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

Abeer Muhammedali Alnasrawi

University of Arkansas, Fayetteville

Follow this and additional works at: http://scholarworks.uark.edu/etd

Part of the Biochemistry Commons, Molecular Biology Commons, and the Plant Biology Commons

Recommended Citation
http://scholarworks.uark.edu/etd/1319
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

Abeer Alnasrawi
University of Kerbala
Bachelor of Science in Biology, 2006

December 2015
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Professor Fiona Goggin
Thesis Director

Professor Andy Pereira
Committee member

Professor Vibha Srivastava
Committee member

Dr. Ron Sayler
Committee member
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 ($^{1}$O$_{2}$) is a ROS that is formed during photosynthesis in photosystem II (PSII) of the chloroplasts. Levels of $^{1}$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 $^{1}$O$_{2}$ (AAA-ATPase: Luc) was used to compare $^{1}$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 $^{1}$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 $^{1}$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 $^{1}$O$_{2}$.
Acknowledgments

I would like to express my appreciations to the following people for their generous support and assistance throughout the duration of my thesis research.

Firstly, I would like to express my sincere gratitude to my advisor Prof. Fiona Goggin for her support, mentorship and patience. Her guidance helped me in all the time of research and writing of this thesis.

Besides my advisor, I would like to acknowledge the rest of my thesis committee: Prof. Andy Pereira, Prof. Vibha Srivastava, and Dr. Ron Sayler, for their insightful comments and encouragement.

I would like to thank Dr. Carmen Padilla who spent hours in the lab explaining and showing me how to do everything. Special thanks go to Dr. Junhuan Xu, who was willing to help and give his best suggestions. I am also thankful to Dr. Carlos Avila for designing the FAD7 primers. Thanks to Dr. Min Woo Lee for helping with the GC-MS analysis. I am grateful to all my lab members for making my research experience in this lab a memorable one.

Also, I would also like to thank my parents, my sister, and my brother. They were always supporting me and encouraging me with their best wishes.

Finally, I would like to thank my husband, Murtadha Alher. He was always there cheering me up and stood by me through the good times and bad.
Dedication

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

I. Introduction .......................................................................................................................... 1
   A. Reactive Oxygen Species ............................................................................................... 1
   B. ROS and Plant Stress .................................................................................................. 3
   C. Singlet Oxygen ............................................................................................................. 4
   D. Measurement of ROS .................................................................................................. 5
   E. Luciferase as a Reporter for Singlet Oxygen ............................................................... 7
   F. Fatty Acid Desaturases ............................................................................................... 8
   G. ROS and fad7 Plants .................................................................................................. 11
II. Objectives .......................................................................................................................... 13
III. Materials and Methods .................................................................................................... 14
    A. Plant Materials ......................................................................................................... 14
    B. Surface Sterilization of Seeds and Growth Conditions ........................................... 14
    C. DNA Extraction for F2 (flu AAA-ATPase: Luc X fad7/gl1) Generation .................. 15
    D. Screening for fad 7-Luc and Col-Luc Plants ......................................................... 16
    E. The GloMax 20/20 Luminometer Analysis ............................................................... 17
    F. The Synergy HT Multi-Mode Microplate Reader Analysis ....................................... 17
    G. The Lumina XR Analysis .......................................................................................... 19
    H. Statistical Analysis .................................................................................................... 20
IV. Results ................................................................................................................................ 21
    A. Confirmation of Luciferase Activity in the Transgenic Line flu:AAA-ATPase-Luc .... 21
    B. Introduction of the AAA-ATPase-Luc Reporter Gene into fad 7-1 and Col-0 Backgrounds .................................................................................................................. 22
    C. Screening for fad7-Luc and Col-Luc Plants ............................................................. 24
    D. Confirmation of Reporter Gene Activity in fad7-Luc Plants .................................... 25
    E. Confirmation of Reporter Gene Responsiveness to Rose Bengal ............................. 25
    F. Reporter gene activity in response to abiotic and biotic stress in the fad7-luc and wild-type genetic backgrounds ................................................................. 29
       Reporter gene activity in response to high light stress ............................................. 29
       Reporter gene activity in response to aphid infestation ........................................ 33
V. Discussion ........................................................................................................................... 39
    A. Establishing a Suitable Method for Luminescence Measures ............................... 39
    B. Confirmation the Activity of the \(^1\text{O}_{2}\)-Responsive Reporter Gene .................... 41
    C. The Reporter Gene Activity in Response to Biotic and Abiotic Stresses ............... 41
VI. References ......................................................................................................................... 46
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 \((\text{HO}^+)\) (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 bisphosphate 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$_2$O$_2$ (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$_2$O$_2$ 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 H$_2$O$_2$ and 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 $^1$O$_2$, O$_2^-$, and H$_2$O$_2$ (Fryer, Oxborough, Mullineaux, & Baker, 2002; Laloi et al., 2007). However, high light intensities favor $^1$O$_2$ production (Hideg, Kállai, 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, HO$^-$ 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 (Pchlide) in the dark. In light conditions, Pchlide acts as a photosensitizer and produces $^1\text{O}_2$ (Baruah, Šimková, Apel, & Laloi, 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-Serre, & 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 H2DCF-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, $\text{O}^-_2$ and $\text{O}_2$, 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 $\text{H}_2\text{O}_2$ by a yellow fluorescent protein (YFP) that introduced into the regulatory domain of the $\text{H}_2\text{O}_2$-binding protein in *Escherichia coli*. YEP is a $\text{H}_2\text{O}_2$-specific probe and can be used for quantitative measurement of $\text{H}_2\text{O}_2$ in animal and plant cells (Hernández-Barrera et al., 2015). In addition, $^1\text{O}_2$-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 $^1\text{O}_2$ 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 & Szaly, 2002). The luciferin- luciferase reaction is described in the following equation (Crouch, Kozlowski, Slater, & Fletcher, 1993):

\[
\text{ATP}^1 + \text{D-Luciferin} + \text{O}_2 \rightarrow \text{Oxyluciferin} + \text{AMP}^2 + \text{PPi}^3 + \text{Light}
\]

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

---

1. Adenosine triphosphate
2. Adenosine monophosphate
3. 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 ω-3 fatty acid desaturases (ω-3 FADs) are membrane-embedded enzymes that catalyze the alteration of linoleic acid to linolenic acid (Kodama, Akagi, Kusumi, Fujimura, & Iba, 1997). Fatty acid desaturase 7 (FAD7) is one of the ω-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.,
In addition, the \textit{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 (\textit{Oryza sativa}) plants have shown more resistance to the rice blast fungus (\textit{Magnaporthe grisea}) by the suppression of the \textit{OsFAD7} and \textit{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 \textit{FAD7} were more resistant to high temperatures than untransformed rice plants. Additionally, tomato plants that have \textit{spr2} mutant, in which the \textit{FAD7} function has been disrupted, demonstrated better resistance to potato aphids (\textit{Macrosiphum euphorbiae}) than the wild-type tomato (Avila et al., 2012). Also, two mutants in \textit{Arabidopsis thaliana} (Atfsad7-2 and Atfad7-1fad8) exhibited more resistance to the green peach aphids (\textit{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 \textit{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 (ω-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$_2$O$_2$ 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 & Gogggin, unpublished data). Therefore, we hypothesize that there are higher levels of $^{1}$O$_2$ in FADs disrupted plants than non-disrupted plants. In order to prove our hypothesis, we will measure the $^{1}$O$_2$ 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 $^{1}$O$_2$ 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 $^{1}O_{2}$ 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 $^{1}O_{2}$), 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 gl1 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-
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; Laloi et al., 2006).

**DNA Extraction for F2 (flu AAA-ATPase: Luc X fad7/gl1) 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), 1 mM EDTA). Later, 200 µl 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°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 25 µl PCR reaction prepared by adding 2.5 of MgCl$_2$ (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$_2$O, and 2 µl of the DNA samples.

To detect luciferase positive plants, the forward primer 5’-TTACACGAAATTGC TTCTGGTG-3’ and the reverse primer 5’-CCTCGGGTGTAATCAGAATGC-3’ were used according to Baruah and others (2009). The annealing temperature was 51 ºC, 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’-ATCTGCGGGAAAGATGATG-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’-CCAAGGGAAGTATAGGaAGT-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 leave samples by liquid nitrogen, the weight for every sample was recorded and 1ml µl 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, 5µl of cell lysate was mixed with 20µl 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 µl ddH₂O 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 \textit{fad7-Luc} and \textit{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 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$^2$/sr) that represent the number of photons per second that depart a cm$^2$ 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 $^1$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 $^1$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 $^{1}$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 $^{1}$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).

```plaintext
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 $^{1}$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 *Gl-1* 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, \( \sim 135 \) \( \mu \)mol m\(^{-2}\) 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 \( ^{1}O_{2} \)-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 know to generate \( ^{1}O_{2} \). 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 \( \mu \)M rose bengal. In the first assay, which was conducted with
Figure 4. Selection of \textit{fad 7-Luc} and \textit{Col-Luc} mutants. Out of 115 F2 plants that originated from crossing \textit{flu:AAA-ATPase-Luc} and \textit{fad7-1/gl1}, screening identified 1 \textit{fad7-Luc} plant (homozygous for the \textit{fad7-1} mutation and the wild-type \textit{FLU} allele, and positive for the \textit{AAA-ATPase-Luc} reporter gene and the wild-type \textit{Glt-1} allele), and 3 \textit{Col-Luc} plants (homozygous for the wild-type \textit{FAD7} and \textit{FLU} alleles, and positive for the \textit{AAA-ATPase-Luc} reporter gene and the wild-type \textit{Glt-1} allele.
Figure 5: PCR screening of \( \text{fad7-1/gl1 X flu:AAA-ATPase-Luc} \) 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 \( \text{flu:AAA-ATPase-Luc} \) transgene. B) The 28 luciferase-positive plants were screened for the presence of the wild-type (top) and/or mutant (bottom) alleles of the \( \text{FAD7} \) gene. The results identified 8 plants homozygous for the wild-type \( \text{FAD7} \) allele (highlighted in red), 2 plants homozygous for the \( \text{fad7} \) 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.

![Graph](image)

**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 7B). 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 µM, 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 µM 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 µM 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 $^1O_2$ that can cause damage to our reporter system; therefore, we chose to conduct our experiments (Figures 7 and 8) with 500 µM 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 $^1O_2$ 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 $^1$O$_2$ generation in plants (Krieger-Liszkay, 2005), and so reporter gene activity in *fad7-Luc* and

![Figure 7](image_url)

**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-l* 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 (500µM) (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 µmol m⁻² s⁻¹ and 1000 µmol m⁻² 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 (~135 µmol m⁻² 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 µmol m⁻² s⁻¹), plants exposed to high light had significantly higher reporter gene activity than controls maintained at moderate light levels (Figures 9A and 9B). There was no significant difference in reporter gene activity between the different levels of high light (400 and 1000 µmol m⁻² s⁻¹) at either time point.
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 µmol m$^{-2}$ 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 ± 559,474 p/s/cm²/sr for the plants under high light stress, and 5,332,700 ± 583,291 p/s/cm²/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

33
± 1481.953 luminescence units for the wild-type plants that are inoculated with aphids and 19.000± 1.954173 luminescence units for the controls. The fad7-Luc transformed plants were 9-727-fold higher 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± 711777.8 (p/s/cm²/sr) for the plants inoculated with aphids, and 14729000± 1597621.5 (p/s/cm²/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 proof 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 µmol m⁻² sec⁻¹ or ~135 µmol m⁻² 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; Laloi, 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 3B). 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 3B). 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 $^{1}$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 $^{1}$O$_2$ accumulation.

Firstly, the plants were treated with 500 µM 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 µM 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 µM 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 µM 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 $^{1}$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 $^{1}$O$_{2}$ to be increased in *fad7-1* plants that are generated during photosynthesis.

$^{1}$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 $^{1}$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 $^{1}O_{2}$, 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.

<table>
<thead>
<tr>
<th>Figure #</th>
<th>Luminescence levels status for <em>fad7-luc</em> and <em>Col-Luc</em> plants</th>
<th>Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 8</td>
<td>Numerically higher luminescence for <em>fad7-luc</em> than <em>Col-Luc</em> plants.</td>
<td>Rose bengal 500 µM</td>
</tr>
<tr>
<td>Figure 9 A</td>
<td>Numerically higher luminescence for <em>fad7-luc</em> than <em>Col-Luc</em> plants.</td>
<td>1 h of (1000, and 400 µmol m⁻² s⁻¹) light stress</td>
</tr>
<tr>
<td>Figure 9 B</td>
<td>Numerically higher luminescence for <em>fad7-luc</em> than <em>Col-Luc</em> plants with significant difference between the two genotypes.</td>
<td>2 h of (1000, and 400 µmol m⁻² s⁻¹) light stress</td>
</tr>
<tr>
<td>Figure 10 B</td>
<td>Numerically higher luminescence for <em>fad7-luc</em> than <em>Col-Luc</em> plants with marginally higher P value (P=0.06)</td>
<td>2 h of 400 µmol m⁻² s⁻¹ light stress</td>
</tr>
<tr>
<td>Figure 11 A</td>
<td>Numerically higher luminescence for <em>fad7-luc</em> than <em>Col-Luc</em> plants with significant difference between the two genotypes.</td>
<td>48 h of aphid’s infestation</td>
</tr>
<tr>
<td>Supplemental Figure 2</td>
<td>Numerically higher luminescence for <em>Col-Luc</em> than <em>fad7-Luc</em> plants with significant difference between the two genotypes.</td>
<td>Rose bengal 1 mM</td>
</tr>
</tbody>
</table>
References


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 µM 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).
Supplemental Figure 3. Visualization the plant symptom development in response to rose bengal application (500 µM 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 µl of toluene were added to every sample to solubilize the oil. In addition, 100 µl of the pentadecanoic acid (1µg/ul) were added to every sample as an internal standard. The samples were incubated at 70 ºC 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 µl 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.

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