GLUTATHIONE DYNAMICS IN ARABIDOPSIS SEED DEVELOPMENT AND GERMINATION

By

Mae Rose S. Sumugat

Thesis submitted to the faculty of Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

Masters of Science
In
Horticulture

Gregory E. Welbaum, Chairman
Ruth Grene
Vladimir Shulaev

December 2004
Blacksburg, Virginia

Keywords: *Arabidopsis thaliana*, seed development, seed germination, glutathione, glutathione reductase

GLUTATHIONE DYNAMICS IN ARABIDOPSIS SEED DEVELOPMENT AND GERMINATION

Mae Rose S. Sumugat

Abstract

Seed desiccation and germination have great potential for oxidative stress. Glutathione, one of the most abundant antioxidants in plant cells, is a crucial to the plant's defense mechanisms. To better understand glutathione's responses during these two stages, we examined its dynamics in wildtype Arabidopsis seeds and in a transgenic line containing an antisense glutathione reductase2 (anGR2) cDNA insert. Seeds from the two genotypes were compared morphologically. Glutathione levels in maturing and germinating seeds were measured by HPLC, and GR activity by native PAGE. Cytosolic glutathione was measured *in situ* by confocal laser scanning microscopy. Stress in the form of natural and accelerated ageing, and germination at high and low temperature and at low water potential was applied to both WT and anGR2 seeds to test vigor. Results show similar glutathione levels and GR activity (except during late imbibition) in WT and anGR2. In both genotypes, GSH/GSSG ratio increased and GR activity decreased during seed maturation. During imbibition, the glutathione pool becomes very reduced (<1% GSSG) and in WT seeds, GSH levels increase mostly by GSSG recycling. Cytosolic GSH in embryonic epidermal cells was estimated to be 1.1-1.6 mM. AnGR2 seeds aged faster, and were less tolerant of heat and drought stress than WT. Accumulation of glutathione during maturation indicated that glutathione is a major antioxidant in the seed during storage. Changes in GSH levels during imbibition coincided with ROS production during radicle protrusion. Under stress conditions, anGR2 seeds showed lower vigor, indicating perturbations in the ROS scavenging systems particularly GR2.

ACKNOWLEDGEMENTS

My unending gratitude to the people who make this project possible:

- Jianghong Qian and the crowd at VBI whose patience and hard work had made the HPLC protocol possible.
- Cecilia Vasquez, Shrinivasrao Mane, Jonathan Watkinson, and Verlyn Stromberg, who had made learning new laboratory techniques fun.
- Andreas Meyer and the members of Dr. Rüdiger Hell's lab who had made my stint in Germany truly memorable.
- Dr. Jerry Williams who never got tired of urging me to visit my parents and to do my work on time
- To my professors: Dr. Greg Welbaum, for the encouraging talks and for putting up with a student who is always somewhere else; Dr. Ruth Grene, for adopting me almost like her own student; and Dr. Vladimir Shulaev for his patience and generosity.

Kudos to the FSA crowd (Allan, Roy, Bibins, Botch, Vanini, Jess, Bing, Daisy, Rob) who took me in when I didn't know anybody around and morphed into the best friends one could have. I hope to see you all sometime in the Philippines.

Ming, when do we get to follow those dreams of ours?

Lastly, to my family, Pa, Ma, Darl and Ton who were all love and support.

CONTENTS

TABLES

CHAPTER 2

CHAPTER 3

FIGURES

CHAPTER 2

FIGURES

CHAPTER 3

CHAPTER 1: GLUTATHIONE AND SEED PHYSIOLOGY

I. Literature Review

A. Glutathione

Glutathione (GSH; γ-glutamylcysteinyl glycine) is a multifunctional metabolite that has drawn considerable interest due to unique structural properties, abundance, broad redox potential, and wide distribution in most living organisms. The single thiol group in the central cysteine provides its functionality as a cellular reductant and its stability is derived from the uncommon γ-glutamyl linkage. Along with ascorbate, glutathione is considered as one of the most abundant redox couple in plant cells (May et al. 1998a).

Numerous physiological functions have been attributed to glutathione in plants (for reviews, see Noctor et al. 1998; May et al. 1998a). These include serving as the cell's reservoir of non-protein reduced sulfur. Glutathione functions in plant defense mechanisms against heavy metal as a precursor of phytochelatins (Cobbett and Goldsbrough 2002), and in xenobiotic stresses as a substrate for glutathione-S-transferases (Noctor et al. 1998). Glutathione has also been implicated as a regulator of gene expression during pathogen attack and environmental stresses (Noctor et al. 1998; Noctor and Foyer 1998). GSH has also been shown to have redox control of the cell cycle (reviewed by Potters et al. 2002; May et al. 1998a).

The most well known function of glutathione is as an antioxidant participating in the ascorbate-glutathione cycle (Halliwell and Foyer 1976; Alscher 1989; Noctor et al. 1998, 2002). The cycle functions in the reduction of hydrogen peroxide (H_2O_2) to water through the action of ascorbate, glutathione, and participating enzymes. Ascorbate is oxidized during suppression of H_2O_2 and forms dehydroascorbate (DHA). Glutathione, in turn, provides the electrons needed to reduce DHA back to ascorbate through the action of dehydroascorbate reductase (DHAR). Glutathione reductase (GR), at the expense NADPH, converts oxidized glutathione (GSSG) back to its active reduced form (GSH) (Foyer and Halliwell 1976; for review, Noctor and Foyer 1998).

The ascorbate-glutathione cycle is one of the ROS (reactive oxygen species) scavenging mechanisms in cells. The ROS scavenging systems serve to protect the cell from oxidation damage caused by reactive oxygen species through regulation of their cellular levels. ROS are partially reduced forms of atmospheric oxygen and are unavoidable by-products of aerobic metabolism. They typically arise from the excitation of O_2 or transfer of one, two, or three electrons to O_2 to form, respectively, superoxide radical (O_2^-), hydrogen peroxide (H_2O_2) or a hydroxyl radical (OH^-) (Mittler 2002). ROS can react with majority of the biomolecules, resulting in oxidative stress that can be irreversible and disruptive of normal cell processes. Cellular dysfunction can occur through lipid peroxidation (Halliwell and Gutteridge 1999), hydrogen abstraction from the deoxyribose sugars of DNA and RNA (Breen and Murphy 1995), protein oxidation, and enzyme inactivation (Mittler 2002). However, ROS are now being recognized as more than just toxic molecules that need to be eliminated, but also as important redox signals that can activate different signaling pathways and affect gene expression. Being intimately connected to ROS regulation, the antioxidants, ascorbate and glutathione, can function in mediating the signal through changes in the redox state of the antioxidants (Foyer and Noctor 2003). Redox state is commonly represented as the ratio of the reduced and oxidized forms of the redox couple (i.e. GSH/GSSG, AA/DHA). The redox state of a redox couple is more completely defined as the half-cell reduction potential and the reducing capacity of the couple (Schafer and Buettner 2001).

Evidence is accumulating suggesting glutathione's participation in redox signaling, particularly in plant stress response. During abiotic stress, the expression and activity of detoxifying enzymes are affected by the level and redox state of glutathione. In pine needles, cytosolic CuZn-superoxide dismutase transcript levels decreased with application of GSH. Application of GSSG increased activity of GR without accumulation of GR transcripts (Wingsle and Karpinski 1996). During excess light stress, increase in H_2O_2 preceded rapid increase in ascorbate peroxidase (APX1 and APX2) transcripts in Arabidopsis leaves. Treatment with exogenous glutathione abolished this signal, indicating that changes in redox state of cellular glutathione mediate the redox signal (Karpinski et al. 1997; Mullineaux et al. 2000). Comparison of two γ-glutamylcysteine synthetase (γ-ECS) mutants showed 32 genes that were responsive to changes in glutathione metabolism. Under light stress, a larger number of defense-related genes were

altered in the mutants (Ball et al. 2004). γ-ECS catalyzes the rate limiting step of GSH biosynthesis and the mutants, *rax1-1* and *cad2-1/rml1*, contain 20-50% and ~5% of wild type foliar glutathione levels, respectively. Low temperature is proposed to activate redox signaling either directly through changes in H_2O_2 concentration and GSH/GSSG ratio or indirectly by affecting abscisic acid (ABA), Ca^{2+}, or salicylic acid concentrations (Kocsy et al. 2001). Redox signals from H_2O_2 mediated by glutathione and ascorbate are recognized in cross-tolerance responses of plants (Pastori and Foyer 2002). Glutathione is also implicated in biotic defense reactions. Hyper-sensitive response in barley resistant to powdery mildew attack was characterized by transient glutathione oxidation that coincided with H_2O_2 accumulation, and a subsequent increase in total foliar glutathione (Vanacker et al. 2000).

Glutathione affects the activity of several enzymes such as ABI2 – a Ser/Thr phosphatase considered to exert negative regulation on ABA action (Meinhard et al. 2002), vacuolar H^+-ATPase (Tavakoli et al. 2001), triose-phosphate isomerase and aldolase (Ito et al. 2003) through formation of intramolecular disulfide bridges (first two) or glutathionylation (latter two). Formation of disulfide bridges and glutathionylation are proposed mechanisms for glutathione-mediated signal transduction. Regulation of ABI2 activity links glutathione to hormone signaling pathways (Meinhard et al. 2002). Feedback mechanism is also apparent in glutathione metabolism as two enzymes connected to GSH synthesis, 5'adenylyl sulfate reductase (Bick et al. 2001) and γ- ECS (May et al. 1998b), are regulated posttranscriptionally by oxidative stress. This regulation, again, is mediated through changes in the glutathione redox status. Several aspects of transcription and translation are also redox regulated by glutathione. Two plant transcription factors, Hd-ZipII and Hahb-10, are known to have greater DNA binding activity in the presence of glutathione (Tron et al. 2002). PTK, a subunit of the major chloroplast RNA polymerase, responds antagonistically to glutathione and phosphorylation (Baginsky et al. 1999; Baena-Gonzales et al. 2001). Similarly, chloroplast endoribonuclease p54, involved in RNA 3'end processing, is enhanced by phosphorylation and GSSG, and inhibited by dephosphorylation and GSH (Liere and Link 1997).

There is also evidence of redox control by glutathione on the cell cycle. Levels of GSH in Arabidopsis root tips correlate positively with greater rate of cell division in the epidermal and

cortical initials, and with the lower mitotic index in the quiescent center of the root apical meristem (Sanchez-Fernandez et al. 1997). The Arabidopsis *rml1* mutant is unable to establish active postembryonic root meristems and is rescued by application of GSH (Vernoux et al. 2000). Glutathione has been shown to delay cell cycle in tobacco cell cultures through both its depletion and exogenous application, suggesting that a set balance of glutathione levels is needed to maintain normal cell physiology (Potters et al. 2000, 2004). A possible role of glutathione on the differentiation of tracheary elements has also been proposed (Henmi et al. 2001).

B. Glutathione distribution and abundance

Glutathione is the major non-protein thiol in most animals, plants, and prokaryotes. In animal cells, GSH concentrations come in the range of 1-11 mM in the cytosol, and nucleus, ~5-11 mM in the mitochondria, and less than 1 mM extracellularly (Schafer et al. 2001). In plant cells, GSH concentration outside the chloroplast is 1-2 mM in young wheat leaves (Noctor et al. 2002) and 1.8-4 mM in roots (Arabidopsis: Fricker and Meyer 2001). In the chloroplast, GSH concentration is estimated to be 1-4.5 mM (Noctor and Foyer 1998). However, the glutathione pool varies markedly in different cell types (Fricker et al. 2000; Fricker and Meyer 2000; Gutierrez-Alcala et al. 2000). Glutathione is synthesized in two ATP-dependent reactions catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and glutathione synthetase (GSH-S) wherein the constituent amino acids (glutamate, cysteine and glycine) are added stepwise. Rather than the usual α-linkage, cysteine is linked to the γ-carboxyl group of glutamate. Glutathione biosynthesis is primarily regulated by γ-ECS activity and cysteine levels (Noctor et al. 2002).

The cellular and organellar glutathione level is controlled by several factors including *de novo* synthesis, recycling by GR activity, conjugation reactions, transport from the cell, and degradation (reviewed by Noctor et al. 2002). The GSH/GSSG ratio is considered to be more influential in the control of gene expression and protein function than the absolute size of the glutathione pool. Being one of the most abundant redox couples in the cell, the redox state of glutathione (GSH/GSSG) can serve as an important indicator of the cell's redox environment. Changes in the cellular redox environment have been shown to alter signal transduction, DNA and RNA synthesis, protein synthesis, enzyme activation, and cell cycle progression (Schafer

and Buettner 2001; Arrigo 1999). The redox state of glutathione depends on the balance of oxidative processes and *in vivo* GR activity.

GR activity has been described in a number of plant species and are localized in one or more compartments namely chloroplast, mitochondria, peroxisome and cytosol. Pea and Arabidopsis organellar GR (GR1) is targeted to both chloroplast and mitochondria (Creissen et al. 1995; Cleary et al. 2002; Chew et al. 2003). Activity of each compartmentalized isoform is dependent on the plant cell type. Plastid GR from 3-week-old pea plants accounts for 77-82% of GR activity in mesophyll cells, but only 30% in root cells (Bielawski and Toy 1986). GR activity in pea leaves is predominantly in the chloroplast (77%) with the remaining activity distributed in the cytosol and mitochondria (20% and 3%, respectively) (Edwards et al. 1990). In castor bean endosperm (5 days after anthesis), GR total activity is ~90% cytosolic, 5% plastid, and 4% mitochondrial (Klapcheck et al.1990). Extraplastidial (may be assumed as cytosolic) GR comprises 18-20% of total GR activity in green mustard cotyledons (Drumm-Herrel et al. 1989). These observations indicate greater participation of the cytosolic isoform in non-photosynthetic tissues (Mullineaux and Creissen 1997).

Studies on transgenic tobacco with elevated GR activity in the different cellular locations showed higher total glutathione content but not higher GSH/GSSG ratio compared to control plants (Creissen et al. 1994; Broadbent et al. 1995). Most of the transformed lines (carrying constructs targeted to either cytosol or chloroplast or mitochondria) have increased tolerance to paraquat but not to ozone (Creissen et al. 1994). However, half of the lines with increased GR activity in both chloroplast and mitochondria showed greater tolerance to ozone fumigation. These observations demonstrate that different defense mechanisms may function in ozone and paraquat stress, though both cause elevated activity of GR (Broadbent et al. 1995).

C. Metabolism and ROS production in seed physiology

During seed development, the varied functions of glutathione may come into play throughout the whole developmental process, from the cell division involved in seed formation to protection against oxidative stress experienced during desiccation of the mature seed. Seed moisture content

and metabolic activity vary dramatically throughout the lifetime of the seed. Consequently, sources of ROS may vary considerably in different stages of seed development (reviewed by Bailly 2004).

Developing seeds undergo both photosynthesis (at the early stages) and respiration, making the photosystems, the photosynthetic electron transport and the mitochondrial electron transport system sources of ROS (Mittler 2002). During maturation drying, ROS is said to arise from the disruption of mitochondrial electron transport chain (Leprince et al. 1994). Desiccation sensitivity in recalcitrant seeds has been proposed as due to the inability to actively depress their metabolism during drying, thus increasing the chances of oxidative damage (Leprince et al. 1999).

During storage, non-enzymatic autooxidation of lipids may be a source of ROS in dry seeds when enzyme activity and metabolism is negligible. The generated ROS would then be trapped in seed tissues (McDonald 1999). Maillard products, presumably modified proteins, increase during seed storage. The Maillard reaction may be a consequence of the formation of reducing sugars through gradual hydrolysis of oligosaccharides during ageing (Sun and Leopold 1995; Murthy et al. 2002, 2003).

The onset of metabolism during germination has the potential for the greatest production of ROS in the seed lifetime. Upon imbibition, the seeds resume oxygen uptake and oxidative phosphorylation (Attucci et al. 1991; Botha et al. 1992; reviewed by Bewley 1997). At this stage, it is well documented that H_2O_2 accumulates (Puntarulo et al. 1991; Gidrol et al. 1994; Caro and Cumming 1998; Hite et al. 1999; Schopfer et al. 2001; Bailly et al. 2002; Morohashi 2002). The mitochondrial membranes can be the most important source of H_2O_2 where phosphorylation efficiency may be impaired due to poor membrane integrity particularly in aged seeds (Puntarulo et al. 1991; Smith and Berjak 1995; Benamar et al. 2003a, 2003b). Seeds may also undergo β-oxidation and glyoxylate cycles to convert fatty acids to carbohydrates before establishment of photosynthesis. Both processes also serve as sources of H_2O_2 (Bailly 2004). Other ROS such as NO (Caro and Puntarulo 1999), hydroxyl radicals (Schopfer et al. 2001), and superoxide radicals (Puntarulo et al. 1991; Gidrol et al. 1994; Schopfer et al. 2001) are also observed to accumulate

during imbibition. Thus, the antioxidant compounds and enzymes are widely considered to be of particular importance during developmental stages (Bailly 2004).

D. Seed stages and glutathione

Seed maturation and desiccation

Acquisition of tolerance to water loss is part of seed maturation in species that produce orthodox seeds. Seed are considered to be "orthodox" if they can survive dry conditions for long periods of time. A "recalcitrant" seed is defined as one that is sensitive to desiccation (Vertucci and Farrant 1995). Water loss in seed tissues generally results in mechanical stress (Vertucci and Farrant 1995), lipid peroxidation, and build-up of free radicals (Leprince et al. 1990; Hendry et al. 1992; Hoekstra et al. 2003). Two strategies in coping with water loss have been identified: (1) accumulation of non-reducing sugars and LEA (late embryogenesis abundant) proteins, and (2) increased efficiency of free-radical-scavenging systems (Leprince et al. 1993; Vertucci and Farrant 1995; Leprince et al. 1996). Non-reducing sugars may substitute for water by forming hydrogen bonds that contribute to the stabilization of proteins and membranes during desiccation (Crowe et al. 1988; Leprince et al. 1993; Hoekstra et al. 2003). Formation and accumulation of ROS is associated with desiccation-induced impairment of electron flow within the mitochondria (Leprince et al. 1996). Protection from free radical damage during desiccation is mediated by the scavenging system which includes antioxidants glutathione, ascorbic acid and tocopherols, and free-radical processing enzymes such as superoxide dismutase, peroxidases and catalase (Noctor et al. 1998; Noctor and Foyer 1998).

During wheat drying maturation, the redox state of glutathione leans towards the oxidized form lower GSH/GSSG values. However, the highest ratio was found at the end of the desiccation period (De Gara et al. 2003; Rhazi et al. 2003). In wheat kernels, increase in the number of proteic –SH groups was observed in the first 21 d of the kernel maturation, but decreased after the 28^{th} day. Transition of sulphydryl groups to disulfide bridges may explain the decrease in protein-SH groups and further suggest that GSH redox couple, along with ascorbate, participate in protein folding and maturation, besides protection against oxidative stress (Hwang et al. 1992;

De Gara et al. 2003). Protein-glutathione mixed sulfides (PSSG) were reported to accumulate in desiccated tissues, suggesting a GSH-dependent protection of protein thiol groups via formation of intermolecular disulfide bonds (Kranner and Grill 1996; Noctor et al. 2002; De Gara et al. 2003). Glutathionylation, the oxidation of protein sulphydryl groups to mixed disulfides, occurs with the reaction of a GSSG molecule with free thiol groups of proteins producing a protein-glutathione complex and a molecule of GSH (Kranner and Grill 1996; Noctor et al. 2002; De Gara et al. 2003). GSSG may not only modulate protein function by thiolation, but also protein synthesis (Fahey et al. 1980). Exogenous application of glutathione has been shown to affect the composition of seed storage proteins in soybean (Awazuhara et al. 2002).

In bean seeds, acquisition of desiccation tolerance was accompanied by high activities of GR and catalase (Bailly et al. 2001). GR activity was observed to remain constant in the first stages then abruptly decrease during desiccation of recalcitrant *Quercos* embryonic axes (Hendry et al. 1992) and *Triticum durum* kernels (De Gara et al. 2003).

Seed storage and ageing

Prolonged storage generally reduces seed viability, though the rate of deterioration varies among species. Deterioration in aged seeds has long been associated with damage by oxidative reactions. These reactions include free radical oxidations, enzymic dehydrogenations, aldehyde oxidation, and Maillard reaction (Bernal-Lugo and Leopold 1998; McDonald 1999; Murthy et al. 2002, 2003). Free radical oxidations may reflect the amount of lipid peroxidation, accumulation of H_2O_2 and the levels of glutathione in aged seeds. In both artificially and naturally aged cotton seeds, malondialdehyde (MDA), a product of lipid peroxidation, and total peroxide levels increased 3-fold within 18 months of storage (Goel et al. 2003; Goel and Sheoran 2003). A marked loss of GSH and a simultaneous, though usually not proportional, increase in GSSG levels was observed in aged sunflower (De Paula et al. 1996; Torres et al. 1997), neem (*Azadiracta indica*) (Sacande et al. 2000), watermelon cv. Nung Yu-1 (Hsu and Sung 1997), and tomato seeds (De Vos et al. 1994). However, storage conditions and length greatly influenced the seed glutathione pool in the seed. Neem seeds stored at 32% RH showed stable levels of GSH after 24 weeks (Sacande et al. 2000). Prolonged storage (natural ageing) and accelerated ageing

have similar effects in tomato seeds (De Vos et al. 1994). Accelerated ageing was accomplished by storing seeds in high humidity and elevated temperature (McDonald 1999).

Activity of the antioxidant enzymes peroxidase, catalase, ascorbate peroxidase, glutathione reductase, and superoxide dismutase has also been widely examined in ageing experiments. Decrease in germinability in stored seeds correlates with decrease in the activities of these enzymes (Goel et al. 2003; Goel and Sheoran 2003). Decline in GR activity during ageing was observed in seeds of tomato (De Vos et al. 1994), sunflower (Bailly et al. 1996; De Paula et al. 1996; Torres et al. 1997; Bailly et al. 1998) and cotton (Goel et al. 2003; Goel and Sheoran 2003). Non-enzymatic modification of the scavenging enzymes, glutathione reductase, ascorbate peroxidase, and catalase, through the Maillard reaction increases during seed storing particularly at high moisture content and temperature. The resulting lower activity of these enzymes correlates with a decline in seed vigor (Sun and Leopold 1995; Murthy et al. 2002).

The benefits of the post-harvest practice of priming have been attributed to enhanced activity of the antioxidant system. Priming resulted to increased activity of the different detoxifying enzymes (SOD, catalase, GR, ascorbate peroxidase, and dehydroascorbate reductase) in seeds of sunflower (Bailly et al. 1998; 2000) and onion (Basra et al.1994). Priming involves controlled hydration of dried seeds followed by redrying to their original moisture content (Taylor et al. 1998). This process has been shown to improve germination and vigor of seeds of numerous species (Bailly et al. 1998).

Seed imbibition and germination

In dry orthodox seeds, onset of aerobic metabolism starts with water uptake. With the accumulation of ROS, it is generally accepted that the ROS scavenging system is of particular importance at this stage. During the early hours of imbibition, antioxidants – glutathione, ascorbate and tocopherols – rapidly accumulate in the seeds (Simontacchi et al. 2003). Previously undetectable amounts of reduced ascorbate in dry *Pinus pinea* L. seeds increased by reactivation of its biosynthesis within 24 hrs of imbibition. Increase in GSH levels was mostly due to GSSG recycling (Tommasi et al. 2001). In pea seeds, GSH levels was seen to increase

significantly from 3 to 12 hrs of imbibition and afterwards, return to initial levels (Kranner and Grill 1993). GSH levels in wheat embryos decreased within the first 2 hours of imbibition, while GSSG and PSSG decreased within 10 minutes of imbibition (Fahey et al. 1980). In *Chenopodium rubrum* seeds, the increase in GSH and total glutathione coincided with radicle protrusion (Ducic et al. 2003). However, it is also clear that ROS participates in the induction of germination as several experiments have shown that application of hydrogen peroxide promotes radicle protrusion of certain seeds (Fontaine et al. 1994; Ogawa and Iwabuchi 2001). Hydrogen peroxide treatment was accompanied by an increase in the GSH levels (Fontaine et al. 1994).

During germination, increase of activity of ROS-scavenging enzymes including GR has been observed to counteract ROS production at the onset of aerobic metabolism in seeds (Fahey et al. 1980; Cakmak et al. 1993; Kranner and Grill 1993; Tommasi et al. 2001).

II. GR2 antisense line: seed maturation and germination

To better understand the role of glutathione and glutathione reductase in seed physiology, GR antisense Arabidopsis lines were developed against two GR genes characterized in the plant (Donahue et al., unpublished data). GR1 cDNAs encodes the organellar GR, and GR2 the cytosolic isoform (Kubo et al. 1993, 1998). Preliminary data have shown that nearly a third of the seeds produced by the two antisense line have abnormal phenotypes. Nonviable seeds were also found among the abnormal seeds, suggesting that lowered GR levels, and consequently GSH, may be important for seed viability (Donahue et al., unpublished data). This study will provide a more detailed examination of glutathione dynamics in developing and germinating seeds. It will also look into the effect of ageing and germination under stress on both WT and the antisense line.

Several transformants carrying either GR1 or GR2 antisense DNA inserts were created using tDNA transformation (Donahue et al., unpublished data). Antisense GR1 (anGR1) line 3 (T_2 generation) produced aborted seeds and the T_3 plants were initially used in this study. However, the anGR1 line 3 T_4 seeds failed to show a unique phenotype and experiments were discontinued. Among the GR2 transgenic lines, 1F4 showed the least amount of the GR2 mRNA, low GR2

activity in leaves, and a large percentage of seed abortions. The GR2 antisense line, 1F4 (T_5 seeds), was therefore the only line pursued with detailed experimentation.

Arabidopsis is a winter annual and its seeds are characterized by desiccation tolerance and dormancy (Koorneef and Karssen 1994). The plant has been the focus of study by plant scientist because of its small size, short life cycle, and the availability of its sequenced genome. Seed studies, though, are more limited because of the amount of effort required to isolate sufficient material (Meinke 1994). This study will provide some insight on the redox changes that occur during the different seed stages of this model plant. The aims of this study are to characterize the morphological differences between WT and the GR2 antisense line (1F4) seeds, to describe the physiological events pertaining to glutathione dynamics during seed maturation, storage and germination, and to determine germination response of 1F4 seeds to different abiotic stresses.

III. References

Alscher, RG. 1989. Biosynthesis and antioxidant function of glutathione in plants. Physiol Plant 77: 457-464.

Arrigo, A. 1999. Gene expression and the thiol redox state. Free Radic Biol Med 27: 936-944.

Attuci, S; JP Carde, P Raymond, V Saintges, A Spiteri, A Pradet. 1991. Oxidative phosphorylation by mitochondria extracted from dry sunflower seeds. Plant Physiol 95: 390-398.

Awazuhara, M; H Kim, H Hayashi, M Chino, S Kim, T Fujiwara. 2002. Composition of seed storage proteins changed by glutathione treatment of soybeans. Biosci Biotechnol Biochem 66: 1751-1754.

Baena-Gonzalez, E; S Baginsky, P Mulo, H Summer, E Aro, G Link. 2001. Chloroplast transcription at different light intensities: Glutathione-mediated phosphorylation of the major RNA polymerase involved in redox-regulated organellar gene expression. Plant Physiol 127: 1044-1052.

Baginsky, S; K Tiller, T Pfannschmidt, G Link. 1999. PTK, the chloroplast RNA polymerase-associated kinase from mustard (*Sinapsis alba*), mediates redox control of plastid *in vitro* transcription. Plant Mol Biol 39: 1013-1023.

Bailly, C; A Benamar, F Corbineau, D Come. 1996. Changes in malondialdehyde content and in superoxide dismutase, catalase and glutathione reductase activities in sunflower seeds as related to deterioration during accelerated ageing. Physiol Plant 97: 104-110.

Bailly, C; A Benamar, F Corbineau, D Come. 1998. Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. Physiol Plant 104: 646-652.

Bailly, C; A Benamar, F Corbineau, D Come. 2000. Antioxidant systems in sunflower (*Helianthus annuus* L.) seeds as affected by priming. Seed Sci Res 10: 35-42.

Bailly, C; C Audigier, F Ladonne, MH Wagner, F Coste, F Corbineau, D Come. 2001. Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. J Exp Bot 52: 701-708.

Bailly, C; R Bogatek-Leszczynska, D Come, F Corbineau. 2002. Changes in the activities of antioxidant enzymes and lipoxygenase during growth of sunflower seedlings from seeds of different vigor. Seed Sci Res 12: 47-55.

Bailly, C. 2004. Active oxygen species and antioxidants in seed biology. Seed Sci Res 14: 93-107.

Ball, L; G Accotto, U Bechtold, G Creissen, D Funck, A Jimenez, B Kular, N Leyland, J Mejia-Carranza, H Reynolds, S Karpinski, PM Mullineaux. 2004. Evidence of a direct link between glutathione biosynthesis and stress defense gene expression in Arabidopsis. Plant Cell 16: 2448-2462.

Basra, AS; B Singh, CP Malik. 1994. Amelioration of the effects of ageing in onion seeds by osmotic priming and associated changes in oxidative metabolism. Biol Plant 36: 365-371.

Benamar, A; C Tallon, D Macherel. 2003a. Membrane integrity and oxidative properties of mitochondria isolated from imbibing pea seeds after priming or accelerated ageing. Seed Sci Res 13: 35-45.

Benamar, A; J Grelet, C Tallon, E Teyssier, M Renard, P Satour, F Duval, F Montrichard, P Richomme, D Macherel. 2003b. Some observations on seed quality and mitochondrial performance. In: G Nicolas, KJ Bradford, D Come, HW Pritchard (eds), The Biology of Seeds: Recent Research Advances. CAB International, Wallingford, UK, pp. 243-249.

Bernal-Lugo, I; AC Leopold. 1998. The dynamics of seed mortality. J Exp Bot 49: 1455-1461.

Bewley, JD. 1997. Seed germination and dormancy. Plant Cell 9: 1055-1066.

Bick, J; AT Setterdahl, DB Knaff, Y Chen, LH Pitcher, BA Zilinskas, T Leustek. 2001. Regulation of the plant-type 5'adenylyl sulfate reductase by oxidative stress. Biochem 40: 9040-9048.

Bielawski, W; K Toy. 1986. Reduced and oxidized glutathione and glutathione reductase activity in tissues of *Pisum sativum*. Planta169: 267-272.

Botha, FC; GP Potgieter, AM Botha. 1992. Respiratory metabolism and gene expression during seed germination. Plant Growth Regul 11: 211-224.

Breen, AP; JA Murphy. 1995. Reactions of the oxyl radicals with DNA. Free Radical Biol Med 18: 1033-1077.

Broadbent, P; GP Creissen, B Kular, AR Wellburn, PM Mullineaux. 1995. Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. Plant J 8: 247-255.

Cakmak, I; D Strbac, H Marschner. 1993. Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. J Exp Bot 44: 127-132.

Caro A; S Puntarulo. 1999. Nitric oxide generation by soybean embryonic axes: possible effect on mitochondrial function. Free Radic Res 31: S205-S212.

Chew, O; J Whelan, AH Millar. 2003. Molecular definition of the ascorbate-glutathione cycle in Arabidopsis mitochondria reveals dual targeting of antioxidant defenses in plants. J Biol Chem 278: 46869-46877.

Cleary, SP; FC Tan, KA Nakrieko, SJ Thompson, PM Mullineaux, GP Creissen, E von Stedingk, E Glaser, AG Smith, C Robinson. 2002. Isolated plant mitochondria import chloroplast precursor proteins in vitro with the same efficiency as chloroplasts. J Biol Chem 277: 5562-5569.

Cobbett, C; P Goldsbrough. 2002. Phytochelatins and metallothioneins: roles in heavy metal detoxification and homeostasis. Annu Rev Plant Biol 53: 159-182.

Creissen, GP; P Broadbent, B Kular, H Reynolds, AR Wellburn, PM Mullineaux. 1994. Manipulation of glutathione reductase in transgenic plants: implications for plants' responses to environmental stress. Proc R Soc Edin 102B: 167-175.

Creissen, G; H Reynolds, Y Xue, P Mullineaux. 1995. Simultaneous targeting of pea glutathione reductase and of a bacterial fusion protein to chloroplasts and mitochondria in transgenic tobacco. Plant J 8: 167-175.

Crowe, JH; LM Crowe, JF Carpenter, AS Rodulf, CA Wistrom, BJ Spargo, TJ Anchordoguy. 1988. Biochim Biophys Acta 947: 367-384.

De Gara, L; MC de Pinto, V Moliterni, M D'Egidio. 2003. Redox regulation and storage processes during maturation in kernels of *Triticum durum*. J Exp Bot 54: 249-258.

De Paula, M; M Perez-Otaola, M Darder, M Torres, G Frutos, CJ Martinez-Honduvilla. 1996. Function of the ascorbate-glutathione cycle in aged sunflower seeds. Physiol Plant 96: 543-550.

De Vos, CHR; HL Kraak, RJ Bino. 1994. Ageing of tomato seeds involves glutathione oxidation. Physiol Plant 92: 131-139.

Donahue, JL; VK Stromberg, CL Cramer, R Grene. Antisense expression of glutathione reductase in *Arabidopsis thaliana* L. affects seed development, seed viability, and seedling development. Unpublished data.

Drumm-Herrel, H; U Gerhausser, H Mohr. 1989. Differential regulation of phytochrome of the appearance of plastidic and cytoplasmic isoforms of glutathione reductase in mustard (*Sinapsis alba* L.) cotyledons. Planta 178: 103-109.

Ducic, T; I Liric-Rajlic, A Mitrovic, K Radotic. 2003. Activities of antioxidant systems during germination of *Chenopodium rubrum* seeds. Biol Plant 47: 527-533.

Edwards, EA; S Rawthorne, PM Mullineaux. 1990. Subcellular distribution of multiple isoforms of glutathione reductase in leaves of pea (*Pisum sativum* L.). Planta 180: 278-284.

Fahey, RC; DL Di Stefano, GP Meier, RN Bryan. 1980. Role of hybridization state and thiol-disulfide status in the control of thermal stability and protein synthesis in wheat embryo. Plant Physiol 65: 1062-1066.

Fontaine, O; C Huault, N Pavis, J Billard. 1994. Dormancy breakage of *Hordeum vulgare* seeds: effects of hydrogen peroxide and scarification on glutathione level and glutathione reductase activity. Plant Physiol Biochem 32: 677-683.

Foyer, CH; B Halliwell. 1976. The presence of glutathione and glutathione reductase in chloroplasts: a proposed role in ascorbic acid metabolism. Planta 133: 21-25.

Foyer, CH; G Noctor. 2003. Redox sensing and signaling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119: 355-364.

Fricker, MD; M May, AJ Meyer, N Sheard, NS White. 2001. Measurement of glutathione levels in intact roots of *Arabidopsis*. J Microsc 198: 162-173.

Gidrol, X; WS Lin, N Degousee, SF Yip, A Kush. 1994. Accumulation of reactive oxygen species and oxidation of cytokinin in geminating soybean seeds. Eur J Biochem 224: 21-28.

Goel, A; AK Goel, IS Sheoran. 2003. Changes in oxidative stress enzymes during artificial ageing in cotton (*Gossypium hirsutum* L.) seeds. J Plant Physiol 160: 1093-1100.

Goel, A; IS Sheoran. 2003. Lipid peroxidation and peroxide scavenging in cotton seeds under natural ageing. Biol Plant 46: 429-434.

Gutierrez-Alcala, G; C Gotor, AJ Meyer, MD Fricker, JM Vega, LC Romero. 2000. Glutathione biosynthesis in *Arabidopsis* trichome cells. Proc Natl Acad Sci USA 97: 11108-11113.

Halliwell, B; JMC Gutteridge. 1999. Free Radicals in Biology and Medicine, 3rd ed. Oxford University Press, New York, USA.

Harada, J. 1997. Seed maturation and control of tolerance of seeds. BA Larkins, IK Vasil (eds), Cellular and Molecular Biology of Plant Seed Development. Kluwer Academic Publishers, Netherlands, pp. 545-592.

Hendry, GA; WE Finch-Savage, PC Thorpe, NM Atherton, SM Buckland, KA Nilsson, WE Seel. 1992. Free radical processes and loss of seed viability during desiccation in the recalcitrant species *Quercus robur* L. New Phytol 122: 273-279.

Henmi, K; S Tsuboi, T Demura, H Fukuda, M Iwabuchi, K Ogawa. 2001. A possible role of glutathione and glutathione disulfide in tracheary element differentiation in the cultured mesophyll cells of *Zinnia elegans*. Plant Cell Physiol 42: 673-676.

Hite, DRC; C Auh, JG Scandalios. 1999. Catalase activity and hydrogen peroxide levels are inversely correlated in maize scutella during seed germination. Redox Rep 2: 29-34.

Hsu, JL; JM Sung. 1997. Antioxidant role of glutathione associated with accelerated ageing and hydration of triploid watermelon seeds. Physiol Plant 100: 967-974.

Hwang, C; A Sinskey, H Lodish. 1992. Oxidized redox state of glutathione in the endoplasmic reticulum. Science 257: 1496-1502.

Ito, H; M Iwabuchi, K Ogawa. 2003. The sugar-metabolic enzymes aldolase and triose-phosphate isomerase are targets of glutathionylation in Arabidopsis thaliana: detection using biotinylated glutathione. Plant Cell Physiol 44: 655-660.

Klapcheck, S; I Zimmer, H Cosse. 1990. Scavenging of hydrogen peroxide in the endosperm of *Ricinus communis* by ascorbate peroxidase. Plant Cell Physiol 31: 1005-1013.

Kocsy, G; G Galiba, C Brunold. 2001. Role of glutathione in adaptation and signaling during chilling and cold acclimation in plants. Physiol Plant 113: 158-164.

Koorneef, M; CM Karssen. 1994. Seed dormancy and germination. In: EM Meyerowitz, CR Somerville (eds), Arabidopsis. Cold Spring Harbor Laboratory Press, New York, USA, pp. 313-334.

Kranner, I; D Grill. 1993. Content of low-molecular-weight thiols during the imbibition of pea seeds. Physiol Plant 88: 557-562.

Kranner, I; D Grill. 1996. Significance of thiol-disulfide exchange in resting stages of plant development. Bot Acta 109: 8-14.

Kubo, A; T Sano, H Saji, K Tanaka, N Kondo, K Tanaka. 1993. Primary structure and properties of glutathione reductase from *Arabidopsis thaliana*. Plant Cell Env 34: 1259-1266.

Kubo, A; M Aono, N Nakajima, H Saji, N Kondo, K Tanaka. 1998. Genomic DNA structure of a gene encoding glutathione reductase from *Arabidopsis thaliana*. Plant Physiol 117: 1127.

Leprince, O; R Deltour, RC Thorpe, NM Atherton, G Hendry. 1990. The role of free radicals and radical processing systems in loss of desiccation tolerance in germinating maize (*Zea mays* L.). New Phytol 116: 573-580.

Leprince, O; GAF Hendry, BD McKersie. 1993. The mechanisms of desiccation tolerance in developing seeds. Seed Sci Res 3: 231-246.

Leprince, O; GAF Hendry, NM Atherton. 1994. Free-radical processes induced by desiccation in germinating maize – the relationship with respiration and loss of desiccation tolerance. Proc R Soc Edin 102B: 211-218.

Leprince, O; GAF Hendry, NM Atherton, C Walters-Vertucci. 1996. Free radicals and metabolism associated with the acquisition and loss of desiccation tolerance in developing seeds. Biochem Transac 24: 451-455.

Leprince, O; J Buitink, FA Hoekstra. 1999. Axes and cotyledons of recalcitrant seeds *Castanea sativa* Mill. exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. J Exp Bot 50: 1515-1524.

Liere, K; G Link. 1997. Chloroplast endoribonuclease p54 involved in RNA 3'end processing is regulated by phosphorylation and redox state. Nucleic Acids Res 25: 2403-2408.

May, MJ; T Vernoux, C Leaver, M Van Montagu, D Inze. 1998a. Glutathione homeostasis in plants: environmental sensing and plant development. J Exp Bot 49: 649-667.

May, MJ; T Vernoux, R Sanchez-Fernandez, M van Montagu, D Inze. 1998b. Evidence for posttranscriptional activation of γ-glutamylcysteine synthetase during plant stress response. Proc Natl Acad Sci USA 95: 12049-12054.

Meinhard, M; PL Rodriguez, E Grill. 2002. The sensitivity of ABI2 to hydrogen peroxide links the abscisic acid-response regulator to redox signaling. Planta 214: 775-782.

Meinke, DW. 1994. Seed development in *Arabidopsis thaliana*. In: EM Meyerowitz, CR Somerville (eds), Arabidopsis. CSHL Press, New York, USA, pp. 253-296.

Meyer, AJ; MD Fricker. 2000. Direct measurement of glutathione in epidermal cells of *Arabidopsis* roots by two-photon laser scanning microscopy. J Microsc 198: 174-181.

Morohashi, Y. 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. J Exp Bot 53: 1643-1650.

Mullineaux, PM; GP Creissen. 1997. Glutathione reductase: regulation and role in oxidative stress. In: JG Scandalios (eds), Oxidative Stress and the Molecular Biology of Antioxidant Defenses. Cold Spring Harbor Laboratory Press, New York, USA, pp. 667-713.

Mullineaux, P; L Ball, C Escobar, B Karpinska, G Creissen, S Karpinski. 2000. Are the defense signaling pathways integrated in the regulation of Arabidopsis antioxidant defence gene expression in response to excess excitation energy? Phil Trans R Soc Lond B 355: 1531-1540.

Murthy, UMN; Y Liang, PP Kumar, WQ Sun. 2002. Non-enzymatic protein modification by the Maillard reaction reduces the activities of scavenging enzymes in *Vigna radiata*. Physiol Plant 115: 213-220.

Murthy, UMN; PP Kumar, WQ Sun. 2003. Mechanisms of seed ageing under different storage conditions for *Vigna radiata* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. J Exp Bot 54: 1057-1067.

Noctor, G; AM Arisi, L Jouanin, KJ Kunert, H Rennenberg, CH Foyer. 1998. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49: 623–647.

Noctor, G; CH Foyer. 1998. Ascorbate and glutathione: keeping active oxygen under control. Annu Rev Plant Physiol Plant Mol Biol 49: 249-279.

Noctor, G; L Gomez, H Vanacker, CH Foyer. 2002. Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signalling. J Exp Bot 53: 1283-1304.

Ogawa, K; M Iwabuchi. 2001. A mechanism for promoting the germination of *Zinnia elegans* seeds by hydrogen peroxide. Plant Cell Physiol 42: 286-291.

Pastori, GM; CH Foyer. 2002. Common components, networks, and pathways of cross-tolerance to stress: the central role of "redox" and abscisic acid-mediated controls. Plant Physiol 129: 460-468.

Potters, G; N Horemans, RJ Caubergs, H Asard. 2000. Ascorbate and dehydroascorbate influence cell cycle progression in tobacco cell suspension. Plant Physiol 124: 17-20.

Potters, G; L De Gara, H Asard, N Horemans. 2002. Ascorbate and glutathione: guardians of the cell cycle, partners in crime? Plant Physiol Biochem 40: 537-548.

Potters, G; N Horemans, S Bellone, RJ Caubergs, P Trost, Y Guisez, H Asard. 2004. Dehydroascorbate influences the plant cell cycle through a glutathione-independent reduction mechanism. Plant Physiol 134: 1479-1487.

Puntarulo, S; M Galleano, RA Sanchez, A Boveris. 1991. Superoxide anion and hydrogen metabolism in soybean embryonic axes during germination. Biochim Biophys Acta 1074: 277-283.

Rhazi, L; R Cazalis, E Lemelin, T Aussenac. 2003. Changes in the glutathione thiol-disulfide status during wheat grain development. Plant Physiol Biochem 41: 895-902.

Sacande, M; F Hoekstra, A van Aelst, CHR De Vos. 2000. Is oxidative stress involved in the loss of neem (*Azadirachta indica*) seed viability? Seed Sci Res 10: 381-392.

Sanchez-Fernandez, R; M Fricker, L Corben, N White, N Sheard, C Leaver, M van Montagu, D Inze, M May. 1997. Cell proliferation and hair tip growth in the Arabidopsis root are under mechanistically different forms of redox control. Proc Natl Acad Sci 94: 2745-2750.

Schafer, FQ; GR Buettner. 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic Biol Med 30: 1191-1212.

Schopfer, P; C Plachy, Frahry G. 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in geminating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol 125: 1591-1602.

Simontacchi, M; L Sadovsky, S Puntarulo. 2003. Profile of antioxidant content upon developing of *Sorghum bicolor* seeds. Plant Sci 164: 709-715.

Smith, MT; P Berjak. 1995. Deteriorative changes with the loss of viability of stored desiccation-tolerant and desiccation-sensitive seeds. In: J Kigel, G Galili (eds), Seed Development and Germination. Marcel Dekker, Inc., New York, USA, pp. 701-746.

Sun, WQ; AC Leopold. 1995. The Maillard reaction and oxidative stress during ageing of soybean seeds. Physiol Plant 94: 94-104.

Tavakoli, N; C Kluge, D Golldack, T Mimura, K Dietz. 2001. Reversible redox control of plant vacuolar H^+-ATPase activity is related to disulfide bridge formation in subunit E as well as subunit A. Plant J 28: 51-59.

Taylor, AG; PS Allen, MA Bennett, KJ Bradford, JS Burris, MK Misra. 1998. Seed enhancements. Seed Sci Res 8: 245-256.

Tommasi, F; C Paciolla, MC de Pinto, L De Gara. 2001. A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. J Exp Bot 52: 1647-1654.

Tron, AE; CW Bertoncini, RL Chan, DH Gonzalez. 2002. Redox regulation of plant homeodomain transcription factors. J Biol Chem 277: 34800-34807.

Vanacker, H, TLW Carver, CH Foyer. 2000. Early H_2O_2 accumulation in mesophyll cells leads to induction of glutathione during the hyper-sensitive response in the barley-powdery mildew interaction. Plant Physiol 123: 1289-1300.

Vernoux, T; RC Wilson, KA Seeley, J Reichheld, S Muroy, S Brown, SC Maughan, CS Cobbett, M Van Montagu, D Inze, MJ May, ZR Sung. 2000. The ROOT MERISTEMLESS1/CADMIUM SENSITIVE2 gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. Plant Cell 12: 97-109.

Vertucci, CW; AJM Farrant. 1995. Acquisition and loss of desiccation tolerance. In: J Kigel, G Galili (eds), Seed Development and Germination. Marcel Dekker, Inc., New York, USA, pp. 237-271.

Wingsle, G; S Karpinski. 1996. Differential redox regulation by glutathione of glutathione reductase and CuZn-superoxide dismutase gene expression in *Pinus sylvestris* L. needles. Planta 198: 151-157.

CHAPTER 2: ARABIDOPSIS SEED DEVELOPMENT AND AGEING

INTRODUCTION

Glutathione functions as an antioxidant, a redox buffer, a possible participant in redox-mediated signaling events, and a protector of protein thiol groups during occasions of oxidative stress. As such, glutathione has long been evaluated for its function during seed development, particularly during seed desiccation when unregulated oxidative damage during dehydration may affect seed viability (Leprince et al. 1996). During desiccation, di- and oligosaccharides accumulate and provide the necessary hydrogen bond interactions needed to maintain phospholipid and protein structure (Hoekstra et al. 2001). Seeds also have to deal with cell shrinkage, molecular crowding and active metabolism during dehydration. Membrane folding, vesiculation, and reduced respiration rates have been observed in desiccation tolerant seeds and seedlings (Leprince et al. 1998; Hoekstra et al. 2003). Timely down-regulation of metabolism reduces the generation of ROS and minimizes membrane damage during dehydration (Leprince et al. 1999, 2000).

The ROS that nevertheless are produced can be dealt by the free-radical scavengers including detoxifying enzymes and antioxidant compounds. The ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate-recycling enzymes, catalase and glutathione reductase (GR) are only active up to a certain low water content, whereas molecular antioxidants such as glutathione, ascorbate and tocopherols can also be active at air dry state (Hoekstra et al. 2003). Glutathione is considered the main antioxidant compound during seed maturation and storage since ascorbate increases during early seed development but decreases during desiccation (De Tullio and Arrigoni 2003; De Gara et al. 2003).

Loss of seed vigor during storage and ageing has been correlated with the reduced activity of ROS scavenging enzymes (McDonald 1999; Goel and Sheoran 2003; Goel et al. 2003). Similarly, the recovering effects of priming on aged seeds were associated with increased activity of catalase, SOD and GR, along with increase in GSH content (Bailly et al. 1998, 2000). This chapter examines glutathione redox state and GR activity during Arabidopsis seed maturation and ageing in both WT and anGR2 (1F4) seeds.

METHODOLOGY

I. Plant Material

Plant growth conditions. *Arabidopsis thaliana* ecotype Columbia (WT) and GR2 antisense line, 1F4, plants were grown in a soilless mix (Sunshine Mix) and maintained inside growth chambers (~90 μmol s^{-1} m^{-2} illumination and a 14-hour photoperiod). The plants were watered every 3 days. Humidity was maintained at 70-75% and temperatures were 22°C day/18°C night.

Seed collection and storage. To determine silique ages, flowers were marked with colored ink at the peduncle axils during the day of full bloom (anthers and stigma at the same height). Immature seeds from both WT and 1F4 were collected 10, 16 and 18-20 days after flowering (DAF). Seeds were carefully dissected out of the siliques by slicing through the middle using a needle and sharp forceps. The collected seeds were frozen in liquid N_2 (except those for imaging and germination assay), and stored at -80°C until the different experiments were performed. When flowering had ended, all remaining siliques were allowed to mature and dry. The air-dried seeds were separated from silique material by sieving, and stored in glass vials at ambient laboratory conditions (22-25°C, 40-60% RH). The resulting water content of the seeds was approximately 10% (FW basis).

II. Morphological Characterization

Screening for abnormal seeds. 1F4 seeds were dissected out from 10 or more siliques 10, 16 and 18-20 (dehiscing) days after flowering, and examined for dissimilarity in color and shape as compared to the WT seeds of the same age. The seeds were examined under a binocular stereomicroscope (Olympus VMZ stereomicroscope, 10-40x).

Germination assays. For the immature seeds, 9 or more siliques were chosen and divided into 3 replicates for each stage of development (10, 16 and 18-20 DAF). To test for germinability, the seeds were spread over water-soaked germination paper (Anchor Paper Blue Blotter, St. Paul, MN, US) inside tri-sectioned Petri dishes (Fisher Scientific, Pittsburg, PA, US). The Petri dishes

were sealed with paraffin film (American National Can, Greenwich, CT, US) to prevent desiccation and the seeds were incubated in light (14-hr photoperiod) at 22°C day/18°C night temperature regime. Similar germination tests were conducted for recently harvested and stored dry seeds. Radicle protrusion was recorded as germination and checked every day for 10 days. The same materials (germination paper, Petri dishes, etc.) were used in all germinations tests in this study.

Fresh and dry weight determination. Seed weights were determined as specified in the International Rules for Seed Testing (1985). Arabidopsis seeds of designated stages were dissected from siliques, transferred to pre-weighed aluminum boats and immediately weighed using an analytical balance (Mettler Toledo AE 240, Columbus, OH, US). The seeds were counted afterward and then dried at 101 ± 2°C for approximately 17 hr. After drying, seeds were cooled for 30-45 minutes at 35% RH and then re-weighed.

Seed statistics. Mean time germination (T_{50}) was computed according to the summation equation provided by Leon and Knapp (2004) for samples with more than 50% germination; otherwise, probit analysis was employed (Wilson et al. 1989). All computations were done in Microsoft® Excel Professional 2000 (Microsoft Corporation, US).

III. **Glutathione Dynamics**

A. Determining levels of reduced and oxidized glutathione by HPLC

Extraction of thiols from seeds: The extraction protocol was adapted from Noctor and Foyer (1998). The glutathione redox couple, GSH and GSSG, was measured in 10, 14, 16 and 18-20 DAF seeds, and naturally aged seeds from both WT and 1F4 plants Approximately 15-30 mg seed samples were ground in 1 mL 0.3 N HCl with 1 mM EDTA and 0.1 M ascorbic acid using glass tissue grinders (7 mL Pyrex brand, Fisher Scientific, Pittsburg, PA, US) and centrifuged for 15 min at 14000xg and 4°C. The supernatant was transferred to fresh Eppendorf tubes and centrifuged at the same setting for another 30 min.

Derivatization. Derivatization and chromatography were based on Jones et al. (1998). Three hundred μL aliquots were mixed with 150 μL iodoacetic acid solution (7.5 mg/mL). pH of the samples was raised to 9.0 $\pm$ 0.2 by adding approximately 75 μL of concentrated KOH/tetrahydroborate solution. The borate solution was prepared by mixing 5.6 g KOH, 50 g $K_2B_4O_7 \bullet 4H_2O$, and 100 mL water in a plastic bottle and allowed to stand overnight. The solution was then filtered and stored at room temperature. After checking the pH (9.0 ± 0.2) of the seed extracts using short-range pH paper (Hydrion 8.2-9.8, Fisher Scientific, Pittsburg, PA, US), the extracts were incubated for approximately 15 minutes. Three hundred μL dansyl chloride (Fluka Biochemica, Buchs, Switzerland) in acetone (20 mg/mL) was then added to all samples and vortexed. Derivatization proceeded for approximately 20 hours in the dark at room temperature. Excess dansyl chloride was removed by adding 500 μL chloroform to the seed extracts. The mixtures were then centrifuged for 5 min at 14000xg to separate the aqueous and chloroform layers. The samples were stored in the dark at 4°C until HPLC analysis.

Standards: GSH and GSSG standards were dissolved 0.3 N HCl with 1 mM EDTA and 0.1 M ascorbic acid, and were derivatized similar to the seed samples. Each point in the calibration curve represented individually derivatized solutions. GSSG and GSH were Sigma (98%) (St. Louis, MO, US) and Acros Organics products (98%) (Fisher Scientific, Pittsburg, PA, US) products, respectively. All other chemicals, except the HCl solution (Fisher Scientific, Pittsburg, PA, US), were obtained from Sigma (St. Louis, MO, US).

Recovery: Recovery was determined by taking separate 300 μL aliquots from the seed extracts after centrifugation. The seed extracts were spiked with 20 μL of both GSH and GSSG standards. Dummy samples using 300 μL of the homogenization buffer instead of actual seed extracts were prepared and the same amount of GSH and GSSG standards were added. Measured thiols from the dummy samples were used to calculate % recovery in each sample. Percent recovery was computed using the equation:

$$\% \text{ recovery} = \frac{\text{measured spiked sample}}{\text{measured spike + measured sample}} \times 100$$

Chromatography: From the aqueous layer of the derivatized samples, 150 µL aliquots are taken and transferred to labeled injection tubes. The tubes were then arrayed into the autosampler of a Shimadzu VP series HPLC system (Columbia, MD, US) and kept at 4°C. Injection volume was 20 µL. Two mobile phases were used. Mobile phase A was composed of 80% methanol. Mobile phase B was acetate-buffered methanol solution prepared by mixing 640 mL methanol, 200 mL acetate stock, 125 mL glacial acetic acid and 50 mL water. The time program for each run is described in Table 2-1. The 3-aminopropyl column (4.6mm X 25 cm, 5mm) (from Alltech, Deerfield, IL, US and Waters, Milford, MA, US) was equilibrated with mobile phase A 30 minutes before the start of the HPLC run. Area integration of peaks in the resulting chromatograms was calculated automatically by the operating software, Shimadzu Class VP Version 7.2 (Build 23), of the HPLC system and set for valley-to-valley integration with a threshold of 50 unit areas. Manual integration was done using the manual integration function in the HPLC software when peaks were not automatically recognized.

Table 2-1: Chronological time program for HPLC analysis

Minutes	**Pump A**	**Pump B**	**Event**
0	80%	20%	Initial
10	80%	20%	
15	20%	80%	
32	20%	80%	
34	80%	20%	
44	80%	20%	Return to initial
44.10			Stop

Total flow = 0.8 mL/min

Pump A = mobile phase A

Pump B = mobile phase B

B. Glutathione reductase activity gels

Protein extraction. Soluble proteins from 16 and 20 DAF WT and 1F4 seeds were extracted using glass tissue grinders (7 mL Pyrex brand, Fisher Scientific, Pittsburg, PA, US) from 10-20 mg of 16 and 20 DAF WT and 1F4 seeds in the presence of a homogenization buffer composed of phosphate-EDTA buffer (pH 7.0; 0.05 M KH_2PO_4/K_2HPO_4; 0.001 M EDTA), 0.05% Triton, 0.001 M ascorbic acid and 2% PVPP. The homogenate were centrifuged at maximum speed for 5 min and the supernatant transferred to fresh tubes. The extracts were again centrifuged at maximum speed for 15 minutes. Extraction and centrifugation were performed at 4°C.

Protein quantification. Amount of protein in the extracts were determined by mixing aliquots of the extracts with diluted Bio-Rad (Hercules, CA, US) protein dye according to the Bio-Rad protein assay procedure for 96-well microtiter plates (Fisher Scientific, Pittsburg, PA, US). Absorbance was measured at 595 wv in a microplate reader (Molecular Devices, Sunnyvale, CA, US). A new standard curve based on commercial BSA protein (Sigma, Louis, MO, US) was always created along with each set of samples.

Gels and loading. Fresh discontinuous native acrylamide gels (1.5-2 mm thickness) were prepared in Bio-Rad upright mini-cells for native gel electrophoresis. The separating gels were 10% acrylamide (without SDS and glycerol) and the stacking gel is 4% acrylamide. The same amount (50 for 1.5 mm gels or 70 μg for 2 mm gels) of protein from each sample, mixed with 1/4 vol sample buffer (500 μL 0.5 M Tris-HCl, pH 6.8; 400 μL 100% glycerol; 100 μL 0.5% bromophenol blue) was loaded in each well. The gels were run at 20-22 mA/gel in Laemmli buffer (0.025 M Tris, 0.2 M glycine) for 1.75-2 hr at 4°C.

Gel staining and analysis. To visualize the bands, the native gels were transferred to 100 mL staining solution (0.25 M Tris, pH 7.5-8.0) containing 0.4 mM NADPH, 3.4 mM GSSG, 10 mg DPIP (2,6-dichlorophenol-inophenol), and 10 mg MTT (tetrazolium). The gels were incubated for 30-45 minutes at room temperature and low agitation. Protein bands were visualized by precipitation of formazan, the reduced form of MTT. GSH produced in the staining solution by the activity of the gel-bound GR on GSSG causes reduction of DPIP, which, in turn, reduces

MTT. Gels were developed for GR activity with and without GSSG. GR isoforms were distinguished as the bands that were absent in the gels stained without GSSG. GR2 was identified as the heavier protein compared to GR1 (Donahue et al., unpublished data). Digital images of the gels were taken using the AlphaImager 2000 system (Alpha Innotech Corporation, San Leandro, CA, US). Band intensity was quantified using ImageJ 1.32j software (W. Rasband, NIH, US), utilizing its 'analyze gel' function. All stains, GSSG, β-NADPH (enzyme grade) and other chemicals, unless otherwise stated, were obtained from Sigma (St. Louis, MO, US).

III. Natural ageing and controlled deterioration test for seeds

Controlled deterioration test. The deterioration test used on the Arabidopsis seeds was adapted from Tesnier et al. (2002) and Clerkx et al. (2004). Four replicates of 80 to 130 WT and 1F4 seeds were placed inside folded weighing paper and equilibrated at 85% relative humidity at 15°C (in the dark). The 85% RH environment was created by suspending the wrapped seeds over a saturated solution of KCl at certain temperatures (Greenspan 1977). After 3 days, the seeds were transferred to 40°C and stored for 5 and 7 days. Control 0-day seeds were immediately transferred after equilibrium to 32% RH, resulting in a seed moisture content of approximately 6% (Tesnier et al. 2002; Clerkx et al. 2004). Both control and aged seeds were stored at 4°C (5-7 days) until germination was tested. Germination was tested on water-saturated germination paper in Petri dishes in conditions of 25°C 14-hr day periods. Seeds were examined for radicle protrusion using a binocular dissecting microscope (Olympus VMZ stereomicroscope) twice a day with approximately 12 hr interval for the first 3 days and once every day afterwards till 14 days from start of imbibition.

Freshly harvested and naturally aged seeds. Dried seeds collected from the WT and 1F4 plants were air-dried for 3 days and then germinated over water-moistened germination paper in Petri dishes. The rest of the seeds were transferred into glass vials and stored at ambient laboratory conditions (22-25°C, 40 -60% RH) for 9 months. The water content in the seeds was 10% (FW basis). Germination conditions for both freshly harvested and naturally aged seeds were similar to those used for the immature seeds (Chapter 2, Section II, Germination assays).

RESULTS

SEED MATURATION:

Weight accumulation and desiccation. Examination of Arabidopsis seeds started 10 days after flowering (DAF) and commenced until dehiscence of the fruit (20 DAF) (Figure 2-1). In both WT and 1F4 seeds, no obvious increase in fresh weight was observed from 10 to 18 DAF. WT seeds reached its heaviest at 18 DAF while 1F4 seeds at 16 DAF. The rapid decline in fresh weight, losing approximately 0.01 mg between 18 and 20 DAF, described desiccation of the seeds (Figure 2-2). Accumulation of reserve materials was seen as dry weight increase throughout 10 to 20 DAF. There was no significant difference between the dry weight of WT and 1F4 seeds at dehiscence (Figure 2-3). Comparison for the water content of both seed types showed that WT seeds contain significantly more water through 14 to 18 DAF. At silique dehiscence (20 DAF), WT and 1F4 seeds have 42 $\pm$ 4 % and 27 $\pm$ 8.6 % water content, respectively. Water content in the seeds was more variable at the time of dehiscence (Figure 2-4).

Morphology of maturing seeds. Observed within 10 DAF 1F4 siliques were seeds that appear colorless or white, indicating lack of chlorophyll pigmentation (see Figure 2-1). This seed phenotype was observed only in 10 to 14 DAF 1F4 siliques and not among WT seeds (Table 2-2). All WT seeds at the same stages were photosynthesis-capable. Brown, small incompletely developed seeds, labeled in Table 2-2 as abortions, were also found in both genotypes throughout the days examined. 1F4 siliques had markedly more abortions than WT siliques.

Development of germinability. Seeds 10, 16 and 20 DAF were collected and incubated on moist germination paper to enable precocious germination of immature seeds (Figure 2-5). Indications of delayed maturity in 1F4 seeds were seen in the germination cumulative time courses of the immature seeds. The most prominent difference was observed with the 10 DAF seeds. Seeds germination assays for 10 DAF seeds did not reach 100% germination even after a month of imbibition (80% for WT and 65% for 1F4). 20 and 16 DAF seeds showed almost complete germinability within 12 days of imbibition (Figure 2-5), with, unsurprisingly, the older seeds

having a faster rate of germination. In both genotypes, the T_{50} for 10, 16 and 20 DAF seeds were approximately 10-14 days, 7-9 days and 4-5 days, respectively (Table 2-3). Rate of germination was only significantly different between genotypes in 10 and 20 DAF seeds.

Glutathione redox status in maturing seeds. The changes in water content and dry weight of the seed during maturation render measurement of metabolites on a fresh weight basis misleading in terms of quantity found in individual seeds. For this reason, glutathione levels were presented on a per seed basis. From 10 to 14 DAF, the GSH values in both genotypes ranged 5-6 pmol per seed (Figure 2-6). GSH levels increase (1.4 times for WT and 1.8 times for 1F4 from 14 DAF levels), though not significantly, at 16 DAF with WT seeds containing 8 pmol per seed and 1F4 9 pmol per seed. Over the course of seed maturation, the highest level of GSH was observed at the point where the seeds are almost completely desiccated (18-20 DAF). At this point, the GSH level in both WT and 1F4 was 18 and 14 pmol per seed, respectively. The 18-20 DAF GSH levels were 3 and 2.5 times higher compared to WT and 1F4 10 DAF seeds, respectively. GSSG levels throughout maturation remained constant (2-3 pmol per seed) for both genotypes (Figure 2-6). The accumulation of reduced glutathione occurred as the seeds acquired desiccation tolerance. Concentration of GSH and GSSG due to water loss was accounted for as the glutathione levels were calculated on a per seed basis. GSH/GSSG ratio during maturation also increased steadily as the seeds dehydrate (Figure 2-7). The only stage in which the ratio between WT and 1F4 seeds was significantly different was on 14 DAF where the WT ratio was twice that of 1F4. The redox state of glutathione in 1F4 became more reduced at 16 DAF but at siliques dehiscence, WT and 1F4 seeds had similar GSH/GSSG ratio.

Glutathione reductase activity during seed desiccation. Enzyme activity of the two glutathione reductase isoforms (GR1 and GR2) in Arabidopsis was visualized using native-PAGE. Band intensities were compared and enzyme activity in WT 16 DAF seeds was set as 100%. A slight decrease in activity in both GR1 and GR2 occurred in both WT and 1F4 during seed desiccation (Figure 2-8). At 20 DAF when the siliques dehisce, GR1 and GR2 activity in WT seeds were only 80% of the activity at 16 DAF. A similar response to water loss was also observed in 1F4. GR2 activity in 1F4 seeds was not lower as expected.

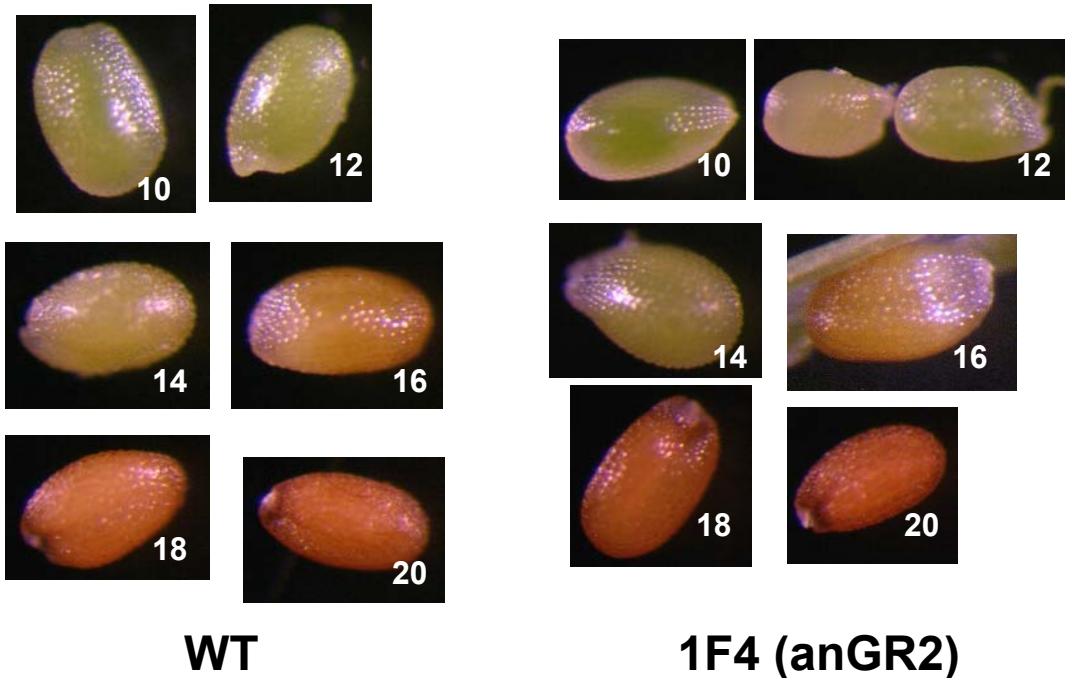


Figure 2-1: WT and 1F4 (antisense GR2 line) maturing seeds. Numbers refer to the days after flowering (DAF). The left seed (pale in color) in the 1F4 12 DAF image was a mutant phenotype. The other 1F4 12 DAF seed was considered similar to WT seeds.

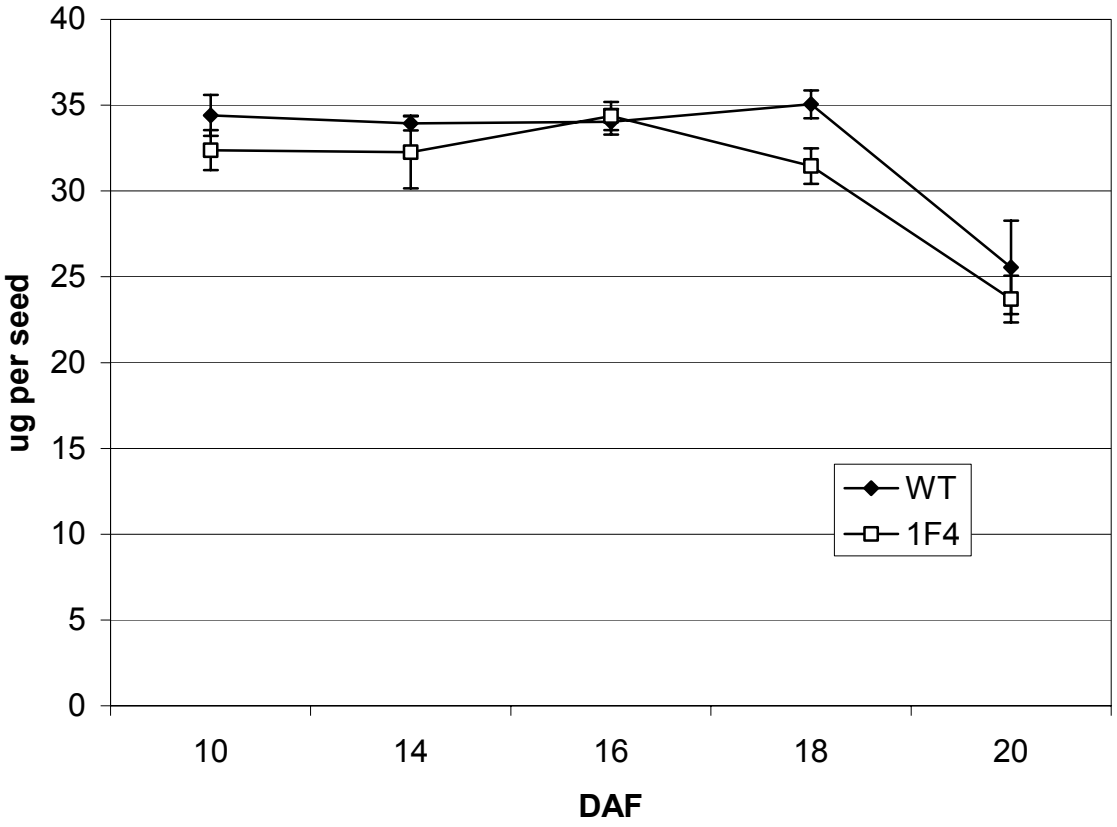


Figure 2-2: Average fresh weight of WT and 1F4 maturing seeds (mean ± SE; n = 3) at different days after flowering (DAF). Only 18 DAF was statistically significant ($p \leq 0.05$) when comparing fresh weight between genotypes.

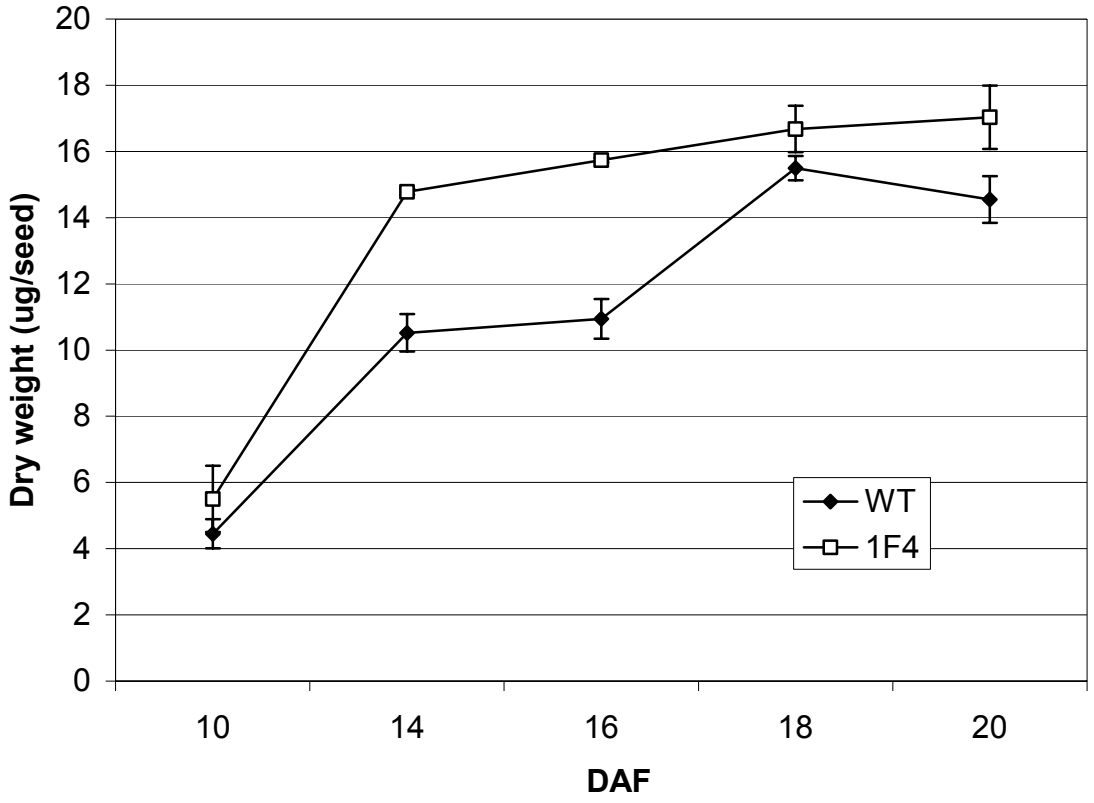


Figure 2-3: Average dry weight of WT and 1F4 maturing seeds (mean ± SE; n = 3) at different days after flowering (DAF). Only14 and 16 DAF were significantly different ($p \leq 0.05$) when comparing between genotypes. Error bars are not visible when they are smaller the height of the point symbol.

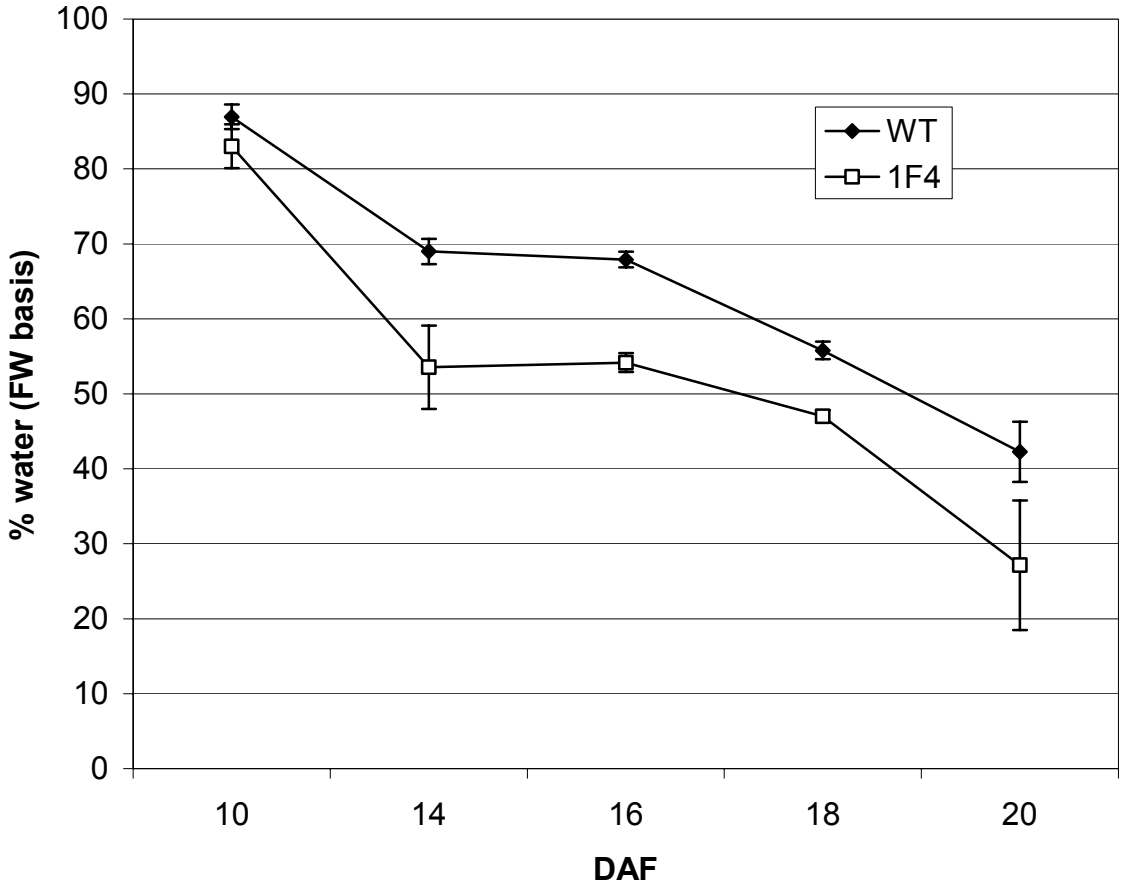


Figure 2-4: Water content of maturing WT and 1F4 seeds on the fresh weight basis (mean $\pm$ SE; n = 3). When comparing between genotypes, 10 and 20 DAF are not significantly different while the rest are at $p \leq 0.05$.

Table 2-2: Morphology of WT and 1F4 maturing seeds at different days after flowering. Seeds were obtained from 2-3 siliques each random plant in a lot of 36 plants. White or colorless seeds are labeled below as pale seeds, and brown, incompletely developed seeds are labeled as abortions.

WT	***Total # seeds examined***	***normal green***	***pale seeds***	***% pale seeds***	***abortions***	***% aborted***
10d	461	457	0	0	4	0.9
12d	502	501	0	0	1	0.2
14d	750	748	0	0	2	0.3
16d	757	756	0	0	1	0.1
18d	629	628	0	0	1	0.2
≥20d	983	974	0	0	9	0.9

1F4	***Total # seeds examined***	***normal green***	***pale seeds***	***% pale seeds***	***abortions***	***% aborted***
10d	1201	1080	114	9.5	7	0.6
12d	941	871	60	6.4	10	1.1
14d	1018	985	9	0.9	24	2.4
16d	909	903	0	0	6	0.7
18d	944	931	0	0	13	1.4
≥20d	1001	979	0	0	22	2.2

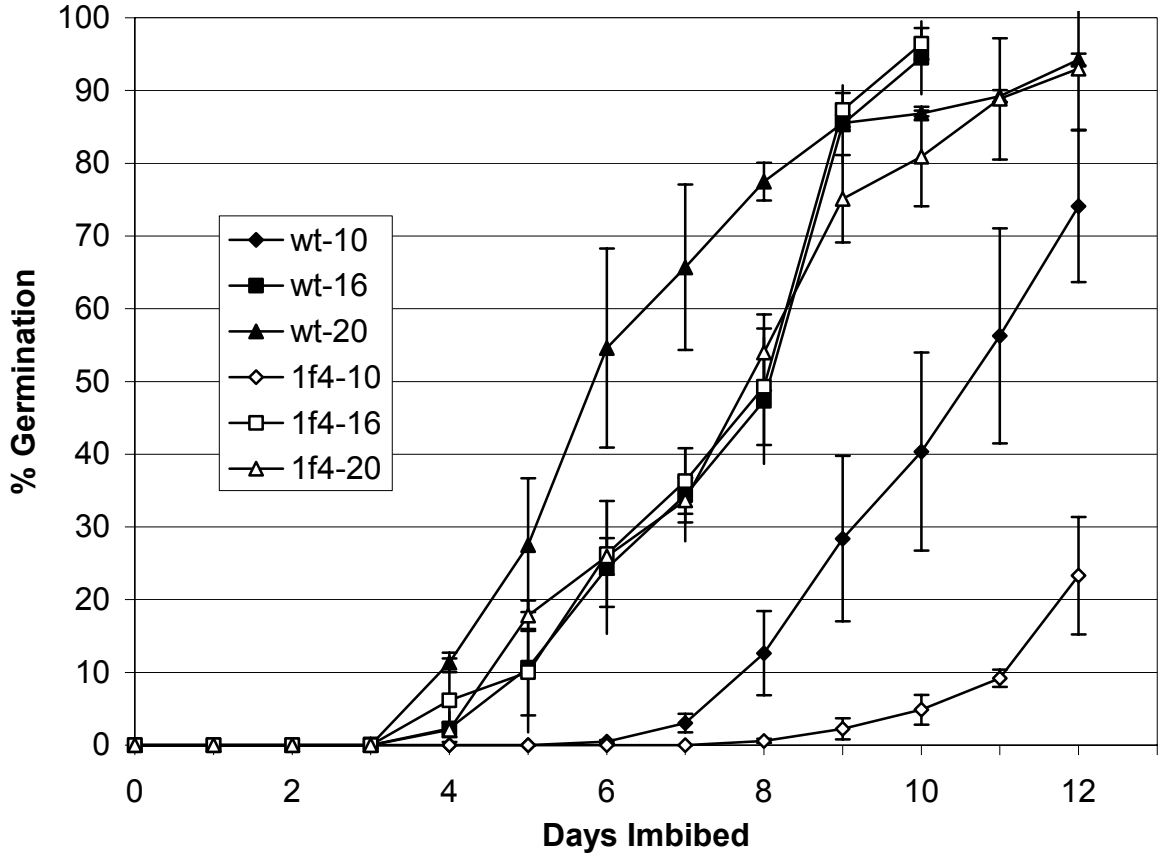


Figure 2-5: Precocious germination of WT and 1F4 seeds. In order to assess maturity of seeds from WT and 1F4, 10 (squares), 16 (diamonds) and 20 (triangles) day old seeds were harvested from the 2 genotypes, and imbibed over germination paper. For the 10 DAF seeds, germinability reached 80% for WT and 65% for 1F4 after a month of imbibition. The data presented are the mean ± SE of 3 experiments.

Table 2-3: Mean time to germination (T_{50}) for precocious germination (mean $\pm$ SE; n = 3 with 70-100 seeds each experiment). At 10 and 20 DAF, WT and 1F4 T_{50} were significantly different ($p \leq 0.05$). The summation equation was used to compute T_{50} values. The germination performance of these seeds is seen in Figure 2-5.

T_{50} **(days)**	***10 DAF***	***16 DAF***	***20 DAF***
WT	9.7 ± 0.7	7.7 ± 0.3	5.7 ± 0.7
1F4	13.7 ± 0.3	7.4 ± 0.3	8.7 ± 0.3

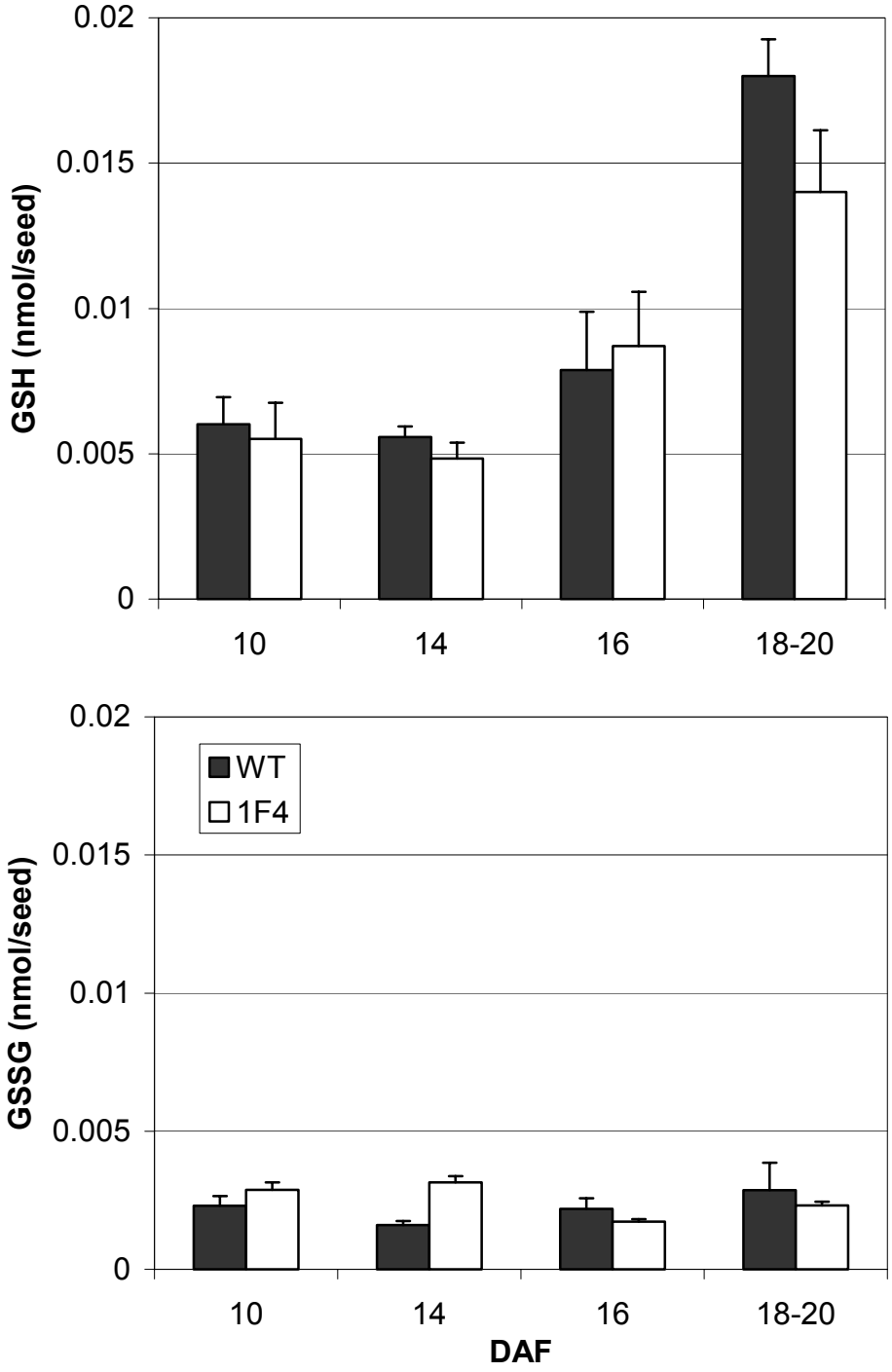


Figure 2-6: GSH and GSSG levels in maturing WT and 1F4 seeds (mean ± SE; n = 3) presented at nmol per seed basis. All values, GSH and GSSG, are statistically similar ($p \leq 0.05$) when comparing between genotypes at a given DAF.

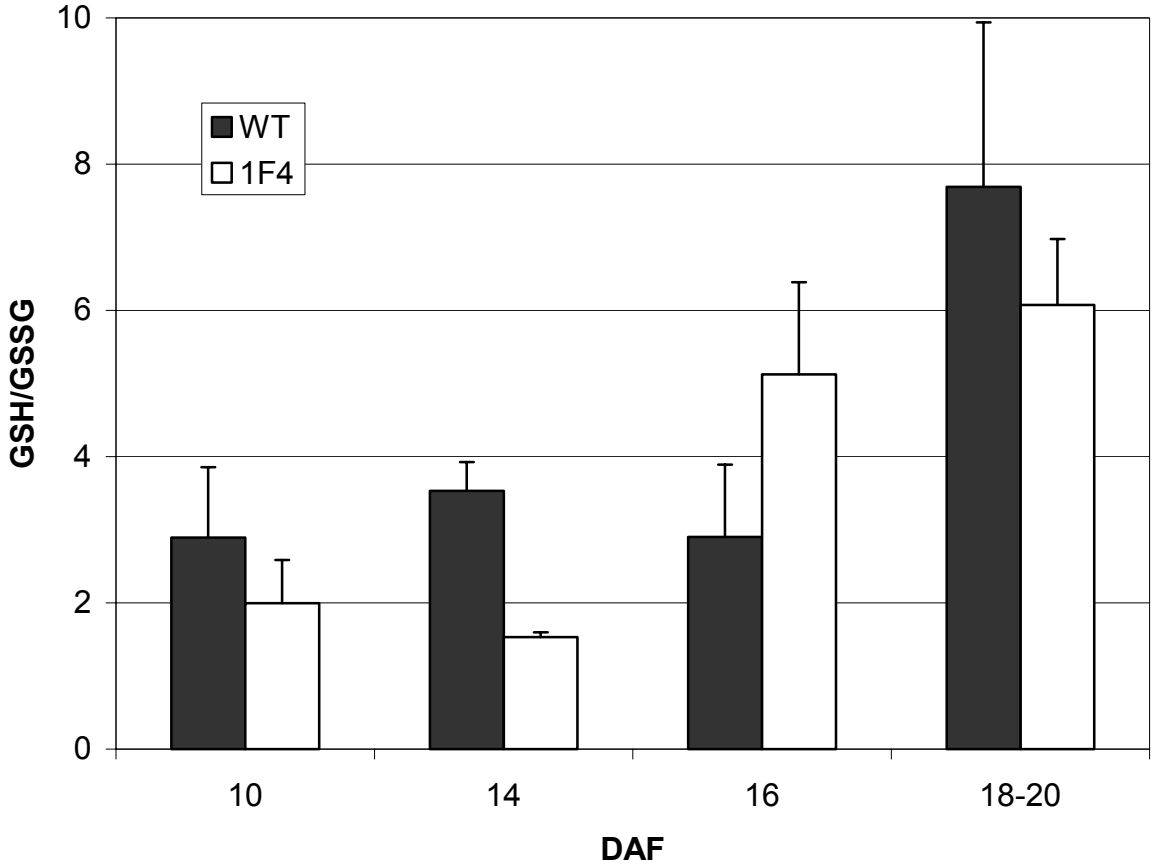


Figure 2-7: GSH/GSSG ratio in WT and 1F4 maturing seeds (mean $\pm$ SE; n = 3). All values, except 14 DAF, were statistically similar ($p \leq 0.05$) when comparing between genotypes at a given DAF. The ratio shown here was calculated from GSH and GSSG values present in Figure 2-6.

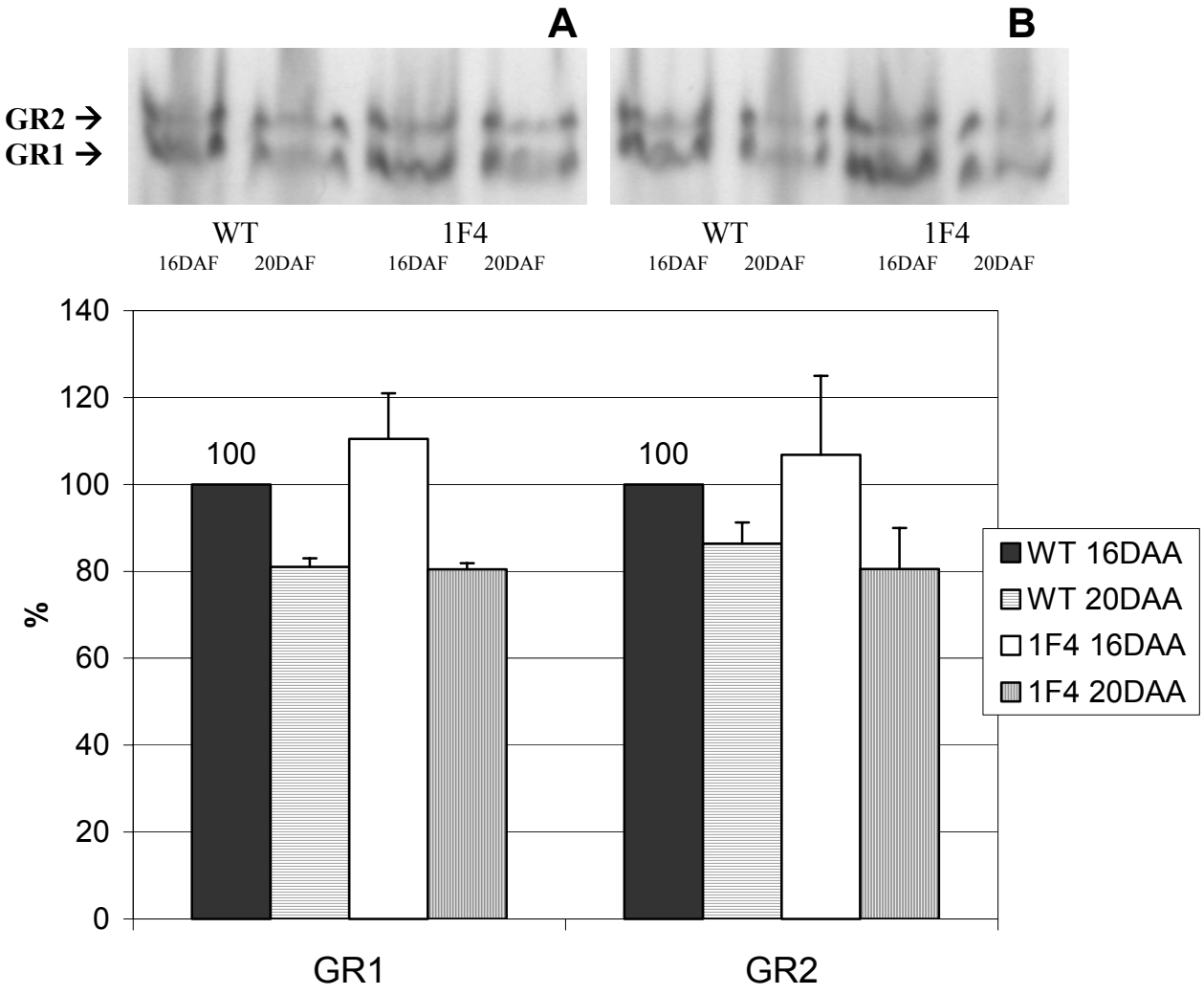


Figure 2-8: Glutathione reductase activity in drying seeds (mean ± SE; n =2). Band intensities of GR1 and GR2 in WT 16DAF were used as norm for each gene and were set as 100%. Values of the other samples are presented here as percentages of the assigned norm.

SEED STORAGE AND AGEING:

Germination of fresh and stored seeds. Freshly harvested WT and 1F4 seeds from dehisced siliques (20-24 DAF) were collected and sown on moist germination paper. Similar germination experiments were conducted on the same lot of seeds after 9 months of storage (natural ageing) at ambient laboratory conditions (22-25°C, 40-60% RH, 10% water content). Total percent germination was determined 9 days after imbibition. For newly harvested seeds, germination percentage of WT and 1F4 seeds did not reach 100% with WT having only 60 ± 13% germination and 1F4, 74 ± 3%. This indicated some residual dormancy in the seeds. Mean time to germination of freshly harvested WT seeds was slightly longer (8.3 ± 1.4 days), though not statistically significant, compared to 1F4 seeds (6.4 ± 0.3 days) (Table 2-4).

Stored WT seeds, in the other hand, reached 93 ± 3% germination and a mean time germination of 2.7 ± 0.3 days, suggesting loss of residual dormancy with storage as compared to the newly harvested seeds. Stored 1F4 seeds had a significantly lower germinability (49 ± 4%) and decreased T_{50} (4.5 ± 0.2 days) compared to 1F4 fresh seeds and to both fresh and stored WT seeds. This response indicated that 1F4 seeds did not tolerate storage at ambient conditions as well as WT seeds.

Controlled deterioration test. To determine whether 1F4 seeds had reduced storage life, accelerated ageing or controlled deterioration tests (CDT) were conducted on WT and 1F4 seeds. Seeds were exposed to 85% RH and 40°C for 5 and 7 days with untreated seeds (0 days) serving as control. In control seeds, the germination percentage was almost 100%, showing high seed germinability under optimal conditions. Exposure to 5 and 7 days CDT resulted in lower germination of both WT and 1F4 seeds. Seven days CDT seeds showed similar <10% germination in both genotypes (6 ± 1% for WT and 3 ± 1% for 1F4). Controlled deterioration for 5 days affected 1F4 seeds more with 45 ± 2% germination compared to WT seeds (57 ± 4%). Both germination % and T_{50} of WT and 1F4 seeds exposed to 5 days CDT (4.4 ± 0.2 days and 6.2 ± 0.3 days, respectively) were statistically different. This supports results in natural ageing where 1F4 seeds aged faster than WT seeds (Figure 2-10).

Glutathione redox status in stored seeds. In naturally aged WT and 1F4 seeds shown in Figure 2-9 (labeled as stored seeds), the glutathione pools (GSH + 2GSSG) were almost completely oxidized with GSSG making up 85% of the pool in WT aged seeds (Figure 2-11). The GSH/GSSG ratio in WT seeds was 0.1 ± 0.01. In the aged 1F4 seeds, no reduced glutathione was detected and GSSG values were similar to WT seeds. Germination experiments earlier showed that the aged 1F4 seeds had lowered seed vigor compared to the WT seeds (Figure 2-9). Glutathione levels were not measured when the seeds were freshly harvested.

Repeated sampling from the same WT and 1F4 seed lots showed that GSH levels declined with time of storage. Figure 2-12 shows a comparison of the calculated GSH/GSSG ratio in seeds from both genotypes between two samplings more than a month apart. The differences in the ratio were not significant ($p \leq 0.05$), however, it can be expected that the difference will increase with longer storage time between samplings.

Table 2-4: Mean time germination in days for fresh and stored seeds (mean ± SE; n = 3). For both genotypes, T_{50} was significantly different ($p \leq 0.05$) when comparing fresh and stored seeds. T_{50} of stored 1F4 seeds was also statistically different from that of the WT stored seeds. Germination percentage of these seeds is shown in Figure 2-10.

T_{50} (days)	**Fresh**	**Stored**
WT	8.3 ± 1.4	2.7 ± 0.3
1F4	6.4 ± 0.3	4.5 ± 0.2

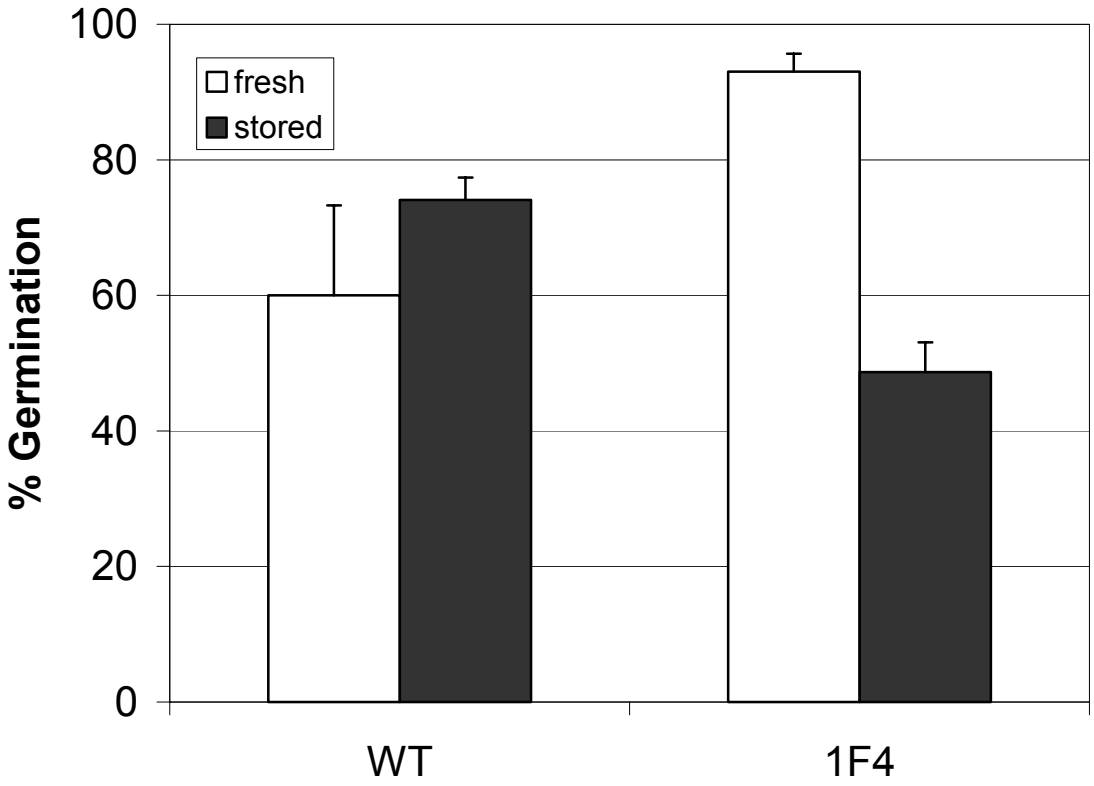


Figure 2-9: Germination percentage of freshly harvested and stored WT and 1F4 seeds. Seeds were stored at ambient conditions (10% water content) for 9 months. Results presented are the mean $\pm$ SE of 3 experiments with 90-100 seeds each. Germination percentage was obtained after 9 days from start of imbibition. Germination percentages between fresh and stored WT seeds were not statistically different ($p \leq 0.05$).

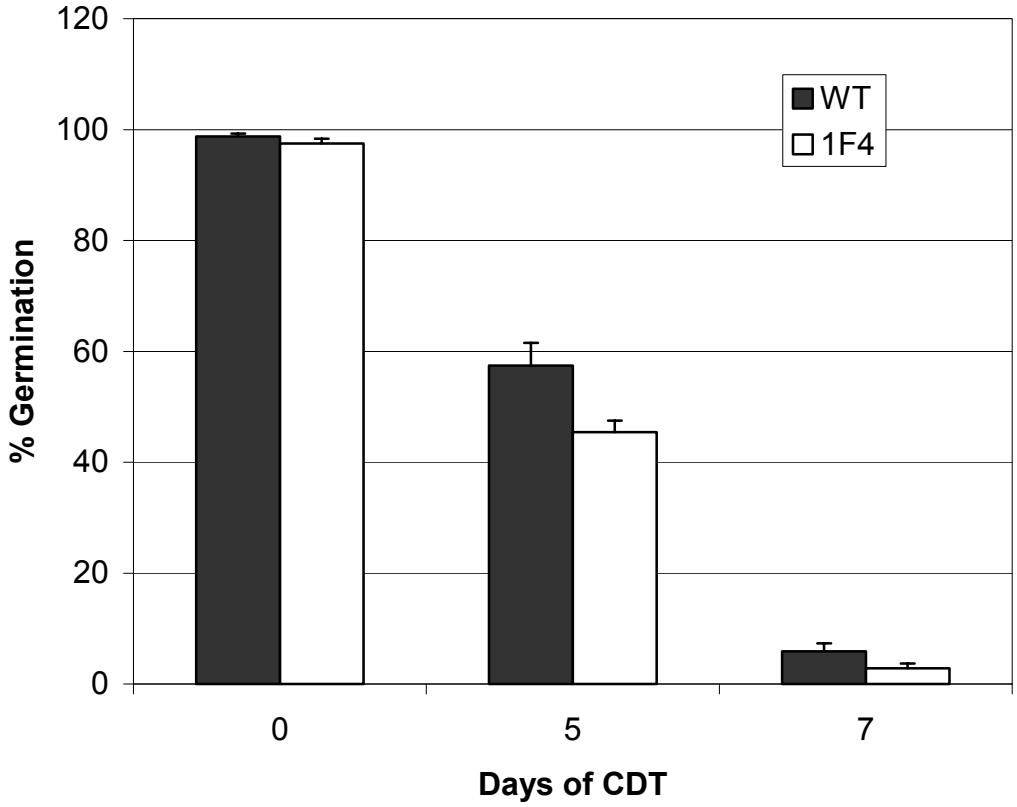


Figure 2-10: Controlled deterioration of WT and 1F4 seeds (mean ± SE; n = 4). The seeds did not receive a cold pre-treatment. At 5 days of CDT, % germination was statistically different at $p \leq 0.05$. T_{50} of WT and 1F4 seeds for 5 days CDT were 4.4 ± 0.2 days and 6.2 ± 0.3 days, respectively and were also statistically different.

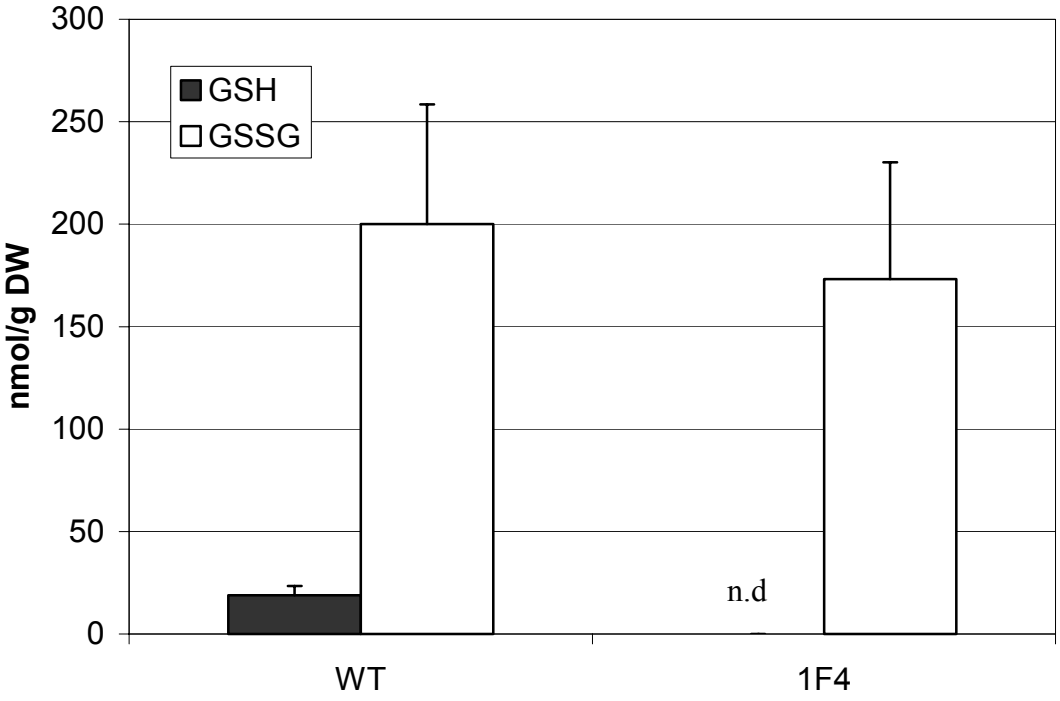


Figure 2-11: Glutathione levels in naturally aged WT and 1F4 seeds. The seeds were stored at ambient conditions for 9 months. No detectable GSH was found in 1F4 seeds (n.d. means not determined). Results are presented as mean ± SE of 3 experiments. The GSH/GSSG ratio of WT seeds was 0.1 ± 0.01. The germination performance of these seeds is reported in Figure 2-9 as stored seeds.

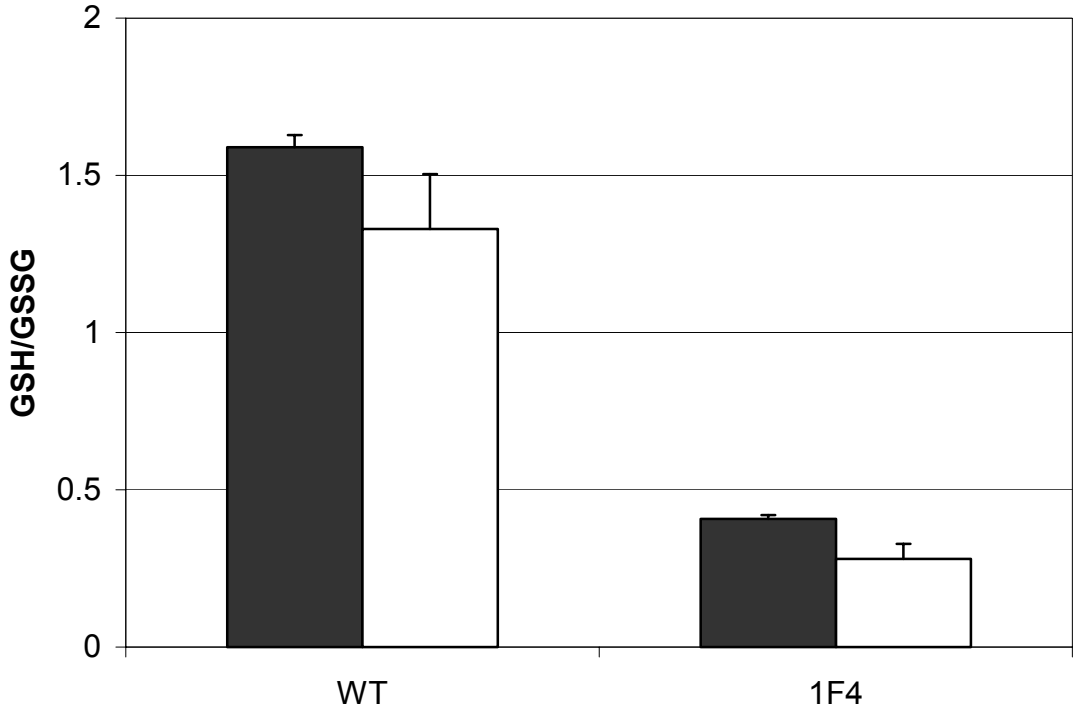


Figure 2-12: Changes in the GSH/GSSG ratio in WT and 1F4 seeds during storage. WT and 1F4 seeds were stored at ambient conditions (10% water content) for 55 and 43 days, respectively (mean ± SE; n = 3). Filled columns refer to the first sampling while white columns refer to values obtained in the later sampling. Differences between fresh and stored seeds of both genotypes were not significant ($p \leq 0.05$).

Table 2-5: Decrease in GSH and GSSG levels during storage. The GSH/GSSG ratio computed from the values below are presented in Figure 2-12. WT and 1F4 seeds were stored at ambient conditions (10% water content) for 55 and 43 days, respectively.

Sampling	*GSH*	*GSSG*	*GSH/GSSG ratio*
WT: 1st	360	226	1.6
WT: 2nd	329	244	1.3
1F4: 1st	90	220	0.4
1F4: 2nd	85	293	0.3

DISCUSSION

Seed maturation in Arabidopsis can be divided into two parts: seed filling (10-16 DAF) and seed desiccation (16-20 DAF). There were limited differences observed between WT and anGR2 (1F4) seeds during seed development. Seed abortions (Table 2-2), difference in seed dry weight and water content (Figures 1-3 and 1-4), and delay in maturation of 1F4 seeds as seen in the low germination percentage and large T_{50} of 10 DAF seeds (Figure 2-5 and Table 2-3) suggest the effects of the GR2 antisense may be more prevalent in earlier stages of seed development or during the seed filling stage. The pale seeds observed in 1F4 10-14 DAF might become the aborted seeds observed in older 1F4 siliques. The pale seed phenotype has been associated with mutant seeds containing globular embryos (Meinke 1994). The greater dry weight of 1F4 seeds indicated a change in the storage accumulation pattern of 1F4 seeds compared to WT (Figure 2-3). Dry weight measurements show that 14 DAF and 18 DAF to be the mass maturity of 1F4 and WT seeds, respectively. The slow precocious germination of 1F4 10 DAF seeds could be the result of a delay in the maturity of the seeds or greater dormancy of the seeds.

During seed maturation, GSH and GSSG levels were similar between the two genotypes (Figure 2-6). GSH/GSSG ratio was also identical between WT and 1F4 except at 14 DAF where 1F4 seeds had a significantly lower ratio than WT (Figure 2-7). This difference in the GSH/GSSG ratio coincided with a greater increase in dry mass of 1F4 compared to WT seeds (Figure 2-3). It is interesting to speculate about the relationship of redox state of glutathione and seed filling but a possible correlation was not investigated experimentally. In soybeans, change in seed protein composition has been observed with exogenous application of GSH or GSSG (Awazuhara et al. 2002).

These differences between WT and 1F4 seeds show slightly lower efficiency of the 1F4 seed development but not enough to greatly reduce the number of viable seeds produced. This may be because both GR1 and GR2 activity in WT and 1F4 were identical during seed desiccation, except for slightly higher GR1 activity in 1F4 16 DAF seeds (Figure 2-8). The GR2 antisense mechanism may be active only at certain stages of plant development such as during seed germination (see Chapter 3) and vegetative growth as GR2 activity was lower in the leaves of

1F4 plants (S. Mane, unpublished data). It is also important to note that the phenotype comparison were of WT and 1F4 seeds produced at optimal conditions. A more distinguishable phenotype in 1F4 may be observed when the plants are grown under stress.

The results in this study show that GSH levels increase while GSSG levels remain constant during the last days of dehydration of wildtype Arabidopsis seeds. The GSH/GSSG ratio, consequently, was a value greater than 2 and increased during seed desiccation. When the siliques dehisced (20 DAF), the seed GSH/GSSG ratio was high. This is surprising given that GR activity was reduced during dehydration. High levels of GSH during late maturation have also been observed in wheat (De Gara et al. 2003; Rhazi et al. 2003). Glutathione biosynthesis and transport into the seed may be possible. However, the water content at 18 and 20 DAF was between 55-30% (Figure 2-4). Unregulated catabolism occurs and respiration stops at 45% water content in seeds (Vertucci and Farrant 1995). It is not known how active the glutathione biosynthetic enzymes and transport proteins are at 55-30% water content.

GSH levels in maturing wheat kernels were almost twice that of GSSG at the end of the maturation period (45-53 DAF) (Rhazi et al. 2003). The GSH/GSSG ratio at desiccation of wheat grain was 1.6 (De Gara et al. 2003). De Gara et al. (2003) and Rhazi et al. (2003) found that GSSG levels are at one point very high during kernel maturation (28 DAF and 48 DAF, respectively). The increase in GSSG coincided with increased disulfide bonds in storage proteins in wheat kernel. Rhazi et al. (2003) found increased protein-glutathione mixed disulfide (PSSG) in the kernels during with grain desiccation rather than during accumulation of storage proteins. In this study, Arabidopsis seeds had constant GSSG levels and increasing GSH levels during seed maturation (Figure 2-6), and this raises questions about the role of the glutathione pair in sulphydryl to disulphide transition in storage proteins of Arabidopsis, which has not been examined here. The glutathione pair, through reduction of cyteine groups and forming reversible disulfide bridges, is involved in protein folding (Orsi et al. 2001), in the assembly of polymeric proteins (Jung et al. 1997) and protection of specific thiol groups from irreversible formation of intramolecular disulphide bonds in seeds (Butt and Ohlrogge 1991; Kranner and Grill 1996). The thioredoxin and glutaredoxin systems are also capable of reduction of disulfide bridges in plants (Rouhier et al. 2002). Measurement of PSSSG during seed maturation and germination would

clarify the relationship between the glutathione pool and disulfide bond formation in Arabidopsis storage proteins.

In some species, activities of several free-radical scavenging enzymes decrease with dehydration of the seed (Vertucci and Farrant 1995; Greggains et al. 2000; Bailly et al. 2001; De Gara et al. 2003; Rhazi et al. 2003) while antioxidants such as tocopherols and glutathione accumulate. This also appears to be the case during the desiccation of Arabidopsis seeds in this study as shown by the accumulation of GSH (Figure 2-6). The high levels of GSH and inactivation of GR (Figure 2-8) during Arabidopsis seed dehydration may reflect a shift in the free-radical scavenging system from detoxifying enzymes to antioxidant compounds. The enzymatic oxidants such as ascorbate peroxidase, catalase, glutathione reductase and superoxide dismutase can only be active under conditions of sufficient water content. Antioxidant compounds such as glutathione, ascorbate, polyols, carbohydrates, tocopherol, quinines, flavonoids and phenolics can be active in the air-dry state (Hoekstra et al. 2003).

The decline of activity in the mitochondria and chloroplast during seed desiccation also provides an explanation for the high GSH/GSSG ratio in drying Arabidopsis seeds (Figure 2-7). The decline in respiration during seed dehydration removes one source of ROS in seeds (Leprince et al. 2000) and that may contribute to maintaining a more reduced glutathione pair at desiccation. Photosynthesis is another source of ROS. Arabidopsis seeds are only photosynthetically active from 10 to 16 DAF color and start to accumulate a flavonoid brown pigment as the seeds dry after 16 DAF (Figure 2-1) (Meinke 1994). Thus, during seed dehydration, less ROS may be produced compared to the seed filling stage and this may contribute to a high GSH/GSSG ratio at desiccation.

The effect of ageing on seeds of WT and 1F4 was examined by natural ageing and by a controlled deterioration test (CDT). 1F4 had a lower germination percentage after 9 months of storage at ambient conditions (22-25°C, 40-60% RH, 10% water content) (Figure 2-9) and after 5 days of CDT (Figure 2-10). Germination of WT seeds (Columbia ecotype) after 5 days of CDT was similar to those results reported for Arabidopsis Landsberg *erecta* ecotype by Tesnier et al. (2002) and Columbia ecotype by Clerkx et al. (2004). After 7 days of CDT, the germination

percentage of our WT seeds was much lower than the previous studies. Clerkx et al. (2004) examined seed longevity in various Arabidopsis mutants including *cad2-1* (Columbia background), which accumulates only 15-30% glutathione of WT levels. When stored for 4 years under ambient conditions, *cad2-1* did not show any reduction in germination. After CDT, germination patterns of *cad2-1* were similar to WT, suggesting that glutathione levels are not crucial in determining sensitivity to ageing in seeds. GSH/GSSG ratio was not determined in the *cad2-1* seeds. In the case of the GR2 antisense line, GSH levels in the 1F4 seeds after desiccation (Figure 2-6) and during natural ageing were similar to WT seeds (Figure 2-11), but 1F4 showed lower vigor than WT (Figure 2-9). Since ageing *cad2-1* and WT was similarly affected by ageing but 1F4 was sensitive to storage, the GSH/GSSG ratio may then be of greater importance than the actual GSH levels itself during germination. In this case, GSSG recycling by GR may prove crucial in recovery of aged seeds. We do not have data on GSH/GSSG nor GR activity ratio in germinating aged seeds but our results in seed stress experiments (Chapter 3) showed that 1F4 seeds were less tolerant of heat and drought stress, suggesting either an insertion artifact or that the redox status of glutathione in stressed 1F4 seeds did not recover adequately or fast enough during germination. Lower GSH/GSSG ratio may decrease enzyme activity and affect cell signaling and gene expression in the germinating seed. Lowered germination percentage was correlated with decline in GR activity with natural and artificial ageing in cotton (Goel and Sheoran 2003; Goel et al. 2003) and sunflower seeds (Bailly et al. 1998; Torres et al. 1997). The presence of the antisense mechanism may have a further debilitating effect on the reduced GSSG recycling capacity of aged seeds and result in a lower GSH/GSSG ratio. The restorative effects of priming on aged seeds were correlated with increase in GR activity along with other detoxifying enzymes (Bailly et al. 1998, 2000).

GSH was observed to oxidize as the seeds aged, either by long-term storage or accelerated aging in sunflower (De Paula et al. 1996; Torres et al. 1997), watermelon (Hsu and Sung 1997) and tomato seeds (De Vos et al. 1994). In the current study, GSH levels in dry Arabidopsis seeds were observed to decrease within approximately 6 weeks of storage (Figure 2-12 and Table 2-5) and were almost completely oxidized after 9 months of storage under ambient conditions (22-25°C, 40-60% RH, 10% water content by FW) (Figure 2-11). Lowered GSH levels may indicate active oxidation of GSH, and thus, oxidative stress in seeds during storage. Oxidative damage in

seeds during storage is likely in the form of lipid peroxidation, sugar hydrolysis and Maillard reactions (McDonald 1999; Murthy et al. 2002, 2003). Seed vigor cannot be correlated with GSH values since both naturally aged WT and 1F4 seeds had similar GSH levels but different germination percentage. De Vos et al. (1994) also had a similar conclusion since aged and control tomato seeds contained, after 2 days of imbibition, comparable glutathione content and redox state, but the times of start of germination were markedly different. Regardless, autooxidation of glutathione by trace metal ions in the cell cannot be completely discounted as the cause for lower GSH levels in ageing seeds (Albro et al. 1986). Looking between genotypes, naturally aged 1F4 seed contained significantly lower glutathione redox states compared to aged WT seeds. The lowered 1F4 GSH/GSSG ratio may suggest that these seeds experience more oxidative stress during storage given that WT and 1F4 glutathione redox state was similar during desiccation (Figure 2-8). A reason for this may be that 1F4 seeds accumulated more ROS during desiccation than WT seeds, thus resulting in a greater amount of oxidized glutathione during storage. Accumulation of ROS may indicate more physiological perturbations during seed maturation in 1F4 than WT.

CHAPTER 3: ARABIDOPSIS SEED GERMINATION AND STRESS TOLERANCE

INTRODUCTION

Seed germination starts with the uptake of water – imbibition – and is completed when a part of the embryo, usually the radicle, emerges from its protective structures (reviewed by Bewley 1997). Imbibition results in temporary structural perturbations, particularly to membranes, which leads to rapid leakage of solutes and low molecular metabolites. Respiration can be detected within minutes of imbibition. The initial high oxygen consumption declines until radicle protrusion where another burst of respiratory activity occurs (Bewley 1997).

Given the damage brought about by desiccation and storage and the respiratory burst during imbibition, Bailly (2004) succinctly describes germination as a potentially harmful process. Reactivation of metabolism after seed imbibition may bring in a large influx of ROS, a factor that can affect success of germination. Therefore, antioxidants and detoxifying enzymes are considered to be of particular importance during germination. Onset of metabolism upon imbibition brings in an influx of ROS that has to be dealt with to ensure smooth resumption of cell processes (Bailly 2004). During seed germination, ROS scavenging enzymes such as SOD, catalase, peroxidase, GR (Leprince et al. 1990; Leprince et al. 1993; Bailly et al. 2001, 2003) and ascorbate recycling enzymes (Cakmak et al. 1993) increase in activity and antioxidants such as glutathione, ascorbate, and tocopherols accumulate (Tommasi et al. 2001; Simontacchi et al. 2003).

Glutathione and glutathione reductase has invariably been associated with stress responses in plants, working as part of the defense mechanisms against oxidative stress. Glutathione has been correlated to adaptation of plants to extreme temperatures, light and drought stress (May et al. 1998; Noctor et al. 1998). In this chapter, focus is directed on glutathione dynamics and GR activity during Arabidopsis seed imbibition and germination in both WT and 1F4 lines. This chapter will also examine the effect of the GR2 antisense insert on germination at high and low temperature, and under drought stress.

METHODOLOGY

I. Plant Material

Plants were grown as described in Chapter 2. Similarly, seeds were harvested and stored as described in Chapter 2. The seeds were stored inside glass vials at ambient laboratory conditions (22-25°C, 40-60% RH) for approximately 2-3 months. The resulting water content of the seeds was approximately 10% (FW basis).

Seeds were imbibed in water for 1, 4, 12, 24, 48 and 72 hr. For the latter 3, seeds were imbibed over moistened germination paper covered with a piece of dialysis membrane. For 1, 4 and 12 hr, seeds were imbibed in Eppendorf tubes containing 100 uL of water. The seeds were imbibed in the presence of light and at 22-25°C. No cold pre-treatment was applied on the seeds.

II. Glutathione Dynamics

A. HPLC methodology

The determination of levels reduced and oxidized glutathione by HPLC was as described in Chapter 2. GSH and GSSG were measured in dry seeds (0 hr), and in seeds imbibed for 1, 4, 12, 24, 48 and 72 hr. The imbibed samples were prepared as described above.

B. Glutathione reductase activity gel

Seeds imbibed as described above for 4 and 12 hr, and dry seeds were used for GR activity gels. Determination of GR activity was done as described in Chapter 2.

C. CLSM imaging

Fluorescent dyes. Stock solutions of 100 mM monochlorobimane (MCB; in ethanol), 100 mM monobromobimane (MBB; in ethanol) and 30 mM propidium iodide (PI; in water) were

prepared and stored at –20°C in 1 mL aliquots until use. Aliquots were thawed and smaller portions used to prepare a 100 μL MCB; 30 μL PI aqueous solution. Dyes were prepared immediately prior to use. All dyes were obtained from Molecular Probes (Eugene, OR, US).

Seed dissection and embryo staining. Dry Arabidopsis seeds from both WT and 1F4 lines were imbibed over filter paper saturated with 5 mM sodium azide for 4 hr at 22 to 24°C. The embryos were dissected out of the imbibed seeds and transferred to prepared slides containing a drop of the dye solution with final concentrations of 100 μM MCB, 30 μM PI and 5 mM sodium azide. Improvised wells were created on the glass slides used by covering the slides with a strip of plastic tape, approximately 170 μm thick, and carving out a rectangular section in the middle. Cover slips were carefully placed over the slides and the embryos were stained for 30 minutes in the dark (Fricker et al. 2000). Cover slips were secured using adhesive tape to prevent the embryos from being damaged by movement. Excess stain was removed by replacing the solution in the slide with 5 mM sodium azide. The slides were immediately placed on ice before imaging. Staining kinetics of MCB is described in Fricker et al. (2000).

Confocal imaging of glutathione S-bimane fluorescence. The embryos were imaged using a Carl Zeiss LSM 510 META (Oberkochen, Germany) inverted confocal laser scanning microscope. The glutathione-bimane conjugate (GSB) was excited at 405 nm by a diode laser set at 2% of maximum output. Fluorescence emission for GSB was collected at 470 to 525 nm using a Zeiss 5x Plan-Neofluar lens or 63x C-Apochromat water-immersion lens. PI labeling was excited at either 514 nm (HeNe laser) (26% output) or 543 nm (Argon laser) (17% output) and emission collected at >600 with a 560 nm dichroic mirror. Pinholes for both collecting channels were maintained between 60 to 78 μm.

Low magnification (5x lens) images were taken of the embryos to determine the amount of damage as indicated by excessive PI staining. High magnification (63x) images were used to measure of GSB fluorescence in the different parts of the embryo. Only images of epidermal cells in the root tip region, hypocotyls and cotyledons were obtained to simplify analysis. Images of epidermal cell do not require computation to compensate for signal attenuation with increasing depth as images from deeper tissues would. The “Find” function in the software LSM

Image Examiner (Carl Zeiss AG, Oberkochen, Germany) and manual adjustments were applied to obtain the optimal detector settings. This ensured that pixel intensity in the images did not saturate beyond the detectable range. The same settings were used for all samples to be compared. Images were captured at a depth where GSB fluorescence was brightest and most defined. For both WT and 1F4 embryos, 3 samples were imaged per embryonic tissue (i.e. cotyledon, hypocotyl and root tip). Tissue and cell integrity was examined in all the samples chosen by PI staining.

A test for auto-fluorescence was performed by staining several dissected embryos with only propidium iodide (30 μM). No auto-fluorescence was detected across all embryo tissues (Figure 3-7), so all signals received in the 470-525 channel were assumed to be from GSB fluorescence. Similar observations were found in intact Arabidopsis roots (Fricker et al. 2000).

GSB fluorescence intensity was calibrated against varying concentrations of GSB standards imaged under the same conditions as the embryos. GSB standards were prepared by mixing 590 μL 20 mM GSH in 200 mM Tris-HCl buffer (pH 8.3) and 10 μL 100 mM monobromobimane (MBB). The mixture was incubated in the dark at room temperature for 15 minutes. The reaction was stopped by addition of 400 μL 10% acetic acid, allowing the pH to drop between 3 and 4. The resulting solution was assumed to contain 1 mM GSB. Dilutions of standard stock solution were prepared and a drop from each solution was transferred to slides similar to those used for the samples. Images of the standards were taken using all the lenses employed in the experiments.

Measurement of glutathione levels in cells. To determine the cytosolic GSH levels in the epidermal cells of the embryonic regions, GSB was quantified by delineating an area of MCB fluorescence in 2 or more cells per image and calculating the mean intensity in the selected areas. Three images corresponding to 3 different samples (embryos) were taken each for the hypocotyl, cotyledon and root tip. Intensity units were converted to mM by using the calibration curve from the GSB standards.

Fluorescence microscopy of MCB-stained embryos. To see overall differences in GSB fluorescence during imbibition and germination, images of GSB-PI stained embryos and seedlings were taken using a Leica MZ FLIII fluorescence stereomicroscope (Bensheim, Germany). Whole embryo images were also taken using the 5x/0.15 Plan-Neofluar lens of the CLSM system. Stains used were the same as above (100 mM MCB and 30 mM PI).

Software. Image processing and analysis were performed using LSM Image Examiner (Carl Zeiss AG, Oberkochen, Germany) and ImageJ 1.32j (W. Rasband, NIH, US) with LSM plug-in. Microsoft® Excel Professional 2000 (Microsoft Corporation, US) was used for computations.

III. Germination under Stress

Germination assays. For control, 80-100 seeds of WT and 1F4 that has been stored for 5 months (22-24°C, 40-60% RH, 10% water content) were spread uniformly on water-saturated germination paper and germinated at 25°C with 14 hr photoperiod. Four replicates were prepared for all experiments. Replicates were set in tri-section Petri dishes sealed with paraffin film. Seeds were examined for radicle protrusion using a binocular dissecting microscope (Olympus VMZ stereomicroscope) every 12 hr for the first 3 d and once every day afterwards till 14 d from start of imbibition. No cold treatment was applied on any of the seeds. T_{50} was computed by using the summation equation provided by Leon and Knapp (2004) for samples with more than 50% germination; otherwise, probit analysis was employed (Wilson et al. 1989). All computations were done in Microsoft® Excel Professional 2000 (Microsoft Corporation, US).

Germination at high and low temperatures. The seeds were spread over water-saturated germination paper and exposed to 30°C day/25°C night for high temperature treatment and 10°C for the low temperature treatment. 14 hr day lengths were provided for both experiments.

Germination at low water potential. PEG 8000 (Sigma, St. Louis, MO, US) solution of -0.56 MPa was prepared according to Michel (1983) (Equation 5) and verified by osmometry (25° C;Wescor, Logan, UT, US). WT and 1F4 seeds were spread over germination paper soaked in the PEG solution and grown at 25°C and 14 hr photoperiod.

RESULTS

SEED IMBIBITION AND GERMINATION

Seed germination. WT and 1F4 seeds germinated to almost 100% after storage for 4 months in ambient (22-25°C, 40-60% RH, 10% water content) conditions (Figure 3-1). However, WT and 1F4 seeds differed significantly in their speed of germination with WT seeds germinating faster. The T_{50} of WT seeds was 1.2 ± 0.1 compared to 1.7 days for 1F4 seeds.

Glutathione pool during imbibition. The levels of reduced (GSH) and oxidized (GSSG) glutathione were examined using HPLC during imbibition (0 to 72 hr) of WT and 1F4 seeds (Figure 3-2). In dry seeds, WT seeds had approximately 4 times more GSH (270 ± 23 nmol/g DW) than 1F4 seeds (64 ± 16 nmol/g DW). After 1 hr imbibition, GSH levels doubled in WT seeds and increased more than 10 times for 1F4. GSSG levels decreased in both seed types to approximately 40% of the dry seed value after 1 hr imbibition. After 4 hr of imbibition, WT and 1F4 had similar amounts of GSH. Both WT and 1F4 seeds reached their highest GSH levels 1055 ± 85 nmol/g DW and 1202 ± 31 nmol/g DW, respectively, after 12 hr imbibition. At 24 and 72 hr imbibition, the majority of the seeds were either still imbibing (24 hr) or germinated (72 hr). Similar to early imbibition, seeds exposed to water for 24 to 72 hr show a highly reduced glutathione pool with GSSG comprising less than 1% of the redox couple. At 24 hr, GSH levels in both WT and 1F4 seeds were in the same range (306 ± 182 and 507 ± 73 nmol/g DW, respectively). The GSH values in the 24 hr imbibed seeds were lower compared to the 12 hr imbibed seed values in both genotype. At 72 hr, GSH levels in both genotypes increased approximately twice from 24 hr, 1F4 was significantly higher than of the WT.

GSSG levels in dry WT and 1F4 seeds were similar (216 ± 23 and 189 ± 51 nmol/g DW, respectively), and decreased by the same percentage (40%) after 1 hr imbibition (Figure 3-2). Four hours of imbibition reduced GSSG levels to approximately 1% of its value in dry seeds. Both WT and 1F4 seeds maintained the low GSSG levels as far as 72 hrs of imbibition.

Looking at the total glutathione pool (GSH +2GSSG), WT dry seeds contained a larger pool compared to 1F4 dry seeds (701 ± 74 and 442 ± 115 nmol g/DW, respectively) (Figure 3-3). After 1 hr imbibition, glutathione pool in 1F4 seeds tripled (1297 ± 206 nmol g/DW) while the change in the glutathione pool in the WT seeds was not significant (852 ± 52 nmol g/DW). Four hours of imbibition showed a lower pool in 1F4 seeds but almost equal amount in WT seeds (871 ± 96 dn 967 ± 108 nmol g/DW). The glutathione pool in both genotypes increased, though not significantly, after 12 hr imbibition (WT: 1070 ± 95 nmol g/DW; 1F4: 1211 ± 33 nmol g/DW), but declined in parallel after 24 hr (424 ± 156 and 509 ± 72 nmol g/DW respectively). At 48 hr imbibition, the pool in WT seeds increased and at 72 hr imbibition was similar to dry seed values. In 1F4 seeds, the pool was twice the 24 hr value at 48 hr imbibition. At 72 hr imbibition, the 1F4 glutathione pool was 3 times the value in dry seeds.

In dry seeds, WT dry seeds have a significantly higher GSH/GSSG ratio (1.3 ± 0.2) compared to 1F4 (0.4 ± 0.06) (Figure 3-4). The GSH/GSSG increased 1 hr imbibition and were statistically similar in both genotypes (6.4 ± 0.7 for WT and 8.8 ± 1 for 1F4). After 4 to 72 hr imbibition, WT seeds maintained a seemingly higher GSH/GSSG ratio, though the difference between WT and 1F4 was not statistically significant ($p \leq 0.05$).

Glutathione reductase activity during imbibition. To compare of GR activity in imbibing seeds, intensities of the bands from the GR activity gels were quantified by densitometry. GR bands from WT dry seeds were chosen as the norm (100%). GR1 activity in WT seeds increased with longer time in imbibition (Figure 3-6). In 1F4 dry seeds, GR1 activity was higher compared to WT dry seeds. This level of GR1 activity was maintained throughout imbibition. GR2 activity in WT remained relatively constant throughout the 24 hr imbibition period. GR2 activity in 1F4 dry seeds and 4 hr imbibed seeds was slightly lower compared to WT seeds. GR2 activity in 1F4 seeds further reduced to 50% of WT dry seeds GR2 activity after 24 hr imbibition.

CLSM imaging of glutathione in Arabidopsis seeds. To facilitate quantification of glutathione from different parts of the embryo, only the epidermal tissue was examined and its glutathione pool measured. Images of cells from the hypocotyl, cotyledon and root tip were taken and used

for analysis (Figure 3-8). For seeds imbibed in sodium azide and incubated at 4°C, GSB measured was assumed to correspond to the concentration of GSH in the cytoplasm of cells of a quiescent embryo or at the very start of imbibition. The embryos were exposed to a short period of imbibition (4 hr), to the metabolic inhibitor – sodium azide, and to low temperature to repress metabolism in the cells and prevent sequestration of GSB conjugates to the vacuole. After dissection, the embryos remained largely unharmed as manifested by the lack of large red PI staining (Figure 3-7). No auto-fluorescence was seen when embryos were stained with only PI (Figure 3-7). In Figure 3-8 and 2-8, GSB was observed to remain in the cytosol in the above conditions. When the seeds were imbibed in water and at 22-25°C, GSB conjugates were sequestered into the vacuole (Figure 3-10). The chloroplastic pool was assumed to be negligibly labeled by MCB (Hartmann et al. 2003). GSB intensity was calibrated against standards and cytoplasmic glutathione in embryonic epidermal tissue was estimated to be 1.1 to 1.6 mM (Figure 3-11). There were no large differences in glutathione concentration between the different embryonic parts, or between tissues of WT and 1F4 embryos.

To assess whether there was an increase in cytosolic GSH levels during imbibition, overall GSB fluorescence was compared between embryos imbibed in water and 0.1 mM BSO for 3 hr and 36 hr (Figure 3-12). No radicle protrusion was observed in the seeds used. BSO (L-buthionine-[*S,R*]-sulfoximine) is an inhibitor of GSH biosynthesis. GSB fluorescence increased slightly, though not significantly, after 36 hr of imbibition for both embryos in water and in BSO (Figure 3-13). No difference was found in bimane fluorescence between seeds imbibed in water and those in BSO at 3 or 36 hr imbibition, indicating that the increase in GSH levels during imbibition may not be due to glutathione biosynthesis. It was observed that GSB fluorescence increased to cover the whole root tip during imbibition. On the other hand, Arabidopsis seedlings germinated in water had greater GSB fluorescence than those germinated in BSO (Figure 3-14), suggesting that significant glutathione biosynthesis may occur after radicle protrusion.

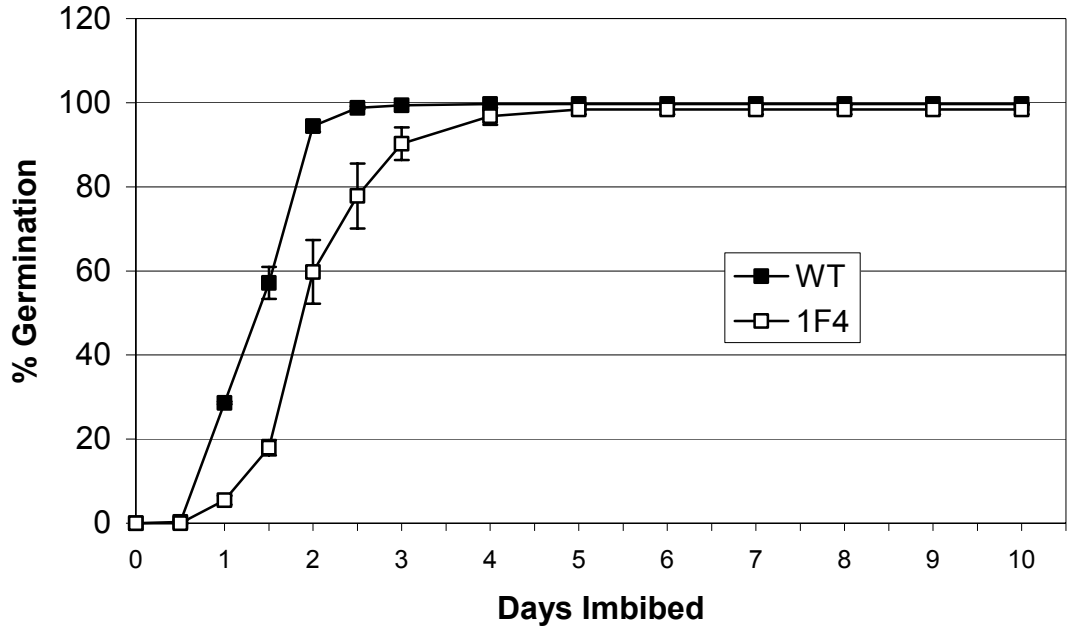


Figure 3-1: Germination pattern of dry WT and 1F4 seeds (mean ± SE; n = 4; 70-100 seeds each). These seeds had been stored at ambient conditions for 4 months. No low temperature pre-treatment had been done to the seeds. WT T_{50} was equal to 1.2 ± 0.1 days and for 1F4, 1.7 ± 0.1 days. The two T_{50} were significantly different at $p \leq 0.05$.

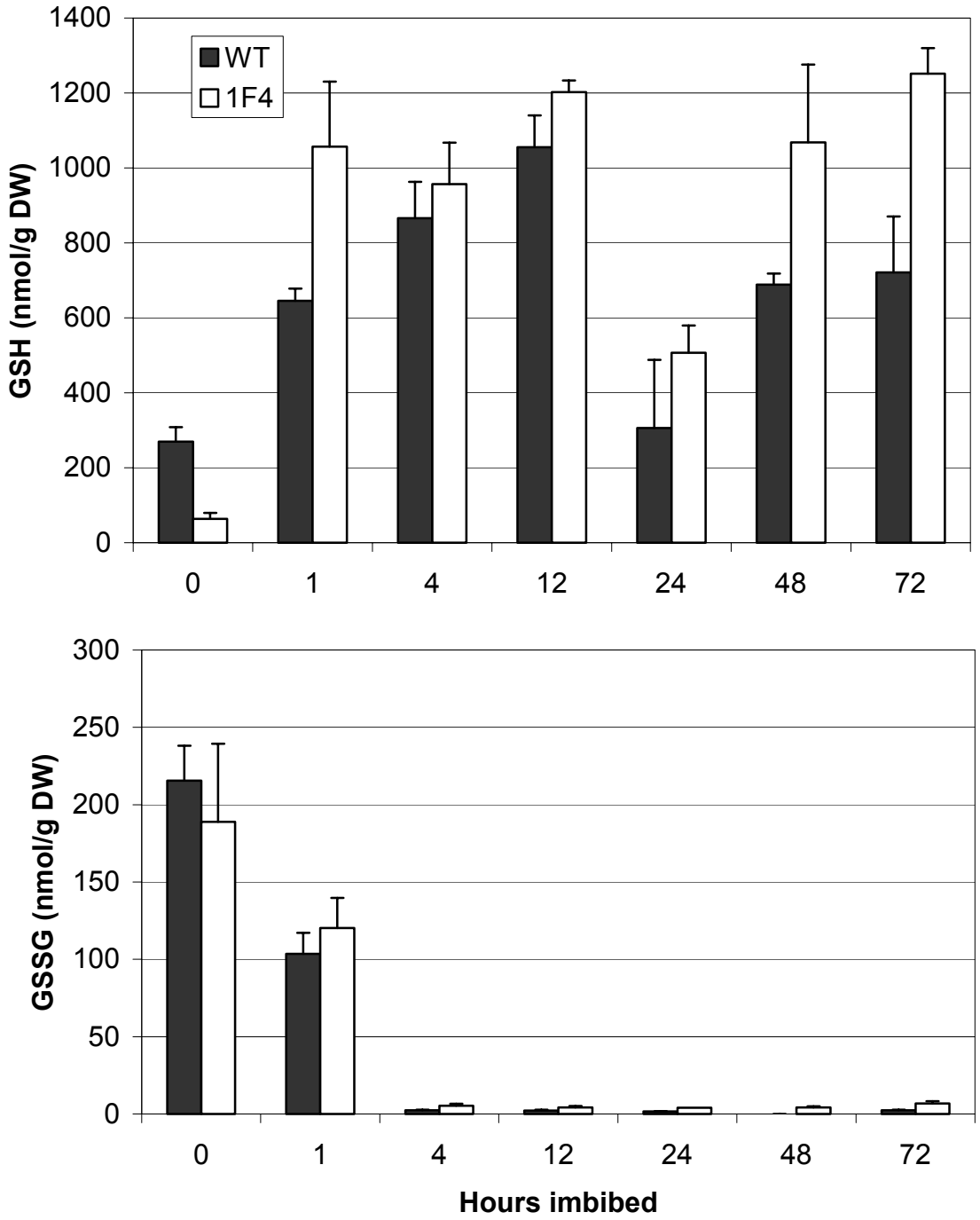


Figure 3-2: GSH and GSSG levels during seed imbibition: 0, 1, 4, 12, 24, 48 and 72 hrs (mean ± SE; n = 3). Seeds were imbibed in distilled water at room temperature. With the GSH values, only 0 and 72 hrs are significantly different ($p \leq 0.05$) between genotypes. All GSSG values are statistically similar ($p \leq 0.05$) between genotypes. No GSSG was detected in WT seeds imbibed for 48 hr.

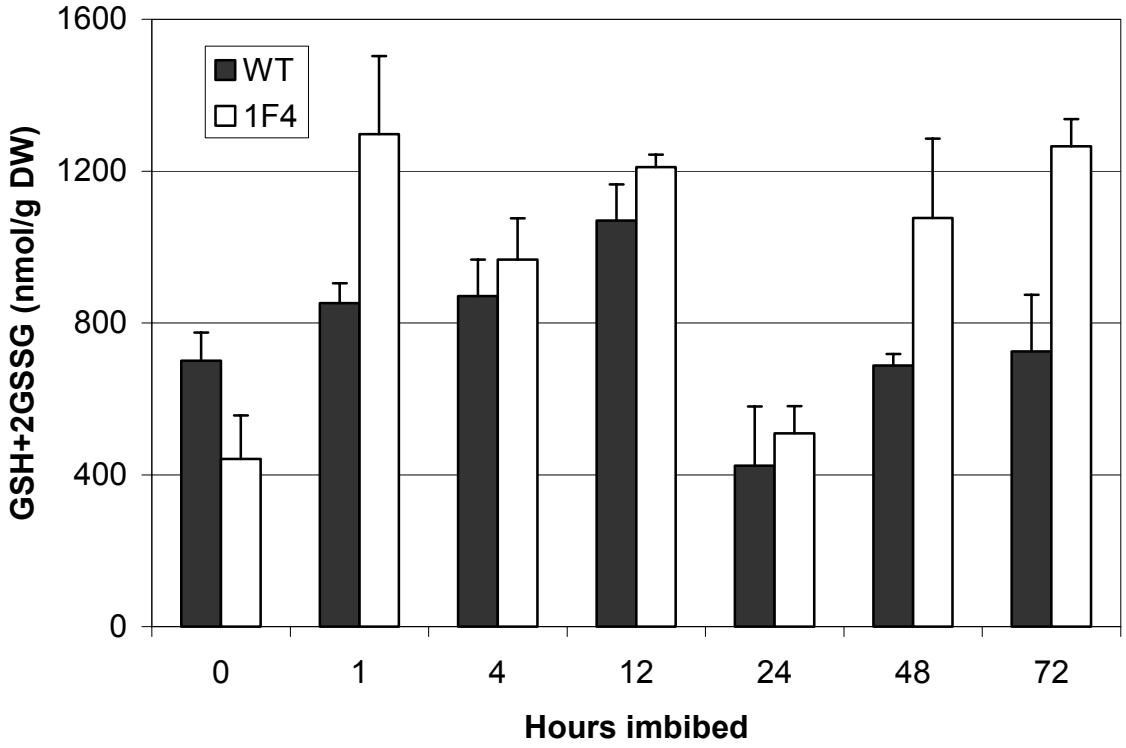


Figure 3-3: Changes in the glutathione pool (GSH+2GSSG) during seed imbibition. Results are pesented as mean ± SE of 3 experiments, except 0 hr where the data are the mean ± SE of 6 experiments. The data above were calculated from the values presented in Figure 3-2.

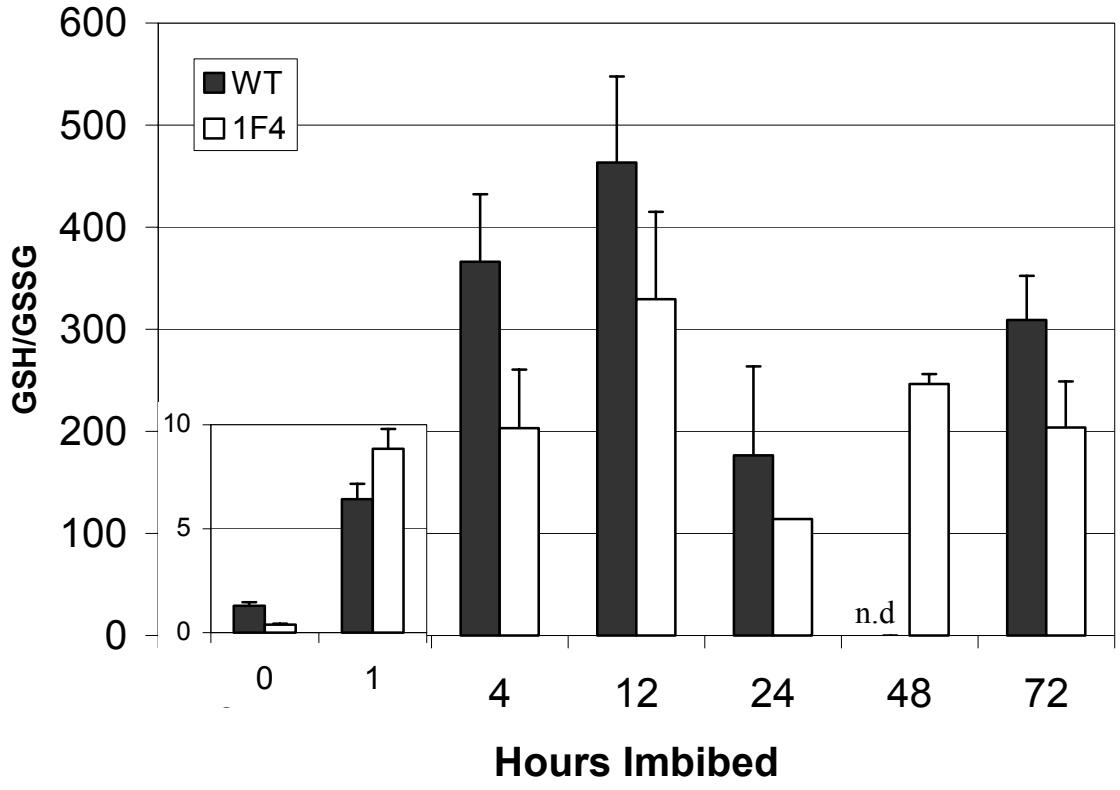


Figure 3-4: GSH/GSSG ratio during seed imbibition: 0, 1, 4, 12, 24, 48 and 72 hrs (mean ± SE; n = 3). The above figures were calculated from values in Figure 3-2. At 48 hrs, no GSSG was detected in WT seeds (n.d. = not determined), hence no ratio can be calculated. Inset shows 0 and 1 hr values in detail.

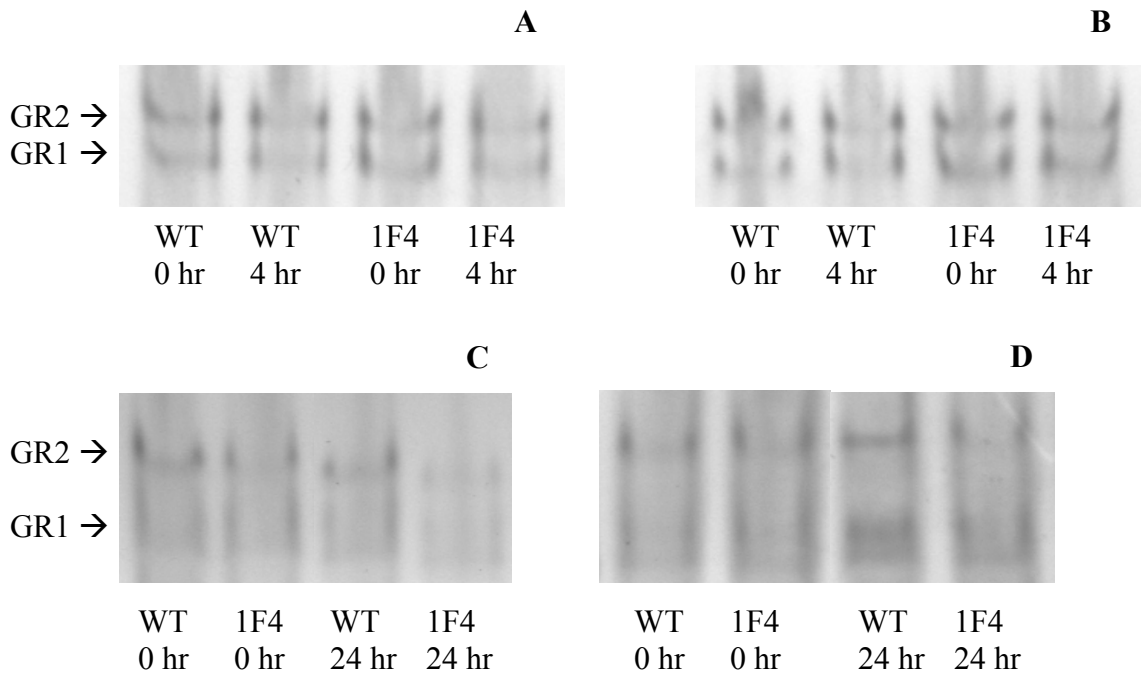


Figure 3-5: GR activity in WT and 1F4 seeds after 4 hr (A and B), and 24 hr (C and D) imbibition (mean ± SE; n = 2). Seeds were imbibed in distilled water at room temperature. Each lane represents a single separate extraction.

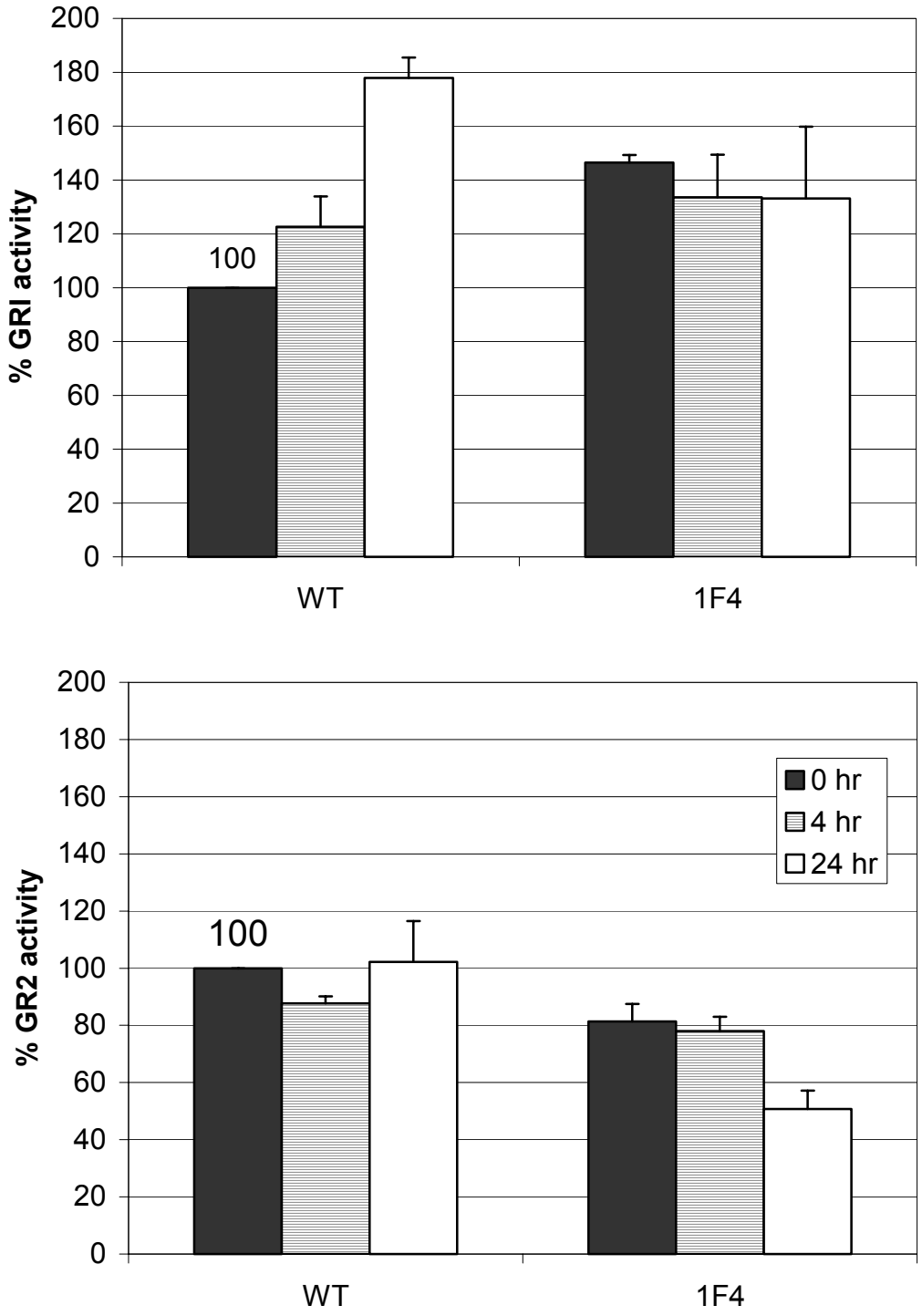


Figure 3-6: Comparison of GR activities in WT and 1F4 seeds during imbibition presented as relative percentages with WT 0 hr values set as 100%. Results shown are the mean ± SE of two experiments At 24 hrs, WT seeds reached T_{50} germination.

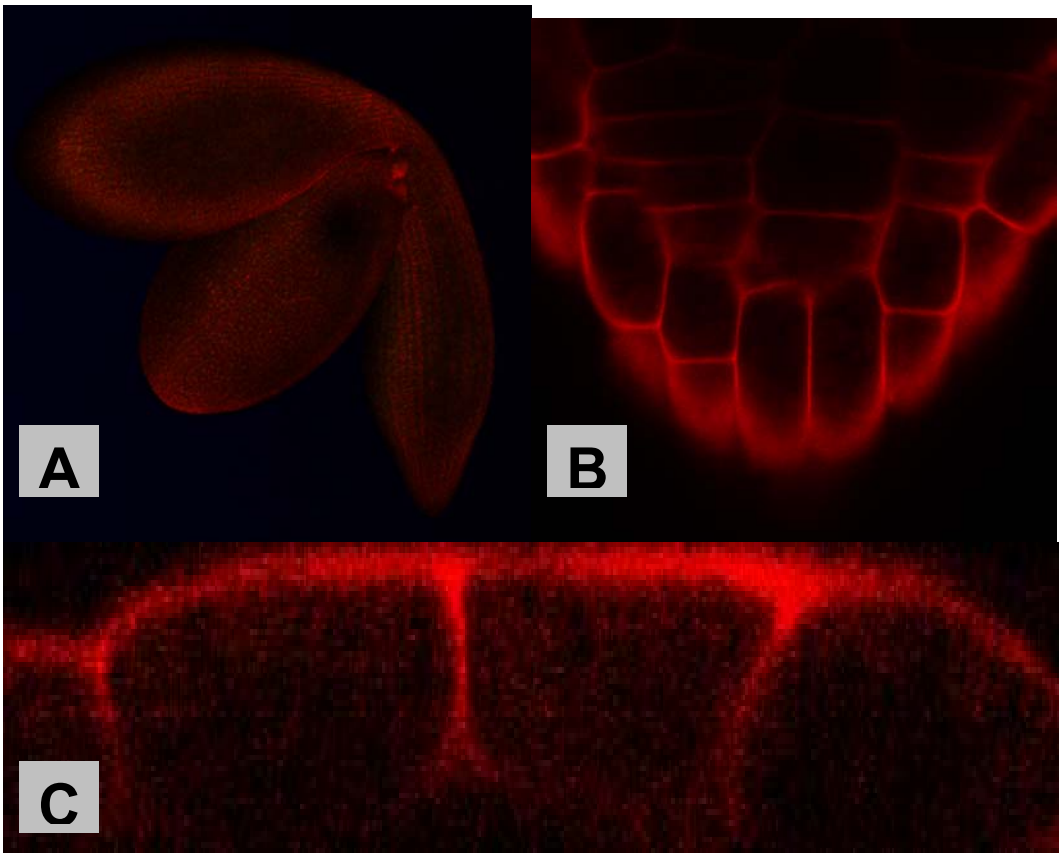


Figure 3-7: Propidium iodide (PI) fluorescence in whole embryo and cells. A, Image of an Arabidopsis embryo stained with 30 uL PI. Lack of large red patches indicate that the embryo was undamaged. B, Root tip cells (X-Y section) with PI staining, showing no autofluorescence. C, Z section of the epidermal layer stained with only PI. These images were captured using similar settings as samples stained with both MCB and PI. PI stains the cell wall and the nucleus. Absence of staining in the nucleus and the intact walls indicate tissue and cellular integrity of the sample. All subsequent samples were stained with PI and checked for damage before being imaged.

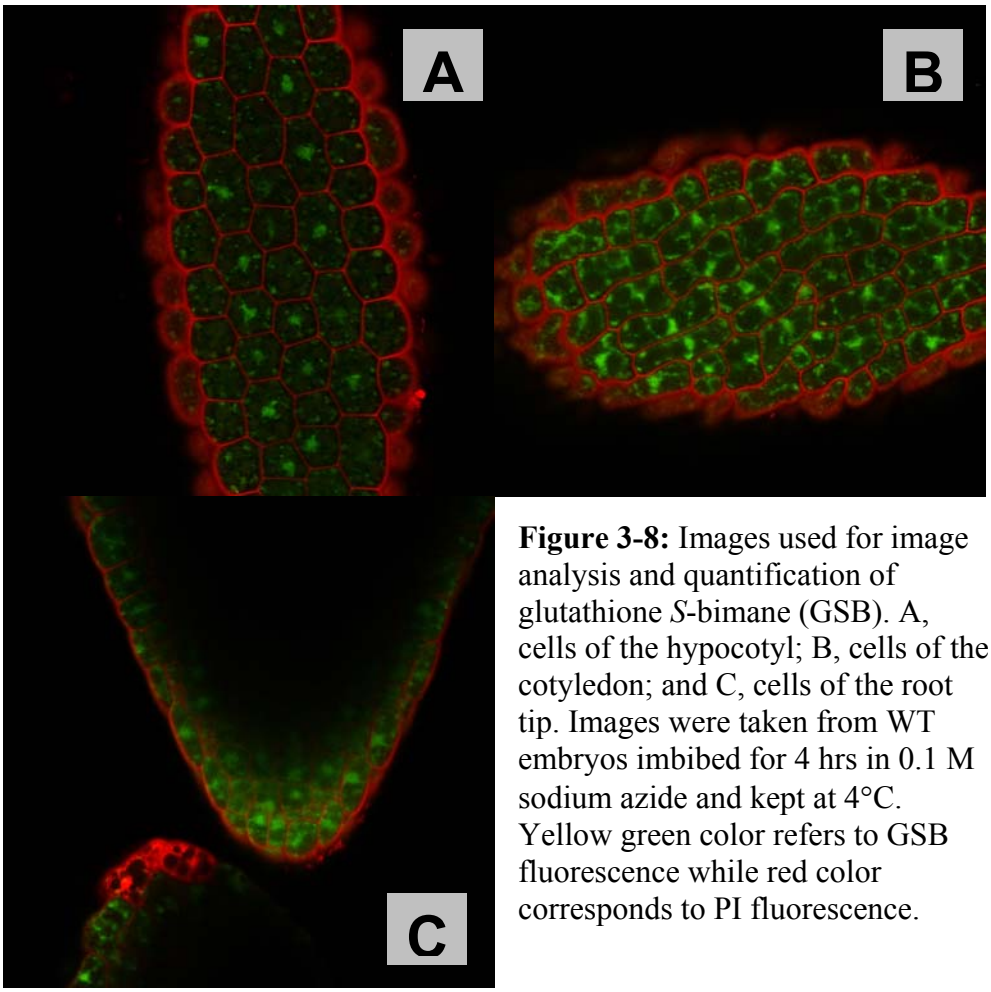


Figure 3-8: Images used for image analysis and quantification of glutathione *S*-bimane (GSB). A, cells of the hypocotyl; B, cells of the cotyledon; and C, cells of the root tip. Images were taken from WT embryos imbibed for 4 hrs in 0.1 M sodium azide and kept at 4°C. Yellow green color refers to GSB fluorescence while red color corresponds to PI fluorescence.

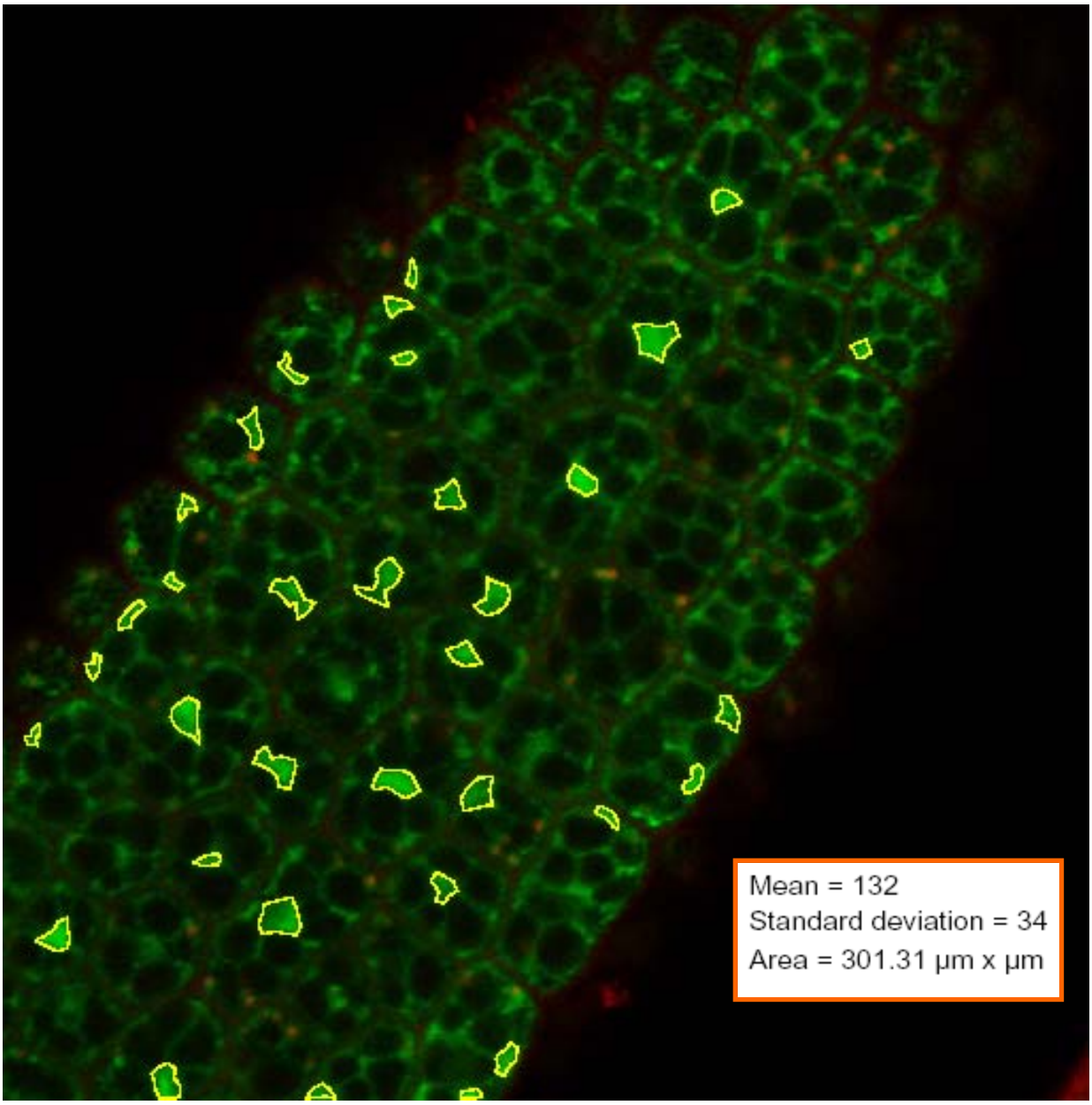


Figure 3-9: Analysis of CLSM images. This is a screenshot of an image where GSB fluorescence is being quantified. Quantification was performed by delineating areas of GSB fluorescence and measuring the average intensity in the chosen areas. The inset is the output from LSM Image Examiner. Final values were obtained from the mean of three images (i.e. 3 samples).

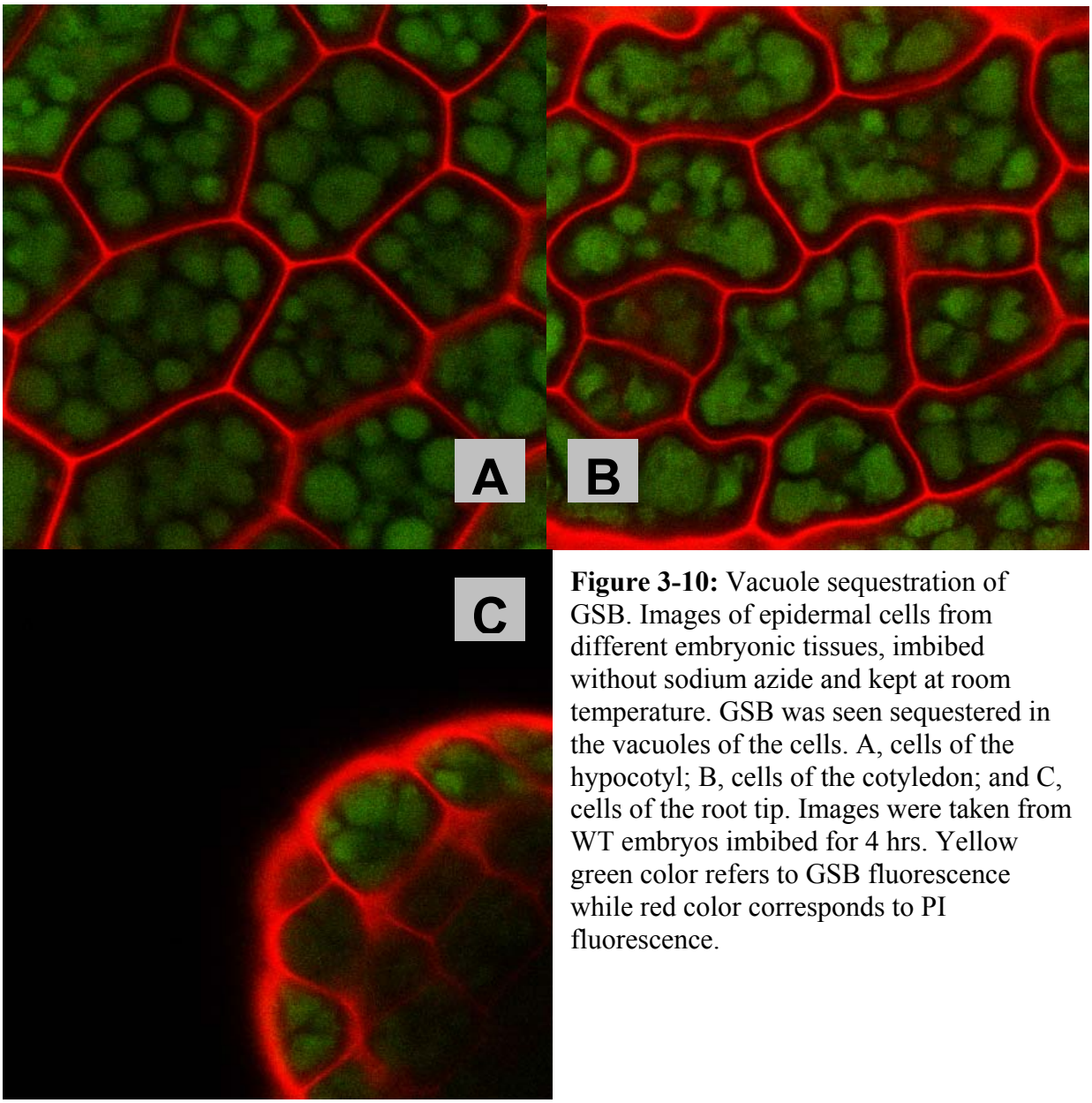


Figure 3-10: Vacuole sequestration of GSB. Images of epidermal cells from different embryonic tissues, imbibed without sodium azide and kept at room temperature. GSB was seen sequestered in the vacuoles of the cells. A, cells of the hypocotyl; B, cells of the cotyledon; and C, cells of the root tip. Images were taken from WT embryos imbibed for 4 hrs. Yellow green color refers to GSB fluorescence while red color corresponds to PI fluorescence.

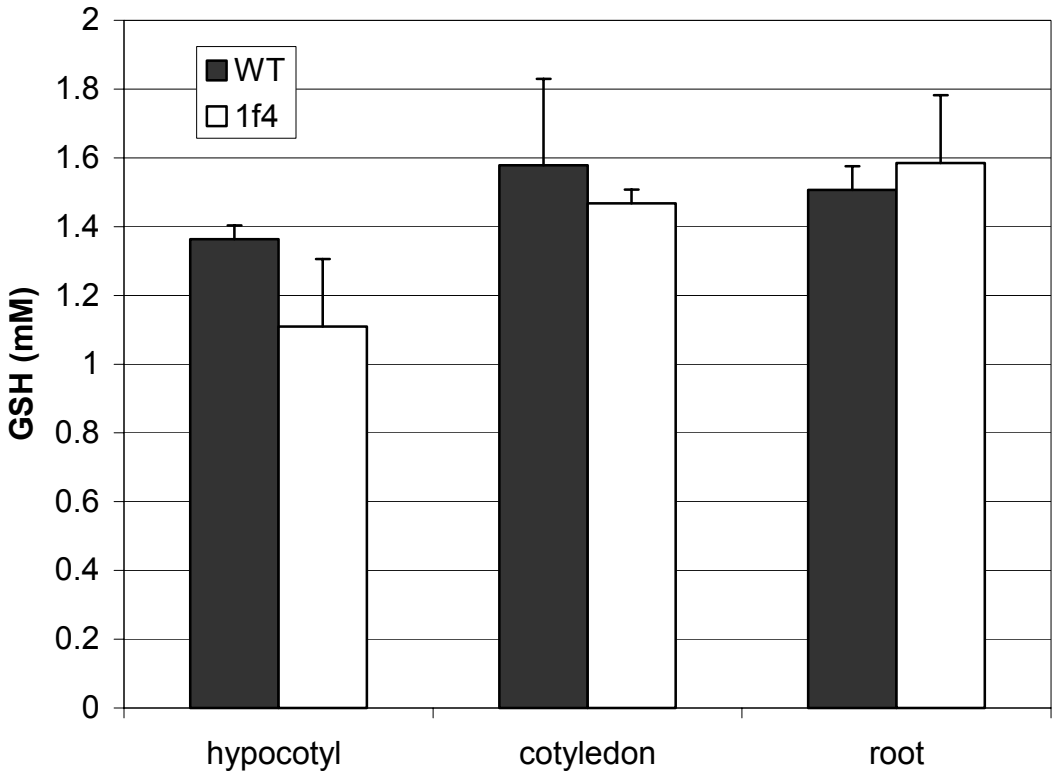


Figure 3-11: Measured glutathione pool from epidermal cells of different embryonic organs of WT and 1F4 seeds. Results presented are mean ± SE of 3 samples.

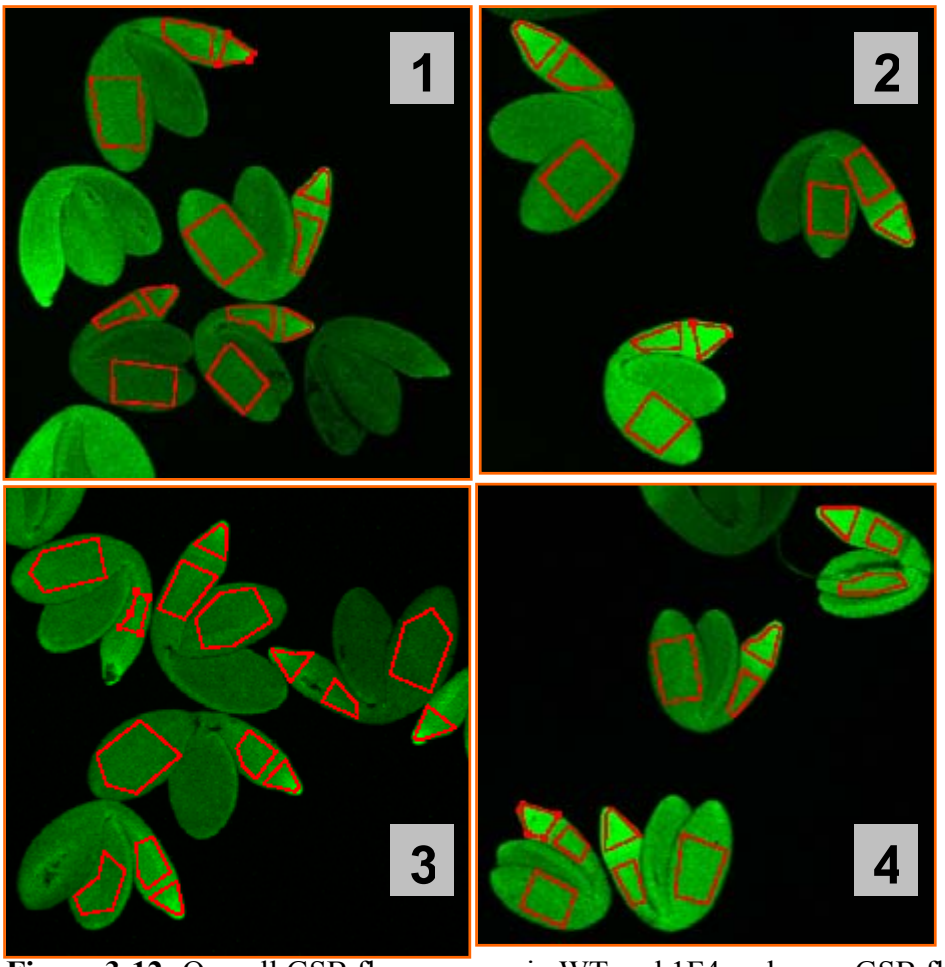


Figure 3-12: Overall GSB fluorescence in WT and 1F4 embryos. GSB fluorescence was measured for embryos imbibed in water to determine changes in size of the glutathione pool during imbibition. Cellular measurement was not done due to sequestration of the GSB to the vacuole. Low temperature and sodium azide were used in the previous experiments to prevent movement of GSB to the vacuole, but these do not allow for normal imbibition of the seeds. The same stains were used on these embryos as with the samples imaged at higher magnification.

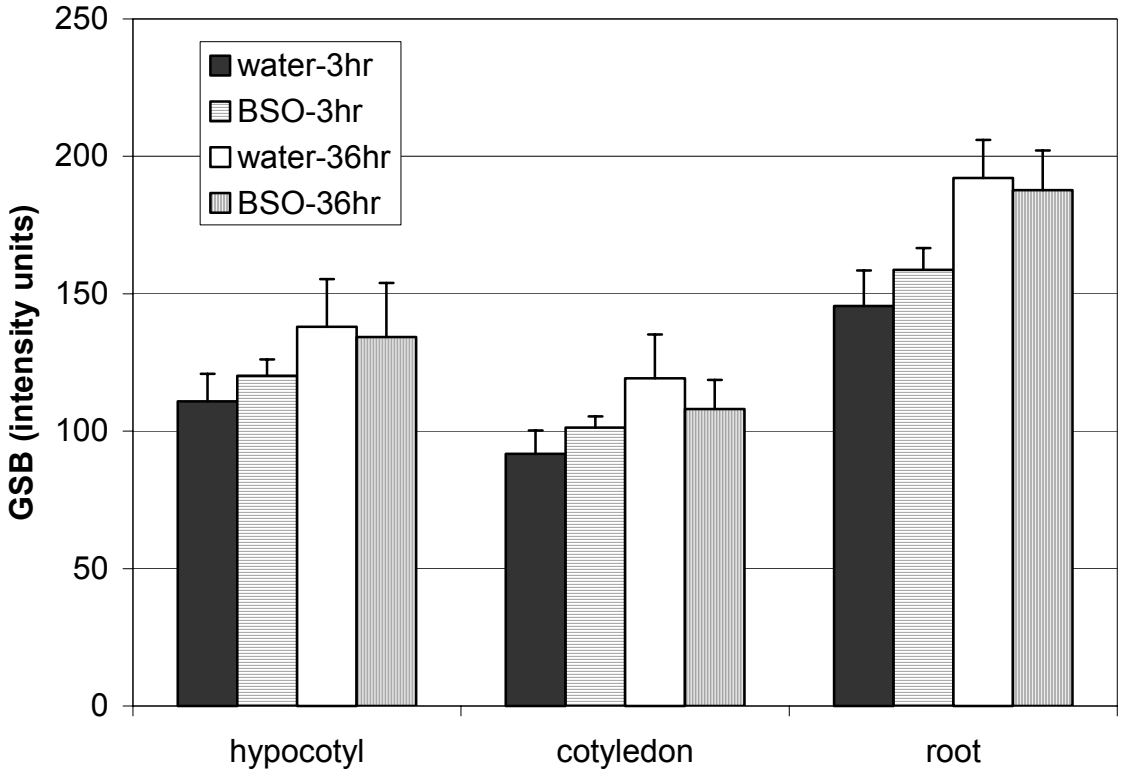
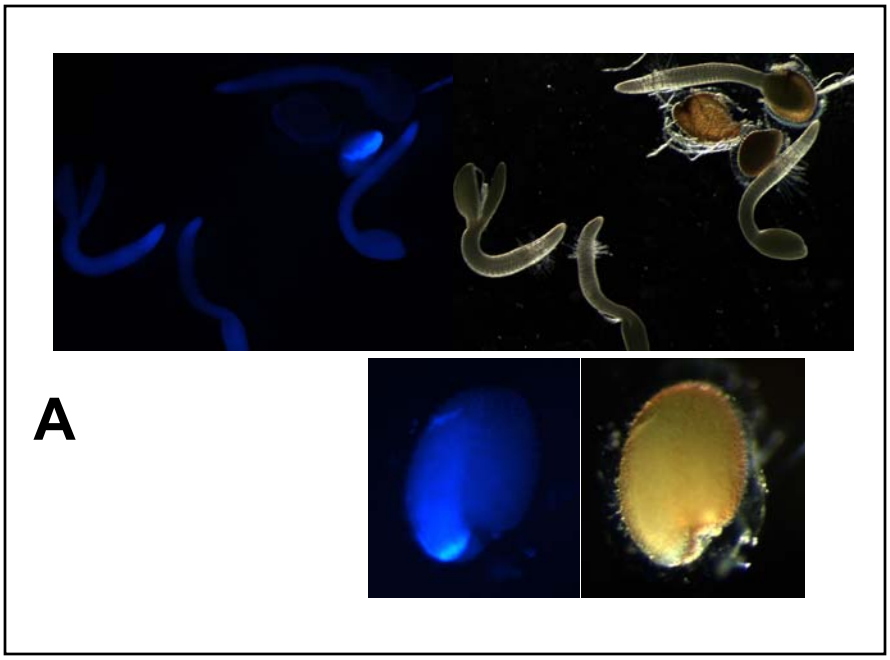


Figure 3-13: Comparison of GSB fluorescence in imbibed embryos. Overall GSB fluorescence in the different embryonic tissues of WT seeds, measured as shown in Figure 6. The WT embryos were imbibed for 3 and 36 hrs in water and 0.1 mM BSO at 24°C. Results presented are mean ± SE of 9 samples.



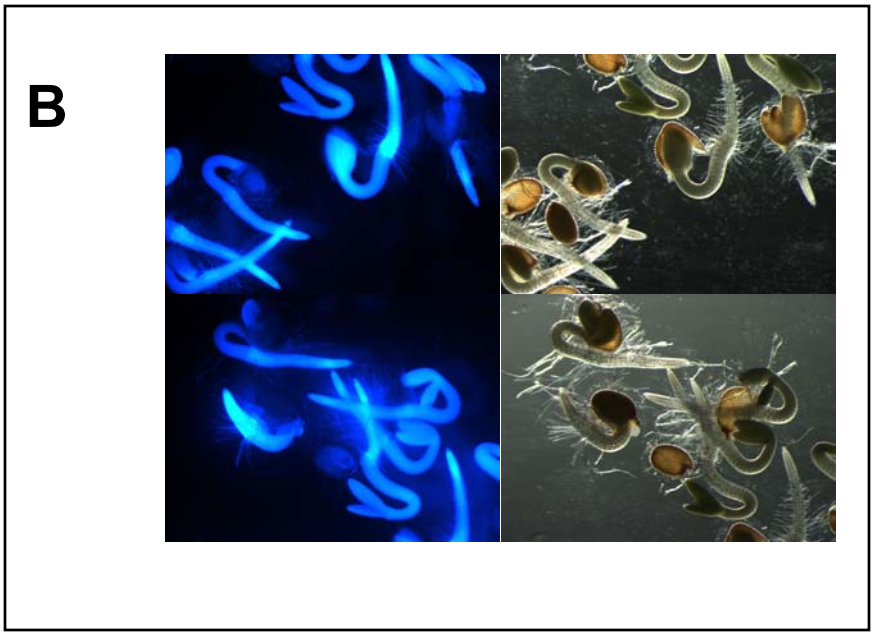


Figure 3-14: Images of WT seeds germinated in 0.1 mM BSO (A) and water (B) using a fluorescence stereomicroscope. The seedlings were stained with MCB and GSB fluorescence was seen as the blue color in the images (images on the left). The same seedlings were also imaged using reflected light (images on the right). The smaller images in A are close ups of a seed germinating in BSO solution.

GERMINATION UNDER STRESS

Germination at high and low temperature. Germination of WT and 1F4 seeds at 10°C had a similar effect to stratification, wherein the seeds were imbibed at a low temperature for several days before they were sown to induce germination. Germination at low temperature was uniform in WT and 1F4 seeds (Figure 3-16). Both genotypes reached 100% germination and a mean germination time of 3.01 days. Germination in high temperature (30°C/25°C) reduced 1F4 seeds germination percentage to 48 ± 2% while WT seeds showed 95 ± 2% germination (Table 3-2). WT seeds reached mean time germination (T_{50}) in less than a day (0.78 ± 0.2 days) (Table 3-1). 1F4 seeds, on the other hand, had a mean germination time of 4.28 ± 0.5 days and only 48 ± 2% germination. This suggests that the GR2 antisense line is more sensitive to heat stress.

Germination at low water potential. To simulate drought stress, WT and 1F4 seeds were germinated on -0.56 MPa PEG solution. Germination in both genotypes slowed and the germination percentage was lowered (Figure 3-15). Total germination of WT seeds was 70 ± 3% (Table 3-2). Germinated WT seedlings developed slowly and looked unhealthy with thin roots and cotyledons. Since only 21 ± 1% of the 1F4 seeds germinated, probit analysis was used to calculate mean time germination (T_{50}) (Figure 3-17). Mean time germination was significantly different between the genotypes with WT T_{50} equal to 3.1 ± 0.3 days and 1F4 9.6 ± 1.4 days (Table 3-1).

As shown earlier, WT and 1F4 controls (25°C in water) showed almost complete germination (Figure 3-1). T_{50} of WT and 1F4 seeds were statistically different ($p \leq 0.05$) with 1F4 longer (1.7 ± 0.1 days) compared to WT (1.2 ± 0.1 days) (Table 3-1).

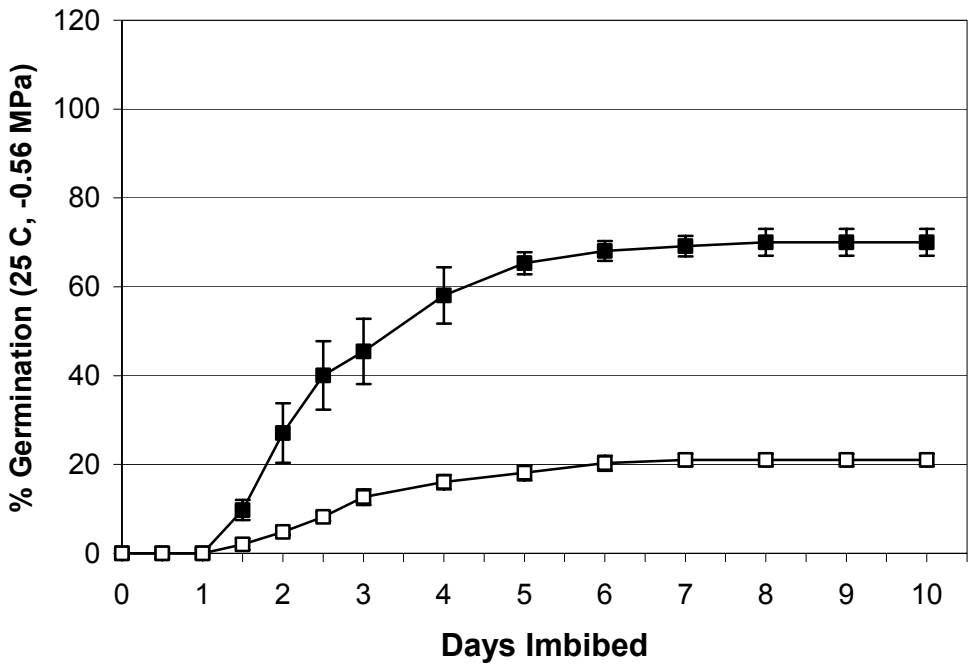


Figure 3-15: Germination of WT and 1F4 seeds at low water potential (-0.56 MPa). All seeds were germinated in light, at 25°C and without cold pre-treatment. Values shown are the mean ± SE of 4 replications containing 80-100 seeds each. Error bars are not visible when they are smaller the height of the point symbol.

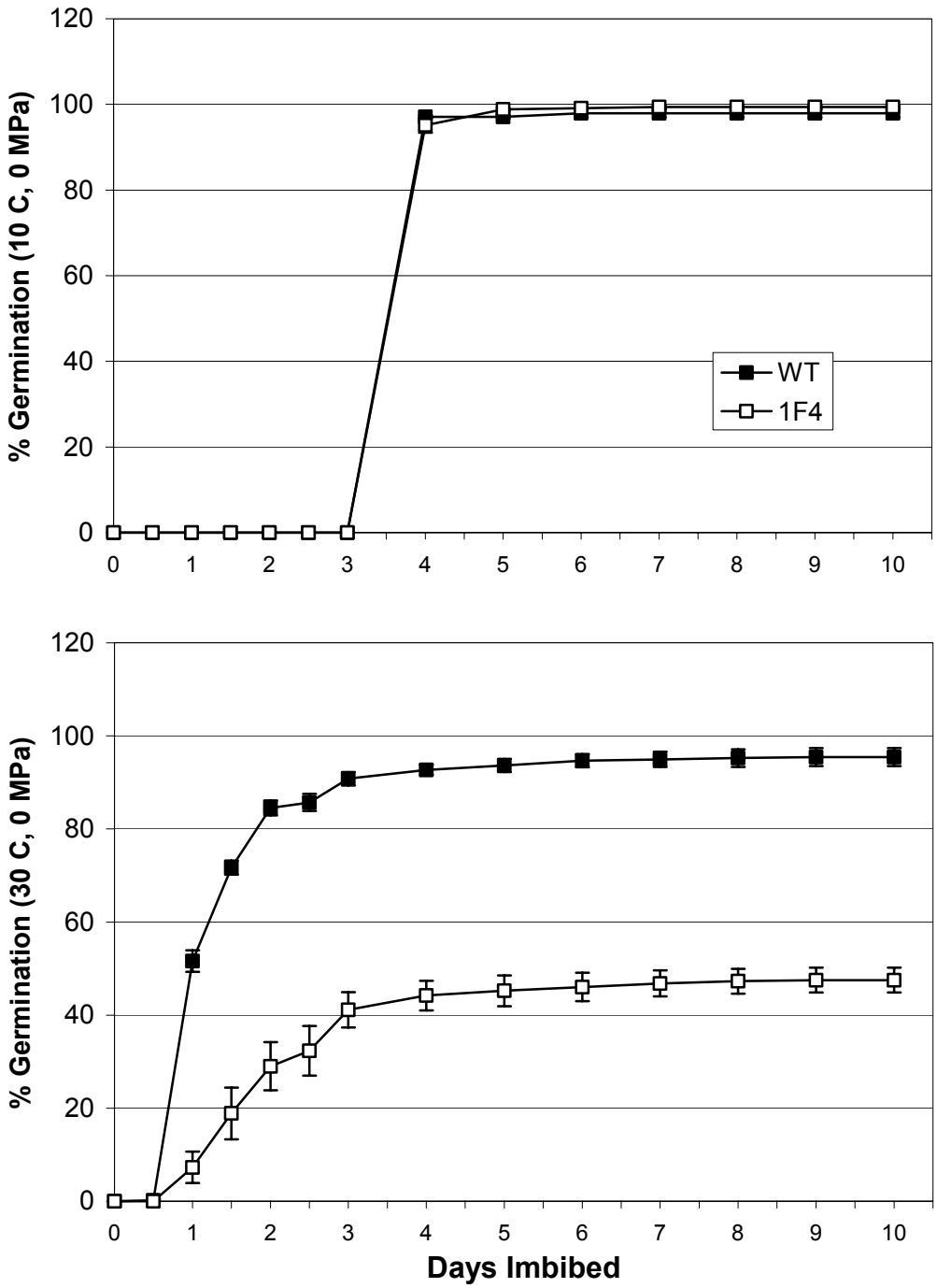


Figure 3-16: Germination of WT and 1F4 seeds at high and low temperatures. Seeds grown at high temperature were exposed to 30°C/25°C regime, while those germinated at low temperature were exposed to 10°C. Seeds were germinated in light and without cold pre-treatment. Values shown are the mean ± SE of 4 set-ups containing 80-100 seeds. Error bars are not visible when they are smaller the height of the point symbol.

Table 3-1: Mean germination time (T_{50}) of WT and 1F4 seeds in stress conditions (mean ± SE). Four replicates of 60-100 seeds each were used. Figures in italics indicate less than 25% germination. Non-similar letters indicate significant difference when comparing between genotypes at each stress condition.

Seed genotype	***25℃ water***	***10℃ water***	***30℃ water***	***25℃ -0.56 MPa***
WT	1.2 ± 0.1^{a}	3.01 ± 0^{c}	0.78 ± 0.2^{a}	3.1 ± 0.3^{a}
1F4	1.7 ± 0.1^{b}	3.01 ± 0^{c}	4.28 ± 0.5^{b}	*9.59 ± 1.4^{b}*

Table 3-2: Total germination of WT and 1F4 seeds at different stress conditions (mean ± SE; n =4; 50 to 100 seeds per replicate) 14 days after start of imbibition. All assays were done in 14 hrs photoperiod and no cold pre-treatment. Seeds for control, low temperature and high temperature set-ups were germinated in distilled water. Means are significantly different ($p \leq 0.05$) between genotypes at 30°C and –0.56 MPa experiments.

Seed genotype	***25℃ water***	***10℃ water***	***30℃ water***	***25℃ -0.56 MPa***
WT	100	98 ± 1	95 ± 2	70 ± 3
1F4	98 ± 1	99	48 ± 2	21 ± 1

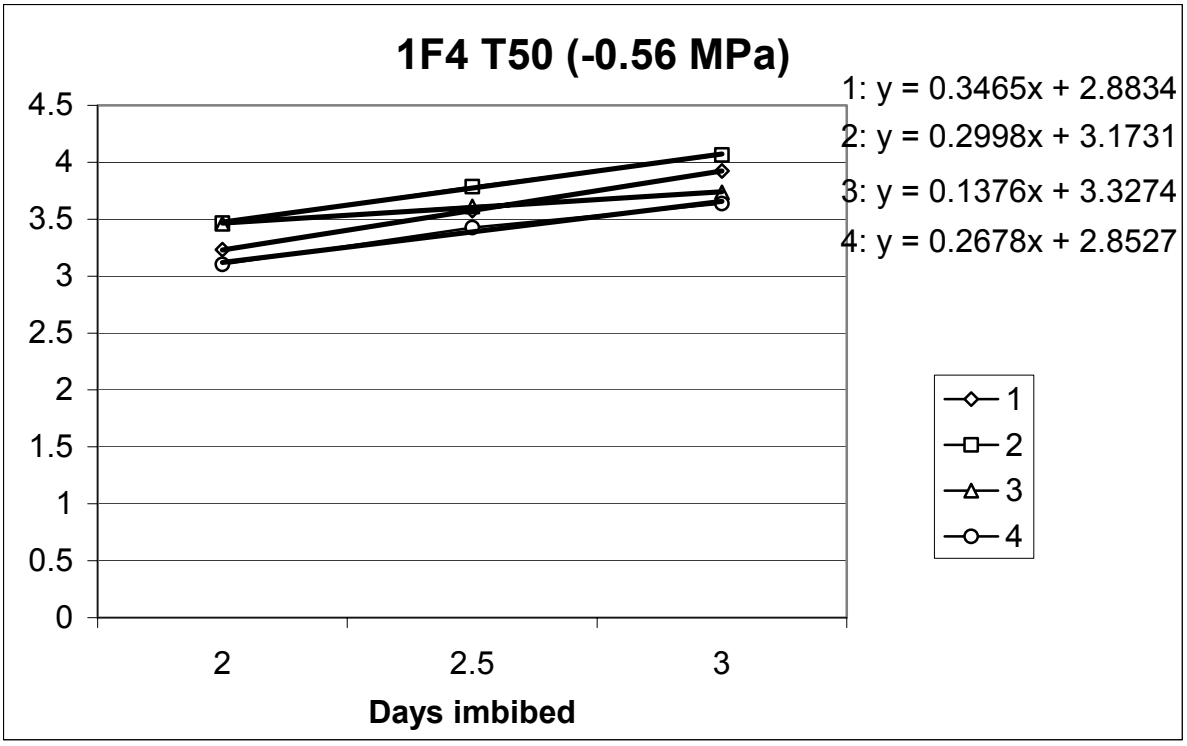


Figure 3-17: T_{50} calculation for 1F4 seeds germinated at –0.56 MPa by probit analysis. The first 3 or 4 data points in the germination curves of each sample were transformed to probit values and the equivalent day for probit value 5 (T_{50}) was computed using linear regression.

DISCUSSION

This study shows that Arabidopsis seed imbibition and germination is characterized by a very reduced glutathione pool. GSSG levels declined to less than 1% of the pool after 4 hr of imbibition and remained at this level throughout germination (Figure 3-2). During the first 12 hr of imbibition, GSH levels in Arabidopsis seeds were between 600 to 1200 nmol/g DW. Higher GSH values were reported in imbibing pea seeds where GSH levels ranged from 1000-1500 nmol/g DW 0 to14 hr of imbibition. GSSG levels in pea seeds started as 24% of the total glutathione pool in dry seeds, declined rapidly after 5 hr and were only 3% after 14 hrs imbibition (Kranner and Grill 1993).

The decline in GSH levels at 24 hr imbibition coincided with the T_{50} of WT Arabidopsis seeds (Figure 3-1 and 2-2). A similar pattern was also observed with *C. rubrum* seeds wherein the GSH levels and the glutathione pool rose just before radicle emergence and then declined back to values similar to dry seeds (Ducic et al. 2003). In pine seeds (without teguments), an increase in GSH levels was also observed after 24 hrs of imbibition and after 48 hr, GSH levels declined to less than dry seed values (Tommasi et al. 2001). In this study's results and those cited above, the decrease in GSH levels during or immediately before radicle protrusion cannot be accounted for by an increase in GSSG concentration since GSSG remained at very low quantities (Figure 3-2). The absence of an upsurge in GSSG levels suggests that not only is oxidative stress magnified during germination, but that glutathione is also being utilized in other processes. Production of ROS such as H_2O_2 (Puntarulo et al. 1991; Gidrol et al. 1994; Caro and Cumming 1998; Hite et al. 1999; Schopfer et al. 2001; Bailly et al. 2002; Morohashi 2002), NO (Caro and Puntarulo 1999), hydroxyl radicals (Schopfer et al. 2001), and superoxide radicals (Puntarulo et al. 1991; Gidrol et al. 1994; Schopfer et al. 2001) was associated with imbibition and germination. The increase in GSH levels just before germination may then serve as buffer against oxidative stress in germinating seeds. Alternatively, regulation of ROS levels in seeds is important as ROS can serve as a signaling molecule during germination and as protection against pathogen attack (Schopfer et al. 2001). Glutathione may still function mainly as an antioxidant during germination, but the loss of glutathione to other processes such as glutathione breakdown and conjugation reactions should also be taken into account. Little is known of glutathione

catabolism in plants, though one of the proposed pathways is breakdown of the tripeptide to its component amino acids (Foyer et al. 2001). Glutathione-*S*-transferase mRNA is known to be upregulated during germination (Bailey et al. 1996) and its overexpression is associated with better stress tolerance in seeds (Roxas et al. 1997). Glutathionylation, catalyzed by glutaredoxin, may be one avenue for GSH when lower GSH levels do not correspond with a marked accumulation of GSSG (Starke et al. 2003). Though glutathionylation was not measured in this study, it can be one of the sources of GSH besides *de novo* synthesis during germination. Glutathionylation refers to formation of a mixed disulfide bond between glutathione and a target protein and is considered an important mechanism for sensing redox perturbation in cells (Foyer and Noctor 2003).

GSSG recycling may account for most of the increase in GSH levels in WT seeds during imbibition, as the total glutathione pool remains similar to that of the dry seeds (Figure 3-3). GSSG recycling, along with GSH biosynthesis, was also observed in pine seeds during germination (Tommasi et al. 2001). An increase in GR activity was correlated with germination of wheat seeds (Cakmak et al. 1993), *Hordeum vulgare* (Fontaine et al. 1994), sunflower (Bailly et al. 2002), and maize (Leprince et al. 1990). In 1F4 seeds, the 3-fold increase in the glutathione pool may be due to glutathione synthesis and/or release of GSH from PSSG (protein-glutathione conjugates) (Figure 3-3). Preliminary data with *in vivo* imaging of glutathione have shown that, in WT seeds, GSH synthesis seems to occur to a large extent after radicle protrusion (Figure 3-14). Glutathione biosynthesis may account for the increase in GSH levels in 1F4 seeds, though we cannot confirm that in this study. Conjugation of glutathione to proteins (glutathionylation) was observed in wheat (Rhazi et al. 2003; Li et al. 2004a, 2004b) and spinach seeds (Butt and Ohlrogge 1991) and accumulation of PSSG was associated with seed desiccation (Kranner and Grill 1996). Conversely, PSSG content declined with imbibition (Fahey et al. 1980; Butt and Ohlrogge 1991).

The 3-fold increase in the glutathione pool in 1F4 seeds may be a form of compensation for lowered GR2 activity caused by the antisense insert, a response to oxidative stress during germination or an insertion artifact. GR2 activity in 1F4 declined to 50% percent of WT after 24 hr imbibition, and this may induce glutathione biosynthesis as compensation. The increase in

1F4 GSH levels may also be induced by increased oxidative stress caused by differences in experiment conditions such as drying of the germination paper on which the seeds were sown. It also interesting to note that during storage, GSH was more oxidized in 1F4 seeds compared to WT, indicating that 1F4 seeds were experiencing greater oxidative stress than WT. It may be a similar case during germination of 1F4 seeds where the greater stress was reflected in induction of high GSH levels. The oxidative stress may be a position effect that causes perturbations in the metabolic processes of the cell. A mapping experiment is needed to determine if the antisense insert has disrupted expression of a gene or genes.

In WT imbibing seeds, GR1 activity increased during germination, perhaps in preparation for onset of photosynthesis in seedling, while GR2 activity remained constant. On the other hand, 1F4 seeds had constant high GR1 activity but a decreasing GR2 activity during imbibition (Figure 3-6). This difference in GR activity suggests that the antisense mechanism may not be constitutively active in seed tissues, though the plant seems to compensate for it by increasing the activity of the other GR isoform. Another factor may also be that the seeds used were T_5 generation. Though the 1F4 line is homozygous, repeated planting eliminates the weakest individual plants, leaving plants with a phenotype similar to WT to survive. During imbibition, the GR system in 1F4 is capable of reducing oxidized glutathione at the same rate as WT seeds given that all GSSG was reduced within 4 hours of imbibition in both genotypes (Figure 3-2).

In situ labeling of GSH in seed embryos estimate cytosolic GSH to be 1.1 to 1.6 mM. Several plant tissues (i.e. Arabidopsis intact root, trichome cells, suspension culture cells), have been examined using this technique and different values were obtained (approximately 0.2 mM culture cells and trichome cells to 2-3 mM in most root cells), emphasizing the physiological differences between tissue types (Fricker et al. 2000; Gutierrez-Alcala et al. 2000; Meyer et al. 2001). Between WT and 1F4 embryos, no significant difference in the GSH levels was observed using CLSM (Figure 3-11). This was not reflected in the HPLC data of dry WT and 1F4 seeds where WT seeds had significantly higher GSH levels (Figure 3-2). The discrepancy may be because the MCB stain in the CLSM technique labels only cytosolic GSH. In poplar leaves, only 50% of the glutathione pool measured *in vitro* was labeled by MCB *in situ* (Hartmann et al. 2003). However, both CLSM (Figure 3-13) and HPLC (Figure 3-2) results show that no

significant GSH biosynthesis occurs during Arabidopsis seed imbibition. This is seen in the lack of effect of BSO on embryo GSB fluorescence and the relatively steady state glutathione pool as determined by HPLC during imbibition. The CLSM method offers a lot of possibilities in seed and seedling studies such as determining the location of GSH synthesis and GSH transport in seedlings and glutathione dynamics including degradation in plant embryos during development and germination.

Germination at low water potential simulates germination under drought stress. Imbibition at low water potential affected both WT and 1F4 seeds, resulting to lower germination percentage. Less than 25% of the 1F4 seeds showed radicle protrusion, compared to WT's 70% germination (Figure 3-15). This shows that 1F4 seeds have greater sensitivity to drought stress. Many studies have reported changes in GR activity in drought stressed plants or cells (Bueno et al. 1998; Anderson and Davis 2004; Keles and Unyayar 2004,), however, there is less information on germinating Arabidopsis seeds under drought stress. *Helianthus anuus* seedlings exhibited lower GR activity when subjected to drought stress (Keles and Unyayar 2004). No changes in GR activity were observed in BY-2 cells grown on high osmoticum (Bueno et al. 1998). WT Arabidopsis seedlings grown at moderate water deficit showed stimulation of root elongation and rate of cell production, and lateral root initiation and elongation were unaffected (van der Weele et al. 2000). Most of these characteristics were suppressed at lower water potential (-1.2 MPa). Stimulation of root growth under mild stress is a coping mechanism that allows roots to plumb the soil for water (van der Weele et al. 2000).

As glutathione is proposed to participate in acclimation of plants to abiotic stress including low (Kocsy et al. 2001) and high temperatures (Dat et al. 1998), germination of both WT and 1F4 seeds at 10 and 30°C was examined. 1F4 seeds reacted differently when germinated at these temperatures with only approximately 40% germination at high temperature and almost 100% at low temperature. Germinating WT and 1F4 seeds at 10°C resulted in a germination pattern similar to stratified Arabidopsis seeds for both genotypes, suggesting that the given temperature was not enough to be stressful to both WT and 1F4 seeds. Germination at 30°C, however, showed a significant difference in performance between WT and 1F4 seeds. Germination of 1F4 seeds was reduced to about 50% while WT seeds were not affected. Several studies of plants

subjected to heat stress have shown that H_2O_2 accumulated and lipid peroxidation increased in stressed plants (Sairam et al. 2000; Rivero et al. 2004). GR activity was observed to decrease in tomatoes and the chilling-sensitive maize line 'Pen' grown at 35°C and 40°C, respectively (Rivero et al. 2004; Kocsy et al. 2004).

One reason for the lower germination percentage of 1F4 seeds under stress conditions may be inadequate activity of GR2 to cope with the GSSG recycling needs in the stressed seeds. This may cause longer mean times germination and even seed death. Another possibility may be that lowered stress tolerance of 1F4 seeds is due to an insertion artifact wherein the GR2 antisense insert disrupted one or more genes resulting in this phenotype. Other GR2 antisense lines or a GR2 knockout have to be studied to confirm this phenotype. We have obtained GR2 knockouts and observed that they are very sensitive to stress, indicating the importance of the gene in stress responses of plants. We have not been able grow the plants to fruiting stage and thus, their seed physiology was not examined in detail.

CHAPTER 4: RETROSPECTION AND POSSIBILITIES

SUMMARY

Seed desiccation and germination is characterized by high potential for oxidative stress. Numerous studies have been done on the activity of the ROS scavenging systems both the detoxifying enzymes and the antioxidant compounds during seed dehydration and germination, but few have been conducted on *Arabidopsis thaliana* seeds. With the number of crop plants and large seeded species available, Arabidopsis seeds are generally not considered an ideal model for genetic and molecular studies on seed development (Meinke 1994). The amount of effort required to obtain enough material for biochemical assays usually proves to be discouraging. However, the advent of genomic information on the plant made Arabidopsis an attractive model organism for molecular and genetic analysis of different stages of plant growth, including seed development.

To better understand antioxidant responses during plant stress, antisense lines against the two glutathione reductase genes were created. Evidence of high percentage of seed abortions in anGR2 (1F4) lead to the start of this project. The biggest stumbling block in this study was the unavailability of seeds from the early generation of transformed antisense plants. T_4 seeds were obtained from anGR1 T_3 plants, and T_5 seeds from anGR2 (1F4) T_4 plants. WT and anGR1 seeds were indistinguishable from each other, both having similar precocious germination percentages, germination rate and percentage in dry seeds, seed abortions and seed weight (data not shown). Focus was then placed on 1F4, which deviated more clearly from WT phenotype in the above features.

There are two novel techniques used in this study. One was the HPLC method we have adapted for studying Arabidopsis seeds. The methodology allowed for simultaneous measurement of GSH and GSSG using a fluorescent detector, which has higher accuracy than UV detection. The derivatized samples can be stored for a year when kept at 4°C and in the dark (Jones et al. 1998). The HPLC method can also be modified to allow measurement of glutathionylation in seed proteins. As far as we know, this is the first study done on glutathione dynamics in Arabidopsis

seed maturation and germination. The second method is the in vivo imaging of glutathione by confocal laser scanning microscopy (CLSM). Though the use of this technique in seeds is still in its preliminary stages, this study has shown that it is feasible. Using CLSM, changes in cytosolic glutathione of the different tissues such the root apical meristem and the developing vascular tissues in the seed embryo can be determined. Native PAGE gels were used to determine GR activity in the seeds because measures of activity of individual isoforms. However, a disadvantage of native PAGE gels is that they cannot show with great accuracy small differences in activity between isoforms.

Examination of GR activity in seeds maturing and germinating under optimal conditions showed slightly higher GR1 activity in 1F4 compared to WT and similar GR2 activity except after 24 hr of imbibition when 1F4 had only 50% of WT activity. GSH and GSSG levels in WT and 1F4 were also similar during seed maturation and germination. However, a unique phenotype was very apparent when the 1F4 seeds were exposed to stress, namely natural and artificial ageing, and germination at high temperature and at low water potential. Unfortunately, we do not have data on the GR activity and glutathione pool during germination of aged or stressed seeds.

Among the results obtained in this study is (1) that during Arabidopsis seed desiccation, glutathione accumulates and GR activity decreases. Glutathione may then function as the major antioxidant in stored seeds. (2) Ageing of Arabidopsis seeds entails oxidation of GSH, indicating oxidative stress during storage. (3) 1F4 seeds to age faster in ambient and CDT conditions indicating higher oxidative stress in these seeds and perhaps a debilitating effect of GR2 antisense insert during recovery at imbibition. (4) During imbibition and germination, the glutathione pool dynamics in Arabidopsis seeds follow a similar pattern to other seeds. The glutathione pool becomes very reduced and GSH levels in WT seeds increase mostly by GSSG recycling during imbibition. (5) A peak in GSH concentration just before germination indicated high capacity of the glutathione system to deal with ROS production associated with radicle protrusion. (6) Our results showed that 1F4 seeds are less tolerant of heat and drought stress, indicating perturbations in the ROS scavenging systems particularly GR2.

Several aspects of this study need to be examined in further detail such as the glutathione levels in aged and stressed seeds during germination, and GSH biosynthesis during germination and seedling growth. Experiments focused on disulfide bridge formation and reduction during seed maturation and germination may provide insight on storage protein accumulation in Arabidopsis and the function of thiols in these key stages. Glutathione has been proposed to serve as part of redox signal transduction in plants (Foyer and Noctor 2003). Further studies of glutathione during stress may provide understanding, at the molecular level, the induction of acclimation and stress tolerance in seeds.

REFERENCE

Albro, PW; JT Corbett, JL Schroeder. 1986. Generation of hydrogen peroxide by incidental metal ion-catalyzed autooxidation of glutathione. J Inorg Biochem 27: 191-203.

Anderson, JV; DG Davis. 2004. Abiotic stress alters transcript profiles and activity of glutathione *S*-transferase, glutathione peroxidase, and glutathione reductase in *Euphorbia esula*. Physiol Plant 120: 421-433.

Awazuhara, M; H Kim, H Hayashi, M Chino, S Kim, T Fujiwara. 2002. Composition of seed storage proteins changed by glutathione treatment of soybeans. Biosci Biotechnol Biochem 66: 1751-1754.

Bailey, PC; GW Lycett, JA Roberts. 1996. A molecular study of dormancy breaking and germination in seeds of *Trollius ledebouri*. Plant Mol Biol 32: 559-564.

Bailly, C; A Benamar, F Conrineau, D Come. 1998. Free radical scavenging as affected by accelerated ageing and subsequent priming in sunflower seeds. Physiol Plant 104: 646-652.

Bailly, C; C Audigier, F Ladonne, MH Wagner, F Coste, F Corbineau, D Come. 2001. Changes in oligosaccharide content and antioxidant enzyme activities in developing bean seeds as related to acquisition of drying tolerance and seed quality. J Exp Bot 52: 701-708.

Bailly, C; R Bogatek-Leszczynska, D Come, F Corbineau. 2002. Changes in the activities of antioxidant enzymes and lipoxygenas during growth of sunflower seedlings from seeds of different vigour. Seed Sci Res 12: 47-55.

Bailly, C; J Leymarie, S Rousseau, D Come, A Feutry, F Corbineau. 2003. Sunflower seed development as related to antioxidant enzyme activities. In: G Nicolas, KJ Bradford, D Come, HW Pritchard (eds), The Biology of Seeds: Recent Research Advances. CAB International, Wallingford, UK, pp. 69-75.

Bailly, C. 2004. Active oxygen species and antioxidants in seed biology. Seed Sci Res 14: 93-107.

Bewley, JD. 1997. Seed germination and dormancy. Plant Cell 9: 1055-1066.

Bueno, P; A Piqueras, J Kurepa, A Savoure, N Verbruggen, M van Montagu, D Inze. 1998. Expression of antioxidant enzymes in response to abscisic acid and high osmoticum in tobacco BY-2 cell cultures. Plant Sci 138: 27-34.

Butt, AD; JB Ohlrogge. 1991. Acyl carrier protein is conjugated to glutathione in spinach seed. Plant Physiol 96: 937-942.

Cakmak, I; D Strbac, H Marschner. 1993. Activities of hydrogen peroxide-scavenging enzymes in germinating wheat seeds. J Exp Bot 44: 127-132.

Caliskan, M; Cuming AC. 1998. Spatial specificity of H_2O_2-generating oxalate oxidase gene expression during wheat embryo germination. Plant J 15: 165-171.

Caro, A; S Puntarulo. 1999. Nitric oxide generation by soybean embryonic axes: possible effect on mitochondrial function. Free Radic Res 31: S205-S212.

Clerkx, EJM; H Blankestijn-De Vries, GJ Ruys, STPC Groot, M Koornneef. 2004. Genetic differences in seed longevity of various *Arabidopsis* mutants. Physiol Plant 121: 448-461.

Dat, JF; CH Foyer, IM Scott. 1998. Changes in salicylic acid and antioxidants during induced thermotolerance in mustard seedlings. Plant Physiol 118: 1455-1461.

De Gara, L; MC de Pinto, V Moliterni, M D'Egidio. 2003. Redox regulation and storage processes during maturation in kernels of *Triticum durum*. J Exp Bot 54: 249-258.

De Paula, M; M Perez-Otaola, M Darder, M Torres, G Frutos, CJ Martinez-Honduvilla. 1996. Function of the ascorbate-glutathione cycle in aged sunflower seeds. Physiol Plant 96: 543-550.

De Tullio, MC; O Arrigoni. 2003. The ascorbic acid system in seeds: to protect and to serve. Seed Sci Res 13: 249-260.

De Vos, CHR; HL Kraak, RJ Bino. 1994. Ageing of tomato seeds involves glutathione oxidation. Physiol Plant 92: 131-139.

Donahue, JL; VK Stromberg, CL Cramer, R Grene. Antisense expression of glutathione reductase in Arabidopsis thaliana L. affects seed development, seed viability, and seedling development. Unpublished data.

Ducic, T; I Liric-Rajlic, A Mitrovic, K Radotic. 2003. Activities of antioxidant systems during germination of *Chenopodium rubrum* seeds. Biol Plant 47: 527-533.

Fontaine, O; C Huault, N Pavis, J Billard. 1994. Dormancy breakage of *Hordeum vulgare* seeds: effects of hydrogen peroxide and scarification on glutathione level and glutathione reductase activity. Plant Physiol Biochem 32: 677-683.

Foyer, CH; FL Theodoulou, S Delrot. 2001. The functions of inter- and intracellular glutathione transport systems in plants. Trends Plant Sci 6: 486-492

Foyer, CH; G Noctor. 2003. Redox sensing and signalling associated with reactive oxygen in chloroplasts, peroxisomes and mitochondria. Physiol Plant 119: 355-364.

Fricker, MD; M May, AJ Meyer, N Sheard, NS White. 2001. Measurement of glutathione levels in intact roots of *Arabidopsis*. J Microsc 198: 162-173.

Gidrol, X; WS Lin, N Degousee, SF Yip, A Kush. 1994. Accumulation of reactive oxygen species and oxidation of cytokinin in germinating soybean seeds. Eur J Biochem 224: 21-28.

Goel, A; AK Goel, IS Sheoran. 2003. Changes in oxidative stress enzymes during artificial ageing in cotton (*Gossypium hirsutum* L.) seeds. J Plant Physiol 160: 1093-1100.

Goel, A; IS Sheoran. 2003. Lipid peroxidation and peroxide-scavenging in cotton seeds under natural ageing. Biol Plant 46: 429-434.

Greenspan, L. 1977. Humidity fixed points of binary saturated aqueous solutions. Journal of Research of the National Bureau of Standards – A. Physics and Chemistry 81A: 89-96.

Greggains, V; WE Finch-Savage, WP Quick, NM Atherton. 2000. Putative desiccation tolerance mechanisms in orthodox and recalcitrant seeds of the genus *Acer*. Seed Sci Res 10: 317-327.

Gutierrez-Alcala, G; C Gotor, AJ Meyer, MD Fricker, JM Vega, LC Romero. 2000. Glutathione biosynthesis in *Arabidopsis* trichome cells. Proc Natl Acad Sci USA 97: 11108-11113.

Hartmann, TN; MD Fricker, H Rennenberg, AJ Meyer. 2003. Cell specific measurement of cytosolic glutathione in poplar leaves. Plant Cell Env 26: 965-975.

Hite, DRC; C Auh, JG Scandalios. 1999. Catalase activity and hydrogen peroxide levels are inversely correlated in maize scutella during seed germination. Redox Rep 2: 29-34.

Hoekstra, FA; EA Golovina, J Buitink. 2001. Mechanism of plant desiccation tolerance. Trends Plant Sci 6: 431-438.

Hoekstra, FA; EA Golovina, J Nijsse. 2003. What do we really know about desiccation tolerance mechanisms? In: G Nicolas, KJ Bradford, D Come, HW Pritchard (eds), The Biology of Seeds: Recent Research Advances. CAB International, Wallingford, UK, pp. 259-270.

Hsu, JL; JM Sung. 1997. Antioxidant role of glutathione associated with accelerated aging and hydration of triploid watermelon seeds. Physiol Plant 100: 967-974.

International Seed Testing Association. 1985. International rules for seed testing (Rules 1985). Seed Sci Technol 13: 299-355.

Jones, DP; JL Carlson, PS Samiec, PJr Sternberg, VCJr Mody, RL Reed, LA Brown. 1998. Glutathione measurement in human plasma: evaluation of sample collection, storage and derivatization conditions for analysis of dansyl derivatives by HPLC. Clin Chim Acta 275: 175-184.

Jung, R; YW Nam, I Saalbach, K Muntz, NC Nielsen. 1997. Role of the sulfhydryl redox state and disulfide bonds in processing and assembly of 11S seed globulins. Plant Cell 9: 2037-2050.

Kocsy, G; G Galiba, C Brunold. 2001. Role of glutathione in adaptation and signaling during chilling and cold acclimation in plants. Physiol Plant 113: 158-164.

Kocsy, G; K Kobrehel, G Szalai, M Duviau, Z Buzas, G Galiba. 2004. Abiotic stress-induced changes in glutathione and thioredoxin *h* levels in maize. Env Exp Bot 52: 101-112.

Kranner, I; D Grill. 1993. Content of low-molecular-weight thiols during the imbibition of pea seeds. Physiol Plant 88: 557-562.

Kranner, I; D Grill. 1996. Significance of thiol-disulfide exchange in resting stages of plant development. Bot Acta 109: 8-14.

Leon, RG; AD Knapp. 2004. Effect of temperature on germination of common waterhemp (*Amaranthus tuberculatus*), giant foxtail (*Setaria faberi*), and velvetleaf (*Abutilon theophrasti*). Weed Sci 52: 67-73.

Leprince, O; R Deltour, PC Thorpe, NM Atherton, GAF Hendry. 1990. The role of free radicals and radical processing systems in loss of desiccation tolerance in germinating maize (*Zea mays* L.). New Phytol 116: 573-580.

Leprince, O; GAF Hendry, BD McKersie. 1993. The mechanism of desiccation tolerance in developing seeds. Seed Sci Res 3: 231-246.

Leprince, O; GAF Hendry, NM Atherton, C Walters-Vertucci. 1996. Free radicals and metabolism associated with the acquisition and loss of desiccation tolerance in developing seeds. Biochem Soc Trans 24: 451-455.

Leprince, O; FA Hoekstra. 1998. The responses of cytochrome redox state and energy metabolism to dehydration support a role for cytoplasmic viscosity in desiccation tolerance. Plant Physiol 118: 1253-1264.

Leprince, O; J Buitink, FA Hoekstra. 1999. Axes and cotyledons of recalcitrant seeds of Castanea sativa Mill. Exhibit contrasting responses of respiration to drying in relation to desiccation sensitivity. J Exp Bot 50: 1515-1524.

Leprince, O; FJM Harren, J Buitink, M Alberda, FA Hoekstra. 2000. Metabolic dysfunction and unabated respiration preced loss of membrane integrity during dehydration of germinating radicles. Plant Physiol 122: 597-608.

Leprince, O; FA Hoekstra, FJM Harren. 2000. Unraveling the responses of metabolism to dehydration points to a role for cytoplasmic viscosity in desiccation tolerance. In: M Black, KJ Bradford, J Vazquez-Ramos (eds), Seed Biology: Advances and Applications. CAB International, Wallingford, UK, pp. 57-66.

Li, W; AA Tsiami, SS Bollecker, JD Schofield. 2004a. Glutathione and related thiol compounds II: The importance of protein bound glutathione and related protein-bound compounds in gluten proteins. J Cereal Sci 39: 213-224.

Li, W; SS Bollecker, JD Schofield. 2004b. Glutathione and related thiol compounds I: Glutathione and related thiol compounds in flour. J Cereal Sci 39: 205-212.

Madamanchi, NR; JV Anderson, RG Alscher, CL Cramer, JL Hess. 1992. Purification of multiple forms of glutathione-reductase from pea (*Pisum sativum*) seedlings and enzyme levels in ozone-fumigated pea leaves. Plant Physiol 100: 138-145.

May, MJ; T Vernoux, C Leaver, M Van Montagu, D Inze. 1998. Glutathione homeostasis in plants: environmental sensing and plant development. J Exp Bot 49: 649-667.

McDonald, MB. 1999. Seed deterioration: physiology, repair and assessment. Seed Sci Technol 27: 177-237.

Meinke, DW. 1994. Seed development in *Arabidopsis thaliana*. In: EM Meyerowitz, CR Somerville (eds), Arabidopsis. CSHL Press, New York, USA, pp. 253-296.

Meyer, AJ; MJ May, M Fricker. 2001. Quantitative *in vivo* measurement of glutathione in Arabidopsis cells. Plant J 27: 67-78.

Morohashi, Y. 2002. Peroxidase activity develops in the micropylar endosperm of tomato seeds prior to radicle protrusion. J Exp Bot 53: 1643-1650.

Murthy, UMN; Y Liang, PP Kumar, WQ Sun. 2002. Non-enzymatic protein modification by the Maillard reaction reduces the activities of scavenging enzymes in *Vigna radiata*. Physiol Plant 115: 213-220.

Murthy, UMN; PP Kumar, WQ Sun. 2003. Mechanisms of seed ageing under different storage conditions for *Vigna radiate* (L.) Wilczek: lipid peroxidation, sugar hydrolysis, Maillard reactions and their relationship to glass state transition. J Exp Bot 54: 1057-1067.

Noctor, G; AM Arisi, L Jouanin, KJ Kunert, H Rennenberg, CH Foyer. 1998. Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. J Exp Bot 49: 623–647.

Noctor, G; CH Foyer. 1998. Simultaneous measurement of foliar glutathione, γ-glutamylcysteine, and amino acids by high-performance liquid chromatography: comparisons with two other assay methods for glutathione. Anal Biochem 264: 98-110.

Orsi, A; F Sparvoli, A Ceriotti. 2001. Role of individual disulfide bonds in the structural maturation of a low molecular weight glutenin subunit. J Biol Chem 276: 32322-32329.

Puntarulo, S; M Galleano, RA Sanchez, A Boveris. 1991. Superoxide anion and hydrogen metabolism in soybean embryonic axes during germination. Biochim Biophys Acta 1074: 277-283.

Rhazi, L; R Cazalis, E Lemelin, T Aussenac. 2003. Changes in the glutathione thiol-disulfide status during wheat grain development. Plant Physiol Biochem 41: 895-902.

Rivero, RM; JM Ruiz, L Romero. 2004. Oxidative metabolism in tomato plants subjected to heat stress. J Hort Sci Biotech 79: 560-564.

Rouhier, N; E Gelhaye, J Jacquot. 2002. Redox control by dithiol-disulfide exchange in plants, II. The cytosolic and mitochondrial systems. Ann NY Acad Sci 973: 520-528.

Roxas, VP; J Wang, S Lodhi, RD Allen. 1997. Engineering stress tolerance in transgenic plants. Acta Physiol Plant 19: 591-594.

Sairam, RK; GC Srivastava, DC Saxena. 2000. Increased antioxidant activity under elevated temperatures: a mechanism of heat stress tolerance in wheat genotypes. Biol Plant 43: 245-251.

Schopfer, P; C Plachy, G Frahry. 2001. Release of reactive oxygen intermediates (superoxide radicals, hydrogen peroxide, and hydroxyl radicals) and peroxidase in geminating radish seeds controlled by light, gibberellin, and abscisic acid. Plant Physiol 125: 1591-1602.

Simontacchi, M; Lsadovsky, S Puntarulo. 2003. Profile of antioxidant content upon developing of *Sorghum bicolor* seeds. Plant Sci 164: 709-715.

Starke, DW; PB Chock, JJ Mieyal. 2003. Glutathione-thiyl radical scavenging and transferase properties of human glutaredoxin, (thioltransferase): potential role in redox signal transduction. J Biol Chem 278: 14607-14613.

Tesnier, K; HM Strookman-Donkers, JG Van Pijlen, AHM van der Geest, RJ Cino, SPC Groot. 2002. A controlled deterioration test for *Arabidopsis thaliana* reveals genetic variation in seed quality. Seed Sci Technol 30: 149-165.

Tommasi, F; C Paciolla, MC de Pinto, L De Gara. 2001. A comparative study of glutathione and ascorbate metabolism during germination of *Pinus pinea* L. seeds. J Exp Bot 52: 1647-1654.

Van der Weele, CM; WG Spollen, RE Sharp, TI Baskin. 2000. Growth of *Arabidopsis thaliana* seedlings under water deficit by control of water potential in nutrient-agar media.

Vertucci, CW; JM Farrant. 1995. Acquisition and loss of desiccation tolerance. In: J Kigel and G Galili (ed.), Seed Development and Germination. Marcel Dekker, Inc., New York, USA, pp. 237-271.

Wilson, DOJr; MBJr McDonald, SK St. Martin. 1989. A probit planes method of analyzing seed deterioration data. Crop Sci 29: 471-476.

VITA

Mae Rose S. Sumugat

Department of Horticulture

Virginia Polytechnic Institute and State University

Blacksburg, VA 24060

Email address: msumugat@vt.edu

Personal History

Born in Tagum City, Philippines on January 6, 1979

Education

1. Cor Jesu College, Digos City, Philippines, June 1992-March 1996

2. B.S., Biology, University of the Philippines, Diliman, Quezon City, Philippines 1101, June1996-April 2000

3. M.S., Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, August 2002-December 2004

Professional Experience

Instructor, Institute of Biology, University of the Philippines, Diliman, Quezon City, Philippines 1101, June 2000-August 2002

Graduate Teaching Assistant, Virginia Polytechnic Institute and State University, Blacksburg, VA 24060, August 2002-December 2004