serra, Gouesbet, & Couee, 2012). Many scavenging systems have evolved in plants to defend against the side effects of  $^1\text{O}_2$ . Plastoquinones, tocopherols, and carotenoids that are located in the thylakoid membranes have a significant effect in quenching  $^1\text{O}_2$  (Laloi & Havaux, 2015; Triantaphylidès & Havaux, 2009). Glutathione, ascorbate, and ubiquinol, may also play a role in quenching  $^1\text{O}_2$  (Laloi & Havaux, 2015).

The role of  $^1\text{O}_2$  as a signal molecule has been studied in the conditional fluorescence (*flu*) mutant of Arabidopsis. The *flu* mutant accumulates protochlorophyllide (Pchl<sub>id</sub>) in the dark. In light conditions, Pchl<sub>id</sub> acts as a photosensitizer and produces  $^1\text{O}_2$  (Baruah, Šimková, Apel, & Laloï, 2009; Flors et al., 2006; É Hideg, Kálai, Kós, Asada, & Hideg, 2006). Directly after the  $^1\text{O}_2$  is generated in the plastids, a signal will be transferred rapidly to the nucleus (op den Camp et al., 2003).  $^1\text{O}_2$  is very reactive and not likely to move out of the plastid; however, it causes the generation of a stable second messenger (the plastid protein EXECUTER) that is found to be involved in signal transduction from plastids to nucleus (Wagner et al., 2004; Baruah et al., 2009). In this research, a  $^1\text{O}_2$ -responsive reporter gene will be used to monitor the levels of  $^1\text{O}_2$  in Arabidopsis plants during biotic and abiotic stresses.

## Measurement of ROS

It is very crucial to study the redox status in plants that are under stress conditions that we can draw a picture on how a particular stress affect plants and how plants recover from this stress. Moreover, the changes in the redox status occur in different parts of the cell. Therefore, knowing the intracellular location in which the redox status has changed will assist the scientists in understanding how ROS will react with the neighbored molecules, and the possible mechanisms that can regulate the levels of ROS. On the other hand, the studying of the effectiveness of ROS is very challenging because of their short lifespan, high reactivity (Shulaev & Oliver, 2006) and their low concentration at normal conditions. Therefore, they are very hard to be detected in complex biological tissues. As well, site-specific detection methods are required to specifically identify ROS in their subcellular localization, because ROS are frequently created and/or detoxified in subcellular parts (Shulaev & Oliver, 2006).

There are several distinct techniques that have been used to measure ROS. In one method, researchers measure ROS according to their ability to cause damage to lipids, DNA and proteins. Therefore, they use DNA, lipids, and proteins as an indicator to oxidative stress. Nevertheless, this technique provides indirect measurement of ROS (Fryer et al., 2002). In addition, electron paramagnetic resonance (EPR) has been used with some success in the detection of oxygen free radicals and organic radicals from their semi-stable adduct (Fryer et al., 2002; Van Doorslaer, Dedonder, de Blocks, & Callens, 1999). However, the precise locations of ROS construction in tissues cannot be detected by using EPR method (Fryer et al., 2002). The third technique is cell fractionation that has been used to determine the locations of ROS generation and the site of ROS detoxification systems (Doulis, Debian, Kingston-Smith, & Foyer, 1997; Fryer et al., 2002; Kingston-Smith, Harbinson, & Foyer, 1999). However, this technique involves massive tissue damage which can lead to the production of ROS. Subsequently, the data that produced by using this damaging technique does not usually produce a precise image of the sites for ROS production and the location of ROS detoxification system (Fryer et al., 2002).

Additionally, imaging of ROS in vivo has been accomplished by using different fluorescent dyes (Halliwell & Whiteman, 2004; Van Breusegem, Bailey-Serres, & Mittler, 2008). Because singlet oxygen accumulation usually occurs in mesophyll tissues, digital imaging with high resolution is used to define the location of the accelerated singlet oxygen in Arabidopsis leaves (Fryer et al., 2002). Fryer and other researchers used tracer dyes that are specific to ROS in combination with using high resolution imaging. This method can be used to determine the location of ROS and their accumulation in the tissues (2002). The most widespread dye is H<sub>2</sub>DCF-DA that can be used to detect the ROS in chloroplast and

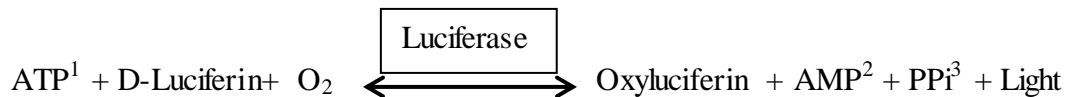
mitochondria (Gao, Xing, Li, & Zhang, 2008; Zhang, Li, Xing, & Gao, 2009). However, these kinds of methods suffer from different drawbacks (Halliwell & Whiteman, 2004). These probes are affected by many chemical interactions. For example, they can interact with different antioxidant molecules,  $O_2^-$  and  $O_2$ , which will have an impact on the signals (Winterbourn, 2014).

Recently, many techniques have been developed in detecting redox status *in vivo* by engineering the redox sensors to be more specific in measuring the levels of the ROS. For example, the reduction–oxidation sensitive green fluorescent protein (roGFP) was targeted by either mitochondrial targeted sequence mt-roGFP or cytosolic targeted sequence c-roGFP in *Arabidopsis* plants to sense the oxidation and reduction status in the mitochondria and the cytosol (Jiang et al., 2006). Additionally, Jiang and his collaborators state that the roGFP is sensitive to the redox status in plants and can be used to monitor the modifications in redox status in real time *in vivo* (2006). Another example is a specific detection of  $H_2O_2$  by a yellow fluorescent protein (YFP) that introduced into the regulatory domain of the  $H_2O_2$ -binding protein in *Escherichia coli*. YEP is a  $H_2O_2$ -specific probe and can be used for quantitative measurement of  $H_2O_2$  in animal and plant cells (Hernández-Barrera et al., 2015). In addition,  $^1O_2$ -responsive promoter that direct the expression of the reporter gene luciferase was developed in *Arabidopsis* fluorescent in blue light (*flu*) mutants to detect the specifically the levels of the  $^1O_2$  in plants (Baruah et al., 2009).

### **Luciferase as a Reporter for Singlet Oxygen**

Bioluminescence is a reaction that occurs at least between two molecules which are formed under usual physiological environments within an organism. The substrate molecule that produces light in this reaction is called luciferin. Luciferin is produced by luciferases which is an

enzyme catalyzes the oxidation of luciferins creating oxyluciferins and photons of light (Greer & Szalay, 2002). The luciferin- luciferase reaction is described in the following equation (Crouch, Kozłowski, Slater, & Fletcher, 1993):



A reporter gene that is selectively stimulated only by  $^1\text{O}_2$  and is not stimulated by hydrogen peroxide or superoxide was developed to detect  $^1\text{O}_2$  in Arabidopsis plants. This reporter gene consists of the promoter of an AAA-ATPase gene (At3g28580) and the luciferase open reading frame (Baruah et al., 2009). The luminescence emitted by transgenic plants acts as an indicator to the levels of the  $^1\text{O}_2$  in the transgenic plants.

The  $^1\text{O}_2$ -responsive promoter gene was introduced into Arabidopsis plants that have an impaired activity of the fatty acid desaturase 7 to see if the loss of function of this enzyme will have an impact on the levels of the  $^1\text{O}_2$  in response to different stresses.

### **Fatty Acid Desaturases**

Fatty acid desaturases (FADs) are enzymes that are widely distributed among all organisms except some types of bacteria such as *Escherichia coli* (Los & Murata, 1998). FADs produce a double bond between two carbon atoms in a fatty acyl chain. The desaturation of fatty acids is crucial for the functioning of biological membranes. For example, at normal temperatures, saturated fatty acids cannot form the bilayer structure which is essential for appropriate working of the biological membrane. Producing a proper number of unsaturated

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<sup>1</sup> Adenosine triphosphate

<sup>2</sup> Adenosine monophosphate

<sup>3</sup> Pyrophosphate

bonds in the fatty acyl chains allows for the proper fluidity of the membrane (Los & Murata, 1998). In turn, changing membrane fluidity will affect the function of certain membrane-embedded proteins (Thompson Jr, 1989). Plants can adjust different types of stresses by having the ability to modify their membrane lipid fluidity (Upchurch, 2008).

The  $\omega$ -3 fatty acid desaturases ( $\omega$ -3 FADs) are membrane-embedded enzymes that catalyze the alteration of linoleic acid to linolenic acid (Kodama, Akagi, Kusumi, Fujimura, & Iba, 1997). Fatty acid desaturase7 (*FAD7*) is one of the  $\omega$ -3 FADs that converts linoleic acid 18:2 to linolenic acid 18:3 (Avila et al., 2012; Liu, Yang, Li, Yang, & Meng, 2006) by producing a double bond between the third and fourth carbon atoms at the end of the fatty acyl chain. Therefore, there are more dienoic fatty acids (the fatty acids that contain 2 double bonds) than trienoic fatty acids (the fatty acids that contain 3 double bonds) in *FAD7* disrupted plants than their wild types. The most abundant fatty acid in the aerial tissues of most plants is linolenic acid and it is the most ample fatty acid on earth. Linolenic acid can ascend to 54 % of the total leaf fatty acids in *Arabidopsis thaliana* (Mene-Saffrane, et al., 2009). It has been found that the transgenic tobacco lines that contain high levels of hexadecatrienoic (16:3) and linolenic (18:3) acids and low levels of their precursors hexadecadienoic and linoleic acids showed more resistance to cold stress than the wild types (Kodama, Hamada, Horiguchi, Nishimura, & Iba, 1994). Also, the presence of *FAD7* can enhance plant resistance to certain conditions such as cold temperature. Similarly, when *FAD7* is overexpressed, it positively affects tomatoes growth in cold temperature. To illustrate, it has been demonstrated that the presence of *FAD7* significantly increases a tomato's tolerance to cold stress. The *FAD7* that is located in the endoplasmic reticulum acts as insulation to the cell handling the normal response of the cell preventing its death, which enhanced tomato plants tolerance to cold temperature (Yu et al.,

2009). In addition, the *fad3 fad7 fad8* triple mutants were more susceptible to pathogens and insects (Mene-Saffrane et al., 2009; Vijayan, Shockey, Levesque, Cook, & Browse, 1998), and they were more vulnerable to environmental stresses than the wild-type plants (Mene-Saffrane et al., 2009; Routaboul, Fischer, & Browse, 2000).

On the other hand, it has been established that the rice (*Oryza sativa*) plants have shown more resistance to the rice blast fungus (*Magnaporthe grisea*) by the suppression of the *OsFAD7* and *OsFAD8* genes in rice (Avila et al., 2012; Yaeno, Matsuda, & Iba, 2004; Yara et al., 2007). Sohn and Back (2007) stated that the transgenic rice plants in which the total linolenic acid amount decreased by 7-32% and the total amount of linoleic acid was increased by the suppression of *FAD7* were more resistant to high temperatures than untransformed rice plants. Additionally, tomato plants that have *spr2* mutant, in which the *FAD7* function has been disrupted, demonstrated better resistance to potato aphids (*Macrosiphum euphorbiae*) than the wild-type tomato (Avila et al., 2012). Also, two mutants in *Arabidopsis thaliana* (*Atfsad7-2* and *Atfad7-1fad8*) exhibited more resistance to the green peach aphids (*Myzus persicae*) than their wild types. The number of aphids on these two mutants was around 42% less than the wild types. Furthermore, the accumulation of salicylic acid, which is a hydroxy-benzoic acid that is contributed to improving plants' immune systems against many pathogens, has been increased by *FAD7* suppression. The interruption of the function of different types of FADs in different plants (soybeans, rice, *Arabidopsis*, and tomato) accounts for increasing salicylic acid accumulation. The accumulation of salicylic acid has been correlated with enhancing plant resistance against aphids (Avila, et al., 2012).



## ROS and *fad7* Plants

It has been reported that the loss of function of fatty acid desaturases ( $\omega$ -3 FADs) in plants contributes to the salicylic acid (SA) accumulation which could induce ROS production (O'Brien, Daudi, Butt, & Bolwell, 2012). Furthermore, O'Brien and other researchers demonstrated that  $H_2O_2$  has been displayed to be accumulated after the application SA, which suggests SA might need other pathway components such as ROS for a robust defense reaction. Also, the accumulation of SA led to a decrease in ROS-scavenging enzymes which in turn leads to higher levels of ROS in response to pathogen attacks. Therefore, plant improving resistance could be related to ROS and their reaction with salicylic acid, which accumulated in the absence of *FAD7*. Hence, ROS may stimulate the accumulation of SA which plays a crucial role in enhancing plant defense mechanism. Also, higher levels of ROS have been shown to increase the accumulation of pathogenesis-related (PR) proteins like PR1 and PR2 (Chen et al., 1995; Kariola et al., 2005; Maleck & Dietrich, 1999; Uknes et al., 1992; Van Loon & Van Strien, 1999).

It has been shown that lipid profiling in Arabidopsis triple mutants *fad3fad7fad8* and *spr2* tomato foliage accrue higher levels of FA-hydroperoxides and that this increase acts as an indicator for oxidative stresses than the wild-type plants (Muller & Goggin, unpublished data). Therefore, we hypothesize that there are higher levels of  $^1O_2$  in FADs disrupted plants than non-disrupted plants. In order to prove our hypothesis, we will measure the  $^1O_2$  produced in *fad7* Arabidopsis plants by using abiotic biotic stresses, and compare it with the wild-type plants.

The effect of green peach aphid's infestation on the levels of  $^1O_2$  will be studied in *fad7* Arabidopsis plants. Green peach aphids are insects that belong to the family *Aphididae*. Aphids attack a wide variety of crops, and feed on the phloem sap of their host crops (Louis, Leung, Pegadaraju, Reese, & Shah, 2010; Tjallingii & Esch, 1993). The phloem sap contains sugars and

carbohydrates as products of photosynthesis. These products are essential for plant nutrition. As a result, sucking the phloem sap from the plants causes aphid-plant interactions, and can cause damaging effects on the plants. Additionally, the damaging effect of aphids comes from their high reproductive ability, their capacity to breed asexually and the ability of some aphids to vector viruses to plants (Kennedy, Day, & Eastop, 1962; Louis et al., 2010). Therefore, they can cause severe damage to their hosts.

In this research, the effect of the *FAD7* gene on  $^1\text{O}_2$  accumulation will be studied in Arabidopsis plants by measuring the luminescence in the transgenic plants with the AAA-ATPase-Luc reporter gene. The luminescence will be measured in response to treatments of rose bengal (a chemical elicitor of  $^1\text{O}_2$ ), high light stress, and aphid's infestation; using different devices such as the LuninaXR, Plate Reader, and Glomax 20/20 luminometer, and all these techniques will be discussed in details in the materials and methods section.

## Objectives

In this research, we studied the levels of the  $^1\text{O}_2$  as one of the ROS that is produced during photosynthesis, and that can also accumulate to higher levels in response to certain stresses. The levels of the  $^1\text{O}_2$  were measured in Arabidopsis plants by using a luciferase-based reporter system (*AAA-ATPase-Luc*) that is selectively stimulated by  $^1\text{O}_2$  but not by other ROS. The levels of the  $^1\text{O}_2$  were measured through measuring the luminescence in the plants with the  $^1\text{O}_2$ -responsive reporter gene. The  $^1\text{O}_2$ -responsive promoter gene was introduced into Arabidopsis plants that have an impaired activity of fatty acid desaturase 7 (*fad7-1*) and into wild-type plants (Col-0). The purposes of this research were (1) to optimize a suitable method for measuring the luminescence in the  $^1\text{O}_2$ -responsive reporter gene containing plants in response to different stresses by using different approaches, and (2) to see if the alterations in fatty acid desaturation have an impact on ROS accumulation in the chloroplast. This was achieved by comparing the levels of the  $^1\text{O}_2$  produced in response to biotic and abiotic stresses between *fad7-1* and Col-0 wild-type Arabidopsis plants through measuring the luminescence in the  $^1\text{O}_2$ -responsive reporter gene containing plants.

## Materials and Methods

### Plant Materials

Experiments were performed by using *Arabidopsis thaliana* wild-type plants, *fad7-1* genotype, and *flu* mutants that express the *AAA-ATPase: Luciferase* reporter gene containing the promoter *AAA-ATPase* (At3g28580) that is only responsive to singlet oxygen and the luciferase open reading frame (Baruah, et al., 2009). The seeds for the *flu: AAA-ATPase: Luc* mutant were provided by Klaus Apel Boyce Thompson Institute. The generation of *fad7-1* and Col-0 plants with the *AAA-ATPase: Luc* reporter gene was done in the laboratory by crossing *Arabidopsis flu AAA-ATPase: Luc* plants (when they start flowering) as a pollen donor with 20 *fad7-1* mutants as maternal parents that lack the trichome differentiation protein GL1. Trichome differentiation protein GL1 is a protein which is expressed in, stems, flowers, and leaves (Kirik et al., 2001). Since the *gll* mutation is recessive, presence one allele of the gene will cause the expression of the GL1 protein. Therefore the crossing was confirmed depending on the presence of the trichomes in the F1 generation.

### Surface Sterilization of Seeds and Growth Conditions

All seeds for all genotypes were surface sterilized prior to every experiment by rinsing the seeds with 70% ethanol for five minutes, and eliminating the ethanol after centrifugation for 10-15 minutes. Then, the seeds were washed with 50% bleach solution (commercial bleach) with 0.05% Tween 20 for 10 minutes. The bleach solution was discarded after the centrifugation for 15-20 seconds, and the seeds were rinsed 6-7 times with autoclaved distilled water until the smell of the bleach was disappeared. Around 50-70 seeds were plated on Murashige and Skoog (MS) media (Murashige & Skoog, 1962) and vernalized at 4°C for 3 days. MS Medium contains 6 g of agarose, 3.22g of MS and 22.5g of sucrose in 750 ml with PH (adjusted by KOH) between 5.5-

5.9. After autoclaving, the MS medium was poured into plates and ready to be used for seeds germination. The vernalized seeds were later moved to the growth chamber (23.0 °C, 16 hours /dark 8 hours photoperiod;  $\sim 135 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity; 65% humidity). After 12-15 days the plantlets were transplanted into potted soil consisting of peat moss, vermiculite, perlite with the ratio 4:3:2.

The *flu:AAA-ATPase: Luc* plants were grown under continuous light conditions because *flu* mutants accrue free protochlorophyllide in the dark. Protochlorophyllide acts as a strong photosensitizer which produces  $^1\text{O}_2$  in plastids during light. Therefore, instantly after the release of  $^1\text{O}_2$ , the growth amount of mature plants declines, whereas seedlings bleach and die (Kim, et al., 2008; Laboi, et al., 2006).

#### **DNA Extraction for F2 (*flu AAA-ATPase: Luc X fad7/gll*) Generation**

DNA extraction protocol developed from (Kasajima, et al., 2004 and Edwards, et al., 1991 with some modifications). The DNA extraction buffer was prepared by adding 1 volume of Edward's Solution (200 mM Tris-HCl (pH 7.5), 25 mM EDTA, and 250 mM NaCl) to 9 volumes of TE buffer (10 mM Tris (pH 8.0), 1mM EDTA). Later, 200  $\mu\text{l}$  of DNA extraction buffer were added to 3-5 mg of Arabidopsis leaf tissues with three glass beads (3 mm). After homogenizing for 30 seconds to 1 minute by the Geno Grinder 2010/Spex Sample Prep., the solution turned to light green and was ready for PCR experiments, and it is stable under  $-20^\circ\text{C}$  for several months.

## Screening for *fad 7-Luc* and *Col-Luc* Plants

PCR analyses were performed on 115 plants of F2 plants that were grown at (16 h L: 8 h D) condition. Therefore, the plants that were homozygotes for the *flu (flu/flu)* mutants were not able to grow, and the plants that were screened were either heterozygote for the *flu (flu/FLU)* mutant or homozygotes for the *Flu* wild-type (FLU/FLU). After the seeds were surface sterilized, they were grown in growth chamber for DNA extraction. 2 µl of DNA samples were used for a 25µl PCR reaction prepared by adding 2.5 of MgCl<sub>2</sub> (25mM), 5 µl of 5x buffers, 1 µl of each forward and reverse primers, 0.5 µl of dNTPs, 0.2 µl of Tag polymerase, 12.8 µl of ddH<sub>2</sub>O, and 2µl of the DNA samples.

To detect luciferase positive plants, the forward primer 5'-TTACACGAAATTGC TTCTGGTG-3' and the reverse primer 5'- CCTCGGGTGTAATCAGAATAGC-3' were used according to Baruah and others (2009). The annealing temperature was 51 °C, and the product size was 139 base pair for the luciferase primers.

The luciferase-positive plants were tested for the presence of *FAD7* wild-type gene by using the forward specific primer for *fad7* 5'-TTTCAGTGGGCTCTCGAAGACT-3', the forward primer for *FAD7* wild-type 5'-TTTCAGTGGGCTCGAAGTCC-3', and the shared reverse primer 5'-ATCTGCGGGAAAAGATGATG-3'. The size of the amplicon was 582 base pair for both the *FAD7* wild type and the *fad7* mutant primers. The *FAD7* primers designed accordingly to Avila & Goggin (unpublished data).

Both *fad7* mutant and *FAD7* wild-type samples were screened for the presence of the *flu* mutant gene using the forward primer (5'-CCAAGGGAAGTATAGG GAAGT-3') and the reverse primer for the *FLU* gene (5'-TGCGGAAGGATCAGTCAGTC-3'). The annealing

temperature was 58 °C, and the product size was 179 base pair for the *flu* mutation's primers. The process of screening for *fad7-Luc* and *Col-Luc* plants is explained in the figure below (Figure 1).

### **The GloMax 20/20 Luminometer Analysis**

First, we tested the variations between the readings of the luminometer to optimize a method with accurate luminescence measures (Supplemental Figure 1). The GloMax 20/20 luminometer analysis was performed by using the Luciferase Assay System protocol (Promega). After grinding the leaf samples by liquid nitrogen, the weight for every sample was recorded and 1ml  $\mu$ l of 1X lysis reagent was added to the tissue samples. After homogenization by using the Geno/Grinder 2010 for 1 minute, the debris was removed by brief centrifugation and the supernatant was transferred to a new tube. Later, 5 $\mu$ l of cell lysate was mixed with 20 $\mu$ l of Luciferase Assay Reagent. The luminescence was measured by using the GloMax 20/20 luminometer.

### **The Synergy HT Multi-Mode Microplate Reader Analysis**

The plate reader analysis was performed by spraying the leaves with the luciferin before 30 minutes of the experiment. Later, the seventh leaf of the Arabidopsis rosette leaves (Farmer, et al., 2013) was placed directly on a 24 well plate, and 400  $\mu$ l ddH<sub>2</sub>O were added to each well. The luminescence were measured by using Bio-tek Synergy HT Multi-Mode Microplate Reader by choosing the settings that allow us to collect all of the photons that are emitted from the samples through blocking all the excitation light and selecting "Hole" setting in the plate reader to capture all the emission light.

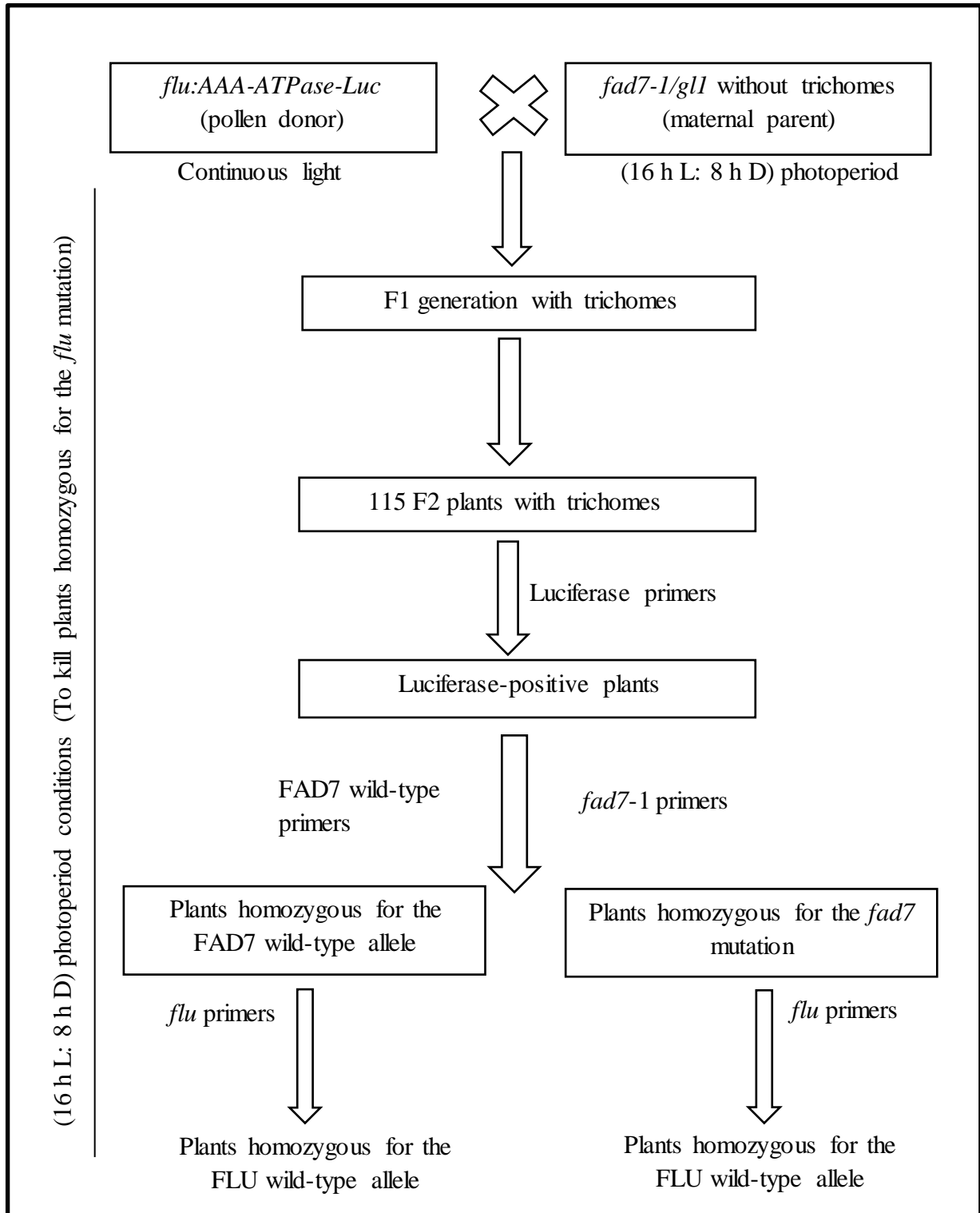
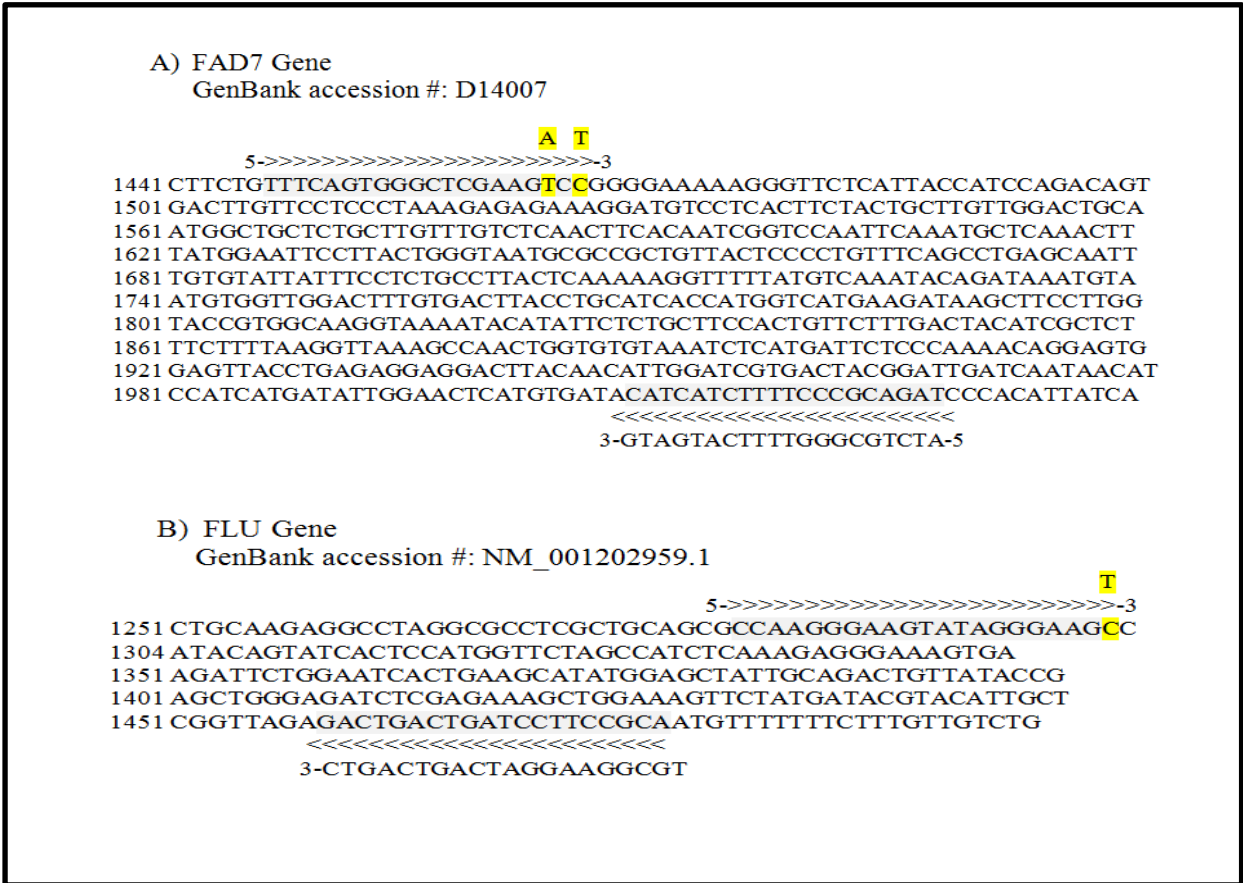


Figure 1: The schematic diagram of the screening process for *fad7-Luc* and *Col-Luc* Plants.





**Figure 2:** The primer design for *FAD7* gene and *FLU* gene. Highlighted areas represent the mutation sights in *FAD7* and *FLU* genes.

In addition, the Luciferase Assay System protocol (Promega) as mentioned previously was also used to measure the luminescence by the plate reader.

**The Lumina XR Analysis**

After the treatment, the intact rosette leaves were collected and imaged by Lumina XR system to visualize the luminescence for the rosette leaves. Then, the leaves were rapidly fresh-frozen by liquid nitrogen and stored under -80 °C to be processed for the GloMax 20/20 data. We got the results from the Lumina XR by selecting the region of interest (ROI) covering the rosette

Arabidopsis leaves. Then, the luminescence was calculated by taking the radiance unit (photons/sec/cm<sup>2</sup>/sr) that represent the number of photons per second that depart a cm<sup>2</sup> of tissue and emit into a solid angle of one steradian (sr) (Living Image Software User's Manual).

### **Statistical Analysis**

Both the one way and two way analysis of variance (ANOVA), and means separation by student's *t* test were performed by using the JMP® Genomics 7.0 software (SAS institute Inc.).

## Results

### Confirmation of Luciferase Activity in the Transgenic Line *flu:AAA-ATPase-Luc*

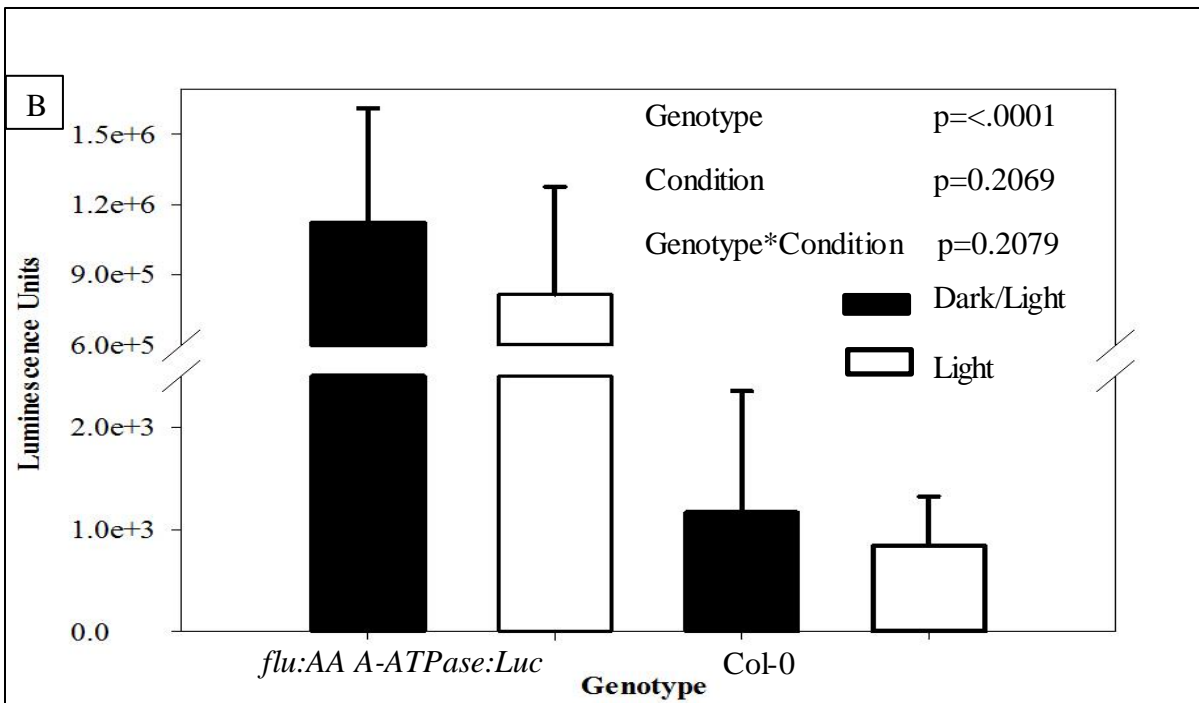
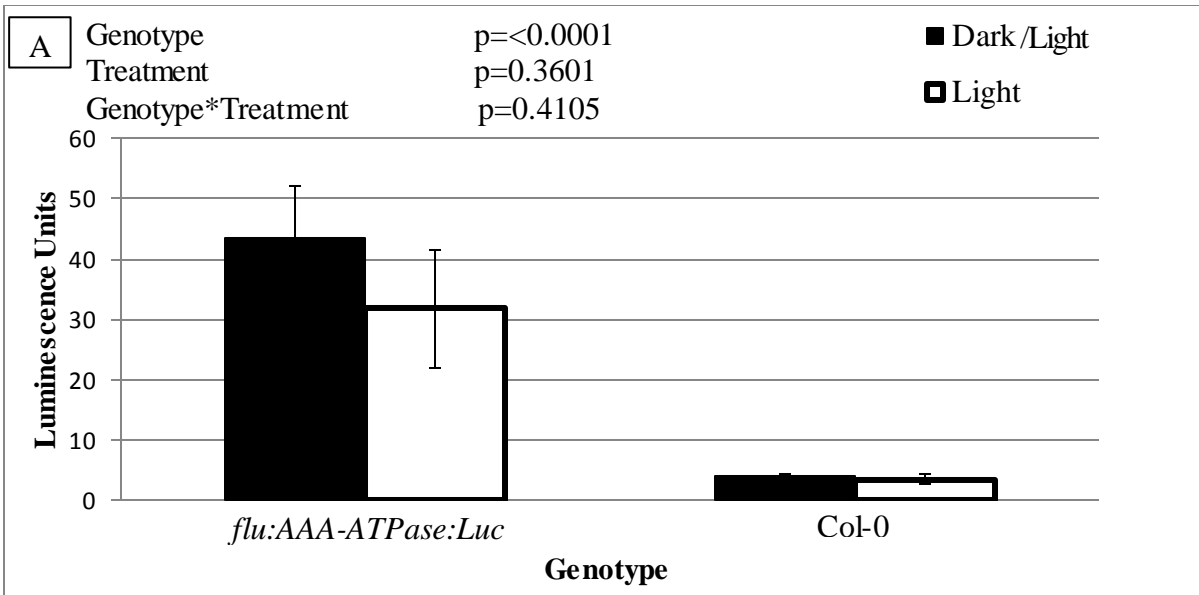
To confirm that the  $^1\text{O}_2$ -responsive reporter gene *AAA-ATPase-Luc* was active in *flu:AAA-ATPase-Luc* (obtained from Dr. Klaus Appel, Boyce Thomson Institute), luciferase activity was compared in this line with untransformed wild-type plants (Col-0). Because the conditional *flu* mutant in which this transgenic line was generated accumulates high levels of  $^1\text{O}_2$  in plastids in response to a shift from dark to light (Meskauskiene et al., 2009), we compared luminescence in plants grown under continuous light with plants that were shifted to dark for 8 hours before the experiment. To optimize a suitable method for measuring the luminescence generated by the luciferase reporter, we compared measures of luminescence in the same plants using foliar extracts between two different methods: microplate analysis of luminescence (Figure 3A), and luminometer-based measurement of luminescence (Figure 3B). For both the microplate measurements and luminometer measurements, luminescence was significantly higher in *AAA-ATPase-Luc* than in untransformed controls, confirming that the transgene was active in the transgenic line.

In addition, the luminometer-based and plate reader-based measurements of the same samples were highly correlated with one another ( $r^2=0.78$ ), indicating good agreement between the two methods. However, whereas the microplate measures of luminescence were 9-fold higher in transgenic plants than in untransformed controls, luminometer-based measurements indicated a 950-fold difference in luminescence between the two treatment groups. This difference suggests that the luminometer-based assay is more sensitive than the plate reader assay.

Both microplate and luminometer-based measures of luminescence were numerically higher for plants that experienced a dark/light transition than for plants grown under continuous light; however, this difference was not statistically significant, and there was no significant interaction between genotype and light treatment (Figures 3A and 3B). The plants were harvested for analysis only 10 minutes after they were transferred from the dark to the light, and this exposure period may not have been long enough for the dark-exposed *flu* mutants to accumulate significantly higher levels of  $^1\text{O}_2$  than the *flu* plants grown under continuous light.

### **Introduction of the AAA-ATPase-Luc Reporter Gene into *fad 7-1* and Col-0 Backgrounds**

To obtain *fad7-1* and Col-0 plants with the AAA-ATPase-Luc reporter gene, the *fad7-1 gl-1* mutant (which is in a Col-0 background) was crossed to the *flu:AAA-ATPase-Luc* transgenic line (also originally developed in a Col-0 background). Plants from the F1 generation were allowed to self-pollinate, and the F2 generation was screened to select for the reporter gene, select against the *flu* and *gl-1* mutations, and track segregation of the *fad7-1* mutation (Figure 4). To aid in selecting against the *flu* mutation, the F2 generation was grown under a normal photoperiod (16 h L: 8 h D), which would cause all plants that were homozygous for *flu* to die. Whereas the maternal parent (*fad7-1 gl-1*) for this cross was glabrous due to the *gl-1* mutation, the F1 plants all had trichomes, demonstrating that they were the product of cross-fertilization rather than self-pollination. PCR with primers specific to the *flu:AAA-ATPase-Luc* reporter gene identified 28 F2 plants that carried the reporter gene. After initial testing of 115 F2 plants, PCR was repeated once on the 28 putative positive plants to confirm that they were positive for the reporter gene (Figure 5A). Then, these 28 plants were tested with primers specific to the wild-type and mutant alleles of the *FAD7* gene, and 8 homozygous *fad7-1* mutants were identified,



**Figure 3. Reporter gene activity in *flu:AAA-ATPase-Luc*, as measured by luminescence.** The *flu:AAA-ATPase-Luc* transgenic line and untransformed controls (Col-0) were either grown in continuous light throughout their growth period (Light), or were grown under continuous light, then exposed to 8 h of darkness, followed by ~10 min of light before collection (Dark/Light). This dark/light transition was previously reported to induce  $^1\text{O}_2$  production in the *flu* mutant. The luminescence was measured in using tissue extracts in the same plants using two different methods: a Synergy HT Multi-Mode Microplate Reader (A) or a GloMax 20/20 Luminometer (B) (n=8).

as well as 2 plants that were homozygous for the wild-type *FAD7* allele (Figure 5B). Plants that were heterozygous at the *FAD7* locus were discarded, and the remaining 10 plants were tested by PCR with primers specific for the wild-type and mutant alleles of the *FLU* gene to select against any plants that might carry the recessive *flu* mutation. This process identified 4 plants that were homozygous for the wild-type *FLU* allele (Figure 5C), including 1 that was homozygous for the *fad7-1* mutation (hereafter referred to as *fad7-Luc*), and 3 that were homozygous for the wild-type *FAD7* allele (hereafter referred to as *Col-Luc*) (Figure 4 and Figure 5). All four plants had trichomes, indicating that all of them possessed a wild-type copy of the *GL1* gene.

### **Screening for *fad7-Luc* and *Col-Luc* Plants**

The screening for the luciferase-positive plants in the F2 generation indicated that 28 out of 115 plants (24%) were positive for the luciferase reporter gene (Figures 4 and 5), which was not consistent with our expected ratio 75%. The Chi Square analysis indicated that there is significant difference between the observed ratio 24% and the expected ratio 75% ( $p < 0.0001$ ). Potentially, the crude DNA extraction method, which does not remove potential PCR inhibitors from the sample and does not allow accurate quantification of DNA content, may have contributed to some false negatives.

32% of the luciferase-positive plants were homozygous for the *FAD7* wild type allele, and 7% were homozygous for the *fad7* mutation (Figures 4 and 5).

28% of the *FAD7* wild type plants were homozygous for the *FLU* wild type allele, and 50% of the *fad7* plants were homozygous for the *FLU* wild type allele. Wild-type *FLU* plants were expected to represent 33% of the F2 generation that survived long enough to be tested, because the plants were grown under a photoperiod that would kill all homozygous *flu* mutants. The Chi Square analysis indicated that there is no significant difference between the observed

ratio and the expected ratio ( $p < 2.216$ ). The overall percentage of homozygous *FLU* plants (40%) was slightly more than the expected segregation ratio, potentially due to the low number of replicates (Figures 4 and 5).

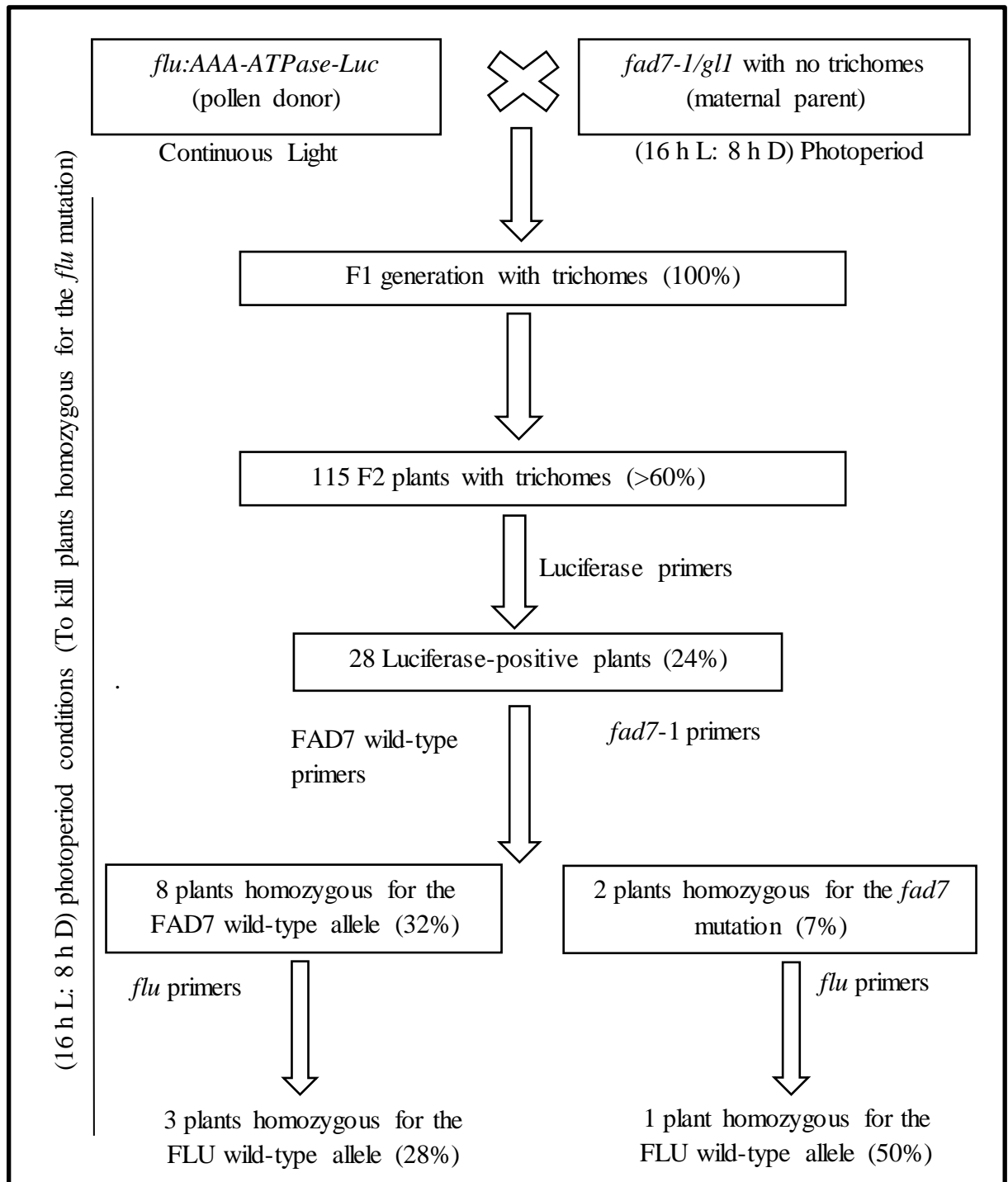
After identifying F2 plants that were homozygous for the *fad7* mutation and positive for the reporter gene, F3 seeds were collected. Most of the subsequent experiments were performed with F3 plants. Luminescence levels in each tested plant were at least 30 standard deviations greater than the mean for the control plants that lacked the reporter gene. The fact that tested plants uniformly had high luminescence strongly suggest that the F2 parents had 2 copies of the transgene so that all F3 also carried the reporter.

#### **Confirmation of Reporter Gene Activity in *fad7-Luc* Plants**

The luminometer-based assay was used to compare levels of luminescence produced by *fad7-Luc* (F3 generation) at normal growth conditions (16 L: 8 D,  $\sim 135 \mu\text{mol m}^{-2} \text{s}^{-1}$  light intensity) to confirm the activity of the reporter gene. The levels of the luminescence for the *fad7-Luc* line, which carries the  $^1\text{O}_2$ -responsive-reporter gene, were 250-fold higher than the levels observed in *fad7-1* mutants without the transgene, confirming that the transgene is active in the *fad7-Luc* line (Figure 6).

#### **Confirmation of Reporter Gene Responsiveness to Rose Bengal**

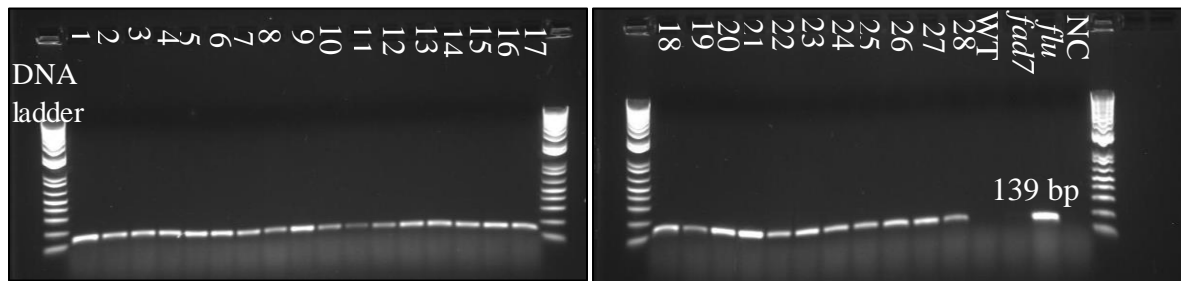
The purpose of these experiments was to confirm the responsiveness of the reporter gene in F3 generation plants in two genetic backgrounds (Col-0 and *fad7-1*) to rose bengal, a chemical treatment known to generate  $^1\text{O}_2$ . One experiment was conducted with a plate reader-based assay using intact leaves (Figures 7A and 7B), and a second was conducted with a luminometer with leaf extracts (Figure 8), using 500  $\mu\text{M}$  rose bengal. In the first assay, which was conducted with



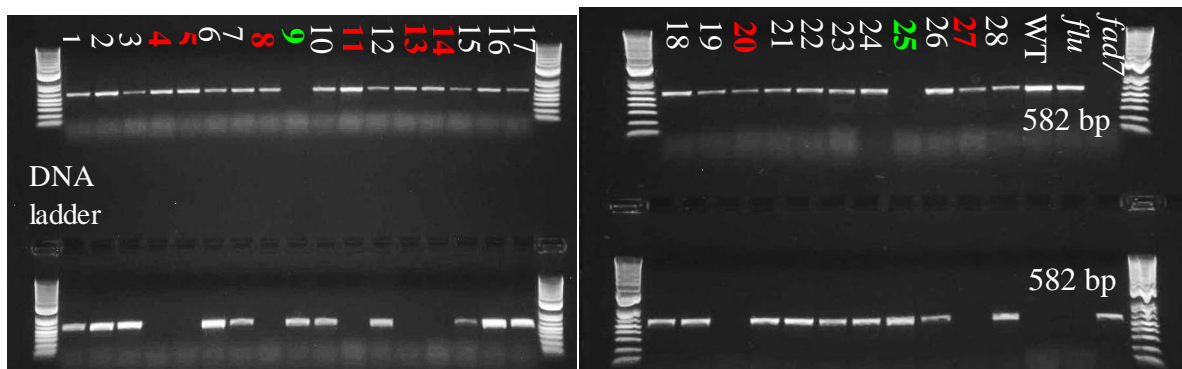
**Figure 4. Selection of *fad 7-Luc* and *Col-Luc* mutants.** Out of 115 F2 plants that originated from crossing *flu*:AAA-ATPase-*Luc* and *fad7-1/gll*, screening identified 1 *fad7-Luc* plant (homozygous for the *fad7-1* mutation and the wild-type *FLU* allele, and positive for the AAA-ATPase-*Luc* reporter gene and the wild-type *Gl-1* allele), and 3 *Col-Luc* plants (homozygous for the wild-type *FAD7* and *FLU* alleles, and positive for the AAA-ATPase-*Luc* reporter gene and the wild-type *Gl-1* allele)



**A) Luciferase primers**



**B) *FAD7* wild-type (top) and mutant (bottom) primers**

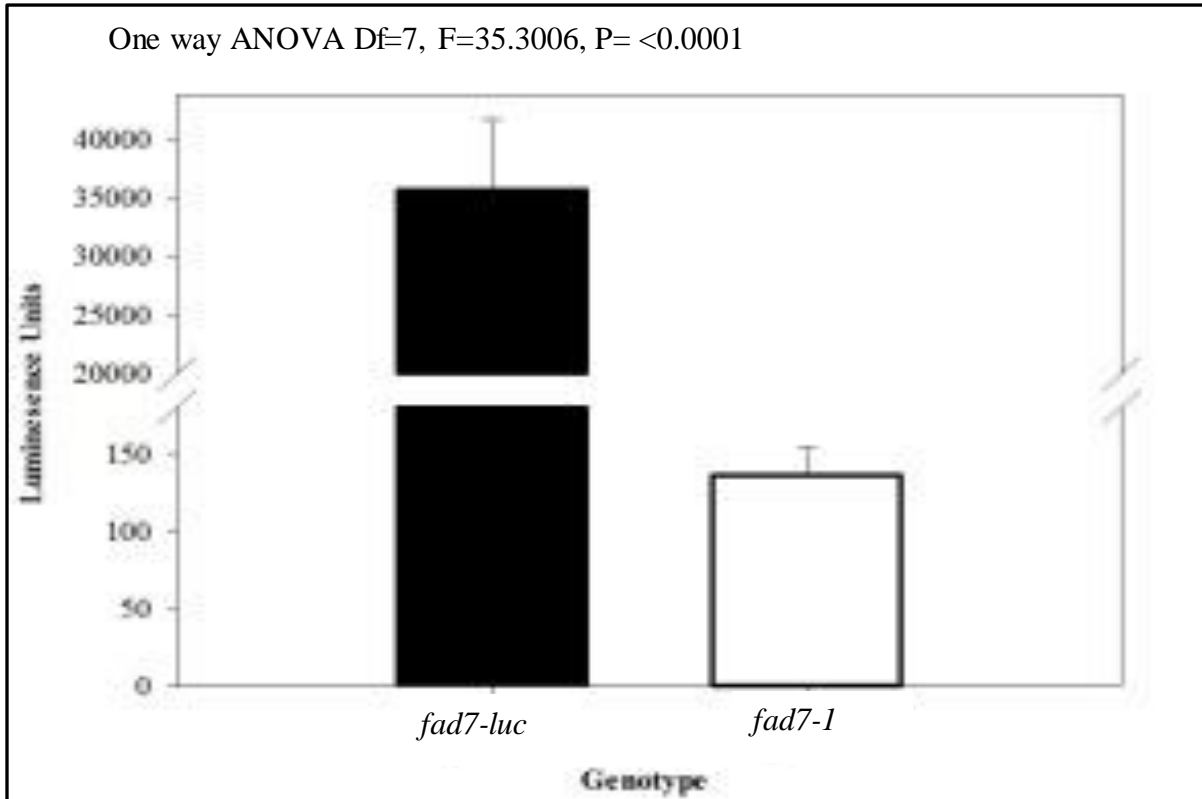


**C) *flu* mutant primers**



**Figure 5: PCR screening of (*fad7-1/g11* X *flu:AAA-ATPase-Luc* F2 plants).** A) Based on prior screening of 115 F2 plants (data not shown), 28 plants were selected for further analysis, and were tested with primers specific to the luciferase reporter gene to confirm the presence of the *flu:AAA-ATPase-Luc* transgene. B) The 28 luciferase-positive plants were screened for the presence of the wild -type (top) and/or mutant (bottom) alleles of the *FAD7* gene. The results identified 8 plants homozygous for the wild-type *FAD7* allele (highlighted in red) 2 plants homozygous for the *fad7* mutation (green), and 18 heterozygotes (white). C. All homozygotes

identified in B were screened for the presence of the mutant allele of the *FLU* gene, which was absent in 3 of the *FAD7* wild-type plants (samples 11, 14, and 20), and 1 of the *fad7* mutants (sample 9). An Invitrogen 1 kb Plus DNA ladder was used to confirm amplicon size.



**Figure 6. Reporter gene activity in *fad7-Luc*, as measured by luminescence.** The levels of luminescence emitted by the *fad7-Luc* (F3 generation) and the *fad7-1* mutants at normal light conditions was measured after luciferin treatment using the GloMax 20/20 luminometer (n=15). The error bars represent the standard error of the mean (SEM). The Y axis is abridged in order to be able to portray transformed and untransformed plants in the same graph.

a plate reader, we also included control groups that did not receive any luciferin, the necessary substrate for luciferase, in order to verify that the luciferin reacts with the luciferase in our reporter gene system. In this assay, the highest levels of luminescence were observed in plants that carried the reporter gene and that were treated with both rose bengal and luciferin; the reporter gene, the enzyme substrate, and the ROS inducer were all required in combination to generate luminescence levels that significantly exceeded the background values for

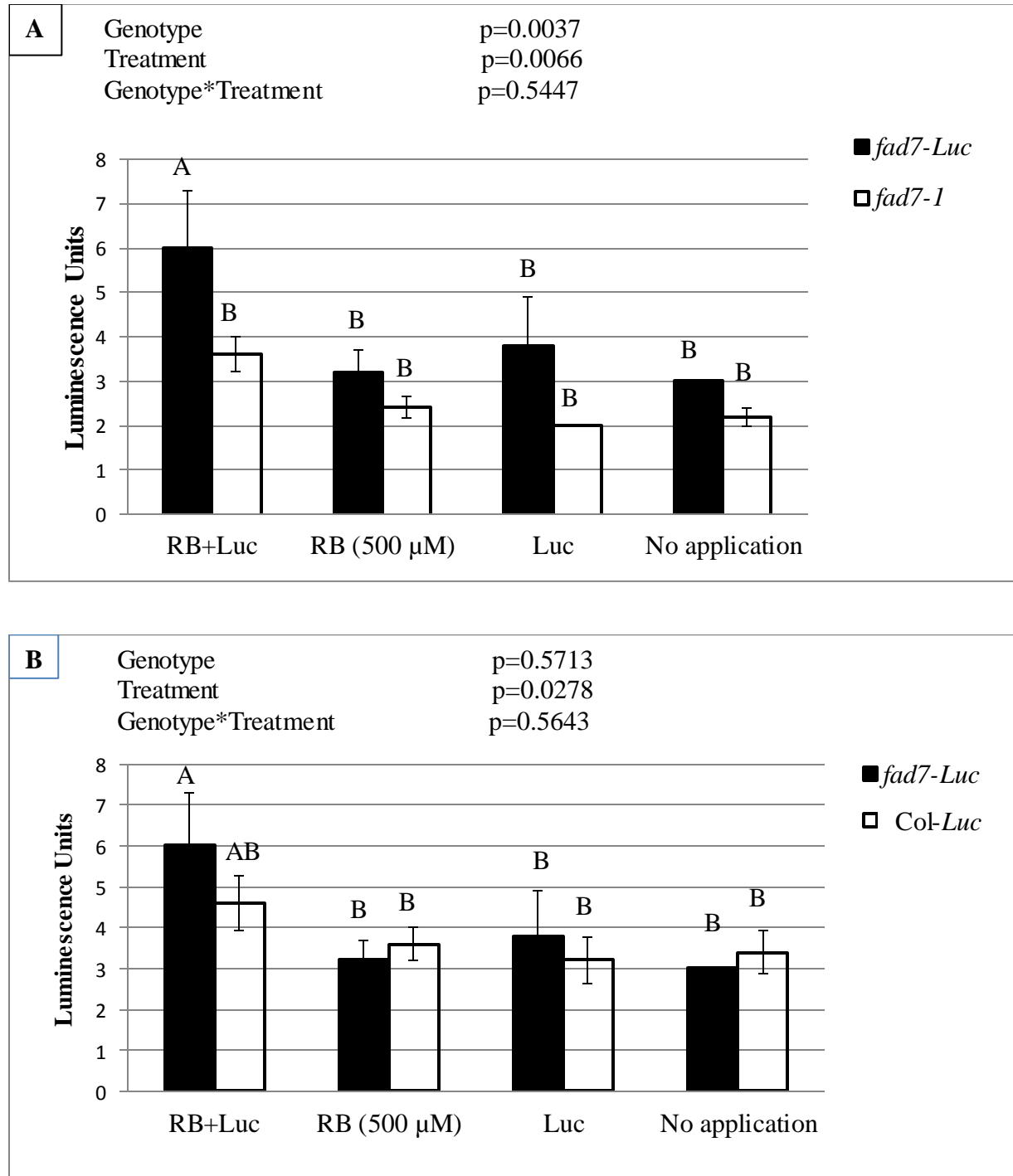
untransformed controls (Figures 7A and 7 B). The luminometer assay also confirmed that the reporter gene in both genetic backgrounds was responsive to rose bengal (Figure 8). In both assays, the levels of luminescence were numerically higher in *fad7-1-Luc* than in *Col-0-Luc*, but this difference was not statistically significant (Figures 7 and 8).

In the process of choosing a concentration of rose bengal for these experiments, we also tested the effects of 1 mM rose bengal on luminescence (Supplemental Figure 2, Appendix II), and compared symptom development in wild-type plants treated with water, 500  $\mu$ M, or 1 mM rose bengal. Surprisingly, 1mM rose bengal did not have a statistically significant effect on luminescence (Supplemental Figure 2, Appendix II), even though the lower concentration of 500  $\mu$ M induced significant increases in light production (Figures 7 and 8). When plants treated with 1 mM rose bengal were observed 48h after treatment, all of the plants displayed extensive necrosis and chlorosis. All of the plants treated with 500  $\mu$ M of rose bengal also showed signs of chlorosis, but damage was less extensive than in the 1 mM treatment group (Supplemental Figure 3, Appendix II). Potentially, in response to 1 mM rose bengal treatment, plants may produce high levels of  $^1\text{O}_2$  that can cause damage to our reporter system; therefore, we chose to conduct our experiments (Figures 7 and 8) with 500  $\mu$ M rose bengal.

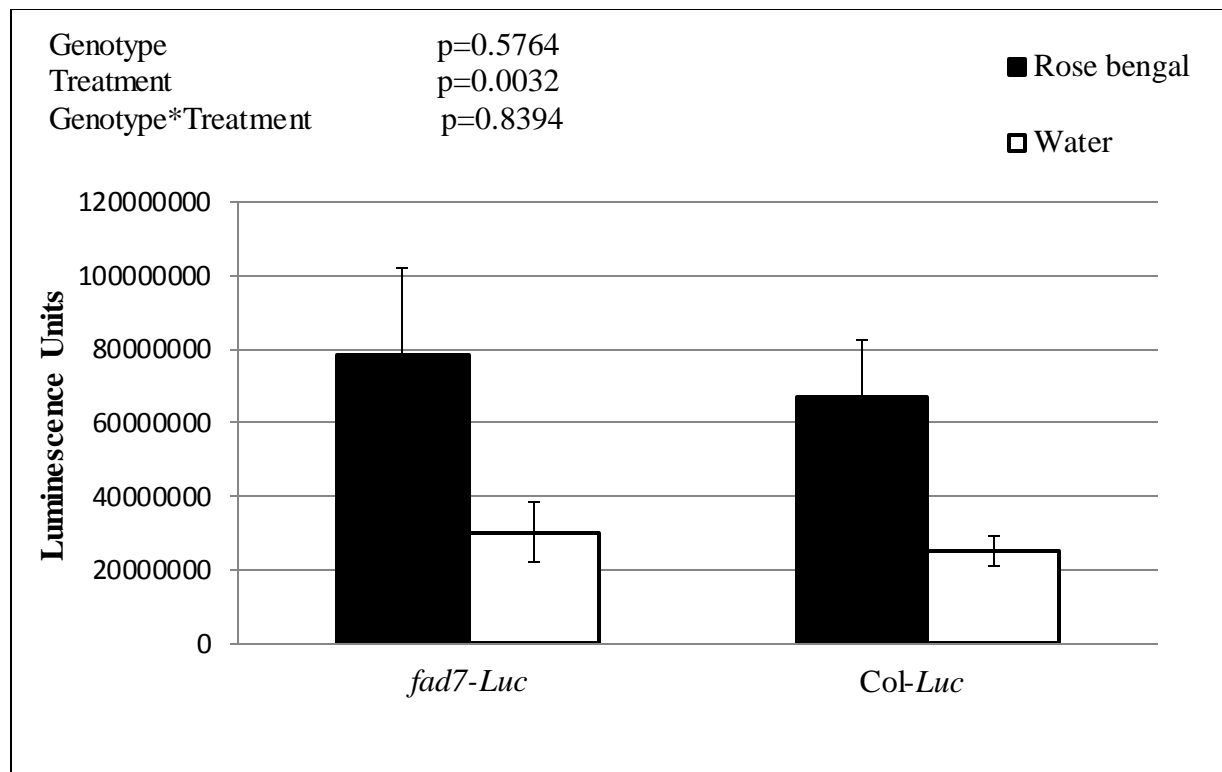
### **Reporter Gene Activity in Response to Abiotic and biotic stress in the *fad7-luc* and Wild-Type Genetic Backgrounds**

The goal of these experiments was to investigate whether  $^1\text{O}_2$  generation in response to abiotic (high light) or biotic (aphid infestation) stresses differed between genotypes with normal (*Col-0*) or impaired (*fad7-1*) fatty acid desaturation.

**Reporter gene activity in response to high light stress:** High light stress is known to induce  $^1\text{O}_2$  generation in plants (Krieger-Liszkay, 2005), and so reporter gene activity in *fad7-Luc* and



**Figure 7. Testing the responsiveness of the reporter gene to rose bengal and luciferin by using intact leaves (n=5).** A) Luminescence was compared in *fad7-luc* and untransformed *fad7-1* plants with and without rose bengal (RB) and luciferin (Luc), the necessary substrate for luciferase, at 2h after rose bengal treatment. B) Reporter gene activity was also compared between *fad7-luc* and *Col-luc* plants in response to luciferin and rose bengal application. Luminescence was measured (in F3 generation) in leaf discs by a plate reader assay. All error bars represent the standard error of the mean (SEM).



**Figure 8. Testing the responsiveness of the reporter gene to rose bengal application (500 $\mu$ M) (n=21).** Reporter gene activity was compared between *fad7-luc* and *Col-luc* plants in response to rose bengal, at 2h after rose bengal treatment. Luminescence was measured (in F3 generation) using a luminometer-based assay. The error bars represent the standard error of the mean (SEM).

*Col-Luc* (F3 generation) was tested after 1h and 2h of exposure to 400  $\mu\text{mol m}^{-2} \text{s}^{-1}$  and 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ , both of which should cause high light stress in *Arabidopsis*. Control plants were maintained under the same light conditions at which all the plants were grown ( $\sim 135 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Luminescence was measured with a luminometer-based assay. At both time periods (1 and 2h) and at both levels of high light exposure (400 and 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ), plants exposed to high light had significantly higher reporter gene activity than controls maintained at moderate light levels (Figures 9A and 9 B). There was no significant difference in reporter gene activity between the different levels of high light (400 and 1000  $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) at either time point

(Figures 9A and 9 B). In both groups of plants (those exposed for 1h and those exposed for 2h), luminescence was numerically higher in *fad7-Luc* than in *Col-Luc*, and this difference was statistically significant in the 2h group. There was no significant interaction between light intensity and genotype, though, which suggests that the consecutive levels of the luminescence were higher in *fad7-Luc* plants were higher than the *Col-Luc* plants.

An additional experiment was performed to test whether a camera-based luminescence detection system, the Lumina XR, could be used to visualize the response to high light stress in intact rosettes. *fad7-Luc* (F4 generation) and *Col-Luc* (F3 generation) plants were exposed to 2h at  $400 \mu\text{mol m}^{-2} \text{s}^{-1}$ , since this exposure produced the greatest reporter gene activity in the previous experiment. Unlike the luminometer, this system did not detect a significant change in reporter gene activity in response to high light; although it suggested that luminescence levels were higher in *fad7-Luc* than in *Col-Luc* (Figure 10 A). The same rosettes that were visualized with the Lumina XR were also extracted to measure with the luminometer, to compare the two methods. Similar to the results with the Lumina XR, the luminometer detected numerically higher levels of luminescence in *fad7-Luc* than in *Col-Luc*, although this difference fell short of statistical significance at  $\alpha= 0.05$  (Figure 10 A;  $P=0.0623$ ). In contrast to the Lumina XR, though, the luminometer-based assay also detected a significant increase in response to high light exposure (Figure 10 B). Also, the correlation between the Lumina XR and the luminometer measurements from the same plants was low ( $r^2=0.46$ ). When we measured background luminescence in wild-type (*Col-0*) plants that lacked the luciferase reporter gene, levels of luminescence were, as expected, very low ( $48.6 \pm 10.2$  luminescence units for the plants under high light stress, and  $40.2 \pm 4.5$  luminescence units for the plants under 16 h L: 8 h D photoperiod). In contrast, the Lumina XR yielded fairly high levels of background fluorescence

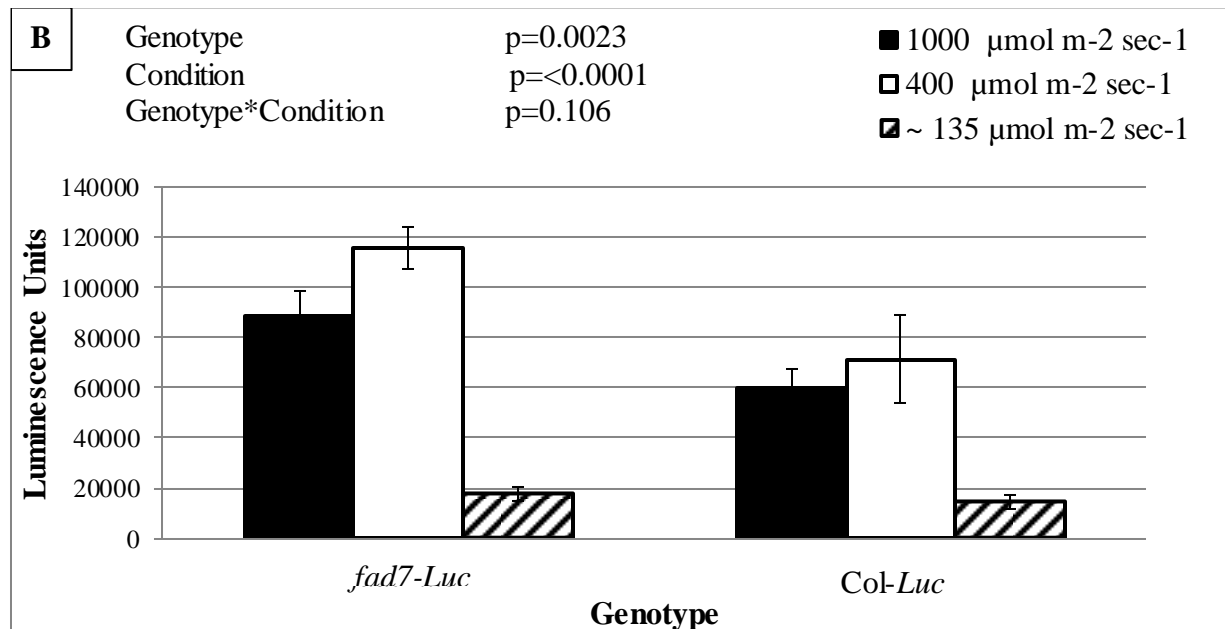
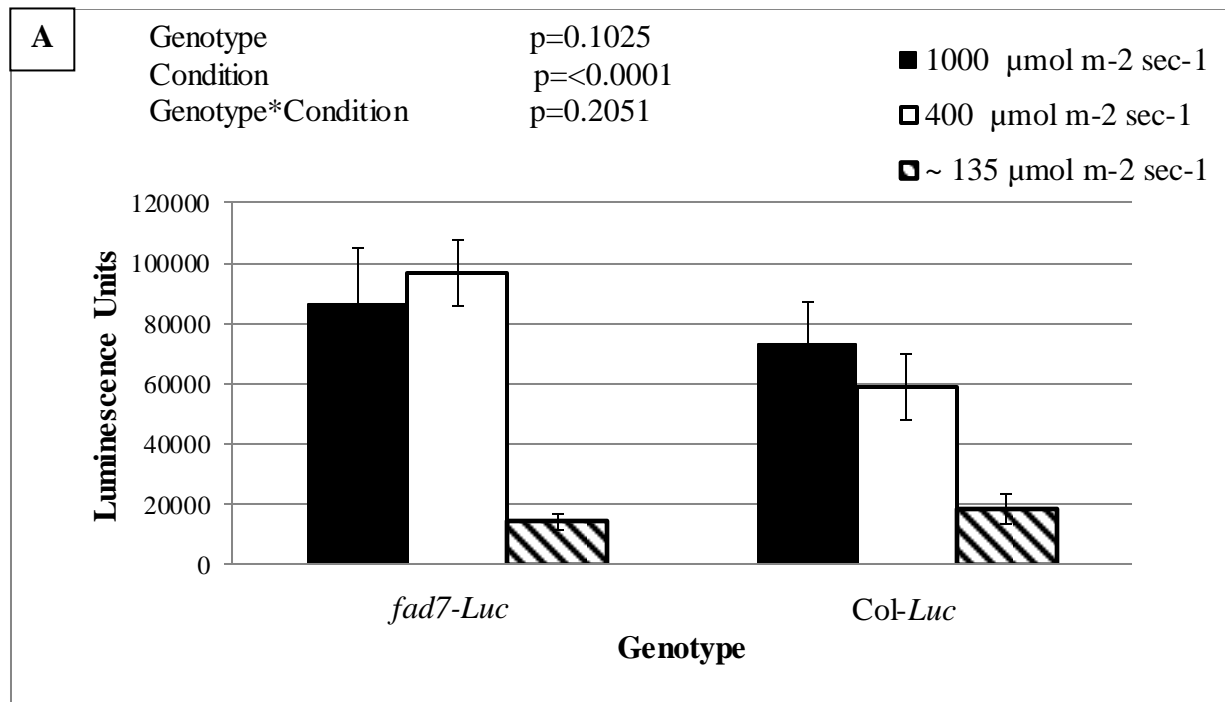
in untransformed plants ( $4,978,100 \pm 559,474$  p/s/cm<sup>2</sup>/sr for the plants under high light stress, and  $5,332,700 \pm 583,291$  p/s/cm<sup>2</sup>/sr for the plants under 16 h L: 8 h D photoperiod). According to the Lumina XR measurements, luminescence from *fad7-Luc* transformed plants was 3-5-fold higher than the luminescence from the untransformed plants, and the values for *Col-Luc* plants were 2-3-fold higher than the values for the untransformed plants. In contrast, the luminometer results showed that the luminescence from *fad7-Luc* transformed plants were 800-900-fold higher than in untransformed plants, and the *Col-Luc* plants produced 100-700-fold higher luminescence than the untransformed plants. These observations suggest that the luminometer is more sensitive than the Lumina XR, with greater differences between signal from the reporter and background levels in controls.

**Reporter gene activity in response to aphid infestation:** We compared the response of *fad7-Luc* (F4 generation) and *Col-Luc* (F3 generation) to aphid infestation because previous research indicated that alteration in fatty acid desaturation could induce ROS production. After a 48h exposure period to aphids, rosettes were harvested, imaged with the Lumina XR, and then processed for analysis with the luminometer. The luminometer-based assay indicated that aphids did not significantly impact reporter gene activity, and that reporter gene activity was significantly higher in *fad7-Luc* than in *Col-Luc* mutants, regardless of the presence or absence of aphids. In contrast, although the Lumina XR measurements were taken on the same plants, this imaging approach yielded very different results, and suggested that the luminescence for the *Col-Luc* plants were significantly higher than the luminescence for *fad7-Luc* plants in response to aphid's infestation (Figures 11A and 11 B).

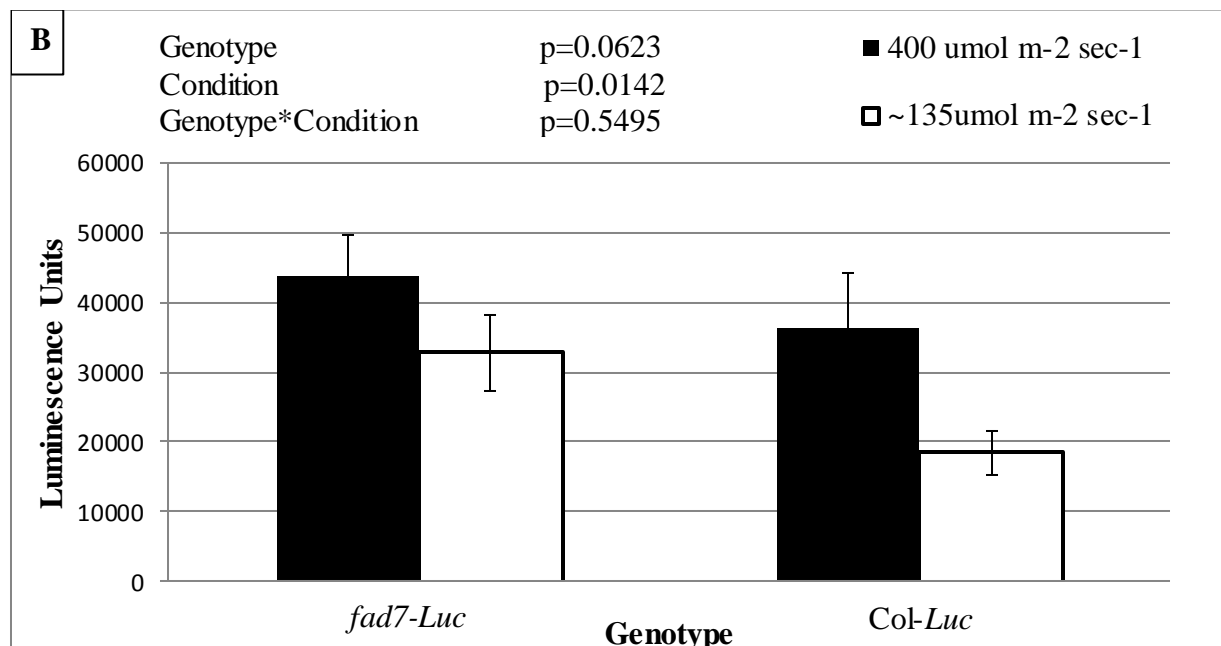
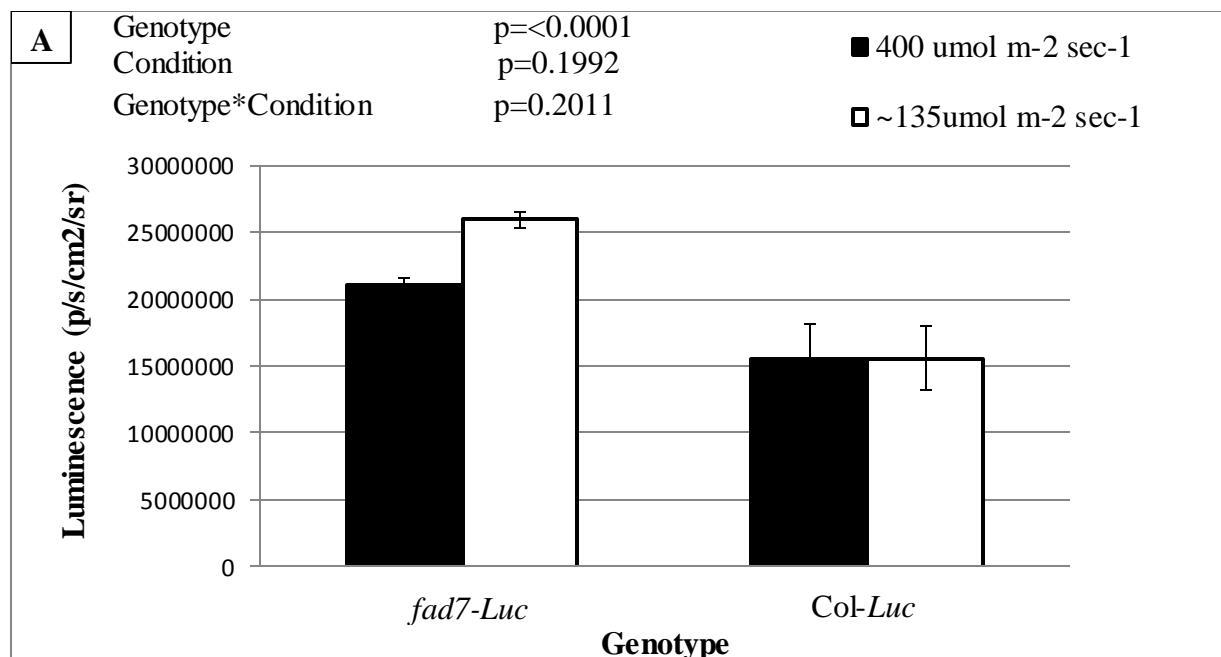
The results for the untransformed controls by the luminometer were 1549.013

$\pm 1481.953$  luminescence units for the wild-type plants that are inoculated with aphids and  $19.000 \pm 1.954173$  luminescence units for the controls. The *fad7-Luc* transformed plants were 9-727-fold higher than untransformed plants, and the *Col-Luc* plants were 5-571-fold higher than the untransformed plants. In contrast, the results for the untransformed controls by the Lumina XR were  $13730000 \pm 711777.8$  (p/s/cm<sup>2</sup>/sr) for the plants inoculated with aphids, and  $14729000 \pm 1597621.5$  (p/s/cm<sup>2</sup>/sr) for the controls. The *fad7-Luc* transformed plants were 1.1-1.2-fold higher than the untransformed plants, and the *Col-Luc* plants were 1.1-1.4-fold higher than the untransformed plants. This is consistent with our previous experiments that prove the high sensitivity of the luminometer.

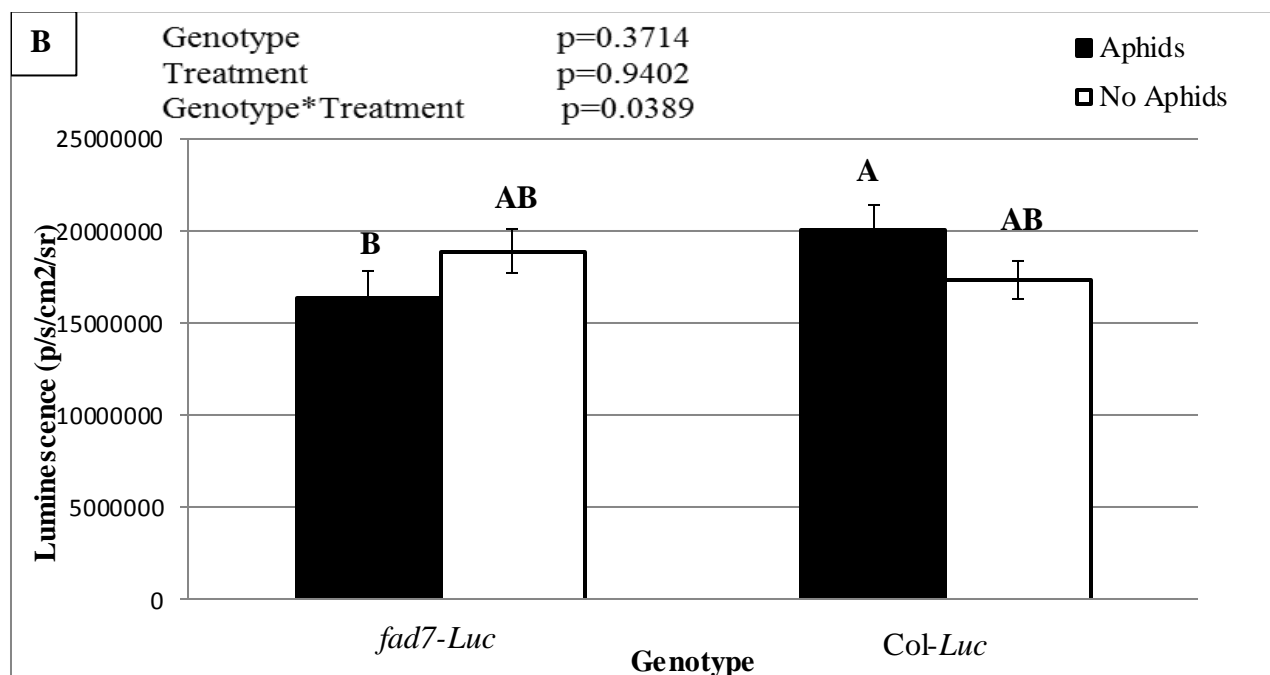
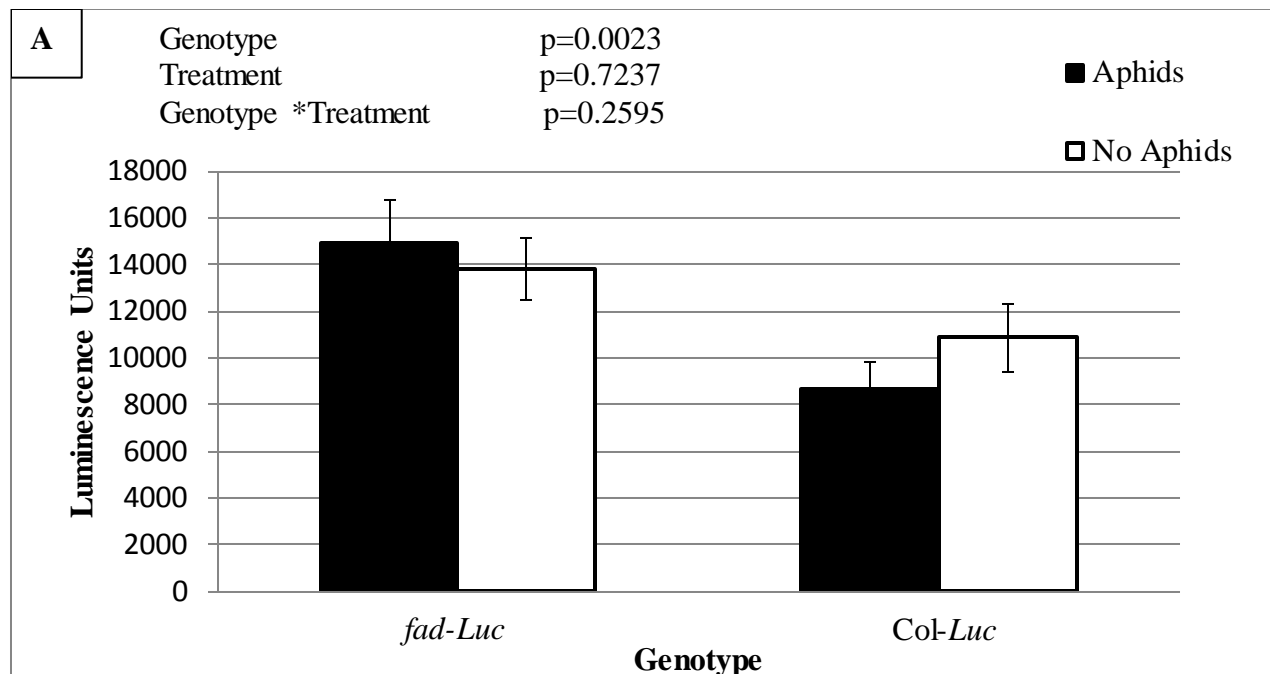




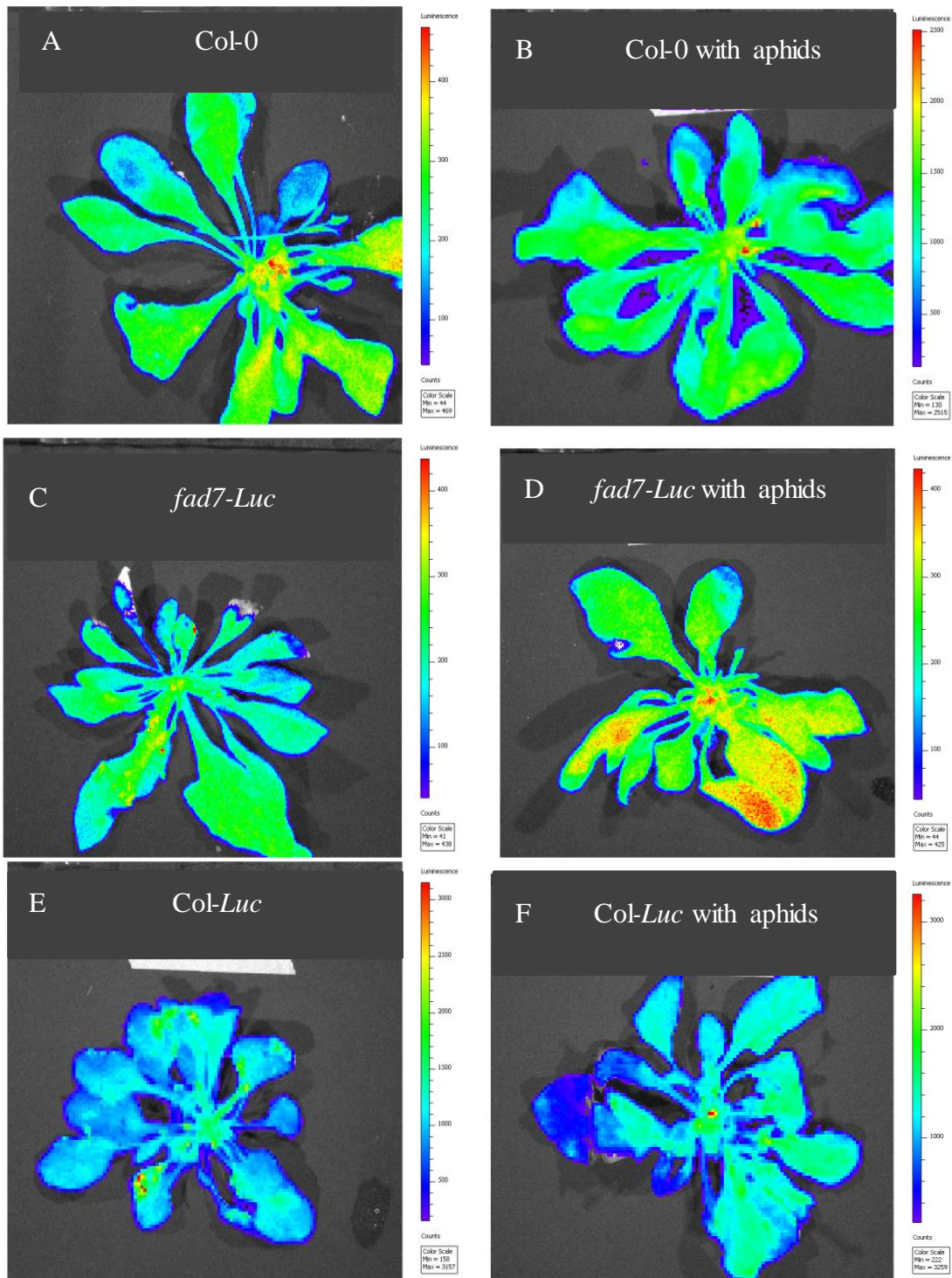
**Figure 9. Reporter gene activity in *fad7-Luc* and *Col-Luc* (F3 generation) in response to high light stress (n=9).** Plants were exposed to either a 1h (A) or 2h (B) light exposure period, and luminescence was measured using a luminometer. The error bars represent the standard error of the mean (SEM).



**Figure 10. Testing the sensitivity of the Lumina XR and the luminometer to the luminescence of the reporter gene in response to the light stress (n=20).** *fad7-Luc* (F4 generation) and *Col-Luc* (F3 generation) plants were exposed for 2h to either a 400  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  or ~135  $\mu\text{mol m}^{-2} \text{sec}^{-1}$  light intensity. Luminescence was measured using the Lumina XR (A) and the luminometer (B). All error bars represent the standard error of the mean (SEM).



**Figure 11. Reporter gene activity in *fad7-Luc* and *Col-Luc* in response to aphid infestation (n=20).** *fad7-Luc* (F4 generation) and *Col-Luc* (F3 generation) plants were inoculated with 10 adult aphids, and the luminescence was compared between *fad-Luc* and *Col-Luc* plants after 48 h by using the Lumina XR (A) the luminometer(B). The error bars represent the standard error of the mean (SEM).



**Figure 12. Samples for the images taken by the Lumina XR.** These images are taken by the Lumina XR system after 48 h of aphid's inoculation. Later, the leaves were fresh-frozen by liquid nitrogen to be processed later for the luminometer assay.

## Discussion

### Establishing a Suitable Method for Luminescence Measures

To optimize a method for measuring the luminescence for the Arabidopsis plants, the luminescence was measured by using a luminometer, a plate reader, and the Lumina XR. Our preliminary experiments with the plate reader were done with leaf discs to allow less destructive methods, but that the difference between the luminescence for transformed plants and untransformed plants was low, so our next experiments were done with tissue extracts.

To compare the luminometer and the plate reader, we used the two systems to measure different aliquots of the exact same samples (Figures 3A and 3B). In both measurements, we used a tissue-destructive method by using the Luciferase Assay Kit (Promega) to eliminate any differences that originated with the sample type. The results for both the luminometer and the plate reader showed numerically higher luminescence for the *flu* mutants that were under the dark for 8 hours period than the untransformed controls. Although the statistical analysis showed that there is a strong relationship between the results for the plate reader and the luminometer with correlation coefficient  $r^2=0.78$  (Figures 3A and 3B), the luminometer results showed higher sensitivity to the luminescence than the plate reader. The differences in luminescence measurements between the transformed plants and untransformed controls were higher for the luminometer than the plate reader. This is consistent with the manufacturers' specifications for the luminometer. According to Promega, the GloMax 20/20 luminometer can detect concentrations of luciferase as low as  $1 \times 10^{-21}$  moles, which making it one of the best luminometers available with high sensitivity (Promega Corporation, 2015).

To confirm the responsiveness of the reporter gene to  $^1\text{O}_2$ , and to test the sensitivity of the luminometer and the plate reader to the rose bengal application, we measured the fold change in response to the rose bengal application (Figures 7 B and 8). The plate reader's results showed that the rose bengal treated plants were 1.3-2 fold higher than the controls for the *fad7-Luc* and *Col-Luc* plants, whereas the luminometer's data analysis showed that the rose bengal treated plants were 2.5-2.6 fold higher than the controls for the *fad7-Luc* and *Col-Luc* plants. The results suggested that the luminometer is more sensitive than the plate reader in measuring the luminescence.

In addition, the comparison between the Lumina XR system and the luminometer revealed that the luminescence measures for the same plants were different with low correlation between the two methods ( $r^2=0.46$ ). That may be because the Lumina XR system is used for *in vivo* imaging, whereas the luminometer requires a tissue-destructive method. Bioluminescence images are surface-based, meaning that the tissues that are closer to the surface appear brighter than the deeper tissue. Also, light production by luciferase requires the enzyme to react with the substrate (luciferin). If the luciferin is applied to the surface of intact tissues, it might not penetrate the tissue deeply enough to provide a correct picture of the luciferase activity (Sadikot & Blackwell, 2005). In contrast, adding the luciferin to plant extracts ensures that the luciferin will be thoroughly homogenized throughout the sample. Therefore, the luminometer assay may provide a more accurate, quantitative measure of the luciferase activity than imaged-based techniques like the Lumina XR.

In short, the luminometer-based assay seems to be more sensitive than both the plate reader-based assay and the image-based assay.

### **Confirmation the Activity of the $^1\text{O}_2$ -Responsive Reporter Gene**

Since The *flu:AAA-ATPase:Luc* mutants accumulate free protochlorophyllide in the dark, and produce  $^1\text{O}_2$  instantly when they are exposed to the light (Kim, et al., 2008; Laloï, et al., 2006), we used this feature of the *flu:AAA-ATPase: Luc* mutants to confirm the expression of the reporter gene in the parental generation. We tested the levels of the luminescence in *flu:AAA-ATPase:Luc* plants and Col-0 wild type plants in response to the dark/light shift by using both the plate reader and the luminometer (Figures 3A and 3 B). As expected, both devices showed higher levels of luminescence in the *flu:AAA-ATPase: Luc* mutants than the wild-type plants. Also, *flu:AAA-ATPase:Luc* mutants that were exposed to the dark for 8 hours accumulate higher levels of  $^1\text{O}_2$  than the mutants that were growing under continuous light conditions. However, there was no significant difference between the two treatments (Figures 3A and 3 B). Having a low number of the replicates ( $n = 8$ ) could have contributed to the lack of significant differences between the light treatments. Also, the time that the plants were transferred to the light for tissue collection could affect the levels of the  $^1\text{O}_2$ . The tissues were harvested within 10 minutes after they were transferred to the light whereas in other studies the tissues were harvested 30 minutes after re-exposing the plants to the light (Meskauskiene, et al., 2009; Meskauskiene, et al., 2001). Possibly, a longer exposure to light was needed to induce significantly higher levels of  $^1\text{O}_2$ .

### **The Reporter Gene Activity in Response to Biotic and Abiotic Stresses**

After confirmation the activity of the reporter gene in the crossed generations, we tested the activity of the reporter gene in both genetic backgrounds (*fad7-Luc* and *Col-Luc*) in response

to rose bengal and high light stress (known elicitors of  $^1\text{O}_2$ ), as well as aphid infestation. The levels of luminescence were compared between *fad7-Luc* and *Col-Luc* to see if the alteration in fatty acid desaturation would have an effect on the  $^1\text{O}_2$  accumulation.

Firstly, the plants were treated with 500  $\mu\text{M}$  rose bengal, and the luminescence was measured with the plate reader and the GloMax 20/20 luminometer. In both experiments we observed numerically higher levels of reporter gene activity for *fad7-Luc* and *Col-Luc* plants in response to rose bengal treatment compared to water-treated controls. In both experiments, the luminescence produced by *fad7-Luc* were higher (but not significantly) than the luminescence in *Col-Luc* (Figures 7 B and 8). Unlike the results for the application of 500  $\mu\text{M}$  rose bengal, there was no statistically significant effect of 1mM rose bengal treatment on reporter gene activity (Appendix II, Supplemental Figure 1). Furthermore, in this assay, *Col-Luc* showed higher luminescence than *fad7-Luc*. Hideg stated that moderate concentrations of rose bengal should be used because high concentrations of rose bengal can cause inhibition of photosynthesis in tobacco plants (2008). We suggest that the 1mM concentration of the rose bengal caused inhibition of photosynthesis in Arabidopsis plants. That was supported by our finding that the application of the 1 mM of rose bengal causes extensive cell death on the rosette leaves after 48 hours of application (Appendix II, Supplemental Figure 2).

Similar to 500  $\mu\text{M}$  rose bengal treatment, high light stress also increased reporter gene activity in both *fad7-Luc* and *Col-Luc* plants, and luminescence levels were higher in *fad7-Luc* than the *Col-Luc* plants in response to the high light stress (Figures 9 and 10).

Unlike the abiotic stresses, aphid infestation appeared to have no effect on reporter gene activity, at least at the time point observed in this study (after 24h of infestation). However,



similar to previous experiments with high light and 500  $\mu\text{M}$  rose bengal, we saw higher levels of luminescence in *fad7-Luc* plants than *Col-Luc* (Figure 11).

In most of the luminometer experiments, the levels of luminescence were higher for the *fad7-Luc* plants than the *Col-Luc* plants. We compared the luminescence between *fad7-Luc* and *Col-Luc* plants in 6 luminometer assays in response to rose bengal, light stress, and aphid infestation (Table 1). Luminescence levels were numerically higher in *fad7-Luc* in 5 of these assays, and this difference was statistically significant in 2 assays and marginally higher ( $P=0.06$ ) in one assay. This suggests that the reduced levels of desaturation in *fad7-1* could promote the constitutive accumulation of  $^1\text{O}_2$ .

The loss of function of fatty acid desaturase 7, a chloroplast-localized enzyme, modifies the desaturation level of the chloroplast membrane, which also influences membrane fluidity (Iba et al., 1993). This modification may affect the proteins, pigments, and other molecules involved in photosynthesis in chloroplast. The chloroplast membrane is the site where the light-dependent reactions of photosynthesis occur. Therefore, electron transport and other photosynthetic processes may be influenced by the fluidity of the lipid-matrix of chloroplast thylakoids (Vigh, Joó, Droppa, Horváth, & Horváth, 1985; Millner & Barber, 1984), and may in turn influence the constitutive levels of  $^1\text{O}_2$  to be increased in *fad7-1* plants that are generated during photosynthesis.

$^1\text{O}_2$  may play an important role in plant defense against pathogen attack. It has been demonstrated that the phytoalexins, compounds synthesized upon infection, use light energy to synthesize  $^1\text{O}_2$ , and this leads to increasing plants defense (Lazzaro et al., 2004; Flors & Nonell, 2006).

The working model that we suggest is that the loss of function of fatty acid desaturase 7 in *fad7-1* plants leads to alteration in the chloroplast membrane that increases constitutive levels of  $^1\text{O}_2$ , which in turn could lead to increased plant defenses against aphid infestation.

**Table 1: Comparing the levels of the luminescence between *fad7-Luc* and *Col-Luc* plants in luminometer assays.**

Figure #	Luminescence levels status for <i>fad7-luc</i> and <i>Col-Luc</i> plants	Treatment
Figure 8	Numerically higher luminescence for <i>fad7-luc</i> than <i>Col-Luc</i> plants.	Rose bengal 500 $\mu\text{M}$
Figure 9 A	Numerically higher luminescence for <i>fad7-luc</i> than <i>Col-Luc</i> plants.	1 h of (1000, and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) light stress
Figure 9 B	Numerically higher luminescence for <i>fad7-luc</i> than <i>Col-Luc</i> plants with significant difference between the two genotypes.	2 h of (1000, and 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ ) light stress
Figure 10 B	Numerically higher luminescence for <i>fad7-luc</i> than <i>Col-Luc</i> plants with marginally higher P value (P=0.06)	2 h of 400 $\mu\text{mol m}^{-2} \text{s}^{-1}$ light stress
Figure 11 A	Numerically higher luminescence for <i>fad7-luc</i> than <i>Col-Luc</i> plants with significant difference between the two genotypes.	48 h of aphid's infestation
Supplemental Figure 2	Numerically higher luminescence for <i>Col-Luc</i> than <i>fad7-Luc</i> plants with significant difference between the two genotypes.	Rose bengal 1 mM

## References

- Arimura, G., Kost, C., & Boland, W. (2005). Herbivore-induced, indirect plant defences. *Biochimica Et Biophysica Acta (BBA)-Molecular and Cell Biology of Lipids*, 1734(2), 91-111.
- Avila, C. A., Arevalo-Soliz, L. M., Jia, L., Navarre, D. A., Chen, Z., Howe, G. A., . . . Goggin, F. L. (2012). Loss of function of FATTY ACID DESATURASE7 in tomato enhances basal aphid resistance in a salicylate-dependent manner. *Plant Physiology*, 158(4), 2028-2041. doi:10.1104/pp.111.191262
- Baier, M., & Dietz, K. (2005). Chloroplasts as source and target of cellular redox regulation: A discussion on chloroplast redox signals in the context of plant physiology. *Journal of Experimental Botany*, 56(416), 1449-1462.
- Baruah, A., Šimková, K., Apel, K., & Laloi, C. (2009). Arabidopsis mutants reveal multiple singlet oxygen signaling pathways involved in stress response and development. *Plant Plant Molecular Biology*, 70(5), 547-563.
- Bolwell, G. P., & Wojtaszek, P. (1997). Mechanisms for the generation of reactive oxygen species in plant defence—a broad perspective. *Physiological and Molecular Plant Pathology*, 51(6), 347-366.
- Chen, Z., Malamy, J., Henning, J., Conrath, U., Sanchez-Casas, P., Silva, H., . . . Klessig, D. K. (1995). Induction, modification, and transduction of the salicylic acid signal in plant defense responses. *Proceedings of the National Academy of Sciences of the United States of America*, 92(10), 4134-4137.
- Crouch, S., Kozłowski, R., Slater, K., & Fletcher, J. (1993). The use of ATP bioluminescence as a measure of cell proliferation and cytotoxicity. *Journal of Immunological Methods*, 160(1), 81-88.
- Dat, J., Vandenabeele, S., Vranová, E., Van Montagu, M., Inzé, D., & Van Breusegem, F. (2000). Dual action of the active oxygen species during plant stress responses. *Cellular and Molecular Life Sciences CMLS*, 57(5), 779-795.
- D'Autréaux, B., & Toledano, M. B. (2007). ROS as signalling molecules: Mechanisms that generate specificity in ROS homeostasis. *Nature Reviews.Molecular Cell Biology*, 8(10), 813-824.
- Davies, D. R., Bindschedler, L. V., Strickland, T. S., & Bolwell, G. P. (2006). Production of reactive oxygen species in *Arabidopsis thaliana* cell suspension cultures in response to an elicitor from fusarium oxysporum: Implications for basal resistance. *Journal of Experimental Botany*, 57(8), 1817-1827.

- Doulis, A. G., Debian, N., Kingston-Smith, A. H., & Foyer, C. H. (1997). Differential localization of antioxidants in maize leaves. *Plant Physiology*, *114*(3), 1031-1037. doi:114/3/1031
- Edwards, K., Johnstone, C., & Thompson, C. (1991). A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. *Nucleic Acids Research*, *19*(6), 1349-1349.
- Farmer, E., Mousavi, S., & Lenglet, A. (2013). Leaf numbering for experiments on long distance signalling in Arabidopsis. *Protocol Exchange*.
- Flors, C., Fryer, M. J., Waring, J., Reeder, B., Bechtold, U., Mullineaux, P. M., . . . Baker, N. R. (2006). Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, singlet oxygen sensor green. *Journal of Experimental Botany*, *57*(8), 1725-1734.
- Flors, C., & Nonell, S. (2006). Light and singlet oxygen in plant defense against pathogens: phototoxic phenalenone phytoalexins. *Accounts of chemical research*, *39*(5), 293-300.
- Foyer, C. H., Lelandais, M., & Kunert, K. J. (1994). Photooxidative stress in plants. *Physiologia Plantarum*, *92*(4), 696-717.
- Foyer, C. H., & Noctor, G. (2003). Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. *Physiologia Plantarum*, *119*(3), 355-364.
- Fryer, M. J., Oxborough, K., Mullineaux, P. M., & Baker, N. R. (2002). Imaging of photo-oxidative stress responses in leaves. *Journal of Experimental Botany*, *53*(372), 1249-1254.
- Gao, C., Xing, D., Li, L., & Zhang, L. (2008). Implication of reactive oxygen species and mitochondrial dysfunction in the early stages of plant programmed cell death induced by ultraviolet-C overexposure. *Planta*, *227*(4), 755-767.
- Greer, L. F., & Szalay, A. A. (2002). Imaging of light emission from the expression of luciferases in living cells and organisms: A review. *Luminescence*, *17*(1), 43-74.
- Gutierrez, J., Gonzalez-Perez, S., Garcia-Garcia, F., Daly, C. T., Lorenzo, O., Revuelta, J. L., . . . Arellano, J. B. (2014). Programmed cell death activated by rose bengal in arabidopsis thaliana cell suspension cultures requires functional chloroplasts. *Journal of Experimental Botany*, *65*(12), 3081-3095.
- Halliwell, B., & Whiteman, M. (2004). Measuring reactive species and oxidative damage in vivo and in cell culture: How should you do it and what do the results mean? *British Journal of Pharmacology*, *142*(2), 231-255.
- Hernández-Barrera, A., Velarde-Buendía, A., Zepeda, I., Sanchez, F., Quinto, C., Sánchez-Lopez, R., . . . Cardenas, L. (2015). Hyper, a hydrogen peroxide sensor, indicates the

- sensitivity of the arabidopsis root elongation zone to aluminum treatment. *Sensors (Basel, Switzerland)*, 15(1), 855-867. doi:10.3390/s150100855
- Hernandez-Barrera, A., Quinto, C., Johnson, E. A., Wu, H. M., Cheung, A. Y., & Cardenas, L. (2013). Using hyper as a molecular probe to visualize hydrogen peroxide in living plant cells: A method with virtually unlimited potential in plant biology. *Methods in Enzymology*, 527, 275-290. doi:10.1016/B978-0-12-405882-8.00015-5
- Hideg, É., Kálai, T., Hideg, K., & Vass, I. (1998). Photoinhibition of photosynthesis in vivo results in singlet oxygen production detection via nitroxide-induced fluorescence quenching in broad bean leaves. *Biochemistry*, 37(33), 11405-11411.
- Hideg, É., Kálai, T., Kós, P. B., Asada, K., & Hideg, K. (2006). Singlet oxygen in plants—its significance and possible detection with double (fluorescent and spin) indicator reagents. *Photochemistry and Photobiology*, 82(5), 1211-1218.
- Hideg, É. (2008). Photosynthesis inhibition by exogenously generated singlet oxygen - a note of caution. *Acta Biologica Szegediensis*, 52(1), 85-88.
- Hideg, E., Spetea, C., & Vass, I. (1994). Singlet oxygen production in thylakoid membranes during photoinhibition as detected by EPR spectroscopy. *Photosynthesis Research*, 39(2), 191-199. doi:10.1007/BF00029386
- Iba, K., Gibson, S., Nishiuchi, T., Fuse, T., Nishimura, M., Arondel, V.... & Somerville, C. (1993). A gene encoding a chloroplast omega-3 fatty acid desaturase complements alterations in fatty acid desaturation and chloroplast copy number of the fad7 mutant of *Arabidopsis thaliana*. *Journal of Biological Chemistry*, 268(32), 24099-24105.
- Jiang, K., Schwarzer, C., Lally, E., Zhang, S., Ruzin, S., Machen, T., . . . Feldman, L. (2006). Expression and characterization of a redox-sensing green fluorescent protein (reduction-oxidation-sensitive green fluorescent protein) in arabidopsis. *Plant Physiology*, 141(2), 397-403. doi:141/2/397
- Kariola, T., Brader, G., Li, J., & Palva, E. T. (2005). Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. *The Plant Cell*, 17(1), 282-294. doi:10.1105/tpc.104.025817
- Kasajima, I., Ide, Y., Ohkama-Ohtsu, N., Hayashi, H., Yoneyama, T., & Fujiwara, T. (2004). A protocol for rapid DNA extraction from *arabidopsis thaliana* for PCR analysis. *Plant Molecular Biology Reporter*, 22(1), 49-52.
- Kennedy, J., Day, M., & Eastop, V. (1962). A conspectus of aphids as vectors of plant viruses. *Publ Commonw.Inst.Ent.*,
- Kim, C., Meskauskiene, R., Apel, K., & Laloi, C. (2008). No single way to understand singlet oxygen signalling in plants. *EMBO Reports*, 9(5), 435-439.

- Kingston-Smith, A., Harbinson, J., & Foyer, C. (1999). Acclimation of photosynthesis, H<sub>2</sub>O<sub>2</sub> content and antioxidants in maize (*zea mays*) grown at sub-optimal temperatures. *Plant, Cell & Environment*, 22(9), 1071-1083.
- Kirik, V., Schnittger, A., Radchuk, V., Adler, K., Hülskamp, M., & Bäumlein, H. (2001). Ectopic expression of the arabidopsis AtMYB23 gene induces differentiation of trichome cells. *Developmental Biology*, 235(2), 366-377.
- Kodama, H., Akagi, H., Kusumi, K., Fujimura, T., & Iba, K. (1997). Structure, chromosomal location and expression of a rice gene encoding the microsomal  $\omega$ -3 fatty acid desaturase. *Plant Molecular Biology*, 33(3), 493-502.
- Kodama, H., Hamada, T., Horiguchi, G., Nishimura, M., & Iba, K. (1994). Genetic enhancement of cold tolerance by expression of a gene for chloroplast [omega]-3 fatty acid desaturase in transgenic tobacco. *Plant Physiology*, 105(2), 601-605. doi:105/2/601
- Krieger-Liszkay, A. (2005). Singlet oxygen production in photosynthesis. *Journal of Experimental Botany*, 56(411), 337-346.
- Laloi, C., Przybyła, D., & Apel, K. (2006). A genetic approach towards elucidating the biological activity of different reactive oxygen species in arabidopsis thaliana. *Journal of Experimental Botany*, 57(8), 1719-1724.
- Laloi, C., Stachowiak, M., Pers-Kamczyc, E., Warzych, E., Murgía, I., & Apel, K. (2007). Cross-talk between singlet oxygen- and hydrogen peroxide-dependent signaling of stress responses in arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America*, 104(2), 672-677. doi:0609063103
- Laloi, C., Apel, K., & Danon, A. (2004). Reactive oxygen signalling: The latest news. *Current Opinion in Plant Biology*, 7(3), 323-328.
- Laloi, C., & Havaux, M. (2015). Key players of singlet oxygen-induced cell death in plants. *Frontiers in Plant Science*, 6, 39-39. doi:10.3389/fpls.2015.00039
- Lamb, C., & Dixon, R. A. (1997). The oxidative burst in plant disease resistance. *Annual Review of Plant Biology*, 48(1), 251-275.
- Lazzaro, A., Corominas, M., Martí, C., Flors, C., Izquierdo, L. R., Grillo, T. A., ... & Nonell, S. (2004). Light- and singlet oxygen-mediated antifungal activity of phenylphenalenone phytoalexins. *Photochemical & Photobiological Sciences*, 3(7), 706-710.
- Liu, X., Yang, J., Li, B., Yang, X., & Meng, Q. (2006). Antisense-Mediated depletion of tomato chloroplast Omega-3 fatty acid desaturase enhances thermal tolerance. *Journal of Integrative Plant Biology*, 48(9), 1096-1107.

- Living Image Software User's Manual. Retrieved from [http://www2.udel.edu/ctcr/sites/udel.edu.ctcr/files/Living\\_Image\\_4-2\\_User\\_Guide.pdf](http://www2.udel.edu/ctcr/sites/udel.edu.ctcr/files/Living_Image_4-2_User_Guide.pdf)
- Los, D. A., & Murata, N. (1998). Structure and expression of fatty acid desaturases. *Biochimica Et Biophysica Acta (BBA)-Lipids and Lipid Metabolism*, 1394(1), 3-15.
- Louis, J., Leung, Q., Pegadaraju, V., Reese, J., & Shah, J. (2010). PAD4-dependent antibiosis contributes to the ssi2-conferred hyper-resistance to the green peach aphid. *Molecular Plant-Microbe Interactions*, 23(5), 618-627.
- Maleck, K., & Dietrich, R. A. (1999). Defense on multiple fronts: How do plants cope with diverse enemies? *Trends in Plant Science*, 4(6), 215-219.
- Mene-Saffrane, L., Dubugnon, L., Chetelat, A., Stolz, S., Gouhier-Darimont, C., & Farmer, E. E. (2009). Nonenzymatic oxidation of trienoic fatty acids contributes to reactive oxygen species management in arabidopsis. *The Journal of Biological Chemistry*, 284(3), 1702-1708.
- Meskauskiene, R., Würsch, M., Laloi, C., Vidi, P., Coll, N. S., Kessler, F., . . . Apel, K. (2009). A mutation in the arabidopsis mTERF-related plastid protein SOLDAT10 activates retrograde signaling and suppresses (1)O(2)-induced cell death. *The Plant Journal: For Cell and Molecular Biology*, 60(3), 399-410.
- Meskauskiene, R., Nater, M., Goslings, D., Kessler, F., op den Camp, R., & Apel, K. (2001). FLU: a negative regulator of chlorophyll biosynthesis in *Arabidopsis thaliana*. *Proceedings of the national academy of sciences*, 98(22), 12826-12831.
- Millner, P. A., & Barber, J. (1984). Plastoquinone as a mobile redox carrier in the photosynthetic membrane. *FEBS letters*, 169(1), 1-6.
- Mittler, R., & Berkowitz, G. (2001). Hydrogen peroxide, a messenger with too many roles? *Redox Report*, 6(2), 69-72.
- Mittler, R., Vanderauwera, S., Suzuki, N., Miller, G., Tognetti, V. B., Vandepoele, K., . . . Van Breusegem, F. (2011). ROS signaling: The new wave? *Trends in Plant Science*, 16(6), 300-309.
- Murgia, I., Tarantino, D., Vannini, C., Bracale, M., Carravieri, S., & Soave, C. (2004). *Arabidopsis thaliana* plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *The Plant Journal: For Cell and Molecular Biology*, 38(6), 940-953.
- Murashige, T., & Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. (1962). *Physiologia Plantarum*, 15(3), 473-497.



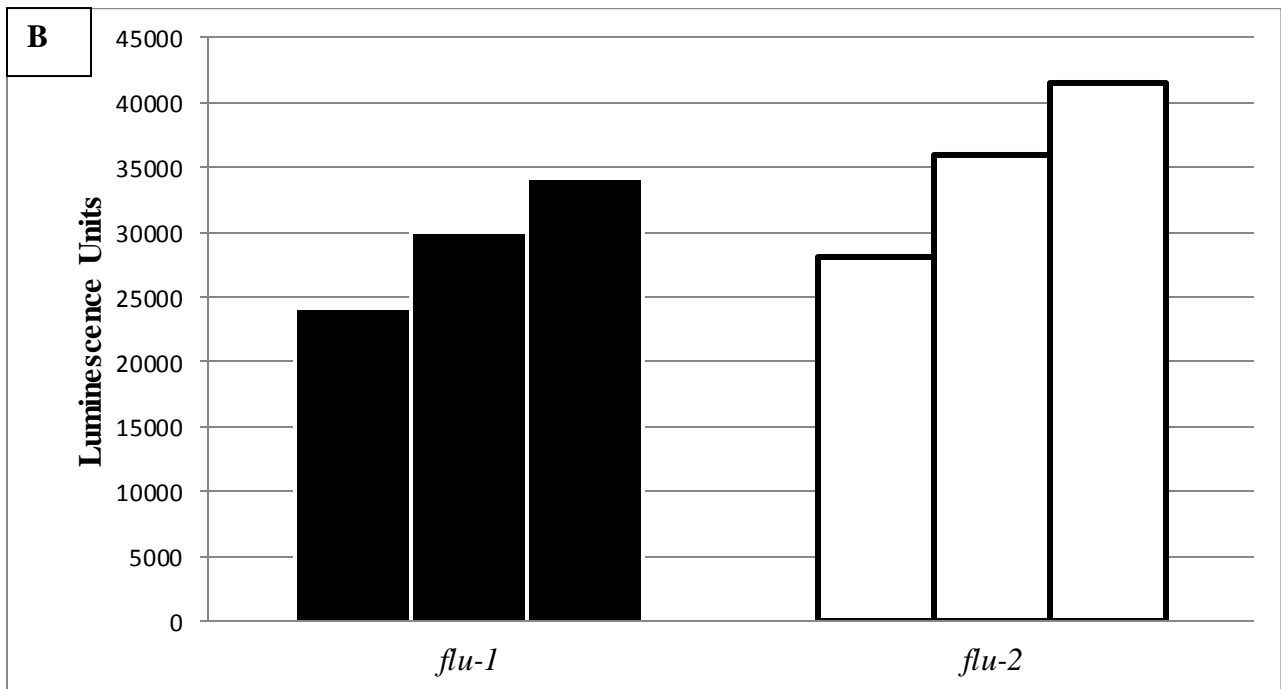
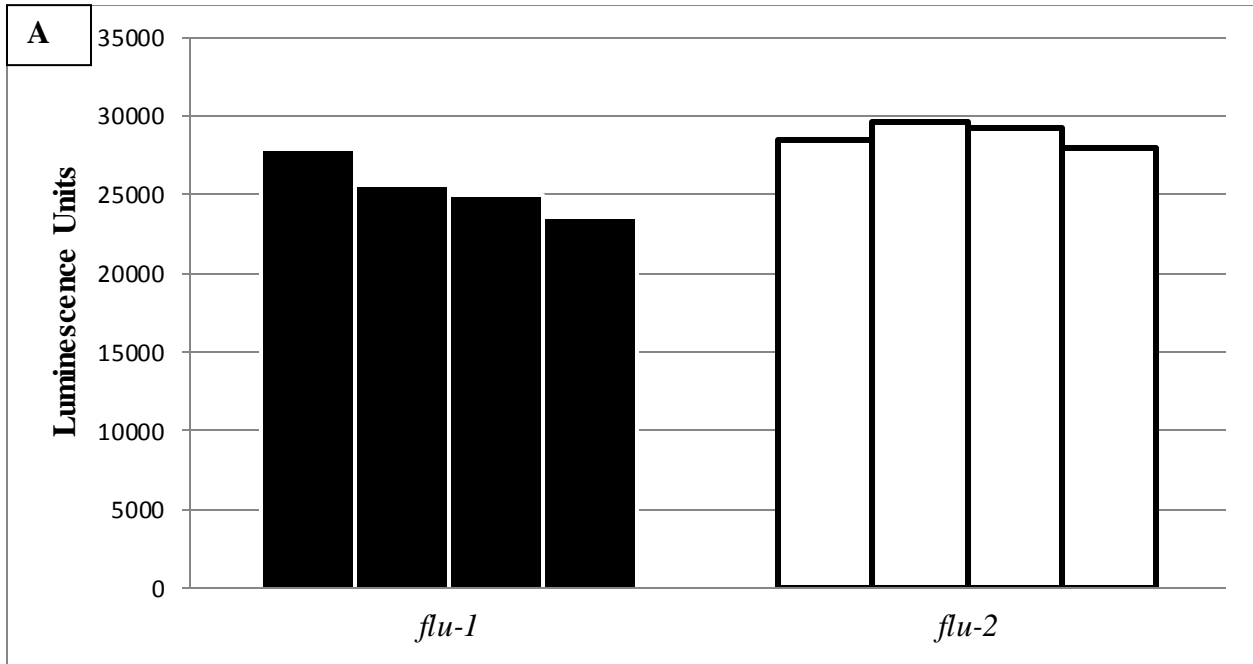
- O'Brien, J. A., Daudi, A., Butt, V. S., & Bolwell, G. P. (2012). Reactive oxygen species and their role in plant defence and cell wall metabolism. *Planta*, 236(3), 765-779.
- op den Camp, R. G., Przybyla, D., Ochsenbein, C., Laloi, C., Kim, C., Danon, A., ... & Apel, K. (2003). Rapid induction of distinct stress responses after the release of singlet oxygen in Arabidopsis. *The Plant Cell*, 15(10), 2320-2332.
- Promega Corporation, (2015). Retrieved from <http://www.promega.com/products/pm/fluorometers-luminometers-multimode-readers/glomax-20-20/>
- Ramel, F., Sulmon, C., Serra, A. A., Gouesbet, G., & Couee, I. (2012). Xenobiotic sensing and signalling in higher plants. *Journal of Experimental Botany*, 63(11), 3999-4014.
- Routaboul, J. M., Fischer, S. F., & Browse, J. (2000). Trienoic fatty acids are required to maintain chloroplast function at low temperatures. *Plant Physiology*, 124(4), 1697-1705.
- Sadikot, R. T., & Blackwell, T. S. (2005). Bioluminescence imaging. *Proceedings of the American Thoracic Society*, 2(6), 537.
- Shulaev, V., & Oliver, D. J. (2006). Metabolic and proteomic markers for oxidative stress. new tools for reactive oxygen species research. *Plant Physiology*, 141(2), 367-372.  
doi:141/2/367
- Sohn, S., & Back, K. (2007). Transgenic rice tolerant to high temperature with elevated contents of dienoic fatty acids. *Biologia Plantarum*, 51(2), 340-342.
- Thompson Jr, G. A. (1989). Membrane acclimation by unicellular organisms in response to temperature change. *Journal of Bioenergetics and Biomembranes*, 21(1), 43-60.
- Tjallingii, W., & Esch, T. H. (1993). Fine structure of aphid stylet routes in plant tissues in correlation with EPG signals. *Physiological Entomology*, 18(3), 317-328.
- Triantaphylidès, C., & Havaux, M. (2009). Singlet oxygen in plants: Production, detoxification and signaling. *Trends in Plant Science*, 14(4), 219-228.
- Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., . . . Ryals, J. (1992). Acquired resistance in Arabidopsis. *The Plant Cell*, 4(6), 645-656.  
doi:10.1105/tpc.4.6.645
- Upchurch, R. G. (2008). Fatty acid unsaturation, mobilization, and regulation in the response of plants to stress. *Biotechnology Letters*, 30(6), 967-977.
- Van Breusegem, F., Bailey-Serres, J., & Mittler, R. (2008). Unraveling the tapestry of networks involving reactive oxygen species in plants. *Plant Physiology*, 147(3), 978-984.  
doi:10.1104/pp.108.122325

- Van Doorslaer, S., Dedonder, A., de Blocks, M., & Callens, F. (1999). Oxidative stress in plants: EPR monitoring in DMPO-DMSO based extracts. *Journal of Plant Physiology*, *154*(1), 132-136.
- Van Loon, L., & Van Strien, E. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiological and Molecular Plant Pathology*, *55*(2), 85-97.
- Vigh, L., Joó, F., Droppa, M., Horváth, L. I., & Horváth, G. (1985). Modulation of chloroplast membrane lipids by homogeneous catalytic hydrogenation. *European journal of biochemistry/FEBS*, *147*(3), 477-481.
- Vijayan, P., Shockey, J., Levesque, C. A., Cook, R. J., & Browse, J. (1998). A role for jasmonate in pathogen defense of Arabidopsis. *Proceedings of the National Academy of Sciences of the United States of America*, *95*(12), 7209-7214.
- Wagner, D., Przybyla, D., op den Camp, R., Kim, C., Landgraf, F., Lee, K. P., ... & Apel, K. (2004). The genetic basis of singlet oxygen-induced stress responses of Arabidopsis thaliana. *Science*, *306*(5699), 1183-1185.
- Wingler, A., Lea, P. J., Quick, W. P., & Leegood, R. C. (2000). Photorespiration: Metabolic pathways and their role in stress protection. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, *355*(1402), 1517-1529.
- Winterbourn, C. C. (2014). The challenges of using fluorescent probes to detect and quantify specific reactive oxygen species in living cells. *Biochimica Et Biophysica Acta*, *1840*(2), 730-738.
- Yaeno, T., Matsuda, O., & Iba, K. (2004). Role of chloroplast trienoic fatty acids in plant disease defense responses. *The Plant Journal*, *40*(6), 931-941.
- Yara, A., Yaeno, T., Hasegawa, M., Seto, H., Montillet, J., Kusumi, K., . . . Iba, K. (2007). Disease resistance against Magnaporthe grisea is enhanced in transgenic rice with suppression of  $\omega$ -3 fatty acid desaturases. *Plant and Cell Physiology*, *48*(9), 1263-1274.
- Yu, C., Wang, H., Yang, S., Tang, X., Duan, M., & Meng, Q. (2009). Overexpression of endoplasmic reticulum omega-3 fatty acid desaturase gene improves chilling tolerance in tomato. *Plant Physiology and Biochemistry*, *47*(11), 1102-1112.
- Zhang, L., Li, Y., Xing, D., & Gao, C. (2009). Characterization of mitochondrial dynamics and subcellular localization of ROS reveal that HsfA2 alleviates oxidative damage caused by heat stress in Arabidopsis. *Journal of Experimental Botany*, *60*(7), 2073-2091.

## **Appendix I. Testing the Variations between Readings of the Luminometer**

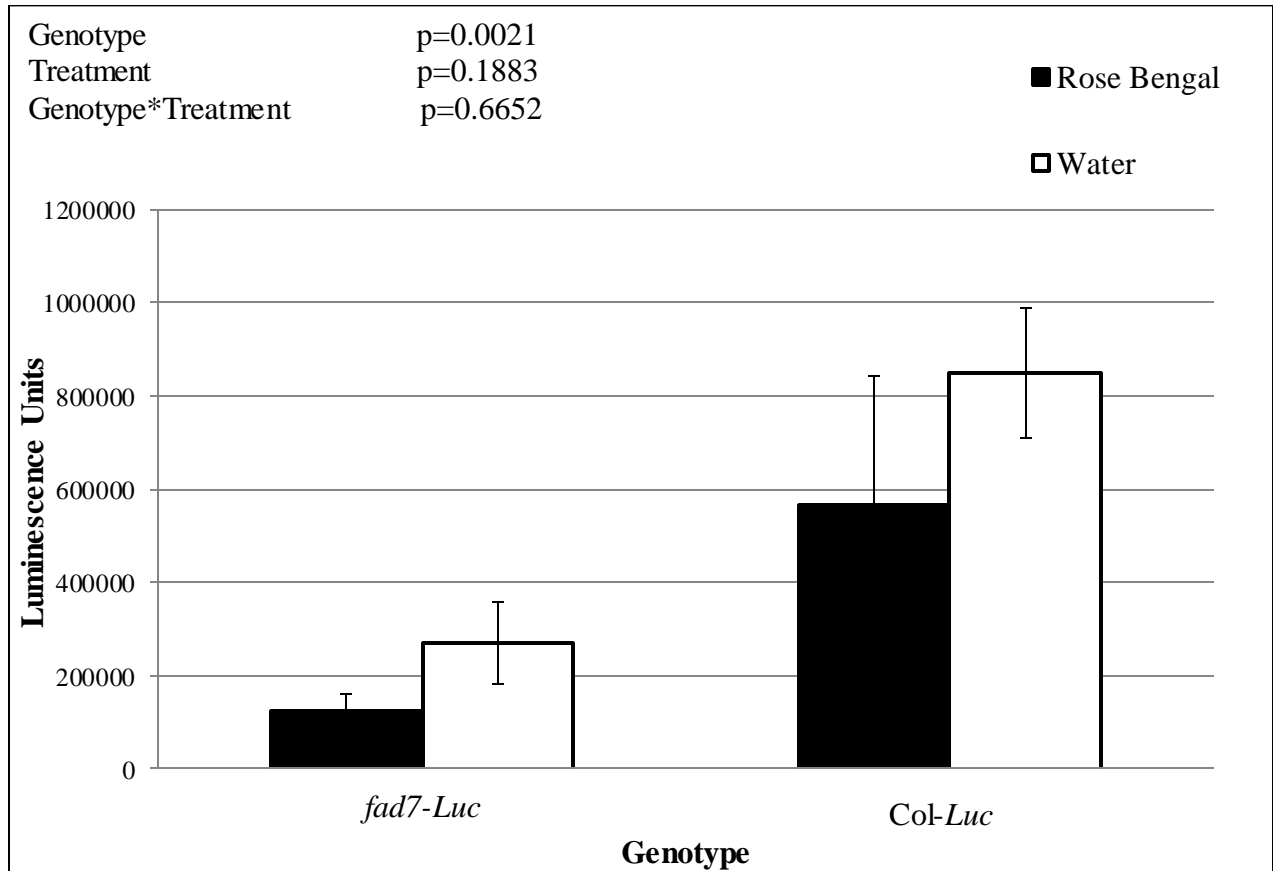
### **Testing the repeatability of the GloMax 20/20 luminometer**

This experiment was performed to test potential sources of technical variation in the luminometer-based assay, to inform our choice of subsampling methods. To see if luminometer performance might vary from one reading to the next, we took 4 consecutive measures of the same 2 sample vials. The results indicated that there was less than 5% variation among different readings for the same sample when the readings are taken sequentially (Supplemental Figure 1A). We also took measurements from multiple aliquots of the same plant extract to determine if there is variation among subsamples, and found 16-28% variation in values for the same extract (Supplemental Figure 1B). These results suggest that the luminometer itself is not a major source of random variation, but that random differences in the composition of different aliquots of the same sample can introduce some noise into the data. Based on these observations, we measured at least 2 subsamples per plant extract in all experiments.



**Supplemental Figure 1. Testing reading variations of the luminometer assay.** (A) The variations between the sequential readings for the two *flu:AAA-ATPase-Luc* plants (B) The variations between the sub-samples for the two *flu:AAA-ATPase-Luc* plants.

**Appendix II. Comparison of Plant Responses to 1mM and 500  $\mu$ M Rose Bengal.**



**Supplemental Figure 2. Reporter gene activity in response to 1mM rose bengal application (n=20).** All plants were treated with luciferin, and luminescence was measured using a luminometer. The error bars represent the standard error of the mean (SEM).

500  $\mu$ M rose bengal

1mM rose bengal

Water



Before  
Treatment



48 hours  
After  
Treatment

**Supplemental Figure 3. Visualization the plant symptom development in response to rose bengal application (500  $\mu$ M and 1 mM) on Col-0 Arabidopsis rosette leaves.** Foliage of four-week old plants were sprayed with water or rose bengal (n= 9). Plants were observed 48 hours after application for necrosis (solid arrow) or chlorosis (dashed arrow). All plants treated with either concentration of rose bengal showed signs of chlorosis, but plants treated with 1 mM displayed more extensive and advanced symptom development.

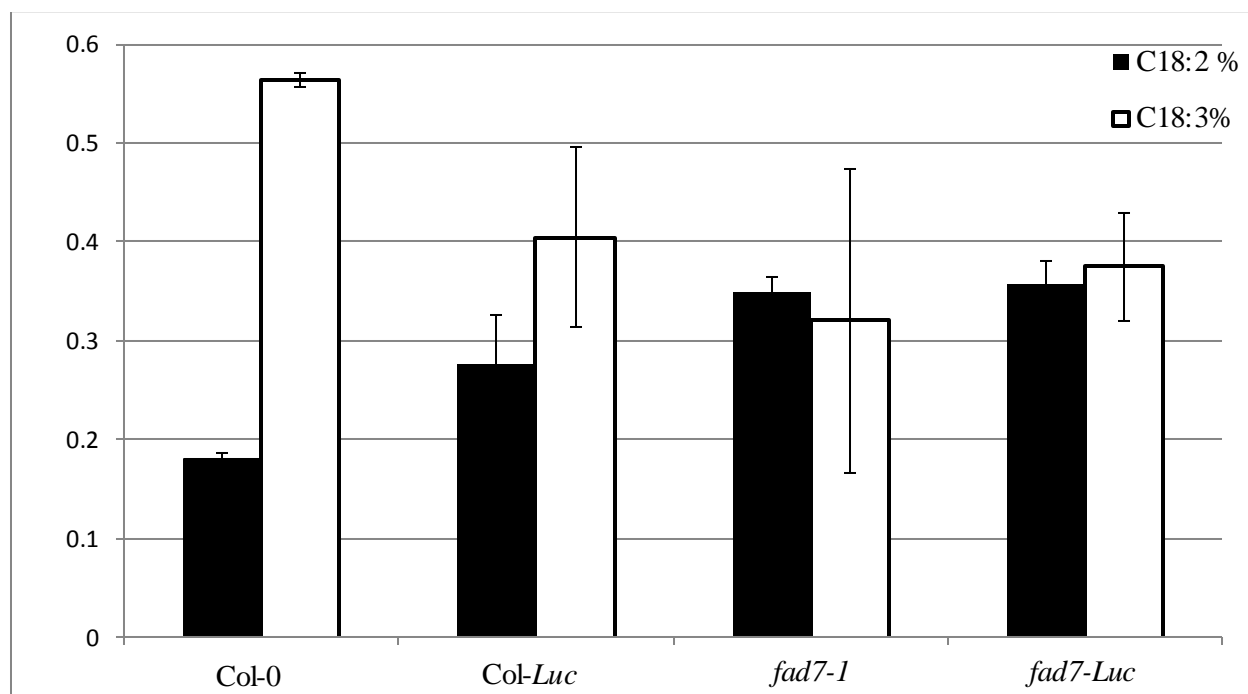
### Appendix III. Lipid Content of the *fad7-Luc* and *Col-Luc* Using Gas Chromatography Mass Spectrometry (GC-MS) analysis

The purpose of this experiment was to test the lipid content of the *fad7-Luc* and *Col-Luc* mutants to verify our PCR results for the *fad7* mutation. The *FAD7* is responsible for conformation the linoleic (18:2) acid to the linolenic acid (18:3). Therefore, the ratio between linoleic acid to the linolenic acid changes toward linoleic acid in the plants that have an impaired activity of *FAD7*.

6 plants of *fad7-Luc*, *Col-Luc*, *fad7*, and Col-0 were used. First, the plants were grinded by the liquid nitrogen, and 1 ml of the 1.5% sodium methoxide was added to every sample. Then, 200  $\mu$ l of toluene were added to every sample to solubilize the oil. In addition, 100  $\mu$ l of the pentadecanoic acid (1 $\mu$ g/ $\mu$ l) were added to every sample as an internal standard. The samples were incubated at 70 °C for 45 minutes. After cooling the samples 1ml of heptane and sterile water were added to every sample and the samples were placed in the centrifuge at 1500 rpm for 2 minutes. After the top layers were transferred to new tubes, and the samples were dried by using the evaporating system. Later, 100  $\mu$ l of hexane were added and the solution was transferred to new vials that are used in the GC-MS system.

The results showed that the wild type Col-0 and *Col-Luc* plants having higher levels of linolenic acid than linoleic acid, which is consistent with our expectations. However, we did not observe noticeable shift toward linoleic acid in the *fad7-1* plants and the *fad7-Luc* plants (Supplemental Figure 4 and Table 2). That may be because our internal standard did not show any peak for the known fatty acid (hexadecanoic acid) in the GC-MS analysis, so we could not compare our results to known standard volume. Therefore, it is very necessary to make sure that

our internal standard working probably, which could be done by optimizing a suitable volume and concentration of the internal standard that can show known peaks in all the tested samples.



**Supplemental Figure 4.** The percentages of the linoleic (18:2) acid and linolenic (18:3) of the total fatty acids for the *fad7-Luc*, *Col-Luc*, *fad7-1*, and *Col-0* genotypes. 200 mg of the leaves of four-week old plants were grinded and processed for the GC-MS analysis (n= 6).

**Table 2:** Sample of the percentages of the main fatty acids in the *fad7-Luc*, *Col-Luc*, *fad7-1*, and *Col-0* genotypes by the GC-MS analysis.

Genotype	C16:0	C18:1	C18:2 %	C18:3 %
<i>fad7-luc</i>	11.666%	4.497%	35.445%	41.872%
<i>fad7-1</i>	11.901%	3.918%	35.175%	38.961%
<i>Col-Luc</i>	20.072%	3.565%	25.535%	46.860%
Col-0	12.531%	1.642%	17.950%	56.706%