Characterization of an anther specific anionic peroxidase and the effects of gibberellic acid on the peroxidase activity in *Brassica rapa*

Charles Thomas Colin Day

Allegheny College

Class of 2011

Senior Comprehensive Project



Characterization of an anther specific anionic peroxidase and the effects of gibberellic acid on the peroxidase activity in *Brassica rapa*

Charles Thomas Colin Day

Allegheny College

Class of 2011

Senior Comprehensive Project

Submitted to Allegheny College Department of Biology for partial fulfillment of the requirements for the degree of Bachelor of Science

Committee Members:
Dr. Ann Kleinschmidt
Dr. Catharina Coenen

I hereby recognize and pledge to fulfill my responsibilities, as defined by the Honor Code, and to maintain the integrity of both myself and the College community as a whole.

Pledge: _____

Acknowledgements

I would like to extend my sincere gratitude to:

Dr. Kleinschmidt, my senior project advisor, for her support and guidance throughout the past three years. She supplied me with not only the countless amounts of knowledge necessary to complete this project, but she provided me with the confidence to excel academically.
"Keep away from people who try to belittle your ambitions. Small people always do that, but the really great make you feel that you, too, can become great." - Samuel Clemens

Dr. Coenen, my second reader, for proposing questions and critiques that helped me revise and successfully complete this project.

The Senior Project Fund for providing the funding that made this research possible.

Dr. Kleinschmidt's Comp Group 2011 for your humor and support.

My family for your support and helping me feel relaxed when I became overwhelmed.

Dan Paul, Britten Keep, Tom Cusimano and Brendan O'Leary for their comic relief and support throughout my time at Allegheny. This college life and experience that we have been living for the past four years would be nothing without you guys.

Abstract

A protein complex displaying anionic peroxidase activity has been discovered in unopened flower buds of *Brassica rapa*. Previous experiments determined the anionic peroxidase activity to be found only within the stamen of unopened flower buds and this activity to be absent in the *ms2* sterile line and have partial activity in the *ms1* mutants. In this study, experiments were performed to further investigate peroxidase activity patterns, to determine if GA_3 treatments can influence this activity pattern and if GA_3 treatments recover the anther specific anionic peroxidase (AS-AnPOX) in *ms1* and *ms2* cytoplasmic male sterile mutants. Native gel electrophoresis showed an increase in the peroxidase activity (isoelectric point of 3.5) 8 days after planting and activity decreases each progressing day until the flower bud is opened. AS-AnPOX activity was found to be absent in the *ms2* mutants and partially present in the *ms1* sterile line. GA_3 treatments were performed on wildtype and cytoplasmic male sterile mutants, and showed an acceleration in the appearance of AS-AnPOX activity in wildtype *B. rapa* and no effect on AS-AnPOX activity in either CMS line. This study confirmed an activity timeline of the AS-AnPOX as well as reaffirmed the absence of this activity in *ms1* and *ms2*. This study also showed that small concentrations of GA_3, when applied to the developing flower buds of wildtype plants, accelerated AS-AnPOX activity, but this concentration of GA_3 could not reverse the absence in activity seen in the CMS plants. The cytoplasmic male sterility may be attributed partially to the absence of the AS-AnPOX which may lead to abnormalities such as premature programmed cell death of the tapetum and the improper maturation of the pollen grains.

Introduction

The most important objective for all living organisms is to reproduce and pass their genes to the next generation. Many higher plants, including *Brassica rapa*, use pollen grains to sexually reproduce. These pollen grains are multicellular male gametophytes containing sperm cells and are differentiated from haploid microspores, which were produced from diploid cells that underwent meiosis. This production of a final reproductive product, pollen, takes place in the anthers of flowering plants (Goldberg et al. 1993). Flower development starts with the specification of floral meristem identity and of floral organ identity within floral meristems flanking an inflorescence meristem. These floral meristems initiate four different types of floral organs: sepal, petals, stamens and carpels. The floral organs are produced as successive whorls starting with sepal and progressing inward (Figure 1). The function of each whorl is determined by three overlapping developmental fields, which correspond to the expression patterns of specific floral organ identity genes. These fields show the three different sets of gene activity (ABC) necessary for proper flower formation. According to the ABC model proposed by Coen and Meyerowitz (1991), organ identity in each whorl is determined by a unique combination of the three organ identity gene activities.

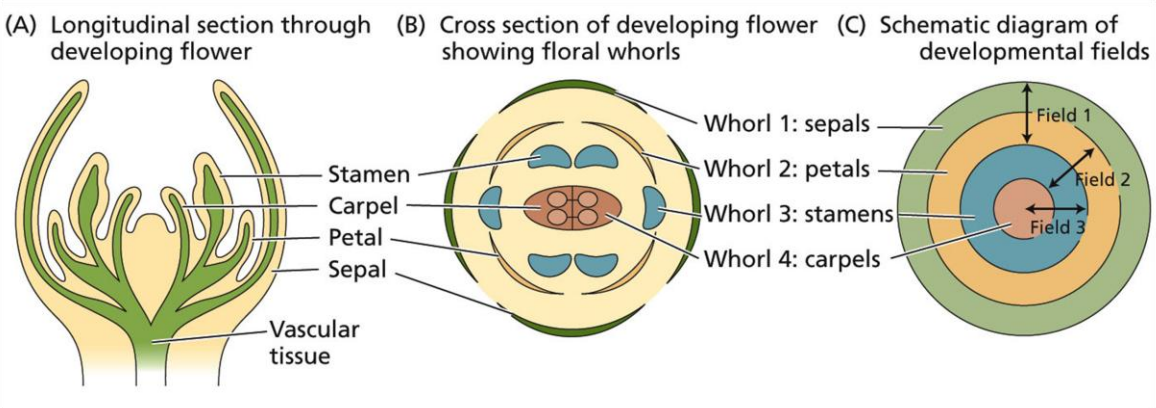


Figure 1: The floral organs initiated by the floral meristem of *Brassica rapa* (A and B). The floral organs are produced as successive whorls starting with sepal and progressing inward. The function of each whorl is determined by three overlapping developmental fields , which correspond to the expression patterns of specific floral organ identity genes. (Taiz and Zeiger 2010)

B*rassica rapa* flowers are organized into four concentric whorls of distinct organs (sepals, petals, stamens and pistils). Stamens, the male reproductive organs of flowering plants, form the third whorl (Zhao et al. 2001). Each stamen consists of a tube of vascular tissue, the filament, and the anther which contains both reproductive and nonreproductive tissues. Anther development occurs in two general phases, the first being the division and differentiation of the anther and the second being the differentiation of the pollen grains and dehiscence, the degradation of the anther to release pollen. The anther is made of four symmetrical lobes that produce the pollen, each of which differentiates into subdivisions of cells that give rise to three distinct cell layers: the endothecium, middle layer, and tapetum (Goldberg et al. 1993). The tapetum surrounds the pollen mother cells (PMCs) that will undergo meiosis and form the haploid microspores (Zhao et al. 2001). These microspores develop into pollen grains, the plant's male gametophyte.

The tapetum (Figure 2), which arises from secondary parietal cells and connective tissues, surrounds developing reproductive cells (Goldberg et al. 1993), plays an important role in pollen development by contributing to microspore release, nutrition, pollen-wall and pollen coat synthesis. Several genes essential for tapetal development and function have been identified in *Arabidopsis*. SPOROCYTELESS/ NOZZLE (SPL/NZZ), EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS (EMS1/EXS), and TAPETUM DETERMINANT 1 (TPD1) all function at an early stage and determine tapetal formation (Schiefthaler et al. 1999; Canales et al. 2002; Yang et al. 2003, 2005; Albrecht et al., 2005). Mutation of SPL inhibits sporocyte development and the *spl* mutant shows complete male and female sterility (Schiefthaler et al. 1999). EMS1/EXS and TPD1 determine the differentiation of the microsporocytes and tapetal cells. Mutation of these genes causes the tapetal cell precursors to differentiate and develop into microsporocytes instead (Albrecht et al. 2005; Yang et al. 2003, 2005).

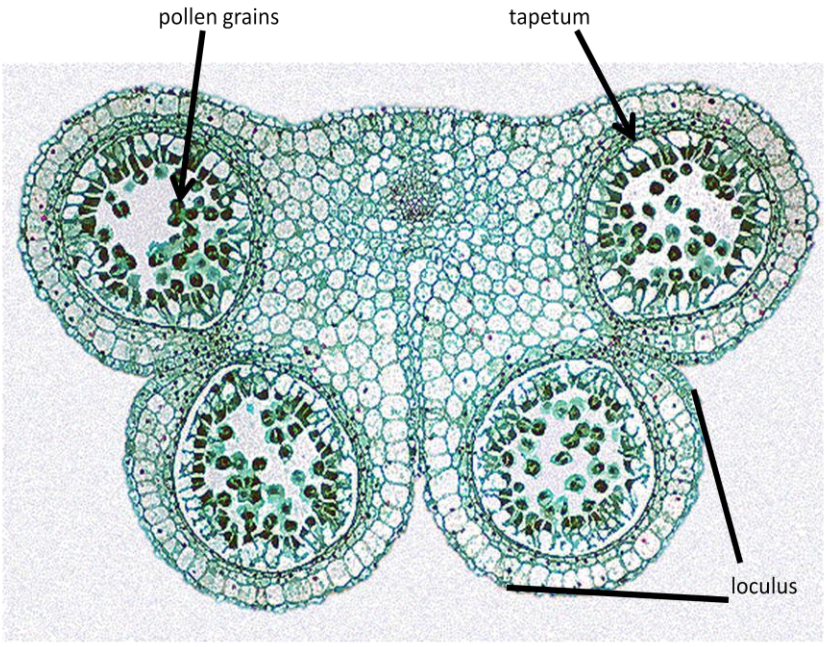


Figure 2: Cross-section of anther, cut transversely. Developing pollen is encapsulated within the four loculus which is surrounded by the tapetum. Image from www.uri.edu.

Following formation of the tapetal cell layer, other genes such as DYSFUNCTIONAL TAPETUM 1 (DYT1), MYB33, and MYB65 become important for tapetal differentiation and early functioning (Millar and Gubler 2005; Zhang et al. 2006). DYT1, which shows strong expression in the tapetum from late stage 5 to early stage 6, encodes a transcription factor important for tapetum-specific gene expression. DYT1 is a crucial component of a genetic network controlling anther development and function (Zhang et al., 2006). In myb33 myb65 double mutant anthers, the tapetum undergoes hypertrophy at the pollen mother cell stage, which results in pre-meiotic abortion of pollen development (Millar and Gubler 2005).

MALE STERILITY 1 (MS1) and MALE STERILITY 2 (MS2) are both essential genes at late stage tapetal development. Both are essential for pollen formation and development and are downstream of both the EMS1 and DYT1 genes. The *ms2* line in *B. rapa* is like the *ms1* line in *A. thaliana*; the MS1 gene in *A. thaliana* encodes a transcription factor with leucine zipper-like and plant homeo domain (PHD) finger motifs, these motifs suggest that MS1 acts as a transcriptional activator. The MS1 transcription factor affects 95 other genes, all of which are located immediately downstream (Ito et al. 2007). In the *ms1* mutant, the tapetum is abnormally vacuolated and microspores collapse soon after they are released from the tetrads. Recent research has shown that MS1 plays a critical role in regulating pollen coat development (Yang et al.2007). MS1 also plays an important role in the breakdown of callose, a structural polysaccharide that breaks down during the late tetraspore stage to allow pollen release. The MS1 gene is also proposed to induce proteases that are important to the progression of program cell death (PCD) (Yang et al. 2007).

The function of the MS1 gene is in *B. rapa* is compatible to the function of the MS2 gene in *A. thaliana*. The MS2 gene product, in *A. thaliana,* shows sequence similarity with fatty acyl-

CoA reductases (FAR enzymes), which convert wax fatty acids to fatty alcohols. This reduction of acyl-CoA is a key step in the synthesis of sporopollenin, which is a major component of the pollens outer most wall, exine (Aarts et al. 1997). Sporopollenin is composed of a mixture of biopolymers, containing mainly long chain fatty acids, phenylpropanoids, phenolics and traces of carotenoids (Boavida et al. 2005). The tapetal cells that surround the developing pollen grain in the anther have a highly active secretory system containing sporopollenin precursors. *ms2* have a limited secretion of these precursors by the tapetal cells causing improper exine formation and nonviable pollen (Aarts et al. 1997).

Many of the genes responsible for stamen and tapetal development are under hormonal control. For example, in *Arabidopsis* mutations affecting biosynthesis of two plant hormones gibberellin (GA) (e.g *ga1-3* mutation) and jasmonate (JA) (e.g *opr3* mutation) both confer male sterile phenotype due to failure of stamen filament elongation and of completion of anthesis and anther dehiscence (Koornneef 1980, Stinitzi et al. 2000). Deficiency in either gibberellin or jasmonate causes male sterility, in part due to the short stamen filament conferred.

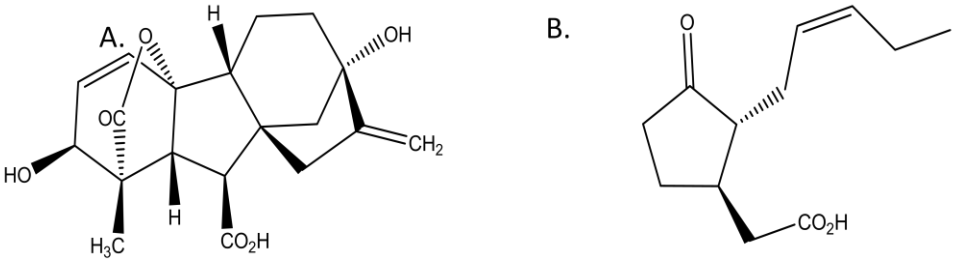


Figure 3. Chemical structure of gibberellic acid (A) and jasmonic acid (B). Structures courtesy of Chem Bio Draw 2007.

Jasmonates are plant signaling molecules that help regulate plant growth and development. Jasmonic acid (Figure 3b) is synthesized in the chloroplasts and peroxisomes and derived from linolenic acid found in membrane lipids (Creelman and Mullet 1995). Jasmonic acid (JA) activates plant defenses against herbivores, fungal pathogens, and regulates growth and

development of anthers. Jasmonic acid induces JA responses by binding directly to the 26S proteosome, which promotes binding and ubiquitination of the JA response repressors such as JAZ proteins (Santner 2009). A lack of JA response can lead to improper pathogen defense and male sterility (Creelman and Mullet 1995). Understanding the JA-dependent phenotype in flower development has come from *Arabidopsis* mutants affected in JA biosynthesis, such as *dad1, opr3* and *aim1* as well as mutants in JA-signaling, such as *coi1*. All these mutants are male-sterile due to delayed anther development and incomplete anther dehiscence, or are impaired in filament elongation (Turner et al. 2002, Mandaokar et al. 2003, Delker et al. 2006). MYB21 and MYB24 transcription factors have been found to be JA-inducible and mediate these jasmonate responses during stamen development (Mandaokar et al. 2006).

Jasmonate treatments have been able to partially rescue the phenotypes of some male sterile mutants. In the CMS opr^3 mutant, which suffers from impaired filament elongation and late anther dehiscence, treatments of 0.03% methyl jasmonate caused a display of partial recovery of normal anther development in stage 13 by elongating the filaments and causing partial dehiscence of anthers (Mandaokar et al. 2002). Some genes seem to be induced fairly late after JA treatment and presumably are involved in downstream processes such as pollen maturation and release. Others, like the OPR flavoproteins involved in the electron transport chain in mitochondrial complex II, are induced immediately after JA treatment. These genes are interesting because they may act early in the signaling cascade and may be required for the downstream processes in anther development. Other mutants JA treatments did not revert phenotypes, even at higher concentrations; twice a day treatments with JA (50 µM) for five days were not able revert the myb21-1 and myb21/myb24 mutant phenotypes (Mandaokar et al. 2006).

Another phytohormone that promotes pollen and anther development is gibberellin. Gibberellins (GAs), are growth hormones with a tetracyclic diterpenoid structure (fig 3a), and are essential regulators of diverse growth and developmental processes throughout the life cycle of the plant (Fleet and Sun 2005). There have been 126 gibberelins identified in plants, fungus and bacteria; the most commonly found GAs in higher plants are GA_1 and GA_3. GA regulates and influences many physiological events throughout a plant's life cycle from promotion of seed germination (Piskurewicz et al. 2008) and stimulation of stem and root growth (Yaxley et al. 2001) to floral initiation and sex determination in unisexual flowers (Remay et al. 2009). Out of the many events that rely on GA, one of the most important is reproductive development, where GA plays a key role in the promotion of anther development (Tsuji et al. 2006) and pollen formation (Aya et al. 2009).

Gibberellin-deficient mutants in *A. thaliana* were shown to have impaired anther development and pollen formation, which both lead to male sterility, and could be reversed with GA treatments (Aya et al. 2009). GA allows for the regulation of male fertility through the transcription factor GAMYB in barley and rice, the sequences in barley were similar to MYB proteins in *Arabidopsis* (Kaneko et al. 2004). GA triggers the degradation of the DELLA repressor protein complex by binding to the GID1 cofactor facilitating substrate recognition by 26S proteosome pathway (Santner et al. 2009). The degradation of DELLA proteins allow for the transcription of GAMYB mRNA, a functioning GAMYB transcription factor and proper GA responses. Plants lacking a functional GAMYB gene develop with shrunken, white anthers that contained no pollen (Kaneko et al. 2004). This lack of pollen is due to the improper death of the tapetal cells within the anther (Aya et al. 2009). In *gamyb* mutants, the tapetal cells never undergo programmed cell death at the tetrad stage, leaving no pollen wall components for the

pollen and causing the grains to collapse (Aya et al. 2009). The modification of specific GAs also plays a role in floral development. The over expression of the GA 2-oxidase, which adds an additional hydroxyl converting GA_3 to GA_8, in *Brassica napus*, has been shown to cause flowering delay, dwarf stature, and reduced and lignification within the growing flower bud (Zhao et al. 2010).

While both of these phytohormones play key individual roles in anther development, pathway intersection is needed and there must be active cross-talk between the two hormones for proper floral development. Studies in *A. thaliana* have shown that GA may be one of the endogenous signals involved in the regulation of JA biosynthesis genes (Cheng et al. 2009). When GA removes the repressor DELLA and allows for the transcription of genes such as DAD1 and LOX1 (fig 4), these transcription factors allow for the transcription of other genes important to JA biosynthesis (Peng 2009). The importance of pathway intersection can also be seen by the fact that JA application can restore the expression of the three *MYBs* in the GA-deficient mutants, strongly suggesting that JA biosynthesis is impaired in the *ga1-3 gai-t6 rga-t2 rgl1-1* mutant (Cheng et al. 2009). There is a linear relationship between GA and JA in that GA up regulates the expression genes that promote JA biosynthesis which in turn induces the expression of *MYB21*, *MYB24* and *MYB57* to control stamen development (fig 4) in *Arabidopsis* (Cheng et al. 2009).

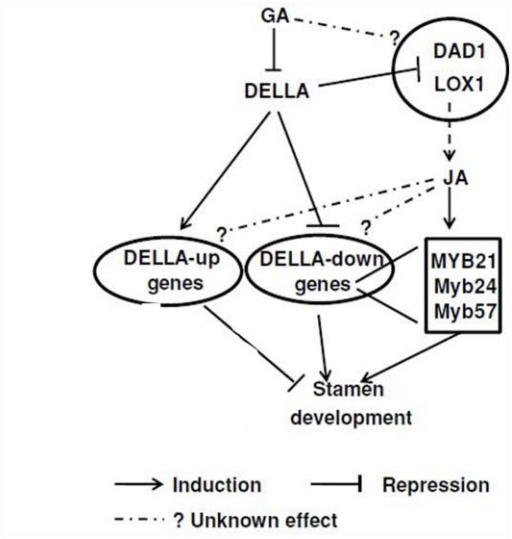


Figure 4. Diagram showing the crosstalk between gibberellin and jasmonic acid during stamen development (Peng 2009)

While phytohormones are important signaling molecules for floral development, higher plants use other signal such as reactive oxygen species to control developmental pathways. Oxygen is an abundant molecule in biological systems. Autotrophs produce O_2 as a product of photosynthesis; oxygen is the fundamental electron acceptor of other essential cellular functions. These useful abilities of O_2 come a price though, the accidental formation of reactive oxygen species by exposure of O_2 to excess energy (Mylona et al. 2007). These reactive oxygen species (ROS) are partially reduced or activated derivatives of oxygen [superoxide ($O_2^{\cdot}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\cdot}$)] are highly reactive and toxic, and if left unchecked ROS could cause serious amounts of damage to or even death to cells (Asada et al. 1987). Organelles, such as chloroplasts, mitochondria and microbodies, are hotspots for the production of ROS in plant cells with a high oxidizing activity or intense flow of electrons (Vranová et al. 2002). The cells use a whole host of antioxidative enzymes such as superoxide dismutase (SOD), ascorbate peroxidase (APX), class III peroxidase (POX) and catalase (CAT) as an intracellular defense system protecting the cells from oxidative stress by reducing ROS.

In the last decade, new roles for ROS have been identified: the control and regulation of biological processes, such as growth (Kovtun et al. 2000), programmed cell death (Overmyer et al. 2003), hormone signaling (Pei et al 2000), biotic and abiotic stress responses and development (Dat et al. 2000). Recent studies also suggest the role of ROS in plant biology as a balance between toxic metabolic byproducts and regulator of growth, development and defense pathways. Depending on which type of cellular signal is received, the cells can amplify ROS action by using ROS-producing pathways, such as NADPH oxidase, leading to programmed cell death (PCD) and oxidative bursts (Overmyer et al. 2003). In contrast ROS-scavenging pathways are responsible for timing and localization of the ROS signals as well as maintaining a low steady-state level of ROS on which the different signals can be registered (Vranová et al. 2002). This balance of ROS toxicity while enabling ROS such as H_2O_2 or O_2- to act as signaling molecules requires a vast gene network consisting of at least 152 genes in Arabidopsis (Mittler et al. 2004).

ROS play a critical role in the regulation of program cell death (PCD) or hypersensitive response. PCD is a plant defense strategy against pathogens, in which the plant cells around a site of infection die rapidly, depriving the pathogen of nutrients and slowing or preventing the pathogens spread (Overmyer et al. 2003). This cascade of cell death is preceded by a rapid increase of ROS, and the production of H_2O_2 and O_2^-, and the membrane protein NADPH oxidase, is key to PCD. This death of cells is very important for the maturation of pollen grains because the tapetal layer under goes PCD and coats the pollen with nutrients and adhesive materials necessary for pollination (Goldberg et al. 1993).

ROS, specifically H_2O_2, also affect the lignin polymerization in the development of secondary anther walls. The formation of the lignin polymer takes place after the activation of

monolignols which require the coupled action of both peroxidase and H_2O_2 (Villarreal et al. 2009). Recent studies show that decreased levels of H_2O_2 results in the failure to properly polymerize enough lignin, thus the anther lacks secondary thickening. This necessity of H_2O_2 for lignification was discovered by the addition of H_2O_2 scavengers to anther tissue, after which there was a dramatic decrease in lignin (Villarreal et al. 2009).

The role of ROS in plants is a complicated one. While ROS is a necessity for proper flower development, high concentrations can be detrimental to the plant cells. ROS react with biological compounds such as proteins, nucleic acids, and lipids, damaging the cells and even causing cellular death. High concentration of ROS in flowers can lead to premature PCD, leaving the pollen grains undeveloped. To defend themselves against the damaging effects of ROS, plants have evolved antioxidant defenses. These antioxidant defenses consist of both non-enzymatic compounds and enzymes. Some non-enzymatic antioxidant defenses are vitamins C and E, glutathione and β-carotene, while some enzymatic defenses are superoxide dismutases, catalases and peroxidases (Overmyer 2003). SODs catalyze the transformation of superoxide in to hydrogen peroxide, while CAT and peroxidases reduce hydrogen peroxide to water (Gupta et al 1993). SOD and CAT use the ROS they reduce as the reductant and oxidant, while peroxidases need different antioxidative compounds to act as their specific reductant (Gupta et al. 1993).

These enzymatic antioxidant defenses are differentially regulated in the process of flower development. The developing flower buds of tomatoes were shown to contain higher mRNA levels of two CuZn SOD genes, while the open flower buds showed substantially lower levels (Perl-Treves 1991). In the anthers of cotton, Mn SOD mRNA levels gradually declined between the sporogenous cell division stage and the mature pollen stage, while APX mRNA levels did not

decrease till the later mature pollen stage (Jiang et al. 2007). The interplay between ROS production and ROS-scavenging mechanisms, both enzymatic and non-enzymatic, determine the steady state ROS level which is important for normal floral development (Miller et al 2008).

Previous experiments have discovered a protein complex showing activity similar to that of an acidic peroxidase in *Brassica rapa* (Kleinschmidt unpublished). The peroxidase activity has been found only in the stamen of unopened flower buds. The protein was shown to have an isoelectric point of 3.5 in native gel assays (Kleinschmidt unpublished). The activity of this protein is only seen while the bud is closed and activity disappears after the flower buds open. The protein complex was found to have a molecular weight of approximately 100-140 kDa. The activity of this protein was also found to be absent in the *ms2* sterile line and have partial activity in the *ms1* mutants (Kleinschmidt unpublished). It is thought that this peroxidase could be performing two tasks. It is either building a structure, such as callose or lignin, necessary for normal tapetal development or it is controlling the timing of tapetal dehiscence by holding back the hydrogen peroxide signal until the pollen is ready. It is more likely that the peroxidase activity is carefully controlling the H_2O_2 signal, because the activity is seen over approximately five days and the construction of a material, such as lignin, would not take this long.

While we do know a great deal about the anionic peroxidase activity, we know very little about how this activity is controlled and there is no research on the relationship between gibberellic acid and peroxidase activity in male sterile mutants. The goal of this study was to determine activity patterns of a flower specific anionic peroxidase, and if GA_3 treatments can influence this activity pattern. Another goal is to determine if GA_3 can recover the flower specific anionic peroxidase in *ms1* and *ms2* mutants.

Material and Methods

Preparation of Plant Tissue Extracts

Brassica rapa fast plants were grown in a water-controlled system that consisted of peat moss pots (Jiffy® Peat Pellets) in a watering tray. Growing media (2 parts promix/1part vermiculite) was sprinkled on top of the pots after the seeds were planted. Plants received nutrients from the contents (1X Peters Professional® fertilizer) of the watering tray, and when needed the tray was refilled. Six seeds were planted into each peat pot, and the plants were then placed in growth boxes. Growth boxes were equipped with one 30W Lights of America Circular light that kept the plants under 24-hour illumination.

Hormone Treatment

Gibberellin treatment was composed from a 50mM GA_3 stock solution diluted 1/100 in ethanol, followed by a 1/100 dilution in 0.05% Triton-X100, resulting in 5µM GA_3 in 0.05% Triton-X100, 1% ethanol. Hormone and surfactant treatments were applied in doses of 10µl to each plant's floral meristem or flower bud. After applications, plants were left standing outside of the growth chambers until all applied liquid was soaked into the plant, at that time the plants were placed back under 24-hour illumination.

Preparation of Samples for Agarose Gel and Electrophoresis

Tissue was harvested from plants and transferred to a microcentrifuge tube (Kontes) on ice. For homogenization, 1.0 volumes, as determined by the mass in mg of tissue, of cold extraction buffer (25 mM Tris, 192 mM glycine, pH 8.5) were added, the tissue homogenized with a pellet pestle (Kontes) for 20 sec, finger vortexed, and then homogenized for another 20

sec. The samples were then centrifuged for 5 min at > 12,000 X G. After centrifugation, 30 µl of the supernatant was mixed with 10 µl of 4X gel loading buffer (100 mM Tris, 770 mM glycine, pH 8.6, 40% glycerol, 0.04% bromophenol blue) in preparation for electrophoresis. The samples were then analyzed by electrophoresis in a 0.8% agarose gel in 1X Tris-Glycine buffer (25mM Tris, 192 mM glycine, pH 8.5) for 25 minutes at 170 volts.

Staining

After electrophoresis, the gel was stained using 20 ml of triethanolamine buffer (128 mM NaCl, 21 mM trientholnamine, pH 7.5), 4 ml of 4-chloro-1-naphthol (3 mg/ml) and 20 µl of 30% hydrogen peroxide; the gel was then agitated for 15 minutes on a shaker and then washed with distilled water.

Analysis

After the staining was completed, isoelectric points (IEPs) of peroxidases were determined by the construction of a standard curve. The standard used contained Cytochrome C, Basic HRP Isoenzyme, Acidic HRP Isoenzyme #1, and Acidic Isoenzymes #2 with IEPs of 10.2, 9.0, 7.1 and 6.4 respectively.

Results

Developmental timing of anionic peroxidase in flower buds

The focus of this investigation is the anionic POX complex that has an IEP of 3.5 and has been shown to be only in anther tissue. The anionic POX complex activity is represented in two bands, IEP 3.6 and 3.2. To determine the time course of this anther specific anionic peroxidase (AS-AnPOX) activity, extracts from wildtype *B. rapa* flower buds were assayed by electrophoresis. AS-AnPOX activity decreased dramatically between day 8 and 9 after germination (fig. 5 lanes 5,6). The first presence of the AS-AnPOX activity was found in flower buds harvested 8 days after germination (fig. 5 lane 5), although it is only a single band as opposed to the activity seen from days 9 to 14 where there was a clear double band present (fig. 5 lanes 6,7,8,9,10,11). After the initial appearance at day 9, AS-AnPOX activity decreased each progressing day until the flower bud opened at day 14.

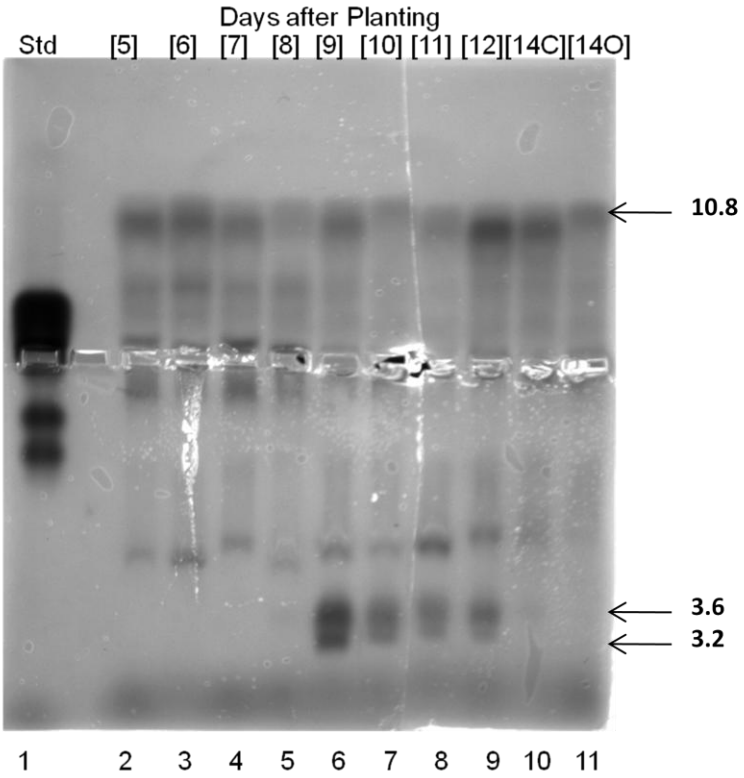


Fig. 5 Time course analysis of peroxidase activity in wild-type flower buds. The flower bud tissues were assayed by electrophoresis on a 0.8% agarose gel and stained for peroxidase activity with 4-chloro-1-naphthol. Numbers above represent the date of harvest in days after planting. Closed (C) and open (O) buds were harvested on day 14. Numbers on the right designate the isoelectric points of peroxidases looked at in this study.

To determine whether the AS-AnPOX activity was present in male sterile mutants, and if it was, the time course of appearance of this activity, a comparison was made between extracts from wild type (fig.6 lanes 2,5,8,12, 15,18), cytoplasmic male sterile 1 (ms1) mutant (fig. 6 lanes 3,6,9,13,16,19), and cytoplasmic male sterile 2 (ms2) mutant (fig. 6 lanes 4,7,10,14,17,20). Wild type plants showed first presence of AS-AnPOX activity at day 9 (fig. 6 lane 5) and a steady increase in activity through day 12 (fig. 6 lanes 8,12,15). Wild type plants showed no AS-AnPOX activity from buds collected from clusters with at least one open flower (fig. 6 lane 18). *ms1* plants showed no AS-AnPOX activity until buds were open, where it can be seen that there is only a single band (fig. 6 lane 19). *ms2* plants showed no AS-AnPOX activity from 8 days

after germination through open flower buds. In both mutants there is an increase in 10.8 cationic peroxidase activity of open flower buds (fig. 6 lanes 19-20).

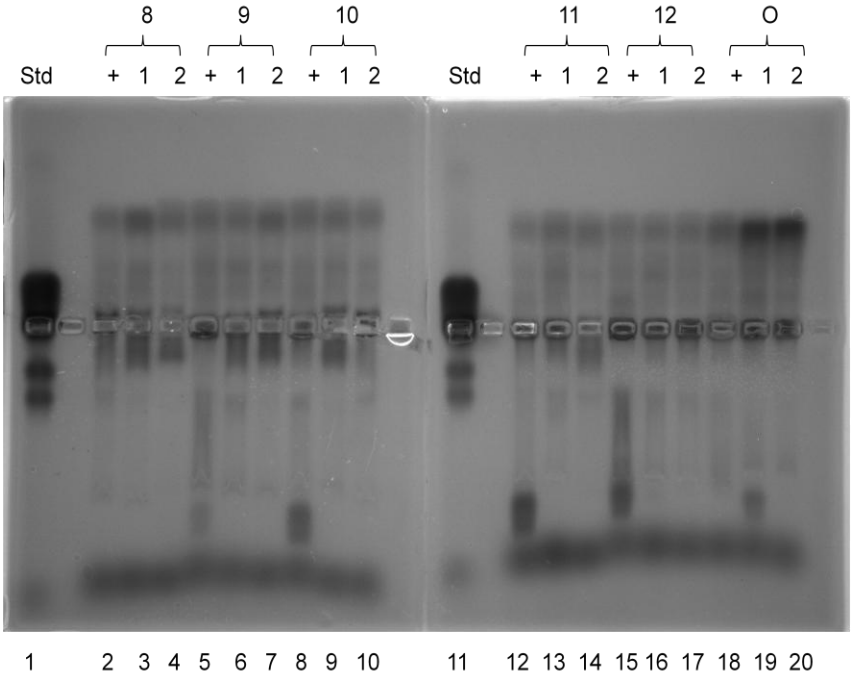


Fig. 6 Time course analysis of peroxidase activity in floral buds of wild-type (+), MS1 (1) and MS2 (2) *Brassica rapa* on a 0.8% agarose gel and stained for peroxidase activity with 4-chloro-1-naphthol. The numbers above represent age of plants in days after germination, closed flower buds (O) were harvested from bud rosettes with at least one completely open flower approximately 14 days after germination for wild type and 15 days after germination for the mutants.

Effect of Gibberellic acid on timing of anionic flower bud peroxidase

To investigate the effects of gibberellic acid on the time course of the appearance of the AS-AnPOX activity, wild-type *B. rapa* flower buds were treated with 0 or 5 µM GA_3, and peroxidase activity was analyzed at 3, 12, 24, and 48 hours after treatment (fig. 7). In control plants, the first presence of the AS-AnPOX activity was seen 12 hours (fig. 7 lane 4) after treatment, which is similar to activity seen from plants with no treatment, this activity then decreased slightly by 48 hours after treatment (fig. 7 lane 8). Flower buds treated with GA_3 showed AS-AnPOX activity 3 hours after treatment (fig. 7 lane 3), which dramatically decreased

by 12 hours after treatment and then slowly increased near its initial activity by 48 hours after treatment (fig. 7 lanes 5,7,9).

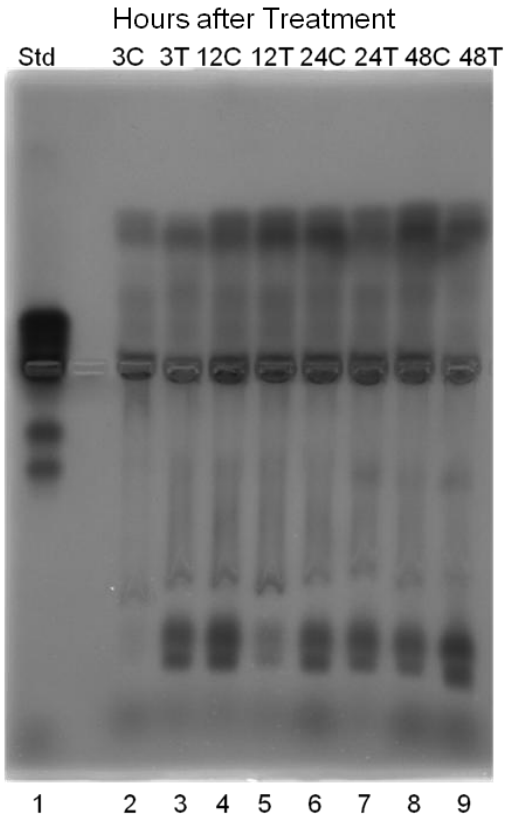


Fig. 7 Peroxidase activity of GA_3 treated wildtype *Brassica rapa*. The flower bud cluster of 8 day old plants were directly treated with 10µl of 5µM GA3 in 0.05% Triton-X100 (T) or 10µl of surfactant (C). The numbers above show the hours after treatment that the flower buds were harvested.

To investigate the effect gibberellic acid on the time course of the peroxidase activity, wildtype and mutant *B. rapa* fast plant flower buds are assayed by electrophoresis after being treated with GA_3. In wildtype plants, initial peroxidase activity was seen 12h after treatment (fig. 8 lanes 8-9), both the control and treated plants showed similar activity intensities at this initial presence. At 24h after treatment, wildtype treated plants showed an increase in the anionic peroxidase activity while the control plants showed a similar level to 12h (fig. 8 lanes 14-15). 48h after treatment, both wildtype control and treated showed an increase in anionic

peroxidase activity (fig. 8 lanes 20-21). Treated wildtype plants showed acceleration in appearance of peroxidase activity when compared to the control, which is similar to what was shown in figure 2. The time course observed in wildtype is different from what was observed in earlier treatment (fig. 7), a greater amount of activity is present in flower buds treated with GA after 24 hr, rather than after 3 hr, but the level does not decrease as observed previously (fig. 7). Both *ms1* and *ms2* mutants showed no anionic peroxidase activity at any point on the treatment timeline (fig. 8 lanes 4-7, 10-13, 16-19, 22-25). Both treated *ms1* mutants showed an increase in 10.8 cationic peroxidase activity 12h after treatment, and remains through 24 h after treatment (fig. 8 lanes 12, 17).

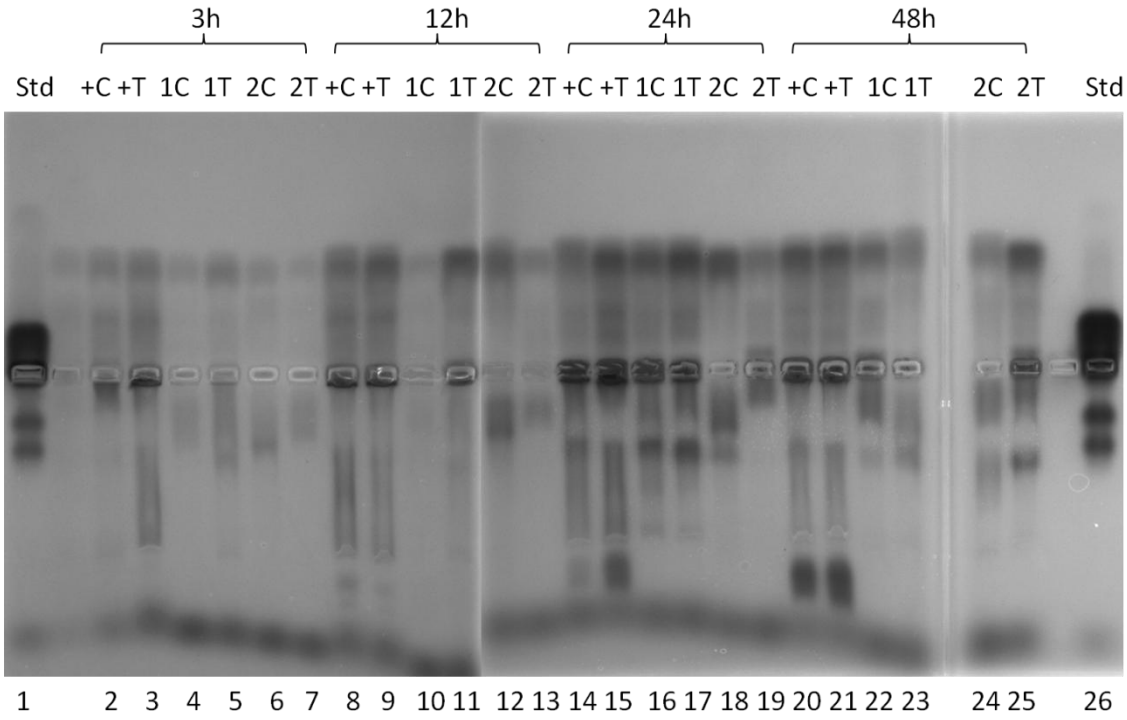


Fig. 8 Peroxidase activity of GA_3 treated wild-type (+), MS1 (1) and MS2 (2) *Brassica rapa* on a 0.8% agarose gel and stained for peroxidase activity with 4-chloro-1-naphthol. The flower bud cluster of 8 day old plants were directly treated with 10µl of 5µM GA3 in 0.05% Triton-X100 (T) or 10µl of surfactant (C). Numbers above show the hours after treatment that the flower buds were harvested.

Localization of anionic flower bud peroxidase

To investigate the localization of anionic peroxidase activity and identify other peroxidase isoenzymes that are localized to the anthers in wildtype, *ms1,* and *ms2 B. rapa*, flower buds are assayed by electrophoresis. Wild type plants showed increased AS-AnPOX activity in the flower buds and some activity in dissected anthers alone (fig. 9, lanes 2-3). *ms2* plants showed no AS-AnPOX activity in dissected anthers and low activity in flower buds (fig. 9 lanes 4-5). *ms1* plants showed relatively equal activity between flower buds and anthers (fig. 9 lanes 6-7). Both mutants showed an increase in activity of cationic peroxidase 10.8 compared to the wildtype.

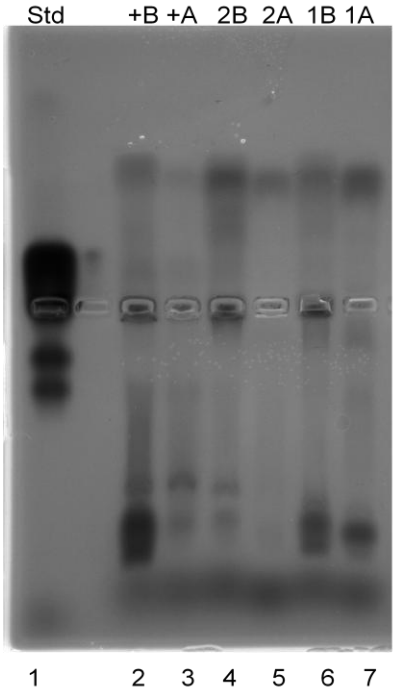


Fig. 9 Comparison of peroxidase activity from entire floral buds and dissected anthers of wild-type (+), MS2 (2) and MS1 (1) in 13 day old *Brassica rapa* on a 0.8% agarose gel and stained for peroxidase activity with 4-chloro-1-naphthol. For mutant plants, floral buds and dissected anthers were from plants harvested at 14 days after germination. Dissected anthers are represented with (A) while the whole flower bud is shown by (B).

Discussion

This study was designed to further characterize the time course and activity control of an anther specific anionic peroxidase (AS-AnPOX) found in closed flower buds isolated from *Brassica rapa* through experimentation with wild type and cytoplasmic male sterile strains *ms1* and *ms2*. The results of an initial native gel electrophoresis assay using extracts from wild type flower buds showed that buds exhibited anther specific anionic peroxidase complex (AS-AnPOX) activity with an isoelectric point of 3.6 (fig. 5). In wildtype *B. rapa* flower buds a dramatic increase in AS-AnPOX activity was seen 9 days after planting. During this time in development, the anther primordial cells are differentiating into their respective tissues, including the tapetum (Goldberg et al. 1993). Possible roles of this AS-AnPOX at this time might be to perform two tasks during this time in development. The AS-AnPOX is either building a structure, such as callose or lignin, necessary for normal tapetal development or it is controlling the timing of tapetal dehiscence by carefully controlling hydrogen peroxide levels until the pollen is ready. It is more likely that the peroxidase activity is carefully controlling the level of H_2O_2 because the activity is seen over approximately five days (fig. 5) and the construction of a material, such as lignin, would not take this long.

A recent study using male sterile rice showed the importance of modulating ROS homeostasis during late stage anther development. The *mads3-4* mutant contains aborted anther walls and improper pollen development, as well as an increase in ROS levels within the developing anther and decreased expression of ROS-scavenging genes, including those encoding peroxidases. The abnormal increase in ROS level in the *mads3-4* mutant is likely the result of the altered expression of genes involved in maintaining ROS level (Hu et al. 2011). It was suspected that a similar increase in ROS levels would be seen in the *ms1* and *ms2* mutants.

After the initial increase in AS-AnPOX activity, a steady decrease in this activity is present until the floral bud opens (fig. 5). This also supports the idea that a H_2O_2 signal is being carefully controlled until the pollen are fully mature, since as the pollen matures the activity decreases allowing for a buildup of H_2O_2 and proper anther dehiscence. Once the pollen is fully mature and the floral bud is open the AS-AnPOX activity is no longer present, even in rosettes of buds, when one bud is open the other closed buds show no AS-AnPOX activity (fig. 6). This suggests that the signal to terminate the AS-AnPOX activity is uniform throughout the rosette of buds. In 13 day old wildtype plants most AS-AnPOX activity is localized outside of the anthers, while very little AS-AnPOX activity is seen in dissected anthers alone (fig. 9). This decrease in AS-AnPOX activity in the anthers could be allowing the buildup of H_2O_2, causing programmed cell death within the anthers and proper dehiscence. It is also possible that during anther isolation the activity was lost due to the mechanical damage and time needed to dissect enough anther tissue.

This localization of activity is very different from many previous experiments which indicate that the activity is localized to the anthers of *B.* rapa and experiments done with floral mutants of *Arabidopsis thaliana* that also suggest that the activity is localized in the stamen (Kleinschmidt unpublished). After proper sequencing of AS-AnPOX, studies involving mRNA levels could show more clearly how the activity is controlled, whether the mRNA is always present in development or if transcription only occurs during a certain time period. These studies would also shed light on where these mRNA are localized in the floral bud. The mRNA may travel from another portion of the floral bud or the plant and the activity is only seen after this transportation has taken place. While the activation of AS-AnPOX may be due to the

appearance of new protein through transcription, many speculate that peroxidase activity may also be controlled by alterations during translation or modifications of the protein itself.

In both strains of cytoplasmic male sterile *B. rapa* the time course of AS-AnPOX activity is different from the wildtype. The *ms1* mutants show no AS-AnPOX activity until the buds are open, at which point only the upper band, IEP 3.6, of the doublet is observable (fig.6 lane 19). In *B.rapa*, the MS1 gene is needed for proper synthesis of sporopollenin, which is a major component of the pollen's outer most wall, the exine (Aarts et al. 1997). The function of the MS1 gene in *B. rapa* is compatible to the function of the MS2 gene in *A. thaliana*. The *ms2* mutant, in *A. thaliana*, has a limited secretion of sporopollenin precursors by the tapetal cells causing improper exine formation and nonviable pollen (Aarts et al. 1997). The lack of activity present during floral development suggests that the MS1 gene product may also control the activation of AS-AnPOX.

While the *ms1* mutants did show some AS-AnPOX activity after floral buds were opened, *ms2* mutants exhibit no AS-AnPOX activity from 8 days after planting through open buds (fig.6). The *ms2* line in *B. rapa* is like the *ms1* line in *A. thaliana*. The MS1 gene, in *A. thaliana*, encodes for a transcription factor with leucine zipper-like and plant homeo domain (PHD) finger motifs, these motifs suggest that MS1 acts as a transcriptional activator. The MS1 transcription factor affects 95 other genes, all of which are immediately downstream (Ito et al. 2007). The lack of activity present during floral development suggests that the MS1 transcription factor plays a role in the regulation of AS-AnPOX activity, most likely as a transcriptional activator of another transcription factor or kinase/phosphatase that activates the AS-AnPOX. In the *ms2* mutants of *A. thaliana*, the tapetum is abnormally vacuolated and microspores collapse soon after they are released from the tetrads. In *A.* thaliana, MS1 also plays an important role in the

breakdown of callose, a structural sugar that breaks down during the late tetraspore stage to allow pollen release, and is also proposed to induce proteases that are important to the progression of PCD (Yang et al. 2007). Along with these two suggested methods, MS1 may also control AS-AnPOX activity for proper PCD and anther dehiscence. The absence of AS-AnPOX activity in the *ms2* mutants is similar to other peroxidase activities in cytoplasmic male sterile (CMS) mutants. In CMS *Gossypium harknessii*, flower bud specific superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities decreased sharply causing premature abortion of pollen. Transcriptional analysis of Mn-SOD and APX genes showed low levels of Mn-SOD and APX mRNA expression in the anthers of the CMS cotton during pollen development (Jiang et al. 2007). In both the *ms1* and *ms2* mutants there is an increase in cationic peroxidase activity of open flower buds (fig. 6 lanes 19-20). This increase in cationic peroxidase activity could be another peroxidase compensating for the buildup of a H_2O_2.

An additional goal of this study was to determine the effect of gibberellin treatment on the time course of AS-AnPOX activity. In wildtype plants, this increase in activity was accelerated by GA_3 treatment of 8 day old plants, while the control only showed an increase 12 hours after treatments (fig. 7 lanes 3,4). This initial increase observed was followed by a decrease in the level of this peroxidase (fig. 7 lane 5). This activity pattern in wildtype plants was also seen in the mutant assay, while the initial AS-AnPOX activity was not seen until 12 hours after treatment, GA_3 treated plants showed AS-AnPOX activity before control plants (fig. 8). The fact that AS-AnPOX activity was absent till 12 hours after treatment as opposed to the 3 hours after treatment in the previous experiment suggests that while GA_3 does play a role in the activation, other factors must play into the activation. Perhaps the plants treated in figure 8 had

not developed to a point where the GA treatment could induce an early AS-AnPOX activity response.

GA_3 applications have shown similar results in the peroxidase activities of other plants. In *Catharanthus roseus*, SOD and APX activity increased under GA_3 treatment across the whole plant; GA_3 treatments also increased levels of non-enzymatic antioxidants such as ascorbic acid and reduced glutathione content (Jaleel et al. 2007). While SOD and APX peroxidases are normally used to defend against oxidative stress, their activities maybe induced in similar ways to AS-AnPOX activity, suggesting that exogenous GA_3 applications at low concentrations not only increase defense mechanisms but can also regulate floral development. Gibberellins are also thought to regulate exine formation and the PCD of tapetal cells and that direct activation of the GA induced transcription factor GAMYB is key to exine formation (Aya et al. 2009). The activation of AS-AnPOX by GA could regulate exine formation and the H_2O_2 signal needed for the proper PCD of the tapetal cells. Perhaps GA controlled transcription factors such as GAMYB allow for the transcription of products that activate AS-AnPOX. The change in activation of AS-AnPOX could also be the product of converting one GA to another isomer, in that GA_3 might not be the isomer that activates AS-AnPOX, but rather after application GA_3 is converted to another isomer. In *Brassica napus,* converting GA_3 to GA_8, has been shown to cause flowering delay, dwarf stature, reduced and lignification within the growing flower bud (Zhao et al. 2010). Further studies involving the application of GA competitor inhibitors could clarify how AS-AnPOX activity is affected by a lack of GA signal.

Gibberellin treatments have been shown to reverse impaired anther development and pollen formation found in CMS plants (Aya et al. 2009), and since this AS-AnPOX is related to anther development, it was thought that GA_3 applications could possibly rescue the AS-AnPOX

activity in *ms1* and *ms2*. Both mutants showed no AS-AnPOX activity in the time course of 3 hours to 48 hours after GA_3 treatment (fig. 8). This suggests that GA_3 application cannot reverse the AS-AnPOX activity in these two CMS mutants and that GA_3 does not directly regulate the MS1 and MS2 genes. Since studies in *A. thaliana* have shown that GA may be one of the endogenous signals involved in the regulation of jasmonic acid (JA) biosynthesis genes (Cheng et al. 2009), perhaps AS-AnPOX activity is not regulated by JA either since the JA biosynthesis genes should have been induced by the GA application. Another explanation could be that the CMS mutants may not have the proper receptors or kinases for GA and JA pathway intersection. If proper GA and JA crosstalk does not occur, the stamen does not develop properly and male sterility occurs (Cheng et al 2009, Peng 2009). Further experiments of JA applications and GA/JA applications would further clarify how these two hormones are involved in the regulation of AS-AnPOX activity and how the hormone's crosstalk controls floral and pollen development. Another possibility is that treatment after the appearance of flower buds is too late in development of the flower bud for proper activation of AS-AnPOX, perhaps earlier treatment with GA_3 might restore the activity.

As in the initial mutant assay (fig. 6), both *ms1* and *ms2* mutants show an increase in cationic peroxidase(IEP 10.8) activity 12h after GA_3 treatment, which remains through 24 h after treatment (fig. 8 lanes 12, 17). This increase in cationic peroxidase activity could be another peroxidase compensating for the lack of AS-AnPOX activity and buildup of a H_2O_2. This could also be an effect of the GA_3 treatment, much like in *C. roseus* when peroxidase activity and non-enzymatic antioxidants increased under GA_3 treatment across the whole plant (Jaleel et al. 2007).

In conclusion, this study provides information about how AS-AnPOX activity is controled in the developing floral buds of *B. rapa* and the relationship between gibberellic acid

and peroxidase activity in *ms1* and *ms2* mutants. This study confirmed an activity timeline of the AS-AnPOX as well as reaffirmed the absence of this activity in *ms1* and *ms2*. This study also showed that small concentrations of GA_3, when applied to the developing flower buds of wildtype plants, accelerated AS-AnPOX activity, but this concentration of GA_3 could not reverse the absence in activity seen in the CMS plants. Perhaps the proposed follow up experiments will be able to more clearly determine the regulation of AS-AnPOX activity in developing floral buds. While this was not the first study done on the AS-AnPOX complex, still more information is needed on the protein complex and activity.

Literature Cited

Albrecht C, Russinova E, Hecht V, Baaijens E, de Vries S. 2005. The Arabidopsis thaliana SOMATIC EMBRYOGENESIS RECEPTOR- LIKE KINASES 1 and 2 control male sporogenesis. **Plant Cell 17:** 3337–3349.

Aarts MG, Hodge, R., Kalantidis, K., Florack, D., Wilson, Z.A., Mulligan, B.J., Stiekema, W.J., Scott, R., and Pereira, A. 1997. The Arabidopsis MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. **Plant Journel 12:**615–623.

Asada K, Takahashi M. 1987 Production and scavenging of active oxygen in photosynthesis. **Photoinhibition: Topics in Photosynthesis 9:**227–287

Aya K, Ueguchi-Tanaka M, Kondo M, Hamada K, Yano K, Nishimura M, Matsuoka M. 2009. Gibberellin modulates anther development in rice via the transcriptional regulation of GAMYB. **Plant Cell 21(5):**1453-72.

Blackmore S, Wortley AH, Skvarla JJ, Rowley JR. 2007. Pollen wall development in flowering plants. **New Phytologist 174:**483-498.

Boavida LC, Becker JD, Feijo JA. 2005. "The making of gametes in higher plants". **The International Journal of Developmental Biology 49**: 595

Canales C, Bhatt AM, Scott R, Dickinson H.2002. EXS, a putative LRR receptor kinase, regulates male germline cell number and tapetal identity and promotes seed development in Arabidopsis. **Current Biology 12:** 1718–1727.

Carlsson J, Leino M, Sohlberg J, Sundström JF, Glimelius K. 2008. Mitochondrial regulation of flower development. **Mitochondrion. 8(1):**74-86

Cheng H, Song S, Xiao L, Soo HM, Cheng Z, Xie D, Peng J. 2009. Gibberellin acts through jasmonate to control the expression of MYB21, MYB24, and MYB57 to promote stamen filament growth in Arabidopsis. **PLoS Genet.** 5(3):e1000440

Coen H, Meyerowitz E. 1991. The war of the whorls: Genetic interactions controlling flower development. **Nature 353:** 31–37.

Creelman RA, Mullet JE. 1995. Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. **Proceedings of the National Acadamy of Science USA 92(10):**4114-9.

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. **Cell Molecular Life Science 57:**779–795

Delker C, Stenzel I, Hause B, Miersch O, Feussner I, Wasternack C. 2006. Jasmonate biosynthesis in Arabidopsis thaliana--enzymes, products, regulation. **Plant Biology 8(3):**297-306.

Fleet CM, Sun TP. 2005. A DELLAcate balance: The role of gibberellin in plant morphogenesis. **Plant Biology 8:** 77–85.

Goldberg, R.B, Beals TP, Sanders PM. 1993. Anther development: basic principles and practical applications. **The Plant Cell 5:**1217-1229.

Gómez-Campo, C. 1999. *Biology of Brassica Coenospecies*. Elsevier. pp. 186-189.

Gupta AS, Webb RP, Holaday AS, Allen RD.1993. Overexpression of Superoxide Dismutase Protects Plants from Oxidative Stress (Induction of Ascorbate Peroxidase in Superoxide Dismutase-Overexpressing Plants). **Plant Physiology. 103(4):**1067-1073

Hu L,Liang W,Yin C, Cui X, Zong J, Wang X, Hu J, Zhang D. 2011. Rice MADS3 Regulates ROS Homeostasis during Late Anther Development. **The Plant Cell 23:** 515–533

Ito T, Nagata N, Yoshiba Y, Ohme-Takagi M, Ma H, Shinozaki K.. 2007. Arabidopsis MALE STERILITY1 encodes a PHD-type transcription factor and regulates pollen and tapetum development. **Plant Cell. 11:**3549-62.

Iwakawa H, Shinmyo A, Sekine M.2006. Arabidopsis CDKA;1, a cdc2 homologue, controls proliferation of generative cells in male gametogenesis. **Plant Journal 45(5):**819-31.

Jiang P, Zhang X, Zhu Y, Zhu W, Xie H, Wang X.2007. Metabolism of reactive oxygen species in cotton cytoplasmic male sterility and its restoration. **Plant Cell Reports 26:**1627–1634

Kaneko M, Inukai Y, Ueguchi-Tanaka M, Itoh H, Izawa T, Kobayashi Y, Hattori T, Miyao A, Hirochika H, Ashikari M, Matsuoka M. 2004. Loss-of-function mutations of the rice GAMYB gene impair alpha-amylase expression in aleurone and flower development. **Plant Cell 16(1):**33-44.

Kleinschmidt A. 2007. unpublished data

Koornneef M, van der Veen JH. 1980. Induction and analysis of gibberellin sensitive mutants in Arabidopsis thaliana (L.) **Theoretical and Applied Genetics 58:**257–263.

Kovtun Y., Chiu W., Tena G., Sheen J. 2000. Functional analysis of oxidative stressactivated mitogen-activated protein kinase cascade in plants. **Proceedings of the National Acadamy of Sciences U.S.A. 97**:2940–2945

Levitin B, Richter D, Markovich I, Zik M. 2008. Arabinogalactan proteins 6 and 11 are required for stamen and pollen function in Arabidopsis. **Plant Journel 56:**351-63.

Mandaokar A, Kumar VD, Amway M, Browse J. 2003. Microarray and differential display identify genes involved in jasmonate-dependent anther development. **Plant Mol Biol. 52(4):**775-86.

Mandaokar A, Thines B, Shin B, Lange BM, Choi G, Koo YJ, Yoo YJ, Choi YD, Choi G, Browse J. 2006. Transcriptional regulators of stamen development in Arabidopsis identified by transcriptional profiling. **Plant Journal 46(6):**984-1008.

Millar A.A, Gubler, F. 2005. The Arabidopsis GAMYB-like genes, MYB33 and MYB65, are microRNA-regulated genes that redundantly facilitate anther development. **Plant Cell, 17:**705–21.

Miller, G. Mittler, R. 2006. Could heat shock transcription factors function as hydrogen peroxide sensors in plants? **Annual Botany 98:** 279–288.

Miller, G. Shulaev, V. Mittler, R. 2008. Reactive oxygen signaling and abiotic stress. **Physiology Plant 133:** 481–489.

Mittler, R. 2002. Oxidative stress, antioxidants and stress tolerance. **Trends Plant Science 7:**405–410.

Mittler R, Vanderauwera S, Gollery ., Van Breusegem F. 2004. Reactive oxygen gene network of plants. **Trends Plant Science 9(**10):491-98

Mylona P.V., Polidors A.N., Scandalios J.G. 2007. Antioxidant gene responses to ROS-generating xenobiotics in developing and germinated scutella of maize. **Experimental Botany 58(6):**1301-12.

Overmyer K, Brosché M, Kangasjärvi J. 2003. Reactive oxygen species and hormonal control of cell death. **Trends Plant Science 8:**335–342

Pei ZM, Murata Y, Benning G, Thomine S, Klüsener B, Allen GJ, Grill E, Schroeder JI. 2000. Calcium channels activated by hydrogen peroxide mediate abscisic acid signalling in guard cells. **Nature 406:**731–734

Peng J. 2009. Gibberellin and jasmonate crosstalk during stamen development. **Journel of Integral Plant Biol. 51:**1064-70.

Perl-Treves R, Galun E. 1991. The tomato Cu,Zn superoxide dismutase genes are developmentally regulated and respond to light and stress. **Plant Molecular Biology 17(4):**745-60.

Piskurewicz U, Jikumaru Y, Kinoshita N, Nambara E, Kamiya Y, Lopez-Molina L. 2008. The gibberellic acid signaling repressor RGL2 inhibits Arabidopsis seed germination by stimulating abscisic acid synthesis and ABI5 activity. **Plant Cell (10):**2729-45.

Remay A, Lalanne D, Thouroude T, Le Couviour F, Hibrand-Saint Oyant L, Foucher F. 2009. A survey of flowering genes reveals the role of gibberellins in floral control in rose. **Theoretical and Applied Genetics 119(5):**767-81.

Santner A, Calderon-Villalobos LI, Estelle M. 2009. Plant hormones are versatile chemical regulators of plant growth. **Nature Chemical Biolology 30:**1-7.

Schiefthaler U, Balasubramanian S, Sieber P, Chevalier D, Wisman E, Schneitz K.1999. Molecular analysis of NOZZLE, a gene involved in pattern formation and early sporogenesis during sex organ development in Arabidopsis thaliana. **Proceedings of the National Acadamy of Science USA96:**11664–11669

Stintzi A, Browse J. 2000. The Arabidopsis male-sterile mutant, opr3, lacks the 12-oxophytodienoic acid reductase required for jasmonate synthesis. **Proceedings National Acadamy Science USA. 97:**10625–10630.

Tsuji H, Aya K, Ueguchi-Tanaka M, Shimada Y, Nakazono M, Watanabe R, Nishizawa NK, Gomi K, Shimada A, Kitano H, Ashikari M, Matsuoka M. 2006. GAMYB controls different sets of genes and is differentially regulated by microRNA in aleurone cells and anthers. **Plant Journel 47(3):**427-44.

Turner JG, Ellis C, Devoto A. 2002. The jasmonate signal pathway. **Plant Cell Supplement:** 153–164.

Vranová E, Inzé D, Van Breusegem F. 2002. Signal transduction during oxidative stress. **Expperimental Botany. 53(372):**1227-36.

Villarreal F, Martín V, Colaneri A, González-Schain N, Perales M, Martín M, Lombardo C, Braun HP, Bartoli C, Zabaleta E.. 2009. Ectopic expression of mitochondrial gamma carbonic anhydrase 2 causes male sterility by anther indehiscence. **Plant Molecular Biology 70:**471-485.

Yang C,. Vizcay-Barrena G, Conner K, Wilson ZA. 2007. Male Sterility1 is required for tapetal development and pollen wall biosynthesis. **The Plant Cell 19:**3530-3548.

Yang SL, Xie LF, Mao HZ, Puah CS, Yang WC, Jiang L, Sundaresan V, Ye D. 2003. TAPETUM DETERMINANT 1 is required for cell specialization in the Arabidopsis anther. **Plant Cell 15:**2792–2804.

Yang SL, Jiang L, Puah CS, Xie LF, Zhang XQ, Chen LQ, Yang WC, Ye D.. 2005. Overexpression of TAPETUM DETERMINANT 1 alters the cell fates in the Arabidopsis carpel and tapetum via genetic interaction with EXCESS MICROSPOROCYTES 1/EXTRA SPOROGENOUS CELLS. **Plant Physiology 139:**186–191

Yaxley JR, Ross JJ, Sherriff LJ, Reid JB. 2001. Gibberellin biosynthesis mutations and root development in pea. **Plant Physiology 125(2):**627-33.

Zhao, D. 2001. Genetic control of reproductive meristems. **Annual Plant Reviews: Meristematic Tissues in Plant Growth and Development.** Sheffield Academic Press, Sheffield, UK, pp 89–142

Zhao XY, Zhu DF, Zhou B, Peng WS, Lin JZ, Huang XQ, He RQ, Zhuo YH, Peng D, Tang DY, Li MF, Liu XM. 2010. Over-expression of the AtGA2ox8 gene decreases the biomass accumulation and lignification in rapeseed (Brassica napus L.). **Journal of Zhejiang University. Science. B 11(7):**471-81.

Zhang W, Sun Y, Timofejeva L, Chen C, Grossniklaus U, Ma H.2006. Regulation of Arabidopsis tapetum development and function by DYSFUNCTIONAL TAPETUM1 (DYT1) encoding a putative bHLH transcription factor. **Development 133:**3085–3095.

Zur I, Dubas E, Golemiec E, Szechyńska-Hebda M, Gołębiowska G, Wedzony M.2009. Stress-related variation in a ntioxidative enzymes activity and cell metabolism efficiency associated with embryogenesis induction in isolated microspore culture of triticale (x Triticosecale Wittm.). **Plant Cell Reports 28(8)**:1279-87