EFFECT OF ETHYLENE SENSITIVITY ON DEVELOPMENT AND GERMINATION OF *Petunia x hybrida* SEEDS

By

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This document is dedicated to my mom, Lynn Roll.

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Past research has proven that several hormones play a role in different stages of development, dormancy, and the germination of seeds. Ethylene, a gaseous plant hormone, is involved throughout many plant processes including the development and germination of seeds, though the action of ethylene is not completely understood with respect to seeds. The goal of this research was to take a more detailed look at ethylene's role in *Petunia x hybrida* seed development and germination.

It was observed in transgenic petunias (44568 CaMV35S::*etr1-1*) reduced in ethylene sensitivity that ethylene primarily acts by stimulating the developmental time-course and thereby increasing germination rates. The full time-course of seed development was delayed in homozygous 44568 seeds by approximately five days compared to wild-type Mitchell Diploid (MD) seeds. Also, when the two lines were reciprocally crossed, only seeds produced on the 44568 maternal plants displayed the phenotype of delayed seed development. When germination was assayed, both

hemizygous and homozygous seeds carrying the *etr1-1* transgene had reduced germination, but both were able to recover to MD germination levels after six months of cold storage. All seeds carrying the *etr1-1* transgene were also more sensitive than wild-type to exogenously applied ABA during an additional germination assay.

Differences in gene expression between 44568 and MD were observed through microarray analysis. The results of the microarray experiments and the observation of a color difference of freshly harvested seeds altered in ethylene perception led to further analysis of proanthocyanidins, or condensed tannins. Expression analysis of genes involved in condensed tannin synthesis did not exhibit any major differences between the genotypes carrying the *etr1-1* transgene versus MD. Overall, the primary findings of this research were that the ethylene sensitivity of the maternal parent had a significant role in the developmental timing of seeds. Conversely, the overall decreased sensitivity of the zygotic tissue to ethylene determined the stronger dormancy induction and heightened ABA germination sensitivity observed in all seeds carrying the *etr1-1* transgene.

CHAPTER 1
INTRODUCTION

Angiosperm seed development is mediated by an assortment of genetic programs involving hormones, fatty acids, storage proteins, carbohydrates and many other components of plant growth and metabolism. The seed is composed of several tissues including the embryo, endosperm, and the testa or seed coat. The embryo contains the tissues that include the root and shoot meristems which develops into a seedling. The endosperm is comprised of an epidermal layer, an aleurone, and nourishing tissue surrounding the embryo. The seed coat provides a protective cover over the other tissues (Harada, 1997). A seed undergoes a complex course of development after fertilization until the point where it is considered a mature seed capable of germination. The development of the embryo after fertilization occurs in three general stages: differentiation of tissues, cell enlargement and maturation (Buchanan et al., 2000; Chaudhury and Berger, 2001).

Some seeds enter dormancy after maturation, while other seeds immediately become ready for mobilization of stored reserves in preparation for germination to begin. There are two types of dormancy: primary and secondary. Dormancy is defined as the inability of mature seeds to germinate under favorable conditions (Bewley, 1997). Primary dormancy occurs in the freshly-harvested seed; it develops during seed development and maturation on the mother plant. The maintenance of primary dormancy is determined by environmental and genetic factors (Bewley, 1997; Gubler et al., 2005). This dormancy prevents the seed from germinating in unfavorable conditions and is

imposed by the embryo itself or the seed coat. Embryo dormancy can be overcome by dry storage or stratification, and seed coat dormancy can be countered by removal of the seed coat (Kepczynski and Kepczynska, 1997). Secondary dormancy is initiated after the seed has been dispersed from the mother plant. This type of dormancy inhibits germination due to a lack of proper environmental cues such as temperature or light needed for the initiation of the germination processes (Foley, 2001).

An overlap of hormone and carbohydrate signaling pathways is apparently integral in seed developmental processes. Currently, a good portion of the dicot seed research is being conducted on Arabidopsis due to the predictable patterns of cell division within the seed and the wide availability of mutants, including those that are insensitive or hypersensitive to many of the hormones (Buchanan et al., 2000). Seeds from different species have widely different proportions of carbohydrates, oils, and stored proteins; therefore, it is important to study seed development and germination in different species to determine where differences may occur (Ruuska et al., 2002). The species used in this research is *Petunia x hybrida*. Petunia seeds are similar to Arabidopsis in structure and components of metabolites; therefore, information gained from mutant analysis research conducted on Arabidopsis seeds may provide a basis for research conducted on seed action in petunia. Petunia, like Arabidopsis, is used as a model system, but petunia serves as a particularly useful model for studies on floriculture species. A short generation time and the ability to make abundant amounts of seeds are also characteristics of petunia that make it a good candidate for seed research.

The hormone of interest in the following research is the gaseous plant hormone ethylene. Ethylene action in seeds is not significantly understood, and further research on

ethylene may provide interesting evidence for interactions with other hormones. In research with petunia transgenic plants reduced in ethylene sensitivity, it was observed that fruit development is affected by ethylene, along with many other plant processes such as senescence, disease tolerance and root development (Wilkinson et al., 1997; Clevenger et al., 2004; Shibuya et al., 2004). In previous research conducted on the effects of altering ethylene synthesis and sensitivity in seeds, it was observed that other hormones such as gibberellic acid and abscisic acid were also impacted (Beaudoin et al., 2000; Ghassemian et al., 2000; Chiwocha et al., 2005). It is likely that interactions between ethylene and other plant hormones play a vital role in seed development (Kepczynski and Kepczynska, 1997). The purpose of this research is to use genetic, molecular, and physiological analyses to help define ethylene's role in seed development and subsequent seed germination, and to also characterize ethylene's interactions with other hormones in petunia seeds.

CHAPTER 2
LITERATURE REVIEW

Ethylene Biosynthesis, Action and Signaling

The plant hormone ethylene is a simple hydrocarbon gas that has been studied for over a century (Abeles et al., 1992). It is involved in many different plant processes including floral and foliar senescence, vascular differentiation, stress response, fruit ripening, and adventitious root formation (as reviewed in Bleecker and Kende, 2000). The synthesis pathway starts with methionine being converted to S-adenosyl-L-methionine (SAM) by the enzyme SAM synthetase (Adams and Yang, 1979; Yang and Hoffman, 1984). Subsequently, SAM is converted to 1-aminocyclopropane-1-carboxylic acid (ACC) by ACC synthase (ACS), which is the first committed step in ethylene biosynthesis (Yu et al., 1979). The last step is the conversion of ACC to ethylene catalyzed by ACC oxidase (ACO) (Hamilton et al., 1991; Spanu et al., 1991).

The genes that encode the enzymes of the ethylene biosynthesis pathway have been cloned and studied in-depth in many species (Sato and Theologis, 1989; Hamilton et al., 1990; Zarembinski and Theologis, 1994). The expression of these genes can be induced by many factors, and expression levels are generally correlated with ethylene production (Acaster and Kende, 1991; Kende, 1993). Ethylene itself in some cases induces expression of ACS and ACO resulting in autocatalytic ethylene synthesis (Abeles et al., 1992). Conversely, ethylene can be auto-inhibitory as well and restrict synthesis through regulating expression of enzymes involved in the synthesis pathway (Abeles et al., 1992).

The previous case of positive feedback regulation of ethylene biosynthesis is known to be a characteristic feature of ripening fruits and senescing flowers (Kende, 1993).

Components of ethylene perception and the subsequent initiation of signaling were able to be identified because of the extremely useful seedling triple response screen. The screen was developed through the phenotype of ethylene response that causes seedlings to grow short, stout and have an exaggerated apical hook in the presence of ethylene in the dark. Seedlings that did not exhibit this phenotype were defective in ethylene perception (Bleecker et al., 1988). The first component discovered in ethylene perception through this screen was a receptor, ETR1 (Bleecker et al., 1988). Four other receptors in Arabidopsis have also been identified since then: ETR2, ERS1, ERS2 and EIN4 (Hua et al., 1995, 1998; Sakai et al., 1998). Analysis of the receptors revealed that there is homology to bacterial two-component receptors. The first component consist of a sensor protein that receives signals through an input domain which autophosphorylates a histine residue. The second component is a response regulator protein that receives the phosphate and mediates responses through an output domain (Chang and Stewart, 1998). Mutant research focused on the receptors, especially ETR1, has provided information about the action and effects of ethylene perception. The first ethylene receptor mutant, *etr1-1*, was identified in Arabidopsis and was discovered to result in a strong decrease in ethylene sensitivity (Bleecker et al., 1988). Some of the results of the loss of ethylene perception were delayed floral and foliar senescense, decreased adventitious rooting, increased susceptibility to pathogens, decreased seed germination and delayed fruit ripening (Bleecker et al., 1988). Additionally, it was observed that the *etr1-1* mutation

from Arabidopsis could be transformed into heterologous species, such as petunia and tomato, and also confer the reduction in ethylene sensitivity (Wilkinson et al., 1997).

The next component identified in the ethylene signaling cascade was CTR1. The loss of function mutant of CTR1 exhibited a constitutive ethylene response in absence of ethylene; therefore, it was deemed a negative regulator of ethylene signaling (Kieber et al., 1993). Double mutants between CTR1 and ETR1 had the phenotype of constitutive ethylene response, so it was concluded that CTR1 acts downstream of the receptors (Kieber et al., 1993; Hua et al., 1998; Sakai et al., 1998). Two other components downstream of CTR1 are EIN2 and EIN3. EIN2 is a membrane bound protein that positively regulates the downstream EIN3 transcription factor (Guzman and Ecker, 1990). The cloning of EIN2 revealed that it is a novel plant specific protein whose exact biochemical function is unknown (Alonso et al., 1999). EIN2 is also known to be a membrane protein with 12 membrane spanning regions, and the amino end of the sequence shows homology to a family of metal ion transporters but transport activity has not been shown to date (Alonso et., 1999). The downstream target of EIN2, EIN3, is another positive regulator of ethylene responses. EIN3 was discovered to belong to a small family of genes because mutant plants of EIN3 only showed partial reduced sensitivity of ethylene; therefore, it was concluded that it is part of a small family with some functional redundancy (Roman et al., 1995). One target of EIN3 is ERF1 (Solano et al., 1998). ERF1 is a member of a family of transcription factors that are known as ethylene-response-element binding-proteins (EREBPs), which initiate transcription of genes involved in the ethylene responses (Ohme-Takagi and Shinshi, 1995).

An interesting aspect of the ethylene signaling cascade is that a mutant receptor is considered a gain of function mutant. This is because CTR1 is continuously repressing the downstream components of the signaling cascade until ethylene binds to a receptor and inactivates the repression. A mutant in one of the receptors is a gain of function mutant because the mutant receptors fail to turn off in the presence of ethylene (Bleecker and Kende, 2000). This gain of function characteristic results in ethylene insensitivity when only one of the five receptors are mutated.

Seed Development

The development of the angiosperm seed is first initiated at the point of fertilization. The process of double fertilization eventually leads to the development of the embryo and endosperm structures of the seed. One sperm cell fertilizes the haploid egg cell and develops into the zygotic embryo tissue. The other half of double fertilization occurs when a second sperm cell joins with a diploid central cell resulting in a triploid endosperm. These processes occur within maternal diploid tissue which eventually becomes the testa, or seed coat (as reviewed in Chadhury and Berger, 2001). Different ploidy tissue and different ratio representations of the maternal and paternal genomes make understanding the regulation of seed development extremely complex.

The seed goes through three main chronological phases during development after fertilization: 1) cell division and differentiation of the cells; 2) cell enlargement through accumulation of assimilates and storage reserves; 3) maturation, acquisition of desiccation tolerance and preparation for dormancy (Chaudhury and Berger, 2001).

After fertilization, the zygote undergoes a period of rapid cell division. The first two cells formed are the apical and basal cells. The apical cell gives rise to the main portion of the embryo which includes the shoot meristem, whereas the basal cell forms

the root and suspensor (Mayer et al., 1993; Mayer and Jurgens, 1998). Following cellularization, the endosperm begins differentiation. The endosperm serves as the nourishing tissue for the developing embryo (Brink and Cooper, 1947). The endosperm also controls the osmotic potential around the embryo, mechanical support during early embryo growth and storage of reserves and hormones (Lopes and Larkins, 1993). Research also indicates the endosperm has a role in providing signals for early development to the developing embryo (van Hengel et al., 1998). Additionally, it is also thought that the maternal tissue and endosperm regulate the development of each other (Lopes and Larkin, 1993; Felker et al., 1985). During this stage carbohydrates begin to be imported into the developing tissue. Sucrose supplied by the endosperm is thought of as the main carbon and energy source of seed metabolism (Schwender and Ohlrogge, 2002). Sucrose is symplastically transported through the phloem of the maternal plant tissue into the seed coat, which is also maternally derived tissue (Weber et al., 1998). In the seed coat, the sucrose is cleaved into hexoses by invertases and transported passively into the endosperm and developing embryo (Weber et al., 1995).

During the expansion phase, the cells begin to accumulate storage reserves. Stored reserves are usually accumulated in the endosperm and in the embryo in the form of proteins and carbohydrates, which break down and are used as carbon and energy sources during the germination process (Lara et al., 2003). This stage is also marked with high respiration rates due to the high levels of metabolic activity occurring during the assimilation process (Zaitseva et al., 2002). Seed storage proteins are specifically synthesized at certain periods of development and are tightly regulated. Certain seed

storage proteins such as albumins and globulins are expressed more during the accumulation phase through the middle of the maturation phase.

The last portion of seed development is the maturation and dessication phase. In this stage of development invertases are inactivated and sucrose is carried by a sucrose transporter protein into the seed coat. The sucrose is not cleaved at this point and is moved symplastically into the embryo through the plasmodesmata; this increase in sucrose concentration in the seed helps signify the end of development is nearing (Borisjuk et al., 2002). This increase in sucrose:hexose concentration helps control seed development and sends signals to the embryo to begin the maturation phase by inducing storage associated gene expression in the final stages of seed development (Smeekens, 2000). Late embryogenesis abundant (LEA) proteins are a class of storage proteins that are highly induced later in this maturation phase due to their role in acquisition of desiccation tolerance (Wobus et al., 1999; Hoekstra et al., 2001). Abscisic acid has also been shown to play a role in inducing genes such as seed storage and LEA proteins during these latter stages of seed development for protection of the seeds in the dessication process (Baker et al., 1988; Dure et al., 1989; Brocard et al., 2003). Direct interaction occurs between ABA signaling transcription factors, such as ABI3, and the transcription factors associated with seed storage proteins, which illustrates abscisic acid's involvement in seed developmental processes (Luerssen et al., 1998; Stone et al., 2001; Lara et al., 2003).

Maternal Plant Role in Seed Development

Before fertilization, the maternal genome controls all aspects of the egg and central cell gene expression, but once fertilization occurs a zygotic mode of gene expression is induced and the paternally derived genes are thought to begin to be

expressed (as reviewed in Chadhury and Berger, 2001). Yet, one study hypothesizes that paternal genes are still silenced during the very early stages of seed development; therefore, the maternal plant has primary control over early seed development. This was observed when 20 paternally inherited loci were not expressed during early seed development in Arabidopsis (Vielle-Calzada et al., 2000).

The maternal plant can have an effect on various other seed developmental processes such as growth potential, the switch from mitotic growth to cell expansion, storage product accumulation, resource allocation, and seed structure (Weber et al., 2005). This area of research is not completely understood and may provide more detailed information about the genetic control of seed development (Chadhury and Berger, 2001). Microarray analysis was used to examine genes differentially expressed in maternal tissue in order to gain more understanding of possible roles of the maternal tissue (Sreenivasulu et al., 2002). It was observed that most of the genes found to be more highly expressed in the maternal tissue encoded enzymes involved in carbohydrate and lipid metabolism, which is expected for the maternal tissue's role in providing a nutrient supply for the developing seed (Sreenivasulu et al., 2002). Several other genes were found to be highly expressed in the maternal tissue, of which the functions are unknown. These genes include a transcription factor related to FILAMENTOUS FLOWER and a methionine synthase that may play a role in transport of nutrients to the embryo (Sreenivasulu, 2002). Another study in petunia showed that normal endosperm development required expression of two MADS box genes, FBP7 and FBP11, in the maternal tissue; therefore, the maternal plant controlled formation of the seed structure (Colombo et al., 1997). Additionally, it was shown that the maternal plant is significant

in the structure of the seed in barley, where a group of endosperm mutants that caused a phenotype of shrunken seeds also exhibited this phenotype irrespective of the paternal genotype (Felker et al., 1985).

ABA influence on seed development originates from both the zygotic tissue and the maternal tissue. It has been shown that the ABA synthesized in the maternal tissue is involved in the switch to the maturation phase of seed development, whereas the zygotic tissue produces the ABA that is involved in the late seed development programs such as acquiring desiccation tolerance (Finkelstein et al., 2002; Frey et al., 2004). Additionally, through mutant studies defective in ABA synthesis in *Nicotiana plumbagnifolia*, it was shown that maternal ABA has critical roles in promoting early seed development, initiating seed coat pigmentation, and capsule dehiscense (Frey et al., 2004). These findings show that maternal tissue have tight developmental controls over the seed. Further investigations in genes expressed preferentially in maternal fruit tissue can provide potentially important information about seed development by revealing interactions between genes originating in maternal tissues and expression of genes controlling development in the zygotic seed tissues.

Ethylene in Fruit and Seed Development and Subsequent Germination

One of ethylene's main effects in fruit development is promotion of fruit maturation and abscission. It has been shown in climacteric fruit, tomato being one example, that ethylene is produced during fruit ripening which, in turn, causes a degradation of chlorophyll, leading to the change in color of fruit through maturation. At this point maturation related proteins increase and begin the conversion process of starches, organic acids and lipids into sugars (as reviewed in Giovannoni, 2001). Studies with mutants altered in ethylene sensitivity such as *Never-ripe* and transgenic

CAMV35S-*etr1-1* have a distinct delay in ripening and senescence which demonstrates that ethylene has a critical role in fruit maturation. These characteristics were also seen in a petunia line strongly reduced in ethylene sensitivity, "44568" (Wilkinson et al., 1997).

Another important role of ethylene throughout fruit development and seed germination is in the process of programmed cell death. Programmed cell death occurs in many different processes during normal progression of cellular maturation. In seeds, programmed cell death occurs in the endosperm in order to allow for the recycling of proteins during seed maturation (Young and Gallie, 2000). Ethylene acts by inducing genes that have a role in breaking down the endosperm tissue surrounding the embryo in the seeds. By using chemical blocking agents that prevented the synthesis of ethylene, programmed cell death in the endosperm was delayed (Kepczynski and Kepczynska, 1997).

Ethylene's role in seed germination is still not completely known and remains a subject of controversy. Early studies suggested that ethylene is involved in breaking primary dormancy (Ketring and Morgan, 1971; van Staden et al., 1973), while others indicated that a rise in ethylene production is merely a consequence of breaking dormancy (Satoh et al., 1984; Kebczynski and Karssen, 1985). More recently it has been suggested that ethylene reduces ABA sensitivity, and therefore reduces ABA-induced seed dormancy and increases germination in Arabidopsis (Ghassemain et al., 2000). Many questions cannot be answered because the interactions between ethylene and other important hormones, such as abscisic acid, are not completely understood.

Additional studies have led to hypotheses on how ethylene may regulate germination. One suggested mechanism developed from studies in cocklebur is that ethylene induces expression of β-cyanoalanine synthase (CAS), an enzyme likely to be involved in cyanide metabolism in the action of seed germination (Hasegawa et al., 1995). These studies indicate that ethylene stimulates the action of the mitochondrial CAS, which down regulates the cyanide level and at the same time causes an increase in the amino acid pool during the pre-germination period (Hasegawa et al., 1995). This supports the idea that ethylene plays a role in a more conducive environment for germination by lowering toxic cyanide levels and allowing for essential amino acids needed in the germination process (Hasegawa et al., 1995; Maruyama et al., 1997).

Another hypothesis is that ethylene promotes germination by stimulation of hydrolytic enzymes that break down the endosperm to provide an available nutrient supply for radicle emergence and subsequent germination. Ethylene was also shown to increase β-1,3-glucanase induction in pea and tobacco (Petruzelli et al., 1995; Leubner-Metzger et al., 1998). The promoter region of this gene was mapped and was found to contain ethylene-response elements, which lead to the idea that the ethylene response element binding proteins are transcription factors necessary for ethylene dependent β-1,3-glucanase induction. The gene was also found to be positively regulated by ethylene and negatively regulated by ABA (Leubner-Metzger et al., 1998). This shows yet another incidence of interacting roles of these two hormones during seed germination.

Bleecker et al. (1988) observed in Arabidopsis *etr1-1* mutants that seed germination was significantly lower than wild-type seeds and that application of GA_3 overcame some of the germination deficiencies. Clevenger et al. (2004) conducted

experiments on the transgenic line 44568, an ethylene-insensitive petunia line expressing CAMV35S-*etr1-1*. Seed production and germination rates were observed. Seed quality was quantified by seed weight. Homozygous 44568 seeds and seeds produced on 44568 maternal plants were slightly lower in seed weight than seeds produced on wild-type plants. Another phenotype observed in both homozygous 44568 and hemizygous seeds produced on a 44568 maternal plant was delayed fruit development. These experiments illustrate the point that the some of the phenotypes of the *etr1-1* transgene are dependent upon the maternal plant (Clevenger et al., 2004).

Seed germination was also observed by Clevenger et al. (2004). Seed germination from seeds produced in two different greenhouse temperatures was measured in homozygous Mitchell Diploid, *etr1-1* and hemizygous seeds produced from reciprocal crosses. In the warmer temperature greenhouse, 29ºC, germination rates from a Mitchell or maternal Mitchell plant were about 95 percent, whereas seeds made on the 44568 maternal parent had a range of germination between 75 and 85 percent. The cool temperature greenhouse, 24ºC, produced seeds with lower germination rates. Mitchell Diploid and maternal Mitchell Diploid seeds had germination rates between 85 and 91 percent, while seeds produced on the maternal 44568 line had between 55 and 65 percent germination. A delay in the germination of seeds produced on 44568 was also seen in this experiment. MD seeds reached their maximum germination levels within the first five days of the study, whereas the seeds produced on the 44568 parent did not reach the maximum germination levels until ten to thirteen days after the seeds were placed in germination media. These experiments show an interesting trend, that the reduction in ethylene sensitivity is affecting something during the fruit or seed developmental

processes on the maternal plant, which subsequently influences seed germination characteristics (Clevenger et al., 2004).

Ethylene, ABA, and Sugar in Seed Development and Germination

ABA has been known to have a direct role in seed development and germination. One major role of ABA in seed development is the promotion of storage protein accumulation (Brocard et al., 2003). ABA is also known to have a major role at the end of seed development by preventing vivipary, or precocious germination (Finkelstein et al, 2002). The transcription factors VP1, or the Arabidopsis homolog, ABI3, have been shown to have a regulatory role in early ovary development, late seed development, and the initiation of seed dormancy (Finkelstein and Somerville, 1990; McCarty et al., 1991 Giraudat et al., 1992; Nambara et al., 1992). This was shown through mutant analysis where the mutant plants of these B3 domain family members resulted in defects in late embryo development and germination and a reduction in storage protein levels (Hoecker et al., 1995; Suzuki et al., 2001). Also, null alleles of ABI3 and VP1 resulted in loss of ABA sensitivity, which caused vivipary in both species, Arabidopsis and maize (McCarty et al., 1989; Nambara et al., 1992). It was also shown that the repression function of VP1 does not require the B3 binding domain; therefore, it is possible that repression is also mediated by protein-protein interactions with other transcription factors (Hoecker et al., 1999). Thus the product of VP1 and ABI3 are likely key regulators in the seed maturation, developmental, and germination programs (McCarty, 1995). The mechanisms by which these genes exactly regulate seed development are still being studied (Ikeda et al., 2004; Lopez-Molina et al., 2002).

Published data show that ethylene has a large role in programmed cell death during seed development. ABA has been shown to regulate this process as well. Plants treated

to block ABA synthesis accelerated programmed cell death and increased ethylene production; this example indicates a possible antagonistic relationship between ethylene and ABA (Young and Gallie, 2000). Recent evidence has also shown that it is likely that ethylene is a negative regulator of ABA during ABA induced seed dormancy. Ethylene has been suggested to act by reducing the sensitivity of seeds to endogenous ABA levels (Ghassemain et al., 2000). The other hypothesis is that ethylene directly decreases ABA biosynthesis (Ghassemain et al., 2000). Another contributing factor to these interactions could be sugar responsive signals. Hexose signals have been implicated in regulating ABA biosynthesis and sensitivity. One specific study showed that glucose can induce expression of the ABI5 gene, a transcription factor that is differentially expressed during seed development (Cheng et al., 2002). ABI5 interacts with ABI3 to regulate ABA responsive element mediated transcription (Hobo et al., 1999). Ethylene may be countering this ABA effect by inhibiting these sugar signals (Koch, 2004).

Relationships between sugars, ABA, and ethylene have been seen in many studies. The ethylene overproduction (*eto1)* and constitutive signaling *(ctr1)* ethylene mutants were found to be glucose insensitive due to the ability of seedlings to grow on levels of glucose that would normally inhibit development (Zhou et al., 1998). On the other hand, *etr1, ein2, ein3* and *ein6* plants, which all are affected in either ethylene perception or signaling, show glucose hypersensitivity and exhibited developmental arrest on lower than normal levels of glucose (Zhou et al., 1998). This relationship between ethylene and glucose may indicate that glucose signaling can inhibit ethylene action during seed germination (Zhou et al., 1998). Mutant analysis has provided information that shows that ethylene acts antagonistically to the glucose response, whereas ABA is a promoter.

Double mutant analysis with *gin1etr1* and *gin1ein2* exhibited a resistance to developmental arrest of seedlings grown on higher levels of glucose, similar to the phenotype seen with double mutants of *gin1/aba2*. Because ABA and ethylene exhibit opposite roles when influencing glucose responses it is likely that ethylene affects glucose signaling through ABA to promote seed development and germination, but the molecular mechanisms of these interactions still remain unclear (Cheng et al., 2002).

Conversely, another study has shown that glucose delays the seed germination process, yet this delay is not affected by ethylene sensitivity. Several transcription factors (ABI2, ABI4, and ABI5) were studied that have been deemed as ABA-responsive due to the fact one of their loss-of-function mutant phenotypes decreased ABA sensitivity in the seed. Hexokinase function, ABI2, ABI4, and ABI5 did not have a role in the glucose delay of germination; therefore, it was determined that there are other signaling cascades that involve glucose signals that could cause the delay in germination (Dekkers et al., 2004).

Condensed Tannins

Proanthocyanidins, condensed tannins, are colorless flavonoids that result from the condensation of flavan-3-ol units (Xie et al., 2003). These pigments are colorless and are found in the seed coat of Arabidopsis seeds but turn brown through a proposed oxidation process, though the genes controlling the oxidation have not been determined (Debeaujon et al., 2001). Additionally, Arabidopsis wild-type seeds also darken with time of storage. This occurs because the proanthocyanidins fill the large vacuole of the endothelium cells which cause the outward darker appearance of the seeds (Debeaujon et al., 2001).

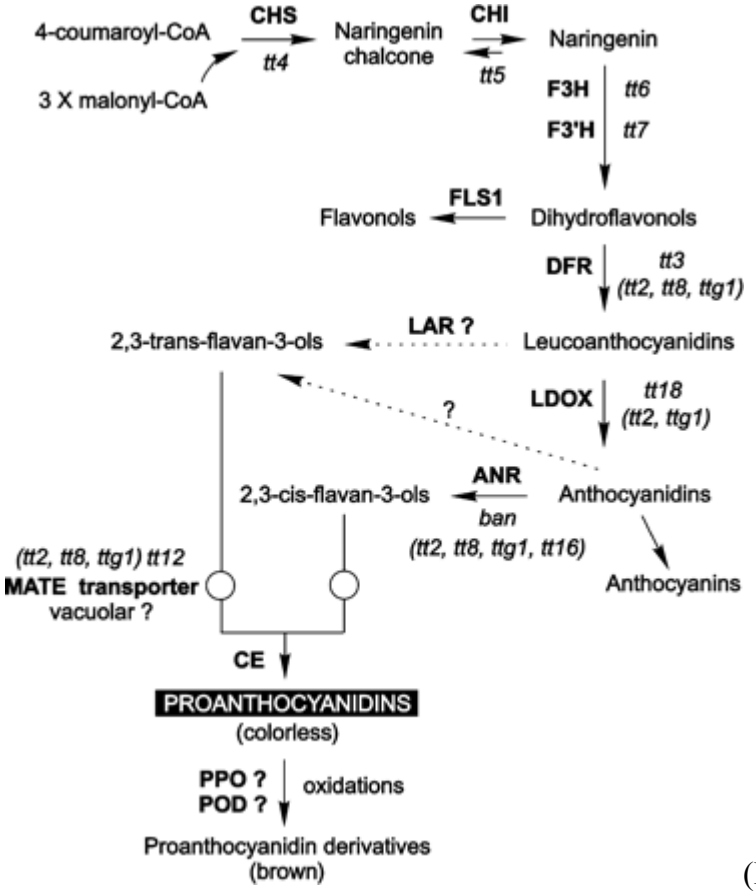
Though the exact function of the tannins has not been determined, it is thought that the tannins in the seed coat aid in protection against pathogens (Winkel-Shirley, 1998).

It is also thought that the proanthocyanidins strengthen seed coat imposed dormancy and extend seed longevity by providing a stronger physical barrier structure and decreasing the permeability of the seed coat to water. (Debeaujon et al., 2000).

The proanthocyanidin synthesis pathway diverges off of the anthocyanin biosynthesis pathway; therefore, many genes are common to both pathways including chalcone synthase, chalcone isomerase, flavonoid 3'hydroxylase (F3'H) and dihydroflavonol reductase (DFR) (Figure 2-1). Several classes of Arabidopsis mutants termed the *transparent testa* (*tt1-tt19*), *transparent testa glabra* (*ttg1 and ttg2*) and *banyuls* (*ban*) mutants are deficient in different areas of the anthocyanin and proanthocyanidin sythesis pathway (Abrahams et al., 2002). These mutants are altered in seed coat color and degree of seed dormancy (Debeaujon et al., 2003). *BAN* is one gene cloned in this pathway of particular importance because it is exclusive to the proanthocyanidin pathway. *BAN* encodes a dihydroflavonol reductase-like protein and it has been shown to function as an anthocyanidin reductase. *BAN* converts anthocyanidins to 2,3-cis-flavan-3-ols which condense into the colorless proanthocyanidins (Xie et al., 2003).

In addition to the synthesis of condensed tannins, the regulation of the proanthocyanidin pathway has also become a focus of research. Several proteins in Arabidopsis have also been identified as regulators of proanthocyanidin biosynthesis. These include TT2, which is a MYB transcription factor, TT8, a MYC/bHLH transcripton factor, and TTG1, a WD40-repeat family protein. All positively influence *BAN,* and mutants in any of these genes results in a colorless seed coat devoid of proanthocyanidins, and the seeds exhibited reduced dormancy and were able to germinate

at higher percentages than wild-type seeds (Debeaujon et al., 2000). Other regulators include TT1 and TT16, which regulate the proanthocyanidin biosynthesis in the seed body but not the chalaza/micropyle region. TT1 is a zinc finger protein, whereas TT16 is



(Debeaujon et al., 2003)

Figure 2-1 Proanthocyanin Synthesis Pathway

the ARABIDOPSIS BSISTER MADS transcription factor, which is homologous to the FBP24 MADS protein in *Petunia x hybrida* (Nesi et al, 2002). *tt16* mutants had a distorted shape of endothelial cells and prevented activation of the BAN promoter in the endothelium layer. Therefore, it was suggested that TT16/ABS is involved in endothelium development. A vanillin stain was used in order to study proanthocyanidin accumulation in developing seeds of *tt16* mutants so that the colorless compounds could be seen by a dark red staining. Vanillin turns red upon binding to flavan-3,4-diols

(leucoanthocyandins) and flavan-4-ols (catechins). Staining was not seen in the endothelial cells but was seen in the chalazal bulb and the micropyle (Nesi et al, 2002).

Most mutants in the *tt*, *ttg* and *ban* classes all exhibited some degree of seed color and dormancy changes, but all of the mutants did not exhibit the same exact phenotype. The seed coat color varied from pale brown of *tt10* and *tt14*, which had progressive browning during storage. Others mutants exhibited a pale yellow color like *tt4*, which is absent of flavonoids. (Debeaujon et al., 2000). *ban* mutants were unique in that they exhibited a grayish-green color. Another characteristic of most of these mutants was that many of the mutant seeds exhibited a reduction in seed weight and seed size, though the reason for this phenotype is not understood (Debeaujon et al., 2000). It has also been shown that the phenotypes of *tt, ttg* and *ban* mutants are all exclusive to the seed coat tissue; therefore, all phenotypes are determined by the maternal parent. Reciprocal crosses resulted in a F_1 generation with phenotypes of the maternal parent (Debeaujon et al., 2000). Most mutants exhibited increased germination over a shorter amount of storage time and are considered to have reduced dormancy (Debeaujon et al., 2000). These mutants include *ttg1*, which germinates at nearly 100 percent after two days in storage. The reduced dormancy is not evident in all testa mutants, though *tt*8, *tt*9, *tt*12 and to a lesser extent, *ban*, all did not germinate as well as wild-type after 27 days of storage (Debeaujon et al., 2000). Germination was also tested in reciprocal crosses and all F_1 progeny acted in a similar manner as their maternal parent (Debeaujon et al., 2000). Though there is not a direct correlation between seed color and the severity of seed coat imposed dormancy, it appears that there is some linkage seen in these mutants affected in proanthocyanidin synthesis. The most important determining factor over the color

phenotype is the actual levels of proanthocyanidins present in the seed coat. It is believed that these colorless tannins are the barrier preventing germination (Debeaujon et al., 2000).

Conclusion

It is known that ethylene is involved in maturation processes of plant development. Ethylene has been widely studied in processes involved in fruit maturation (as reviewed in Giovannoni, 2001). Ethylene's impact on the development of the seed within the fruit has not been studied as extensively as fruit development. Additionally, it is not known whether ethylene's impact on seed development influences seed germination characteristics. The relationship between ethylene and other hormones, such as ABA, in specific seed developmental processes is even more difficult to determine due to the lack of research conducted in this area. Ethylene is thought to promote seed germination by acting as a negative regulator of ABA action, which is known to establish seed dormancy (Ghassemian et al., 2000). The proposed research will provide a more in-depth analysis of ethylene's action in seed development of *Petunia x hybrida* and the subsequent impact on seed germination. The maternal plant is thought to have a crucial role in early seed development; therefore, a focus on ethylene sensitivity of the maternal parent will be highlighted (Vielle-Calzada et al., 2000). The effect of reducing ethylene sensitivity and its result on sensitivity to ABA will also be determined to observe whether there is a relationship between these two hormones in petunia seeds.

CHAPTER 3
EFFECT OF REDUCED SENSITIVITY TO ETHYLENE ON SEED DEVELOPMENT, DORMANCY AND GERMINATION

Introduction

Over the last 75 years seed research on several aspects of seed physiology has contributed to considerable increases in crop yield, and the understanding of seed biology continues to improve as the science of molecular biology advances. However, the seed is a complex structure and therefore, much needs to be learned about genetic interactions in order to completely understand seed development. The complex interactions that occur during seed development and dormancy have strict genetic and hormonal control (Holdworth et al., 2001). One of the hormones that significantly impacts seed development is ethylene which is also involved in many plant developmental processes including floral senescence, abscission, fruit ripening, and seed germination (as reviewed in Bleecker et al., 2000).

The three main components of the angiosperm seed are the embryo, endosperm and the testa. The testa is the only part of the seed structure that is completely developed from the maternal parent; therefore, reciprocal crosses can be used as experimental tools to help determine if certain factors of seed development are more influenced by the maternally derived testa. The seed goes through three main chronological phases during development: 1. Cell division and differentiation 2. Cell enlargement through accumulation of assimilates and storage reserves and 3. Acquisition of desiccation tolerance and preparation for dormancy (Chaudhury and Berger, 2001).

The first phase of seed development begins immediately after fertilization and is commonly a time of rapid cell division and differentiation. Rapid cell division typically will persist through the first half of seed development (Colombo et al., 1997). Genes involved in cell division and differentiation such as beta tubulin, and regulatory genes such as LEC1 (Lotan et al., 1998) are associated with these cell cycle processes and are highly expressed during this period of development.

Once the majority of cell division is complete, the next developmental stage begins with an increase in cell expansion. In this stage seed storage proteins accumulate in the vacuole or as membrane bound protein bodies within the cell (Hoekstra et al., 2001). Lipids and starches are also produced during this phase of development (Norton et al., 1975; Wobus et al., 1999; Hoekstra et al., 2001). This assimilation and cell expansion stage is usually marked by higher expression of known seed storage genes, such as globulins and albumins, and regulatory genes, such as FUS3, that control the synthesis of these storage proteins (Kermode, 1995; Wobus and Weber, 1999). The accumulation of stored reserves continues into the last stage of development but slows down increasingly until the end of seed development. This stage is also marked with high respiration rates due to the high levels of metabolic activity occurring with the assimilation process (Zaitseva et al., 2002).

The transport of assimilates in the embryonic tissue from the maternal parent plant can be used as a gauge to help determine the developmental progress of the seeds. Sucrose is generated from the maternal parent and unloaded into the maternal seed coat tissue from the fruit tissues where it is cleaved by cell wall invertases (Weber et al., 1995). The hexoses are released into the zygotic embryonic tissue by a passive, facilitated

membrane-transport process (Buchanan et al., 2000; Borisiuk et al., 2002). The hexoses are readily taken up and used by the endosperm, cotyledons, and developing embryo during the highly energy taxing cell division phase (Weber et al., 1995). Sucrose import into the developing seed begins early in development and continues throughout the latter stages of maturation, where sucrose is cleaved less frequently than the earlier stages of development. Sucrose is transported directly into the embryonic tissue for seed storage purposes (Heim et al., 1993; Borisiuk et al., 2002).

Desiccation and the acquisition of desiccation tolerance are the primary actions of the last stage of seed development (Finkelstein et al., 2002). The fruit and seeds slowly cease metabolic activity and begin to desiccate in preparation for dormancy or subsequent germination. Dehydrins are a class of genes that are highly expressed during this phase and are hypothesized to function in stabilizing membranes and protecting the cells for dehydration (Black et al., 1999). Abscisic acid has also been shown to have a role in this phase of development by inducing expression of genes, such as LEAs (late embryonic abundant), which are thought to be involved in maturation and desiccation tolerance (Bartels et al., 1988).

Ethylene is known to have a role in many plant processes, either directly or through interactions with other hormonal and genetic factors. Ethylene is known to have some role in the breaking of seed dormancy of certain species (Ketring and Morgan, 1969; Globerson, 1977; Kepczynski et al., 2003), but it has not been extensively shown to have a role in the actual development of the seed (Kepczynski and Kepczynska, 1997). For example ACC content, ACC-synthase activity, ACC-oxidase *in vitro* activity and ethylene production were measured in chick-pea seeds. It was shown that all of these

actions reached a maximum during the expansion phase of seed development and then slowly decreased until maturation was complete (Gallardo et al., 1999). The relationship between ethylene synthesis and development of the chick-pea seed suggests that there is a correlation between developmental progress and ethylene synthesis and action (Matilla, 2001).

Seed dormancy is an important factor established during seed development due to its influence on subsequent germination. Seed dormancy is generally characterized as a state in which a viable seed will not germinate when placed in suitable temperature, moisture, and oxygen conditions which are normally considered to be adequate for germination (Roberts, 1972). There are two types of dormancy established within seeds, primary and secondary (Bewley, 1997). Primary dormancy occurs during development on the maternal plant and prevents the seed from germinating until conditions are favorable (Bewley, 1997). During primary dormancy germination is repressed until an after-ripening period is satisfied through cold storage (Leon-Kloosterziel et al., 1996). Secondary dormancy is initiated after the seed is released from the maternal plant and requires an environmental stimuli, such as light or temperature, to commence the germination processes (Foley, 2001) Seed dormancy can be induced by the embryo, endosperm, testa or a combination of these factors (Bewley, 1997). Dormancy and the subsequent germination processes are under hormonal control, and it is likely a complex interaction of several hormones. Extensive research has been conducted on abscisic acid's role in maintaining seed dormancy (Zeevaart and Creelman, 1988). ABA is synthesized by the zygotic tissues in the mid to latter stages of seed development and is known to be involved in the switch from cell division and differentiation mechanisms to

seed maturation mechanisms. ABA is known to induce a cyclin-dependent kinase inhibitor that leads to cell cycle arrest which ends the rapid growth phase and begins the maturation phase (Wang et al., 1998) Mutant analysis has also confirmed a role for ABA in maintaining seed dormancy. Mutant plants of ABI3, a B3 domain transcription factor involved in ABA signaling of Arabidopsis thaliana, produced seeds with extensively reduced seed dormancy (Koornneef et al., 1984; Nambara et al., 1992; Ooms et al., 1993). ABI3 is also orthologous to the maize Viviparous 1 protein, which when mutated also produced plants with precocious germination (McCarty et al., 1991).

Over the past several years, the interactions between ethylene and ABA have been investigated more intensely. As a result, it is thought that there is an antagonistic relationship between the two hormones and that ethylene inhibits ABA signaling and aids in releasing seed dormancy (Beaudoin et al., 2000). Seed dormancy was investigated in Arabidopsis ethylene-insensitive *ein2-45* seeds, which was discovered in a screen of mutated Arabidopsis seeds that suppressed the ABA resistant seed germination phenotype of *abi1-1* (Beaudoin et al., 2000). EIN2, a membrane protein important to the ethylene signaling cascade, results in ethylene-insensitivity when it is mutated (Guzman and Ecker, 1990). *ein2-45* seeds showed an increased sensitivity to ABA when germinated on various concentrations of ABA and had a significant reduction in seed germination of freshly harvested seeds. The *ein2-45* seeds exhibited arrested germination under lower concentrations of ABA when compared to wild-type germination. It was also determined that these seed exhibited enhanced seed dormancy and were not able to germinate as well as wild-type without any post-harvest treatment (Beaudoin et al., 2000). The dormancy of *ein2-45* seeds was broken after a cold stratification of 5 days

and resulted in restoration of germination to levels comparable to wild-type seeds. Similar phenotypes of a more severe seed dormancy induction and enhanced sensitivity to ABA were also observed with *etr1-1* Arabidopsis seeds (Bleecker et al., 1988; Beaudoin et al., 2000). This evidence demonstrated that there are direct interactions between ABA and ethylene in the regulation of seed dormancy and germination, and that EIN2 may act as a negative regulator of ABA sensitivity (Beaudoin et al., 2000).

Seed germination occurs when a fully developed non-dormant seed is able to imbibe water, commence metabolic processes, and begin growth as a seedling (Debeaujon and Koorneef, 2000). Several parameters are known to affect the breaking of seed dormancy including gibberellic acid, chilling, and light. All of these dormancy breaking mechanisms are known to act through induction of seed germination associated gene expression (Koornneef and Karssen, 1994). It is likely that ethylene plays a major role in the control of gene expression associated with seed germination, but there is mixed results presented in past research as to whether ethylene is directly involved in the control of seed germination or indirectly through its influence on other factors. Many species appear to have increased germination with exogenous application of ethylene. For instance, studies on *Trifolium subterraneum* (Esashi and Leopold, 1969), *Arachis hypogea* (Ketring and Morgan, 1971) and *Avena fatua* (Adkins and Ross, 1981) all concluded that ethylene production during seed imbibition paralleled the breaking of seed dormancy. More recent research indicates that ethylene is likely to induce germination either by inducing ABA catabolism or reducing the seed tissue sensitivity to ABA (Ghassemian et al., 2000; Beaudoin et al., 2000) Another thought is that ethylene is produced as a result of programmed cell death in the endosperm tissue during

germination (Matilla, 2001). In rice, ethylene action during germination is even more complex and seems to even be inhibitory. Since ethephon treatment of rice seeds enhanced seed dormancy, it is likely that the complex mechanisms underlying seed germination are greatly different between monocots and dicots (Southwick et al., 1986).

Research Objectives

The objective of this study was to characterize several physiological differences between MD and transgenic 44568 CaMV35S::*etr1-1* petunia seeds with greatly reduced ethylene sensitivity. Seed development was examined in order to determine whether it was delayed in the 44568 seeds knowing that fruit maturation was visually delayed (Wilkinson et al., 1997; Clevenger et al., 2004). The physiological traits and development of seeds resulting from reciprocal and self pollinations between 44568 and MD were observed to determine if any of the seed characteristics were significantly influenced by ethylene sensitivity in the maternal parent. Physiological characterization was conducted through analysis of seed weight, and seed size measured throughout development, and seed number per fruit. Seeds produced from 44568, MD and the reciprocal crosses were analyzed for total sucrose content, and $C0_2$ evolution was measured from excised seeds through development as a means to characterize respiration. Molecular characterization of seed development was conducted by mRNA expression analysis of the known developmental seed markers including beta tubulin, seed storage proteins, and maturation associated genes.

Another aim of this research was to investigate dormancy and germination of seeds produced from self pollinations of 44568, MD and reciprocal crosses by measuring germination rates of seeds held in cold stratification conditions over long periods of time. Previous research indicated that germination of seeds produced on female 44568 plants

was reduced at one-month after harvest compared to MD (Clevenger et al., 2004). The degree of dormancy induction was investigated in MD, 44568 and the reciprocal crosses to determine whether the reduced germination would be overcome by a post-harvest chilling treatment. An ABA germination sensitivity assay was also used to determine if 44568 petunia seeds produced results like the *ein2-45* ABA sensitivity seen in Arabidopsis seeds (Beaudoin et al., 2000). Investigation of reciprocal crosses helped to determine if ABA sensitivity is influenced exclusively by maternal tissues. Observations on germination of freshly harvested seeds and seeds held in cold storage through one year, and experiments on ABA sensitivity during germination helped determine the level of dormancy induced in all genotypes and whether ABA sensitivity was a factor in the induction and maintenance of dormancy. These results presented here shed more light on ethylene's involvement in seed development and germination.

Materials and Methods

Culture and Growth of *Petunia x hybrida* Plants

Petunia x hybrida "Mitchell Diploid" (MD) and homozygous *etr1-1*-44568 (Wilkinson et al., 1997) plants were grown for seeds used in seed development studies. Seeds were germinated in trays with Fafard #2 soilless potting mix (Conrad Fafard, Inc., Agawam, MA) and placed in a misting house with an intermittent mist of 5 seconds every 2 hours. Approximately twenty-four hours later, a thin layer of vermiculite was applied to the seed trays. After three days in the mist house, the seed trays were placed in the greenhouse. All plants were grown in a year-round temperature controlled glass greenhouses with 24°C/20°C (+/- 2°C) day/night temperatures. Plants were sprayed with a plant growth regulator, daminozide (Uniroyal Chemical Company, Middlebury, Conneticut) at a rate of 2500 ppm at two weeks after sowing to control excessive growth.

Seedlings were transplanted after eight weeks into 1.5L plastic pots and drenched with four ppm of paclobutrazol (Uniroyal Chemical Company, Middlebury, Conneticut). All plants were fertilized 6 days a week (1 day a week water only) with 150 ppm of 20-4.8-16 Cal-Mg Peter's soluble fertilizer (Scotts-Sierra Horticultural Products Co., Marysville OH).

Seed Weight, Seed Size, and Seed Number of *Petunia x hybrida* Developing Seeds

For determination of seed weight, seed size and seed number MD and 44568 plants were self-pollinated and reciprocally cross pollinated on the same plants. Genotypes are designated as MD, 44568, ExM (44568 x MD) and MxE (MD x 44568) (♀x ♂). Self-pollinations were conducted with flowers just before anthesis. Flowers used for reciprocal crosses were emasculated just before anthesis and pollinated the following day. No more than five fruit were allowed to develop on one plant at the same time. Fruit for seed size and seed weight experiments were collected at each time-point in development in 50 mL Falcon tubes (Fisher Scientific) and kept on ice. Seeds were extracted from the three fruit with a scalpel and forceps and combined into lots to reduce variability. Seeds from all genotypes were collected at 15, 20, 25, and 30 days after pollination (DAP). Immediately seeds were weighed in 25 seed lots so that loss of any water within the seeds would not contribute to any seed weight differences. Subsequently all 25 seeds were analyzed for seed size on a dissecting scope and slide with ruler gradations. Seed size was measured by height of the longest side of the seed and width of the opposing side. Thirty-five lots of 25 seeds were used to compute seed weight and seed number averages for all genotypes. Seed number was counted by hand and was obtained by averaging the number of seeds in 35 different fruit per cross collected from different plants. Averages

and standard errors were computed using the mean function of data analysis statistics of Excel, Microsoft Office 2003.

Sucrose Analysis of Developing Seeds

Total sucrose levels of developing seeds were determined using the sucrose enzymatic assay kit **(**Boehringer Mannheim, Darmstadt, Germany). Seeds were collected at 15, 20, 25 and 30 days after pollination from MD, 44568, and reciprocal crosses. The company recommended protocol for tobacco leaves was used for the developing seeds and was reduced as per manufacturer's recommendations to accommodate the small amount of seed tissue. Four replicates, from seeds collected at the same time, of 30 mg of lyophilized seeds per developmental time-point were used to obtain total sucrose means. The level of total sucrose was determined using a light spectrometer (SmartSpec 3000 BioRad, Hercules, CA). Results of the sucrose quantification are presented in two manners: based on sucrose content of total weight of seed lot tested (ng/g of dry weight) and then sucrose content of mature seeds adjusted to a per seed basis since ETR and ExM seeds are lighter in weight at full maturity. Averages and standard errors were computed using the mean function of data analysis statistics of Excel, Microsoft Office 2003.

CO_2 Analysis of Developing Seeds

CO_2 accumulation was measured by weighing out 0.2 grams of fresh MD, 44568, MxE, and ExM seeds from 3 different fruit from different plants. The seeds were collected at 15, 20, 25, and 30 DAP. 5 groupings of seeds were collected per time-point in each genotype for measurement after different amounts of accumulation time. The seeds were placed in a 12mmx32mm clear 1.5ml vial with an air-tight cap with septa (National Scientific Company, Duluth, GA). One sample of 0.5mL was removed per vial at the appropriate collection time, which included 15 minutes, 30 minutes, 1 hour, 2 hours

and 3 hours and measured on a GOW-MAC gas chromatograph Series 580 (GOW-MAC Instrument Company, Bridgewater, NJ). The average respiration rate (measured as CO_2 evolution) of 4 separate groupings of 0.2 grams of seeds were measured for each time-point at each developmental stage. Averages and standard errors were computed using the mean function of data analysis statistics of Excel, Microsoft Office 2003.

Seed Development Marker Analysis

Whole fruit tissue of MD and 44568 was collected at 5, 10, 15, 20, 25 and 30 days after pollination and immediately placed in liquid nitrogen and subsequently a -80º Celsius freezer until used for RNA extraction. RNA was extracted using the phenol-chloroform method and lithium chloride precipitations (Ciardi et al., 2000). RNA was quantified by spectrophotometer readings (SmartSpec 3000 BioRad, Hercules, CA) and quality was checked by gel electrophoresis. RNA was then diluted with RNAse free water and frozen until RT-PCR analysis of the developmental markers. A set of primers was obtained for several seed development marker from Invitrogen Corporation (Carlsbad, California). GenBank Accession is designated as a "CV" number. Number of cycles of RT-PCR replication is in parenthesis: Beta tubulin YF-9-C01 CV300189 (29) primers: Forward-CCACATTTGTTGGCAATTCA; Reverse-CAGCTCCCTCCTCGTCATAC. LEA-D29 RF-1-H08 CV300578 (22) Primers: Forward- AAGGACTTGGCTTTAAATCCAC, Reverse-TCTGCTGCATATTGCCCAC; Seed maturation RF-5-C02 CV300863 primers (LEA4) (22) : Forward- GAGAAGGGGAGAAGATGACAAC, Reverse-ATAGTGTGTCCCAACCTGCC; 2S Albumin RF-5-G08 CV300914 (25) primers: Forward: GGTGACAGACGATGAAGAAAG, Reverse-ATACGGGGAAGGTAACGAG; 11S Globulin RF-5-G10 CV300916 (25) primers:

Forward: TCGCCAAAAACTTCCCATC, Reverse-CCACACCACAAATTCAAATCC; Ubiquitin (22) Primers: Forward- AACATACAGAAGGAGTCAACAC, Reverse-AGAAGTCACCACCACGAAG. RT-PCR analysis was conducted using the One-Step RT-PCR Analysis kit from Qiagen (Qiagen Inc- USA, Valencia, California). Manufacturer protocol was followed. PCR was run with the following program: 50°C for 30 minutes, 94°C for 15 minutes; multiple cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; final incubation at 72°C for ten minutes. The entire RT-PCR reaction was run out on a 1.5% acrylamide gel by electrophoresis. Pictures were taken on a Polaroid Fotodyne camera. (Polaroid Corporation, Pasadena, California). RT-PCR bands were analyzed visually.

Germination Assay

Seeds were tested for the ability (or inability) to germinate after various periods of 4º C cold storage, with dry desiccant to keep moisture to a minimal level. The same sets of seed of each genotype (MD, 44568 and reciprocal crosses) were tested for radicle emergence and cotyledon expansion on freshly harvested seeds and after one month, six months and 1 year of cold storage. Twenty-five seeds were placed on 100x15mm Petri plates (Fisher Corp) containing basal salt media (Jorgensen et al., 1996), which was modified slightly by removing sucrose, which can inhibit germination, and using half the concentration of MS basal salts. Eight replicate plates of each genotype, Mitchell Diploid, *etr1-1*, MD x *etr1-1* and *etr1-1* x MD were examined for each time-point after seed collection. The germination plates were grown in a temperature controlled Percival at 25º Celsius for 22 days in constant light. Radicle emergence and cotyledon expansion were recorded separately every 2 days for the entire duration of the 22 day experiment.

Averages and standard errors were computed using the mean function of data analysis statistics of Excel, Microsoft Office 2003.

ABA Germination Sensitivity Assay

Seeds of MD, *etr1-1*, *etr1-1* x MD and MD x *etr1-1* were tested for the ability (or inability) to germinate on different concentrations of ABA in the germination media mixture. Seeds examined were 1 month old and had been stored in 4º C cold storage as described above which is the average time in storage needed to overcome typical after-ripening restrictions in petunia seeds. Germination media was the same as above, though the media was enhanced with ABA dissolved in 100% ethanol and added to the final concentrations of 0μM, 0.01μM, 0.1μM, 1μM, 2μM and 10μM. Ethanol was equalized between different concentrations. Eight plates of each concentration were used with 25 seeds per genotype described above. The germination plates were grown in a temperature controlled Percival at 25º Celsius in constant light for 14 days. Measurements were taken as described above. Averages and standard errors were computed using the mean function of data analysis statistics of Excel, Microsoft Office 2003.

Results

The fruit of 44568 plants show a distinct delay in development compared to MD. Wilkinson et al. (1997) first discovered the delay in fruit ripening in these plants. The 44568 fruit are slower to grow to full size and begin the browning processes during maturation later than in MD (Figure 3-1). Visually, when the seeds are excised from the fruit, they also appear to be slower to develop because browning of the seed coat due to oxidative processes begin later in 44568 than in MD (Figure 3-1). Additionally, reciprocally crossed fruit and seeds have visual characteristics like their corresponding

maternal plants. A delay in development of the fruit may have significant impacts on the seed; therefore, further characterization of seeds of 44568, Mitchell Diploid, and the reciprocal crosses (44568 x MD (ExM) and MD x 44568 (MxE); ♀x ♂) was conducted.

Seed Characterization by Weight, Size, and Seed Number

Several characteristics of 44568, MD, and reciprocal cross seeds were analyzed to determine if there were any major differences in physiological traits among genotypes. Seed size was measured in seeds through development of MD, 44568, and the reciprocal crosses and the average area of the seeds was computed (Figure 3-2). 44568 seed size was similar to MD throughout development, but once MD seeds reached maturity differences began to occur in the size of the seeds. Seed size increased in all genotypes until 25 days after pollination. The 44568 seeds continued development through a delayed ripening period for an additional five days (days 25-30) which resulted in a reduction in seed size. The reciprocal crosses had a similar result to the respective maternal parent. ExM seeds became reduced in size in the last five days of extended fruit development similar to seeds made by selfing 44568, whereas the MxE seeds did not exhibit any reduction in size and did not endure an extended maturation time-period.

Next it was investigated whether the loss in seed size of 44568 and ExM seeds would also result in a difference in seed weight (Figure 3-3). Seed weight was measured in all genotypes of seeds at full maturity, and the 44568 and ExM seeds had reduced seed weight in comparison to MD and MxE. When water weight was eliminated the dry weight analysis revealed similar results, where 44568 and ExM had significantly less dry weight than MD and MxE seeds (Figure 3-3). Respiration (CO_2 evolution) was also measured to see if 44568 and ExM seeds continued to respire for an additional 5 days compared to MD and MxE (Figure 3-4). All genotypes had similar CO_2 levels and

accumulation trends at 15 and 20 days after pollination throughout the three hour collection period. By 25 days after pollination, MD and MxE seeds produced levels of CO_2 that were extremely low or non-detectable. 44568 and ExM seeds still produced measurable levels of CO_2 at 25 days after pollination, yet did not have measurable levels of CO_2 by the time the seeds reached maturity at 30 days after pollination.

Seed number was also quantified in order to observe whether a reduction in ethylene sensitivity had any effect on the number of seeds in each fruit since it was shown that the seeds produced on a maternal plant with reduced ethylene sensitivity were smaller in size and weight. There was no significant difference between any of the lines in seed number per fruit (Figure 3-5).

Developmental Delay of Seeds Reduced in Ethylene Sensitivity

Due to the delay in fruit maturation of 44568 and ExM plants, it was observed whether the seeds were also delayed throughout development. Through visual observation it did appear that the seeds were developmentally delayed because the oxidation process was slower and the seeds took longer to acquire brown color (Figure 3-1). A more specific approach was taken to confirm that there was a delay in development. Sucrose quantification was performed on developing seeds, and additionally adjusted to a per seed basis since ETR and ExM seeds are lighter in weight at full maturity (Figure 3-6). Sucrose content measured in the developing seeds indicated that homozygous and hemizygous seeds produced on a 44568 maternal plant were delayed in accumulating sucrose. Sucrose levels increased substantially between 20 and 25 days after pollination in seeds produced on the MD maternal plant. Conversely, the seeds produced on the 44568 maternal plant accumulated sucrose more slowly but eventually reached similar sucrose levels at 30 days after pollination. Sucrose levels of

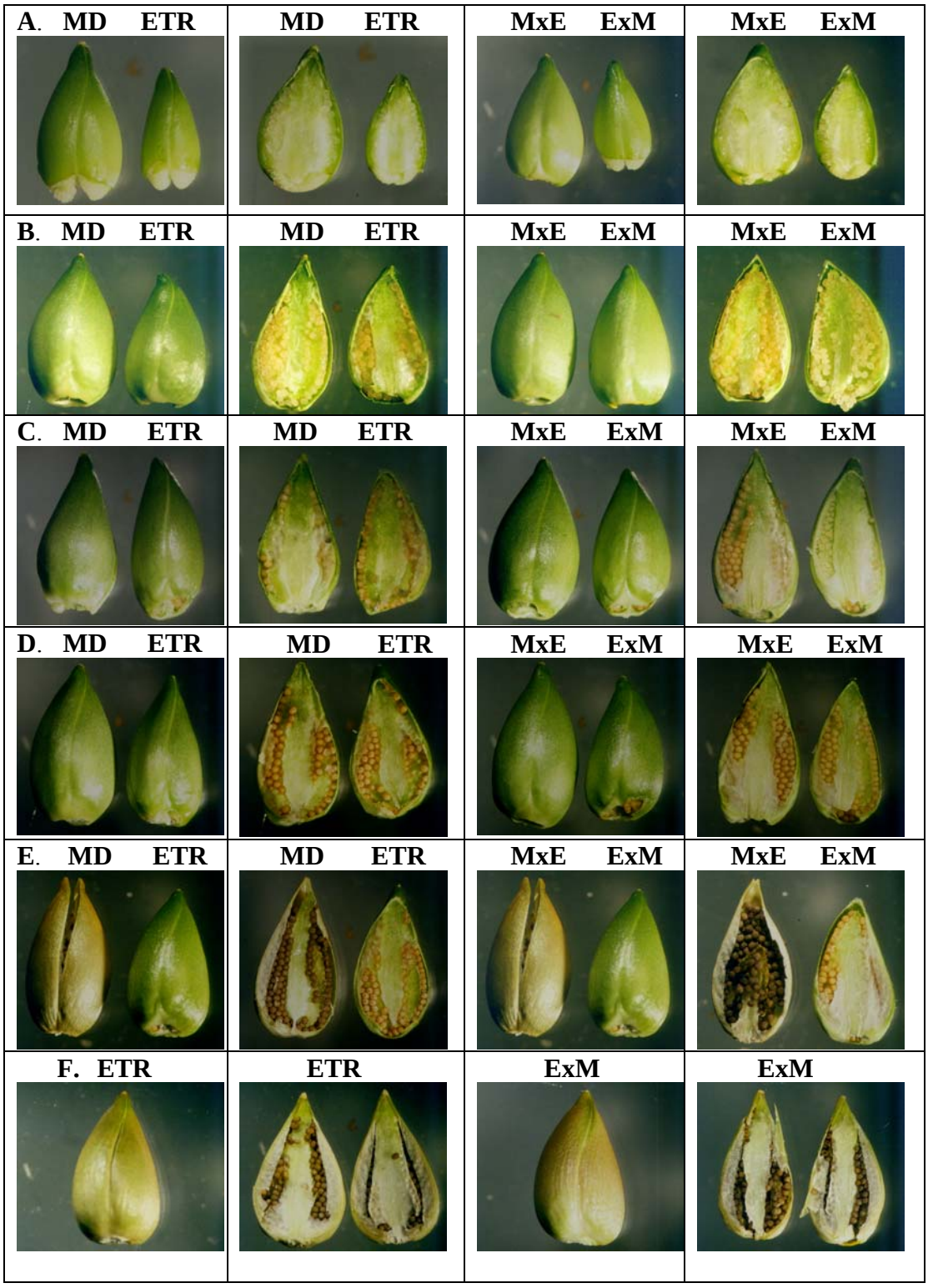


Figure 3-1 A picture series of fruit and seed development of all genotypes. Fruit are shown whole (column 1 and 3) and with longitudinal sections (column 2 and 4) to show developing seeds within fruit. **A**. 5 DAP **B**. 10 DAP **C**. 15 DAP **D**. 20 DAP **E**. 25 DAP **F**. 30 DAP.

A.

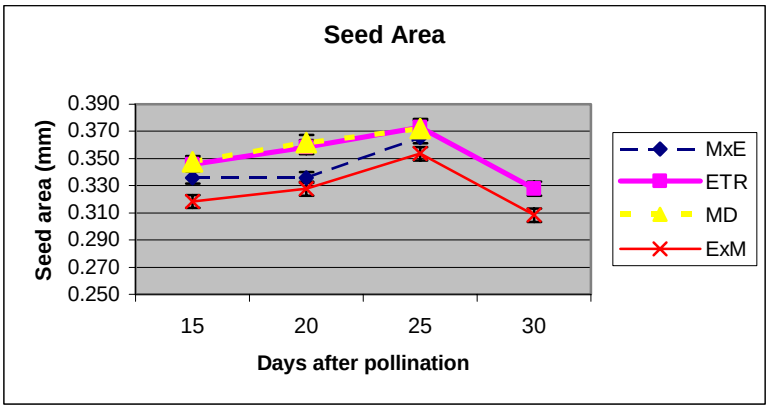
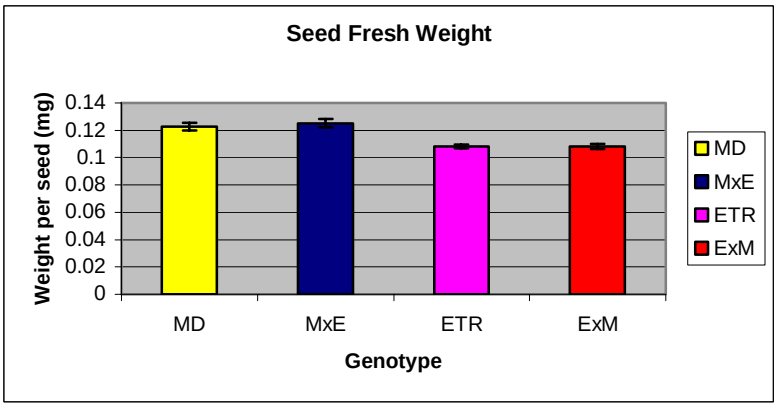


Figure 3-2 Seed size of all genotypes of seeds through development starting at 15 days after pollination through full maturity (25 days after pollination for MD and MxE and 30 days after pollination for 44568 and ExM). Seed size represented as area (height x width).

A.



B.

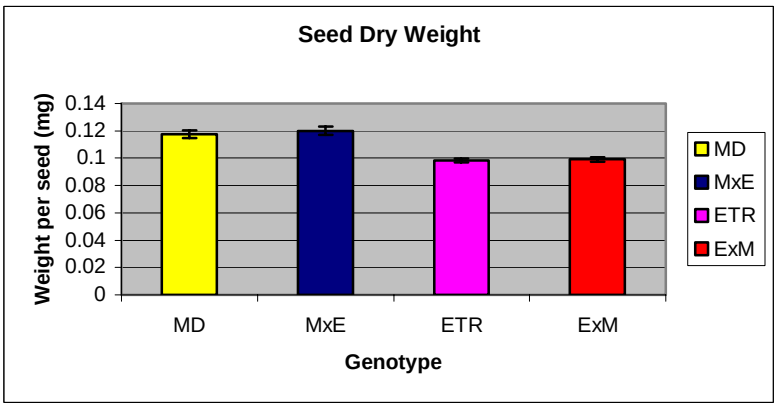
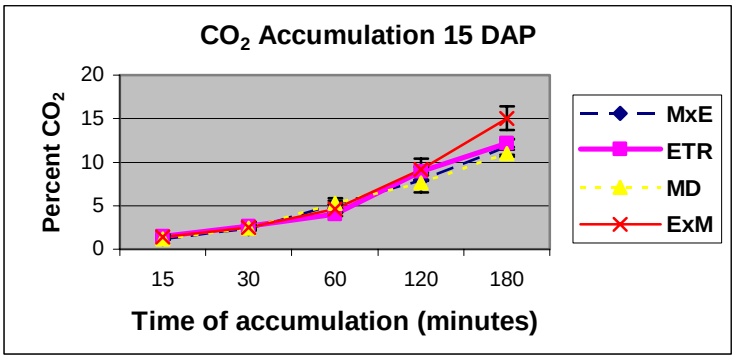
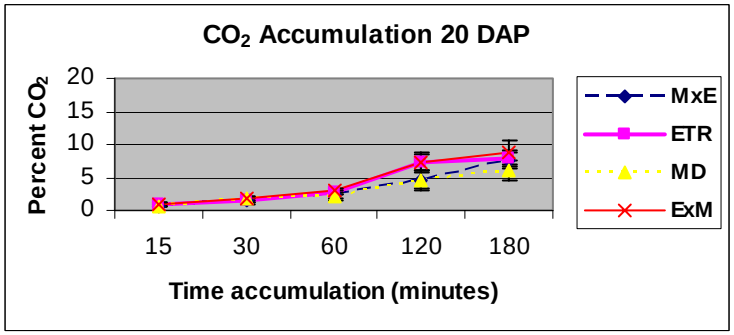


Figure 3- 3 Average weight of individual seeds of MD, MxE, ETR (44568) and ExM **A**. Weight of seeds at fresh harvest **B**. Weight of seeds after moisture content is removed.

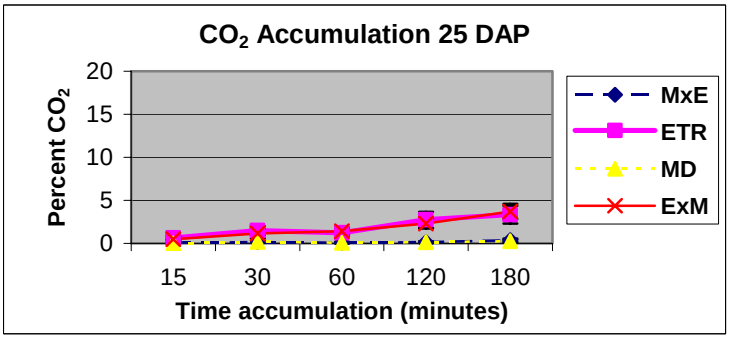
A.



B.



C.



D.

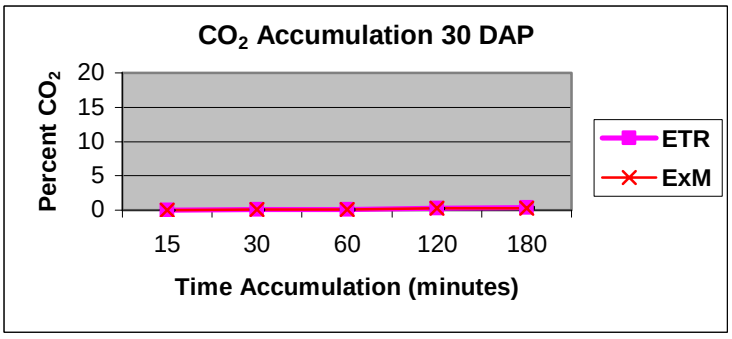


Figure 3-4 CO_2 Accumulation throughout 3 hours of developing seeds of MD, ETR (44568), MxE and ExM. **A**. CO_2 accumulation at 15 days after pollination. **B**. CO_2 accumulation at 20 days after pollination. **C**. CO_2 accumulation at 25 days after pollination. **D**. CO_2 accumulation at 30 days after pollination.

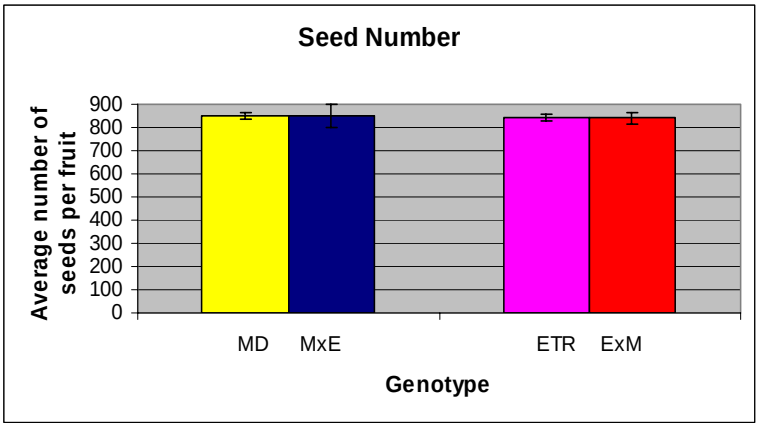
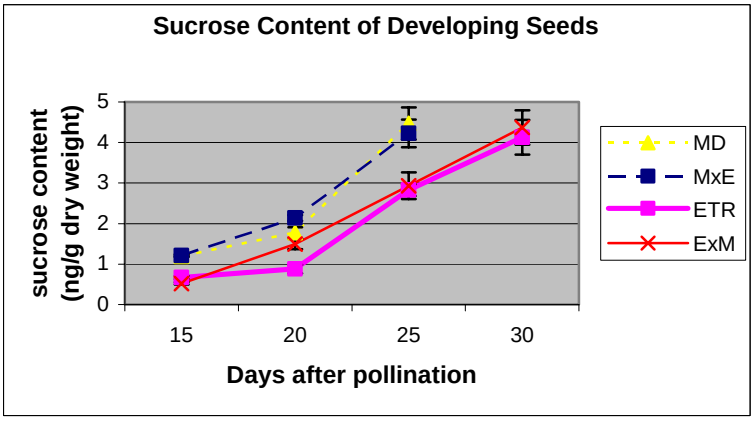


Figure 3-5 Average number of seeds per fruit of MD, MxE, ETR (44568) and ExM.

A.



B.

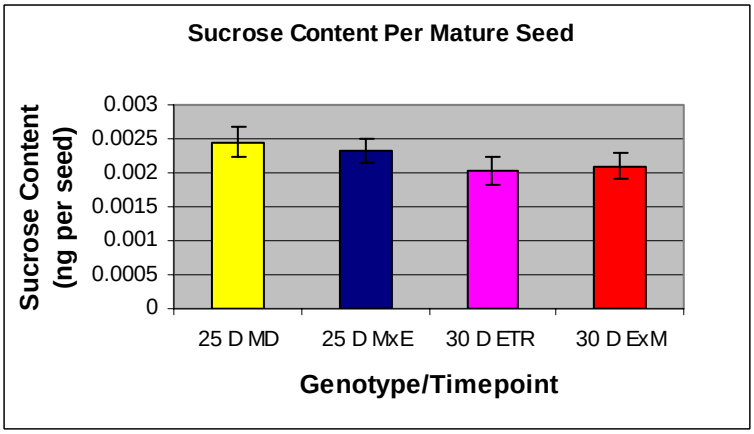


Figure 3-6. Sucrose content of seeds of all genotypes. A. Total sucrose content of developing seeds from 15 days after pollination through full maturity. (25 days after pollination for MD and MxE; 30 days after pollination for ETR (44568) and ExM). A. Sucrose content of mature seeds adjusted to a per seed basis.

mature seeds adjusted to a per seed content indicated that 44568 and ExM seeds did not have a statistical difference in sucrose content at the end of development compared to MD and MxE.

To further confirm that 44568 and ExM seeds were developmentally delayed, mRNA expression analysis of known developmental markers was conducted (Figure 3-7). Markers were chosen based on their differing expression patterns through development and their availability from sequenced petunia cDNA libraries. Beta tubulin, a cell-cycle related structural protein, is used as a seed developmental physiological marker in pepper since expression consistently decreased just before complete seed desiccation tolerance (Portis et al., 1999). The seed storage genes, 11S globulin and 2S albumin, are known to begin accumulation slightly later in seed development and continue through the final maturation phases (Norton and Harris, 1975; Pomeroy, 1991; Wobus et al., 1999; Hoekstra et al., 2001). 11S globulin and 2S albumin are predicted to be the most predominant storage proteins in petunia seeds as seen by the extreme redundancy in the petunia cDNA libraries (personal observations). LEA proteins also begin to accumulate in the mid to latter stages of seed development. In many cases, the timing of LEA mRNA and protein accumulation is correlated with the start of the seed-desiccation process and associated with elevated *in vivo* ABA levels. The products of these genes are thought to function in protecting cells from dehydration (Baker et al., 1988; Dure et al., 1989; Brocard et al., 2003). When expression of these markers was conducted it was observed that beta-tubulin mRNA expression continued later in 44568 fruit and seed tissues compared to MD. 11S Globulin mRNA expression was visible at 5 days after pollination in MD but was not observed in the 44568 line until 10 days after

pollination. 2S albumin expression was also slightly delayed in 44568, a small amount of expression can be observed in MD at 5 days after pollination but is not seen in 44568. LEA4 and LEA D-29 mRNA expression did not have as much of a discrepancy between the two genotypes and the expression patterns appear to be induced in a similar manner.

The Effect of Ethylene Sensitivity on Seed Germination

Since it was observed that the seeds produced on a 44568 plant have delayed development, it was investigated whether this delay would also have any impact on dormancy and subsequent germination. Dormancy can be measured by observing germination percentages after specified periods of dry storage (Leon-Kloosterziel et al., 1996). Seeds produced from self-pollinated MD, 44568, and reciprocal crosses of the two were tested for germination at fresh harvest and after 1 month, 6 months, and 1 year of 4°C storage (Figure 3-8). Seeds from both reciprocal crosses and 44568 had lower germination percentages than MD, with homozygous 44568 having the lowest germination rates at fresh harvest. After one month of storage in 4°C, all seeds containing the *etr1-1* transgene germinated at similar rates, and all had significantly lower germination than MD. After six months and one year of storage, all genotypes had similar germination rates, and seeds containing the *etr1-1* transgene did not germinate differently from MD.

ABA Sensitivity and Germination

Since the after ripening requirement and dormancy were both impacted in all homozygous and hemizygous 44568 seeds, another germination assay was conducted to see if these genotypes were also altered in their sensitivity to exogenous ABA. Seed germination of all genotypes was tested on increasing concentrations of ABA (Figure 3-9). Homozygous and hemizygous 44568 seeds had similar levels of ABA sensitivity, and

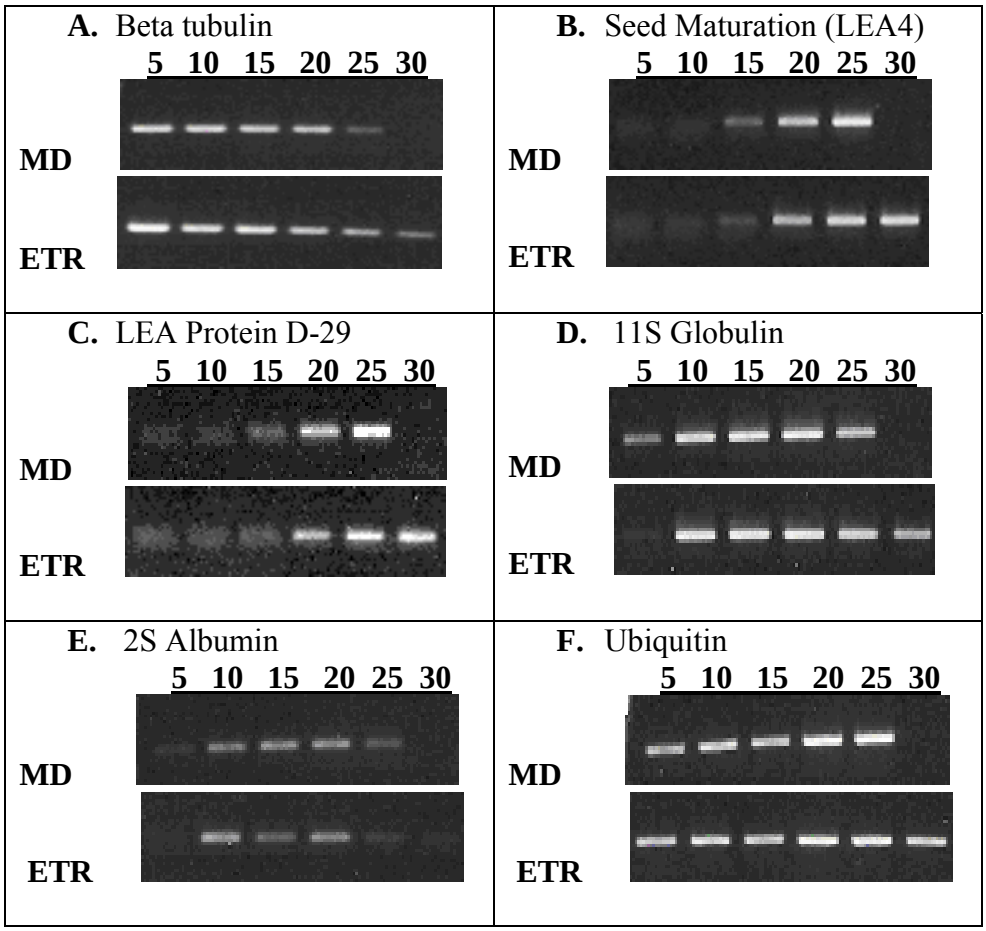


Figure 3-7 RT-PCR analysis of seed developmental markers of whole MD and ETR (44568) fruit tissue. Lanes are: ladder, MD 5, MD 10, MD 15, MD 20, MD 25, space, ETR 5, ETR 10, ETR 15, ETR 20, ETR 25, and ETR 30 days after pollination whole fruit. **A**. Beta tubulin. **B.** Seed Maturation (LEA4). **C.** Late Embryogenesis Abundant Protein D-29. **D.** 11S Globulin storage protein. **E**. 2S Albumin storage protein. **F**. Ubiquitin- loading control

all of these genotypes had increased sensitivity to ABA compared to MD. When observing MD germination as cotyledon expansion, the germination rates of MD were significantly higher at 0, 0.01, and 0.1 µM of ABA when compared to 44568, ExM and MxE. Germination of all genotypes reduced dramatically at 1 and 2 µM of ABA. When germination was observed as radicle emergence MD seeds had an even more dramatic tolerance to ABA than the other genotypes. MD radicles were able to emerge at all concentrations of ABA at significantly higher levels than 44568, ExM and MxE.

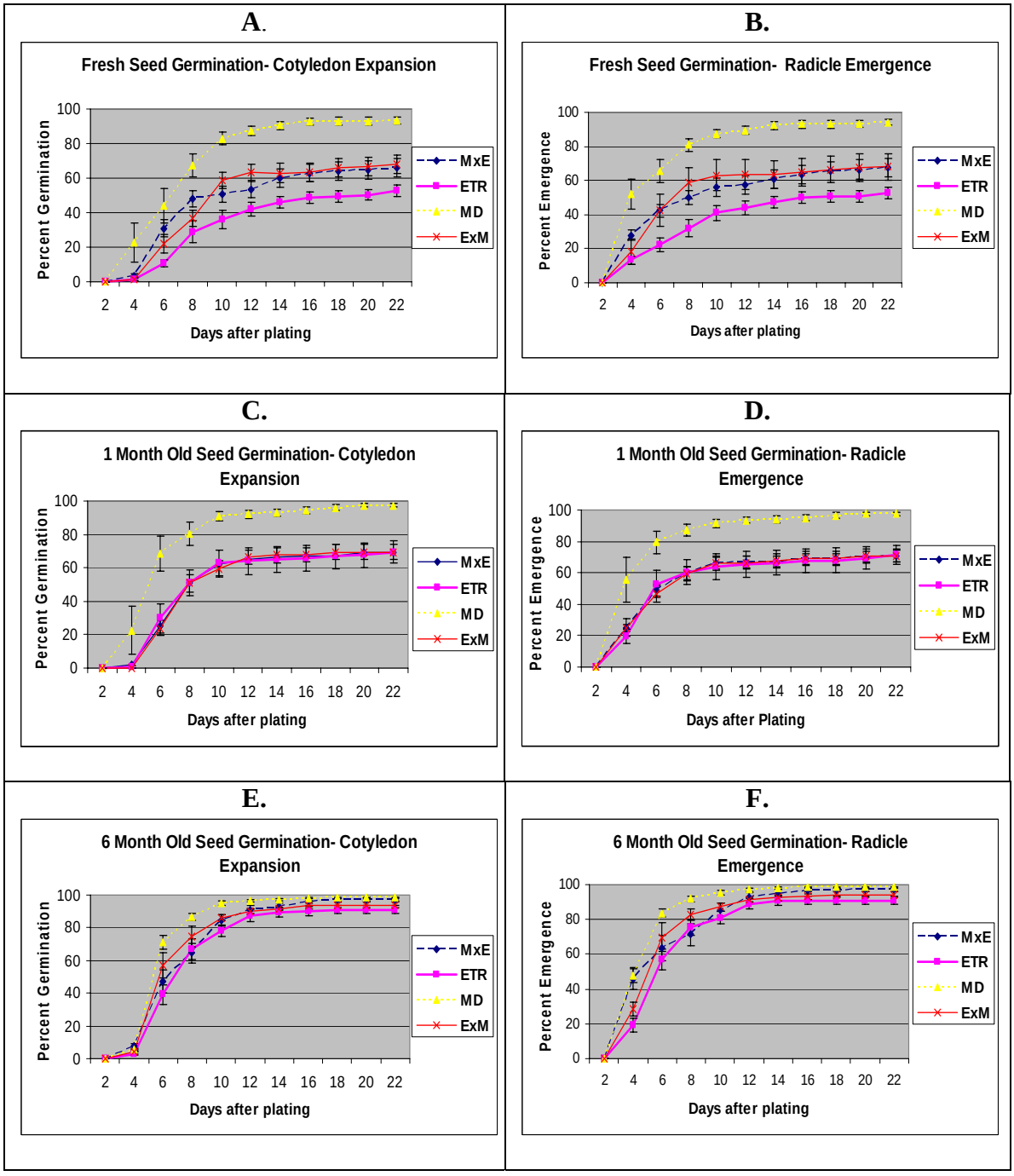


Figure 3-8 Germination of seeds of all genotypes after various storage periods. Germination measured by cotyledon expansion and radicle emergence separately. A. and B. Germination of freshly harvested seeds.C. and D. Germination of seeds after one month of cold storage.E. and F. Germination of seeds after six months of cold storage. G. and H. Germination of seeds after 1 year of cold storage.

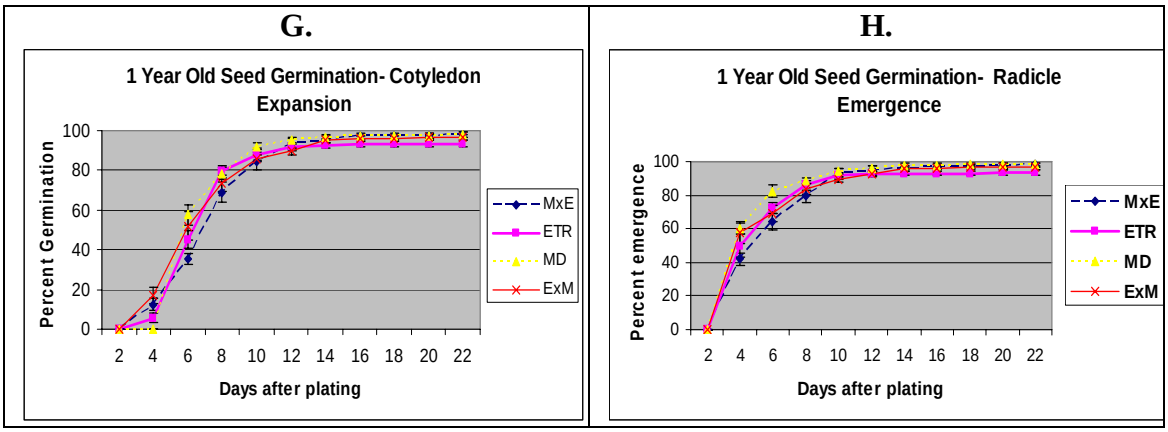
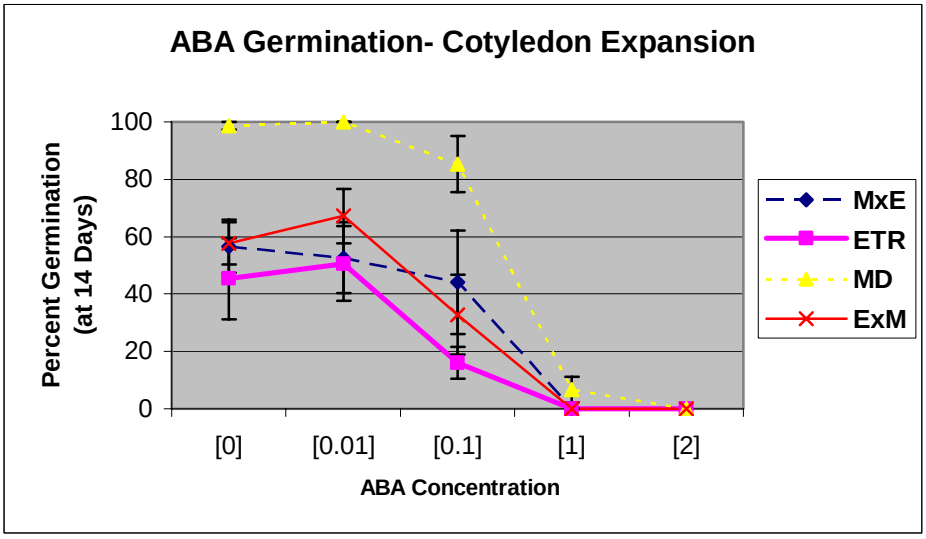


Figure 3-8 Continued

Discussion

The physiological characteristics of seeds altered in their sensitivity to ethylene were analyzed by seed size, weight, and number. It was observed that seed size and weight was only affected in seeds produced on a maternal parent carrying the *etr1-1* transgene. The consequence of the reduction of ethylene sensitivity of the maternal parent resulted in delayed fruit and seed ripening, and subsequently a loss in seed size during the extended ripening time-period. Seeds with the extended ripening period were also lower in seed weight. This loss in seed weight occurs in the five days of extended development since the loss of seed size was observed during this period (Figure 3-2). An explanation for the loss in seed weight of the 44568 and ExM seeds is that these seeds had to endure an additional five days of metabolic activity since fruit ripening and seed dessication was not completed. This extended metabolic period used additional stored material which could include sugars, lipids or any other form of reserve, resulting in the loss of seed size. Metabolic activity in these samples was observed through CO_2 evolution throughout a developmental time-course in seeds of all the genotypes. 44568 and ExM seeds continued to respire throughout the additional five day extended ripening period. These results parallel physiological traits seen in other systems where, as the

A.



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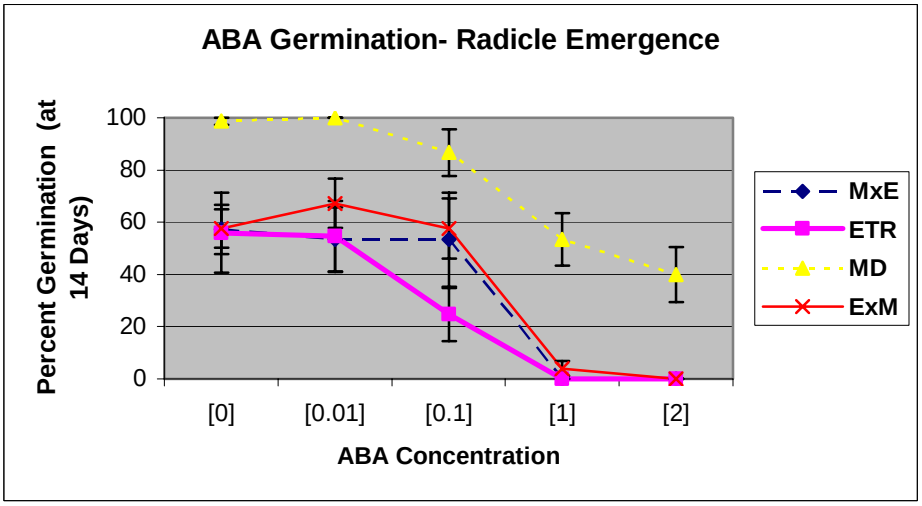


Figure 3-9 ABA sensitivity of germinating 1 month old seeds of MD, ETR (44568), MxE and ExM. Measurement of germination by cotyledon expansion and radicle emergence separately. **A**. Germination measured based on cotyledon expansion 14 days after plating. **B.** Germination based on radicle emergence after 14 days after plating.

development of the seed progresses, respiration decreases (Weber et al., 2005). Therefore, the CO_2 levels given off by the seeds are a good indication of the stage of development, indicating that 44568 and ExM seeds are delayed in development compared to MD and MxE.

Analysis of sucrose levels was also conducted on developing seeds of all genotypes in order to determine the progress of development over time. Sucrose levels were slower to accumulate in seeds produced on a maternal plant carrying the *etr1-1* transgene. From previous research conducted on sucrose levels through maize seed development it was shown that sucrose levels are lower at the beginning of seed development and then levels increase once the cell division phases slow. The higher sucrose levels generate embryonic sink strength and further embryo development (Weber et al., 1996; 2005). Sucrose likely has several roles in seed development, one to act as a nutrient sugar for the developing embryo and the other to act as one of the signaling molecules that induce storage assimilation gene expression (Koch, 2004). Sucrose accumulation may also be associated with stress tolerance during seed dessication (Hoekstra et al., 2001). The sucrose levels increase towards the end of seed development in order to aid in membrane protection during the drying down of the seeds (Hoekstra et al., 2001). Since sucrose levels were delayed in accumulating within the seeds of 44568 and ExM, it is likely that the developing embryo does not receive signals to begin storage protein accumulation and maturation until a later time-point in development.

Lastly, developmental timing in seeds reduced in ethylene sensitivity was observed through mRNA expression analysis of known seed developmental markers. Beta tubulin expression in pepper seeds consistently decreased just before complete seed desiccation tolerance (Portis et al., 1999). Since higher expression of beta tubulin was extended in 44568 seed tissue through 25 DAP in comparison to MD, it can be inferred that seed desiccation tolerance may be acquired later in development in the 44568 seeds than it does in MD. mRNA for 11S globulin was slightly slower to accumulate in 44568

compared to MD which indicates that the cell expansion/storage accumulation phases started slightly later in 44568 in comparison to MD. 11S Globulin expression was visible at 5 days after pollination in MD, but was not shown in the 44568 line until 10 days after pollination. This five day discrepancy between 44568 and MD developmental marker expression parallels the five day extended fruit ripening delay observed much later. These data, together with the sucrose and CO_2 results, clearly indicate that 44568 and ExM seeds are developmentally delayed compared to MD and MxE; thus the loss of ethylene sensitivity of the maternal plant is the most significant factor in causing this delay.

Since development was delayed in seeds produced on a maternal plant carrying the *etr1-1* transgene, the germination phenotype of all the genotypes was observed. The germination results revealed that all genotypes carrying the *etr1-1* transgene were affected in germination characteristics. 44568 and reciprocally crossed seeds all had significantly reduced germination rates compared MD at fresh harvest and after one month of storage. Germination rates of 44568 and reciprocally crossed seeds all recovered after six months and one year of cold storage. These data provide two interesting observations. First, hemizygous and homozygous 44568 seeds have a longer after-ripening requirement than MD. Since after-ripening is a dormancy breaking agent, the greater after-ripening requirement of lines with reduced sensitivity to ethylene confirms that there is heightened primary dormancy (Koorneef and Karssen, 1994). Second, unlike previous findings with the maternal role in developmental delay, the maternal parent does not completely determine subsequent germination characteristics. The level of ethylene sensitivity of the seed's zygotic tissue also plays an important role on the impact of germination. Through previous research conducted on maternal tissue

role in seed developmental processes, it has been shown that the maternal tissues play a significant role in early developmental processes (Vielle-Calzada et al., 2000). Additionally, studies with ABA have revealed that early ABA synthesis, which occurs at the end of the cell division phase, in maternal tissues is involved more in earlier seed developmental processes such as preventing early germination and aids in progression into the maturation phase of embryogenesis (Finkelstein et al., 2002). Conversely, latter ABA synthesis in zygotic tissues is involved in seed maturation programs such as acquiring dessication tolerance (Finkelstein et al., 2002; Frey et al., 2004). These observations help explain the results of this research, where the sensitivity of the maternal tissue had more of an affect than the overall ethylene sensitivity of the zygotic tissue on the developmental timing of the seeds. Yet, later processes in seed development, such as dormancy acquisition, were affected more by the ethylene sensitivity of the zygotic tissue. This would explain the longer after-ripening requirements of all genotypes reduced in ethylene sensitivity, regardless of the maternal parent genotype.

Lastly, since seeds carrying the *etr1-1* transgene exhibited enhanced seed dormancy, an additional germination assay was conducted to determine the sensitivity to exogenously applied ABA. The results of this assay were similar to the results of the standard germination test in that the overall ethylene sensitivity of the zygotic tissue played a major role in the phenotype. It was determined that all seeds carrying the *etr1-1* transgene appeared to exhibit increased sensitivity to ABA. An important issue to consider about the results of this experiment is that endogenous levels of ABA may have an impact on the sensitivity assay. If levels of endogenous ABA are significantly higher in the transgenic lines compared to wild-type, then it would be difficult to determine that

the transgenic seeds are hypersensitive to ABA compared to wild-type. Yet, when mature petunia *etr1-1* seeds where measured for ABA content it did not appear to have significantly different levels of ABA than MD seeds (Barry, 2004). Hemizygous seeds did not exhibit different sensitivity to the ABA than the homozygous 44568 seeds; therefore, the genotype of the maternal plant does not play a major role in the ABA sensitivity phenotype. These data confirm results from previous research conducted on Arabidopsis seeds reduced in ethylene sensitivity, which also exhibited increased sensitivity to exogenous ABA during a germination assay (Beaudoin et al., 2000). This evidence demonstrates that there likely is a direct interaction between ABA and ethylene in the involvement of seed dormancy and germination in petunia seeds similar to Arabidopsis (Beaudoin et al., 2000).

An item of interest that arises from the germination assays is that there still exists a percentage of seeds reduced in ethylene sensitivity that have the capability of germinating. For example, approximately 50-60% of seeds carrying the *etr1-1* transgene still germinate at fresh harvest without any after-ripening time-period. Similarly, 50-60% of the seeds reduced in ethylene sensitivity are able to germinate with the lowest concentration of ABA, 0.01µm, and approximately 20-50% at 0.1µm of ABA. These observations lead to the question as to why some seeds do not exhibit as strong as a phenotype as other seeds when all of the seeds are reduced in ethylene sensitivity. One explanation for the phenomenon is the position of the seeds within the fruit. The petunia fruit is attached to the maternal plant at the base. Additionally, when fruit maturation begins the tip at the top of the fruit opens and begins to dry down from the top to the bottom. Therefore, seeds positioned at the top of the plant may be receiving less

maternal resources. One of these resources could be the germination stimulatory hormone, gibberellic acid. A theory exists in which GA is also involved in an antagonistic relationship with ABA in order to break dormancy and induce germination (Karssen, 1995). It is also known that when homozygous *etr1-1* petunia seeds are imbibed in GA_3 that germination levels increase dramatically (Bleecker et al., 1988). Therefore, some seeds may receive more resources, such as GA, from the maternal plant than others and this could contribute to the discrepancy in phenotype between the seeds reduced in ethylene sensitivity.

Conclusion

Ethylene has been shown to play a significant role in several aspects of plant development. However, there has been little conclusive evidence that it plays a major role in several aspects of seed development, germination and dormancy in a single plant species. The data presented here provide evidence that ethylene plays an important role in all of these developmental processes. Seed physiological characteristics that were altered by the reduction of ethylene sensitivity included a reduction in seed weight and size of seeds produced on a maternal plant carrying the *etr1-1* transgene. Seed development is greatly influenced by a reduction in ethylene sensitivity of the maternal plant as seen through delayed sucrose accumulation and an extended time-period of respiration. Since maternal tissue has been shown to have some control of early seed development in petunia (Colombo et al., 1997), it is not surprising that the maternal tissue's sensitivity to ethylene plays a major role in the developmental timing of the seeds.

Conversely, the maternal plant does not completely determine the subsequent germination and ABA sensitivity phenotypes seen in the seeds reduced in ethylene

sensitivity. Dormancy was extended in all seeds carry the *etr1-1* transgene. ABA sensitivity during seed germination was also heightened in the homozygous and hemizygous 44568 genotypes compared to MD. These data confirmed the concept that hormone interactions in both maternal and zygotic tissues play a major role in the severity of dormancy and subsequent initiation of seed germination.

All of the data presented in this research provided evidence that ethylene is indeed intricately involved in seed developmental timing and germination processes of petunia seeds, and it is likely through interactions with other hormones such as ABA. The extent of the maternal plant's role on seed developmental processes varies. The maternal plant likely plays more of a major role in the beginning of seed development and less at the end of development when dormancy is induced.

CHAPTER 4
MICROARRAY ANALYSIS AND CONDENSED TANNIN CONTENT OF PETUNIA SEEDS AFFECTED IN ETHYLENE SENSITIVITY

Introduction

Angiosperm fruit and seed development is mediated by an assortment of factors including hormones, storage proteins, fatty acid and carbohydrates; therefore, it has been exceedingly difficult to monitor all or even several of the genes involved in these processes at one point in development (Harada, 1997). It is also known that ethylene has diverse roles during growth and development of plants. Ethylene is especially integral to fruit development and ripening processes (Ecker, 1995; Giovannoni, 2001). Ethylene's substantial role in fruit ripening is illustrated in the delayed fruit ripening phenotype observed in ethylene perception mutants in various species including Arabidopsis, tomato and petunia (Bleecker et al., 1988). Since ethylene is involved in an array of plant responses, it is likely a complex interaction of gene regulation and expression occur during different plant processes. Identification of novel genes associated with ethylene's function in ripening fruit and seeds will help develop a more complete understanding of the physiological role of ethylene in late fruit and seed development.

Microarray analysis is a powerful tool to examine the expression of hundreds of genes at the same time. This technology has tremendous advantage over traditional mRNA expression methods that usually analyze one gene at a time (Ekins and Chu, 1999). Microarray analysis has already been used to examine many plant growth and

development processes including light regulation, wounding response, pathogen infection and hormone defense responses (Zhong and Burns, 2003).

A cDNA microarray was developed to help screen for gene expression differences between MD and 44568 fruit and seeds at 25 days after pollination. A set of 384 cDNAs from sequenced petunia cDNA libraries made from fruit, seeds, and whole flowers were used to make the microarray. The focus of the study was to identify a subset of genes with high levels of differential expression between 44568 and MD. A goal was to also determine if the mRNA expression patterns of these genes paralleled the developmental delay seen in seeds with a maternal parent with reduced ethylene sensitivity or contribute to the stronger induction of dormancy in the homozygous 44568 and hemizygous seeds (Chapter 3).

Additionally, the results of the microarray experiments led to further investigation into a pathway involved in secondary metabolism. Proanthocyanidins, or condensed tannins, are compounds found in the seed coat that turn brown upon oxidation (Debeaujon et al., 2000; Nesi et al., 2001). These tannins are known to help provide a protective barrier for the seed, but they are also thought to be involved in altering seed coat imposed dormancy by reducing the permeability of the seed coat (Debeaujon et al., 2000). To the best of our knowledge, there are no present published works that focus on any kind of ethylene involvement in the proanthocyanidin pathway. Several characteristics of the 44568 transgenic seeds suggest that there may be altered levels of proanthocyanidins. These include increased seed dormancy, visual differences in seed coat color at the end of development, and differential regulation of expression of genes

encoding enzymes and transcriptional regulators of the anthocyanin/proanthocyanidin pathway.

Research Objectives

The main objective of this research was to isolate a set of genes with expression differences between 44568 and MD fruit and seeds at a late time-point in fruit and seed development through microarray analysis. The time-point chosen, 25 days after pollination, represents the point in fruit development where 44568 and MD are the most visually different, where MD is fully brown and ripe and 44568 fruit are just beginning the browning process. A smaller subset of genes chosen from the array results will be investigated in further detail through RT-PCR expression analysis in a seed developmental time series from 20 days after pollination to maturity. The last objective was to further examine expression of genes involved in the proanthocyanidin synthesis pathway to determine if this pathway is altered and could contribute to the stronger induction of dormancy in the 44568 and hemizygous lines resulting from reciprocal crosses with MD.

Material and Methods

Culture and Growth of *Petunia x hybrida* Plants

Petunia x hybrida "Mitchell Diploid" (MD), *etr1-1*-44568 (Wilkinson et al., 1997), and *ein2* RNAi (Shibuya et al., 2004) plants were grown for fruit and seeds used in the microarray experiments and the subsequent confirmation by RT-PCR. Seeds of the three lines were imbibed in 100 ppm of GA_3 overnight to promote uniform germination. Seeds were then sown in 72 cell trays with Fafard #2 soilless potting mix (Conrad Fafard, Inc., Agawam, MA) and placed in a misting house with an intermittent mist of 5 seconds every 2 hours. Approximately twenty-four hours later, a thin layer of vermiculite was applied

to the seed trays. After three days in the mist house, the seed trays were placed in the greenhouse. All plants were grown in a year-round temperature controlled glass greenhouses with day/night temperatures of 24/20°C (+/- 2°C). Plants were sprayed with a plant growth regulator, daminozide (Uniroyal Chemical Company, Middlebury, Connecticut) at a rate of 2500 ppm at two weeks after sowing to control excessive elongation of seedlings. The seedlings were transplanted after eight weeks into six-inch plastic pots and drenched with four ppm of paclobutrazol (Uniroyal Chemical Company, Middlebury, Connecticut). Growth regulators were stopped after this point to allow for pollination of plants, so that the growth regulators did not have an effect on fruit or seed development. Plants were placed on greenhouse benches in a completely randomized design. All plants were fertilized 6 days a week (with 1 day/week water only) with 150 ppm of 20-4.8-16 Cal-Mg Peter's soluble fertilizer (Scotts-Sierra Horticultural Products Co., Marysville OH). All lines were self-pollinated for whole fruit tissue (WF), maternal fruit tissue (MT) and seeds for all experiments. Additionally, 44568 and MD were reciprocally crossed for fruit and seed tissue: MxE and ExM (♀x ♂).

***Petunia x hybrida* cDNA Libraries**

A subset of cDNA clones were selected from five different Petunia x hybrida libraries: Young Fruit (YF), Ripe Fruit (RF), Developmental Flower Stages (DevA), Ethylene Treated Whole Flowers (C2H4), and Post Pollination (PP) (Underwood, 2003). A list of the 384 cDNA clones and source libraries used for microarray analysis is outlined in Appendix A. The genes were selected based on their putative involvement in transcription, hormones, metabolism, stress response and seed development. Focus was placed on genes involved in transcription, such as MADS box transcription factors, 14-3-3 genes, bZip transcription factors and other unknown or putative transcription factors, to

identify altered transcriptional control during seed development. Also, genes encoding enzymes of metabolic pathways were highlighted in the array list, such as enzymes involved in carotenoid synthesis, gibberellin synthesis, and jasmonic acid synthesis, to identify increases in metabolic substrates that could be affecting seed development and germination.

cDNA Microarray Fabrication

Clones chosen for microarray analysis were picked from glycerol stocks and grown in 96-well plates containing 150 µL Luria Broth with Ampicillin (50 mg/mL). Cultures were grown overnight in a 37°C incubator, without shaking or agitation. PCR was conducted to amplify the cDNA product by inoculating 49 µl of PCR mix (containing T3/T7 primers, dNTP's and 10X PCR buffer) with 2 µl of the bacterial culture. The PCR temperature cycling program used was: 95°C for five minutes, followed by 35 cycles of 94°C for one minute, 53°C for one minute, and 72°C for one minute, and a final step of 72°C for seven minutes. PCR products were then analyzed by gel electrophoresis to verify the presence of intact PCR product. The PCR product was then stored in a -20°C freezer until arraying. A portion of the PCR reaction, 22 µL, was transferred to 384-well plates and mixed with a spotting solution of 20X SSC and 20% sarkosyl immediately before array construction.

Microarray Gold Seal glass slides (Corning, Toledo, OH) were processed using a modified protocol of Eisen and Brown (1999). The following steps are the modified steps: slides were gently agitated in 100% ethanol for two hours using a metal slide rack and glass chamber (Shandon Lipshaw, Pittsburg, PA). Immediately after the ethanol rinse, the slides were quickly transferred to several glass chambers full of filter-sterilized double distilled water and rinsed with agitation for 30 seconds at room temperature. The

slides were coated by placing them in a separate glass chamber of 10% poly-L-lysine/10%PBS (Sigma-Aldrich Corp, St. Louis, MO) for one hour. The slides were removed and placed on a table-top bench at room temperature and allowed to dry overnight, with a cover to prevent dust from settling on the freshly coated slides.

cDNAs were arrayed onto poly-L-lysine coated slides using an Affymetrix 418 Robotic four-pin Arrayer (Santa Clara, CA). Each spot had a distance of 550μm away from the neighboring spot, and the entire 384 well plate was replicated three times on one slide. Arrayed slides were stored in a dark black slide box within another plastic box containing Drie-Rite® dessicant (Xenia, OH). Array slides were not stored for more than two weeks without use. Directly before array hybridization the slides were processed by waving them over hot steam for 10 seconds, followed by a 10 minute rinse with 0.2% SDS with agitation and several rinses in filter-sterilized double distilled water. Slides were placed in boiling water for denaturation for 10 minutes, and lastly slides were placed in a cold ethanol rinse for 30 seconds.

Microarray Hybridization

Whole fruit of 44568 and MD were collected at 25 days after pollination (DAP). Three whole fruit (WF) of each genotype were ground in liquid nitrogen with a mortar and pestle for RNA extraction and stored in a -80°C freezer. For the maternal tissue array experiments, an additional five fruit were opened and the seeds were removed by scalpel, and the internal maternal pith tissue (MT) was frozen for RNA extraction and stored in a -80°C freezer. Five fruit were combined to reduce variability between each fruit. 0.5 gram of each tissue was ground in a mortar and pestle and used for RNA extraction using the phenol-chloroform method and lithium chloride precipitations (Ciardi et al., 2000). RNA was cleaned using the Qiagen RNeasy kit and manufacturer

protocol was followed (Qiagen Inc, Valencia, CA). RNA was quantified by spectrophotometer readings SmartSpec 3000 (BioRad, Hercules, CA) and quality was checked by gel electrophoresis. The array probes were labeled using an Array 900 labeling kit (Genisphere, Hatsfield, PA). 2.5µg of total RNA from each sample was used for labeling each dye and the manufacturer protocol was followed. Two arrays slides each were used for the following experiments: 1. Probe 1- MD 25 DAP WF vs. Probe 2- 44568 25 DAP WF and 2. Probe 1- MD 25 DAP MT vs. Probe 2- 44568 25 DAP MT. Hybridized microarrays were scanned by an Agilent DNA Microarray scanner and dye signals were analyzed using Agilent's Feature Extraction Software (Agilent Corp., Palo Alto, CA). Data were analyzed for the 44568 experiments as an average ratio of 5-6 spots. For the two slide 44568 experiments 5 to 6 spots had to show higher expression to be included as differentially regulated. Data in tables are presented as the average ratio. The fold difference in expression is computed as: $2^{\wedge \text{average ratio}}$ (2 to the power of the average ratio of spots). cDNAs with an average ratio of 1.0 or higher are considered differentially expressed, which would represent a 2 fold difference in expression. .

RT-PCR Confirmation of Microarray Experiments

In order to verify the results of the microarray hybridizations, RT-PCR was conducted on several of the cDNA clones that showed putative differential expression in the array experiments. For this analysis total RNA was extracted from whole fruit tissue of MD 20 DAP, MD 25 DAP, 44568 20 DAP, 44568 25 DAP and 44568 30 DAP. RNA was quantified by using a SmartSpec 3000 spectrophotometer readings (BioRad, Hercules, CA) and quality was checked by gel electrophoresis. RT-PCR analysis was conducted following the manufacturer protocol of the One-Step RT-PCR Analysis kit from Qiagen (Qiagen Inc- USA, Valencia, California). The entire RT-PCR reaction was

electrophoresed on a 1.5% acrylamide gel. A set of custom primers was obtained for each individual cDNA from the Invitrogen Corporation (Carlsbad, CA). The number of thermal cycles of RT-PCR varied based on the amount of expression of each gene in the fruit tissue. Genes that were highly expressed in the fruit tissue required fewer cycles in order to visualize the RT-PCR band. The numbers of cycles used in the RT-PCR reaction are represented in parenthesis, and NCBI accession numbers are given following description of gene: Beta Xylosidase GV298846 (25 Cycles) Primers: Forward-TGTGGGTTGGTTATCCTGGT Reverse- ACTGGGCCCCTGTAAAATCT; FBP24 CV299636 (32 Cycles) Primers: Forward- GGGTATCTGGGCAGTGAAAC, Reverse-TAAATCGGCCATAACCCAAA; Ent-Kaurene Oxidase CV299619 (25) YF-3R-F07: Forward- GGCTTGAAGTTGCAGTAGTTC, Reverse-CGAATCCACATGATAAAGAGC; Dehydration Induced Protein CV300614 (27 Cycles) Primers: Forward-GAGGCCAGAAAATGGGAAAT Reverse-TCAGGAAGGAAATGGCAAAC; 4-hydroxypheylpyruvate dioxygenase CV294459 (25) Primers: Forward GAAGATGTTGGCACTGCTGA, Reverse-ACATCCCCTGCCCTACTCTT; Glutathione-S-transferase CV299433 (28) Primers: Forward- CATAGCAGCAGCACAAGGAG, Reverse-TTGCCTTTGCTGCAATTCTT; Beta carotene hydroxylase CV301281 (29) Primers: Forward- AACTGCCATCACTCCACTCC, Reverse-TCATCCTCGAGAACAAAGCA; Pectinerase CV300751 (23) Primers: Forward: TGCAAGCAGTGAGTGTGTGA, Reverse- TCTCGTTTGTGTCCCCTTTC; RPT2 CV298420 (29) Primers: Forward- GTGGACGGAAGAGCTATCCA, Reverse-TCCCTGAGTGGTCACGTACA; Alcohol Dehydrogenase CV292993 (25) C2H4-5-A02

Primers: Forward- ATAGCAGGGGCTTCAAGGAT, Reverse-AGCCATCATGGACACATTCA; Expansin CV300919 RF-5-H01 (25) Primers-Forward- CTTGCTTCTACCTGCGCTTT, Reverse-CCACAACCAGCTCCATTCTT; Oxygen evolving enhancer protein CV299423 PP-4-E12 (30) Primers: Forward-GCAGCCAGGCTATCTTGTTC, Reverse- GGCAAAGCTTTTCAACACCTC; Seed imbibition CV298461 PP-8-A11 (30) Primers: Forward-CCTGGTCGACCTACAAAGGA, Reverse- ACATCACTGCGCCTGACATA; Seed maturation CV300863 RF-5-C02 primers (LEA4) (21) : Forward-GAGAAGGGGAGAAGATGACAAC, Reverse- ATAGTGTGTCCCAACCTGCC; LEA-D29 CV300578 RF-1-H08 (21) Primers: Forward-AAGGACTTGGCTTTAAATCCAC, Reverse- TCTGCTGCATATTGCCCAC Ubiquitin Primers: Forward- AACATACAGAAGGAGTCAACAC, Reverse-AGAAGTCACCACCACGAAG. PCR was run with the following program: 50°C for 30 minutes, 94°C for 15 minutes; multiple cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; final incubation at 72°C for ten minutes. The entire RT-PCR reaction was run out on a 1.5% acrylamide gel by electrophoresis. Pictures were taken on a Polaroid Fotodyne camera. (Polaroid Corporation, Pasadena, California). Data was analyzed visually.

RT-PCR of Condensed Tannin Synthesis Genes

RT-PCR was conducted on genes encoding enzymes involved in the condensed tannin synthesis pathway. RNA was extracted from seed tissue produced from self-pollinations and reciprocal crosses at: 20, 22, 24, 26, 28 and 30 days after pollination in 44568, MD, MxE and ExM. RNA was extracted using a phenol-chloroform method with lithium chloride precipitations (Ciardi et al., 2000). RNA was quantified by

spectrophotometer readings SmartSpec 3000 (BioRad, Hercules, CA) and quality was checked by gel electrophoresis. RT-PCR analysis was conducted with a One-Step RT-PCR Analysis kit from (Qiagen Inc- USA, Valencia, California). Manufacturer's protocol was followed. The entire RT-PCR reaction was electrophoresed on a 1.5% acrylamide gel. A set of primers was obtained for each individual gene verified from Invitrogen Corporation (Carlsbad, CA) and the numbers of cycles in the RT-PCR reaction are designated in parenthesis: Dihydroflavonol reductase-like CV295572 (DFR-like-32) Petunia-3-C03 Primers: Forward- TTGATCAAGCGCCTTCTCTT; Reverse-GGCAGTGTGGAAAACACCTT Dihydroflavonol reductase CV292934 (DFR-32) C2H4-4-D01: Forward- CTCGCCCCACTGTACTCTTC; Reverse-GGCTCTGTTCGTTCATCCAT; FBP24 CV299636 (32 Cycles) Primers: Forward-GGGTATCTGGGCAGTGAAAC, Reverse- TAAATCGGCCATAACCCAAA. PCR reactions were conducted with the following program: 50°C for 30 minutes, 94°C for 15 minutes; multiple cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 1 minute; final incubation at 72°C for ten minutes. The entire RT-PCR reaction was run out on a 1.5% acrylamide gel. Pictures were taken on a Polaroid Fotodyne camera. (Polaroid Corporation, Pasadena, California). Pictures were analyzed visually.

Vanillin Staining of Seeds

Vanillin stains red upon contact with condensed tannins. Fresh and 1-month old seeds of MD, 44568, MD x 44568 and 44568 x MD were imbibed in 1% vanillin in 6M HCl acid for 30 minutes (Aastrup et al., 1984). Afterward they were rinsed in distilled water and stored in a -20ºC freezer until pictures were taken. Pictures were taken with a Leica DC 300 (Leica Camera, Solms, Germany) digital camera fixed to a Wild

Heerrbrugg microscope (Leica Geosystems, Heerrbrugg, Switzerland) and pictures were analyzed visually for red staining.

Results

Microarray Analysis

A microarray experiment was conducted in order to determine gene expression differences between MD and 44568 fruit and seed tissue at 25 days after pollination. At this time-point MD fruit are brown, mature and dehiscing the fully developed seeds. Conversely, 44568 fruit are still green and continue to develop for another five days before complete maturity (Figure 3-1). There were two primary microarray experiments conducted, the first compared 44568 to MD whole fruit at 25 DAP, and the second compared maternal fruit tissue only (without seed tissue) of 44568 to MD at 25 DAP. The experiments were conducted on whole fruit and maternal fruit tissue for several reasons. Whole fruit tissue included the seeds and the results were expected to isolate genes involved in the developmental delay and the stronger induction of dormancy in the homozygous and hemizygous 44568 seeds. The maternal fruit tissue experiment included all fruit tissue except the seeds; therefore, only maternal plant tissue is represented. The maternal fruit tissue experiments were also expected to help delineate genes expression differences in the seeds since putative differentially regulated genes found in the whole fruit experiments but not the maternal experiments were likely seed expressed genes.

Genes were considered differentially regulated if their corresponding microarray hybridized spot showed a Cy3:Cy5 ratio of 1.0 or higher (2-fold difference in expression after normalization). The most differentially expressed clones, with a spot ratio of approximately 2.0 or higher, are shown for each experiment (Table 4-1 and Table 4-2).

Many of the cDNAs have not been studied in petunia; therefore, their exact function is not certain in petunia. The percent identity to genes in other species was used, the highest NCBI Blast matches are provided in the table along with the percent identity to the corresponding petunia cDNA. Full results of the all genes, not just the most differentially regulated genes, expressing at least a two-fold difference (spot ratio of 1.0) in expression in either tissue are presented in Appendix A. The differentially regulated genes in the microarray results are predicted to be involved in various cellular and metabolic processes including stress response, seed storage accumulation, and hormone biosynthesis and function.

RT-PCR analysis of mRNA was conducted on a subset of genes with a whole-fruit time series in MD and 44568 (20 days after pollination through full maturation) to verify that genes that had differential expression in the microarray actually exhibited expression differences between 44568 and MD (Table 4-3). Out of the subset of 15 genes that were analyzed with RT-PCR, 14 confirmed that the results from the microarray experiments were valid. One gene, an alcohol dehydrogenase was shown to be more highly expressed in MD fruit tissue and was not confirmed by RT-PCR, but it is likely that human error may have factored into the results observed for this gene.

Many of the same differentially regulated genes had the same results in both whole fruit and maternal tissue experiments. Genes that were expressed more in 44568 in the microarray experiments showed homology to an expansin, an oxygen evolving enhancer protein and ent-kaurene oxidase (Tables 4-1 and 4-2). Expansins are involved in cell wall loosening, which allows for cell expansion during fruit tissue growth (Cho and Kende, 1997; Chen et al., 2001). RT-PCR mRNA expression of the expansin gene were

markedly higher in 44568 whole fruit tissue at 20 and 25 days after pollination. Expression of the expansin in MD and 44568 was not visible in mature fruit of both genotypes, 25 days after pollination and 30 days after pollination respectively (Table 4-3). The oxygen evolving enhancer protein is part of PSII and is involved in oxygen evolution during photosynthesis (Ko et al., 1990). When RT-PCR was conducted on this gene, it was observed that mRNA expression was slightly higher in 44568 whole fruit tissue compared to MD whole fruit tissue at 25 days after pollination (Table 4-3). Ent-kaurene oxidase is a gene that encodes an enzyme involved in the first committed step of GA biosynthesis (Hedden and Kamiya, 1997). It was expressed at higher levels at 25 DAP in 44568 than MD in the maternal tissue experiment. When expression was checked in whole fruit tissue, ent-kaurene oxidase mRNA was shown to be more predominant in the 44568 whole fruit tissue at 25 DAP also (Table 4-3).

Other genes that exhibited clear differential regulation between the two genotypes were genes that showed homology to beta xylosidase, two genes encoding late embryonic abundant proteins, a seed imbibition gene, and a dehydration-induced gene. The microarray results showed that these genes were more highly expressed in the MD fruit tissue over 44568 fruit tissue at 25 days after pollination. Beta xylosidase is involved in the metabolism of the xyloglucans in the secondary cell wall (Goujon et al., 2003). When expression was observed by RT-PCR, mRNA levels were higher in MD at 25 days after pollination in whole fruit tissue compared to 44568. Expression of beta xylosidase in the 44568 tissue was delayed until 30 days after pollination when the fruit are fully ripe. Late embryonic abundant proteins accumulate in the embryos in the late stages of seed development at the time when seed dessication processes are conducted (Baker et al.,

1988; Dure et al., 1989). mRNA expression of two LEA genes exhibited markedly higher expression in MD at 25 days after pollination in whole fruit tissue compared to 44568 fruit. Expression increased in the 44568 whole fruit tissue at 30 days after pollination but still did not appear to be as high as MD 25 day after pollination expression (Table 4-3).

A smaller subset of genes was analyzed with RT-PCR in a more detailed late seed development time series in all genotypes. This was conducted to further verify the results of the array experiments and to examine expression in reciprocal cross genotypes to determine any maternally regulated expression differences (Figure 4-1). The genes examined showed homology to an expansin, a dehydration induced gene, seed imbibition gene and a LEA protein. The expression of these genes was examined in the latter stages of seed development, from 20 days after pollination until seed maturity (26 DAP for MD and MxE; 30 DAP for 44568 and ExM), in order to observe expression of these genes specifically in the seed just before and during the developmental time-point used in the microarray experiments. mRNA expression for the gene that shows homology to the expansin decreased as seed development progressed. 44568 and ExM mRNA expansin expression was higher at 20 DAP and decreased more slowly than MD and MxE seed mRNA expression (Figure 4-1). The gene that showed homology to the dehydration-induced protein exhibited similar mRNA expression between all genotypes except expression decreased slightly in 44568 and ExM seeds at maturity, but the decrease in expression was not observed in MD and MxE seeds (Figure 4-1). mRNA expression of the LEA gene increased over developmental progress. The increase in expression of the

LEA gene in the seeds was delayed in the 44568 and ExM genotypes compared to MD and MxE (Figure 4-1).

Condensed Tannin Analysis of Seeds Carrying The *etr1-1* Transgene

Two genes involved in the anthocyanin synthesis pathway were determined to be differentially regulated in the results of the microarray experiment. One of these genes showed homology to a gene that encodes a myb transcription factor. It was shown to be more highly expressed in 44568 fruit than MD at 25 days after pollination. Myb 305 activates transcription of the gene encoding the first enzyme of phenylpropanoid metabolism, phenylalanine ammonia-lyase (Jackson et al., 1991; Sablowski et al., 1994, 1995). The other differentially regulated gene from the array data that was more highly expressed in 44568 whole fruit tissue than in MD whole fruit tissue at 25 DAP was FBP24, a MADS box transcription factor involved in proanthocyanidin synthesis pathway (Nesi et al., 2002). Condensed tannins are thought to enhance seed coat imposed dormancy by decreasing permeability of the seed coat (Debeaujon et al., 2000). Further expression analysis of other genes (Figure 4-2) involved in the condensed tannin synthesis pathway was examined to determine if condensed tannins might be a contributing factor to the increased dormancy in the 44568 seeds. Additionally, a slight color difference was also observed in the 44568 and ExM seeds at fresh harvest and after one-month of cold storage compared to MD and MxE (Figure 4-3). Since condensed tannins greatly contribute to the brown color of Arabidopsis seeds (Debeaujon et al., 2000), it was thought that this pathway would be interesting to investigate. mRNA expression analysis of a late seed developmental time series was examined with flavonol 3'hydroxylase (F3'H), dihydroflavonol reductase (DFR), FBP24 (Nesi et al., 2002) and a

Table 4-1 Highest ranked differentially expressed cDNAs of a microarray experiment of whole fruit tissue of ETR (44568) compared to MD at 25 days after pollination. A. Petunia cDNA library ID number B. NCBI Blast X match C. % identity of cDNA clone to Blast X match D. Average microarray spot ratio E. Description of function of cDNA. F. Comparison of results to other microarray experiments (Maternal- 44568 maternal fruit tissue vs. MD maternal fruit tissue at 25 DAP)

Whole Fruit 25 DAP ETR vs MD			**D. Average Spot Ratio**	**E. Description**	**F. Is it same in:**
A. Library ID	**B. Blast-X Match**	**C. % Identity**	**5-6 Spots**		**Maternal**
Higher in 44568					
RF-5-H01	expansin related protein- 023547 Arabidopsis	54	4.54	Cell Wall	yes
YF-1RCA-D11	oxygen evolving enhancer protein- Z11999 tomato	96	3	Photosynthesis	yes
YF-6-F03	floral defensin like protein- AAN64750 Petunia	100	2.87	Stress response	no
YF-5-C10	lipid transfer protein- AP000414 Arabidopsis	59	2.77	Seed Storage	no
YF-1RCA-E11	glutathione S-transferase- AF288191 Arabidopsis	64	2.23	Stress response	yes
YF-3R-H03	FBP24 MADS box transcription factor	97	2.2	Seed Coat Tannins	no
Higher in MD					
C2H2-1-E06	GA-2 oxidase- BAD17855 tobacco	89	2.47	GA	yes
C2H2-11-D05	late embryogenesis protein 5- AF053076 tobacco	71	2.1	Seed Storage	yes
PP-8-A11	raffinose synthase family protein din10- NM122032 Arabidopsis	78	1.96	Stress Response	yes

Table 4-2 Highest ranked differentially expressed spots of microarray experiment of maternal fruit tissue of ETR (44568) compared to MD at 25 days after pollination. A. Petunia cDNA library ID number B. NCBI Blast X match C. % identitiy of cDNA clone to Blast X match D. Average microarray spot ratio E. Description of function of cDNA. F. Comparison of results to other microarry experiments (Whole fruit- 44568 whole fruit vs. MD whole fruit)

MD 25 Maternal vs ETR 25 Maternal			**D. Average Spot Ratio**	**E. Description**	**F. Is it same in:**
A. Library ID	**B. Blast-X Match**	**C. % Identity**	**5-6 Spots**		**Whole Fruit?**
Higher in 44568					
RF-5-H01	expansin related protein- 023547 Arabidopsis	54	6.07	Cell Wall	yes
YF-1RCA-E01	hypothetical protein- NM001003451 zebra fish	38	2.64	Misc	no
YF-1RCA-H02	probable lipoxygenase- X06405 potato	83	2.62	JA	yes
RF-4-H06	dehydrin- BAD13500 tobacco	82	2.51	Stress Response	yes
YF-1RCA-E11	glutathione S-transferase- AF288191 Arabidopsis	64	2.27	Stress Response	yes
PP-9-E07	thiazole biosynthetic enzyme- AAP03875 tobacco	87	2.13	Stress Response	no
YF-1RCA-D11	oxygen evolving enhancer protein- Z11999 tomato	96	2.1	Photosynthesis	yes
YF-3R-F07	ent-kaurene oxidase- AA023063-pea	46	2.04	GA	no
YF-3R-B07	betaine aldehyde dehydrogenase- AAC06242 tomato	83	1.94	Stress Response	yes
Higher in MD					
RF-8-G09	nectarin 1 precursor-Q95PVS tobacco	53	3.78	Nectary/Defense	no
PP-14-B10	lipoxygenase- CAA58859 tobacco	84	2.5	JA	yes
PP-13-B12	beta xylosidase- BAD98523 pear	60	2.38	Cell Wall	yes
C2H2-1-B09	late embryogenesis protein LEA5- AAC06242 tobacco	70	2.28	Seed storage	yes
PP-18-H07	sucrose synthase- AAA97571 potato	97	2.08	Seed storage	yes
PP-19-F12	phi-1- BAA33810 tobacco	92	2.01	Stress Response	no

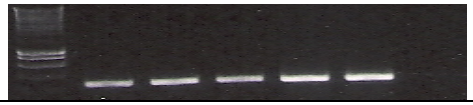
Table 4-3 RT-PCR confirmation of microarray differentially regulated clones. Whole fruit tissue is examined at 20 and 25 DAP in MD and 20, 25, and 30 DAP in 44568. Stars designate lanes that are compared in the array. Larger stars are the proposed higher expressed tissue from the microarray data. A short description is also given of the gene function.

		Higher in Array Data ★ Lower in Array Data ☆
Higher in 44568 25 DAP vs. MD 25 DAP	**Ladder 20 25 20 25 30**	
Oxygen evolving enhancer protein (30)		**OEE1**: part of oxygen evolving complex of PSII - mutant in Chlamydomonas is deficient in photosynthetic oxygen evolution; been shown to be involved in salt tolerance of Mangrove
Expansin (25)		**Expansin-Related (AtEXPR1)**- different from family of four expansin genes in Arabidopsis, therefore deemed Expansin-related; expansins are involved in cell wall loosening-allowing for cell expansion (Chen et al., 2001).
Glutathione S-transferase (28)		**glutathione S-transferase**- detox of herbicides; oxidative stress response; induced by Aluminum treatment; auxin responsive-binds auxin; involved in cell proliferation (Gronwald and Plaisance, 1998).
Myb 305 (32)		**Myb 305** - Myb305 is specifically expressed in flowers of Antirrhinum and can activate transcription from a conserved motif in the promoters of genes encoding the first enzyme of phenylpropanoid metabolism, phenylalanine ammonia-lyase (PAL) (Jackson et al., 1991; Sablowski et al., 1994, 1995).
Ent-Kaurene Oxidase (25)		**Ent-Kaurene oxidase**- GA synthesis enzyme; first committed step of GA biosynthesis-catalyzes the three steps of gibberellin biosynthesis from ent-kaurene to ent-kaurenoic acid; can be a target of negative feedback inhibition (Hedden and Kamiya, 1997).
FBP24 (32)		**FBP24**- MADS Box transcription factor; Homologous to ABS in Arabidopsis-mutant results in colorless seed TT16/ABS is likely to be involved in the control of endothelium development; BANYULS is not activated in the mutant therefore proanthocyanidins do not accumulate in the endothelium (Nesi et al, 2002).

Table 4-3 cont.

Higher in 44568 30 DAP vs. MD 25 DAP	Ladder 20 25 20 25 30	
Beta-Carotene Hydroxylase (29)		**Beta Carotene hydroxylase**- oxygenated carotenoids (xanthophylls) with 2 hydroxylation steps by this enzyme convert beta-carotene to cryptoxanthin and then to zeaxanthin
RPT2 (29)		**RPT2**- light inducible- role in early phototropic signaling; necessary for root phototropism; part of NPH3 family (large family); involved in stomatal opening;encodes a novel protein with putative phosphorylation sites, a nuclear localization signal, a BTB/POZ domain, and a coiled-coil domain
Higher in MD 25 DAP vs. ETR 25 DAP	Ladder 20 25 20 25 30	
Alcohol dehydrogenase (25)		**ADH3**- Most research focuses on adh role in hypoxia because it carries out the terminal electron transfer in anaerobic glycolysis; might have other roles- expressed in maternal anther tissues, stigma, petals, and hypoxic root in Petunia (Garabagi and Strommer, 2004).
Seed Imbibition (30)		**Seed Imbibition**- din10 (dark-induced10) upregulation starts as early as 3 hours in dark; sugar suppress din genes' expression by phosphorylation of hexose by hexokinase- similar to what it seen with the glyoxylate cycle genes; transcripts also in imbibed seeds; has 37% identity with stachyose synthase and raffinose synthase- enzymes involved in metabolism of raffinose family oligosaccharides- RFO has a role in dessication tolerance, cold tolerance, C storage therefore din10 might play a role in the metabolism of RFO in sink leaves caused by cessation of photosynthesis (Fujiki et al., 2001).

Table 4-3 cont.

Seed Maturation (21)		**LEA4**-LEA proteins are highly accumulated in the embryos at the late stage of seed development; In many cases, the timing of LEA mRNA and protein accumulation is correlated with the seed-desiccation process and associated with elevated in vivo ABA levels. The products of these genes are thought to function in protecting cells from dehydration (Baker et al., 1988; Dure et al., 1989
Beta-Xylosidase (25)		**Beta Xylosidase**- secondary cell wall metabolism and plant development; metabolism of xyloglucans in the cellulose microfibril network is believed to be important for cell wall expansion; reduced expression of BXL1 resulted in smaller siliques with less seeds (Goujon et al, 2003).
Late Embryonic Abundant Protein (21)		**RF-1-H08/LEA D-29**; LEA proteins are highly accumulated in the embryos at the late stage of seed development; In many cases, the timing of LEA mRNA and protein accumulation is correlated with the seed-desiccation process and associated with elevated in vivo ABA levels. The products of these genes are thought to function in protecting cells from dehydration (Baker et al., 1988; Dure et al., 1989),
4-hydroxypheylpyruvate dioxygenase (25)		**4-hydroxypheylpyruvate dioxygenase (25)** - catalyzes the formation of homogentisate (2,5-dihydroxyphenylacetate) from p-hydroxyphenylpyruvate and molecular oxygen; homogentisate, is the aromatic precursor of all plastoquinones and tocopherols, essential elements of the photosynthetic electron transport chain and of the antioxidative systems, respectively
Higher in MD 25 DAP vs. ETR 30 DAP	**Ladder 20 25 20 25 30**	
Dehydration-Induced Protein (27)		**Dehydration Induced Protein (RD22 like)** - ABA inducible- rd22 expression was blocked in *srk2e* mutant which is required to control induction of ABA responsive genes; also induced by salt stress but not temp stress; expression found in early and middle stages of seed development (Yu et al., 2004)
Pectinerase-like Protein		**Pectinerase-like protein**- catalyse the demethylation of pectin therefore structural interactions among cell wall components during cell wall turnover and loosening are affected; several pectinerase genes have been found to be involved in fruit ripening and senescence; ethylene reduced EXP1 in strawberry
Ubiquitin		

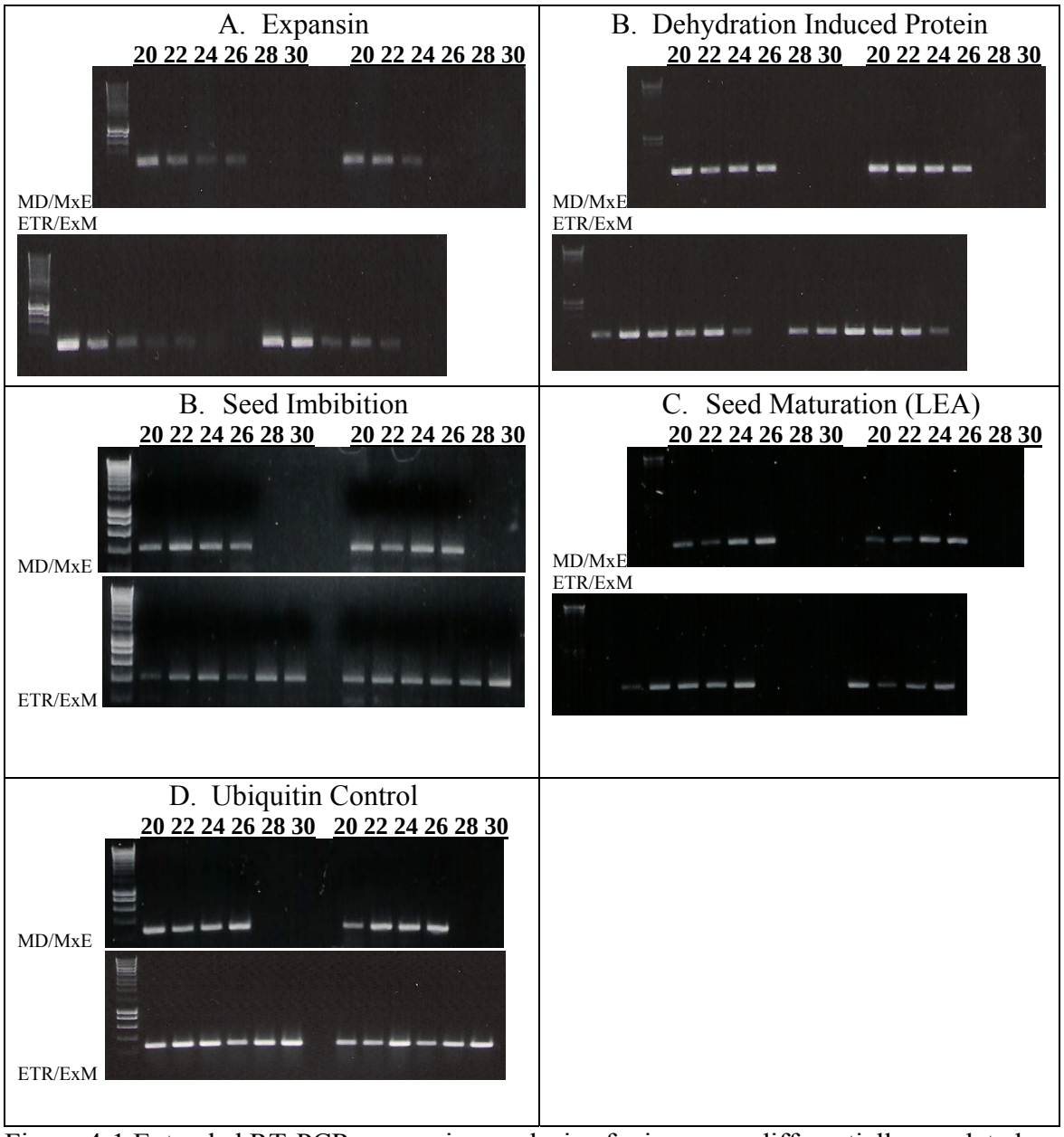


Figure 4-1 Extended RT-PCR expression analysis of microarray differentially regulated clones. Seed tissue was analyzed at 20, 22, 24, 26, 28, and 30 days after pollination in all genotypes.

dihydroflavonol reductase-like gene (DFR-like) (Xie et al., 2003) (Figure 4-4).

Expression analysis of genes encoding the more upstream enzymes, flavonol 3' hydroxlase and dihydroflavonol reductase, was conducted for all genotypes. Flavonol 3'hydroxylase did not exhibit many differences in expression between the genotypes except for a slight delay in induction in the 44568 and ExM genotypes. Expression of flavonol 3'hydroxylase was lower in 44568 and ExM seed tissue at 20 days after pollination and increased at 22 days after pollination through 30 days after pollination. Expression was consistently high in MD and MxE seed tissue from 20 days through 26 days after pollination (Figure 4-4 A). DFR expression was slightly different than the rest of the results because expression decreased in 44568 and ExM genotypes compared to the other two genotypes. mRNA expression was very low at 20 days after pollination in 44568 and ExM seed tissue. Expression slowly increased in these two genotypes until 24 days after pollination but decreased dramatically after 24 days after pollination until seed maturity at 30 days after pollination. Expression of DFR was consistent in MD and MxE seed tissue from 20 days after pollination through seed maturity at 26 days after pollination (Figure 4-4 B).

Examination of RT-PCR analysis of seed tissue from 44568, MD, and the reciprocal crosses was also conducted on genes involved in the downstream portion of the condensed tannin pathway, DFR-like and FBP24. mRNA expression of FBP24 appeared to be similar in all genotypes throughout the seed developmental series. mRNA expression appeared to decrease slightly in MD and MxE at 26 days after pollination compared to expression in 44568 and ExM (Figure 4-4 C). This decrease in not significant; therefore, it is not definitive whether there are expression differences between

any of genotypes. There did not appear to be any major differences in mRNA expression of the DFR-like gene in any of the genotypes. Expression remained constant from 20 days after pollination through seed maturity in all genotypes (Figure 4-4 D).

An experiment was conducted to stain for condensed tannins in all genotypes of seeds with vanillin, which under acidic conditions, turns red upon binding to flavan-3,4-diols (leucoanthocyanidins) and flavan-3-ol (catechins), which are present as monomers or as terminal subunits of proanthocyanidins (Aastrup et al., 1984; Deshpande et al., 1986). (Figure 4-5). Seeds of all genotypes were stained at fresh harvest and after 1-month of after ripening, the time-points when seed color differences were observed in 44568 and ExM compared to MD and MxE. This is also the time period when most of the major differences in germination between the genotypes occurred in previous experiments focused on germination after different storage periods (Figure 3-8). After staining with 1% vanillin, the seeds in all genotypes were still dark brown in color. No dramatic red staining was seen in any of the genotypes at fresh harvest or after one month of storage (Figure 4-5).

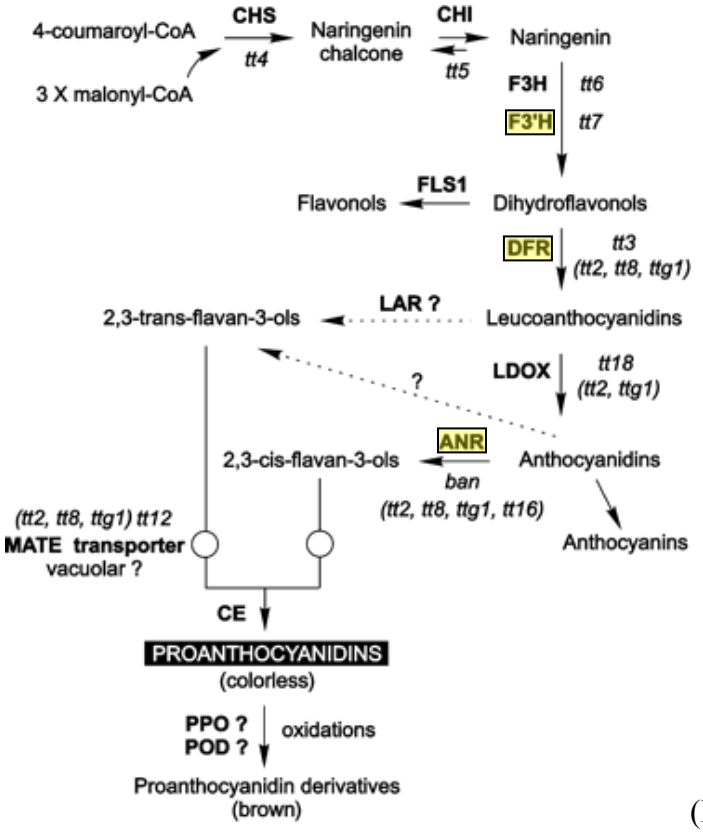
Discussion

Microarray Analysis

Microarray analysis was used in this research as a tool to screen for differentially regulated genes between MD and 44568 fruit and seed tissue at 25 days after pollination, the time-point when these two tissues are visually dramatically different. MD fruit at this time-point are fully ripened, completely brown, and dehiscing the mature seeds. Conversely, 44568 fruit are still completely green at this time-point in development and do not ripen fully until 30 days after pollination. Additionally, at 25 days after

pollination the seeds of MD are mature and viable, whereas 44568 seeds are still immature and are not capable of germination.

Several of the differentially regulated genes from the results of the whole fruit and maternal tissue microarray experiments are likely related to the developmental delay of 44568 fruit and seed tissue (Chapter 3). For example, the gene with the highest expression in 44568 whole fruit compared to MD whole fruit at 25 DAP was an expansin. Expansins are involved in the extension of cell walls during the time of rapid growth by disrupting non-covalent linkages (Chen et al., 2000). Several expansins in tomato have been shown to be expressed during both fruit and seed development (Brummell et al., 1999). One tomato expansin, LeEXP10, was shown to have expression during the earlier



(Debeaujon et al., 2003)

Figure 4-2 Highlighted proanthocyanidin synthesis genes observed through RT-PCR expression analysis in all genotypes.

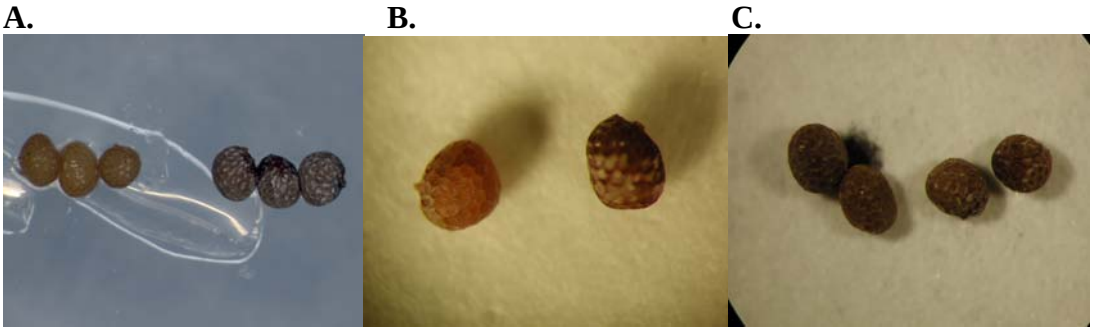


Figure 4-3 Seed pictures of 44568 and MD. (44568 left; MD right) **A**. Fresh seed **B**. 1-month old seed **C**. 1 year old seed

stages of seed development and decreased expression during the maturation and dry-down phase of seed development (Chen et al., 2000). It is likely that the 44568 tissue had higher expression of the expansin gene due to the fact that at 25 DAP the 44568 fruit and seeds are still developing and have not reached the maturation phase; therefore, the cells are continuing to expand in 44568 tissue, whereas the MD fruit and seed tissue are in the maturation and desiccation phase of development.

A more detailed expression analysis, with all genotypes including reciprocal crosses, was conducted in a late seed developmental time series to see if seed tissue alone had altered expansin gene expression and also to see if any maternal regulation was apparent in the seed tissue gene expression (Figure 4-1). Indeed, the seeds at the beginning of the 44568 and ExM time-course series had higher expansin mRNA expression levels than wild-type and MxE. The latter two genotypes continued through development more rapidly; therefore, expression of expansin mRNA had already declined by 20 days after pollination. The 44568 and ExM seeds were less developed; therefore, expansin mRNA expression did not begin to decline in these tissues until 24 days after pollination (Figure 4-1 A).

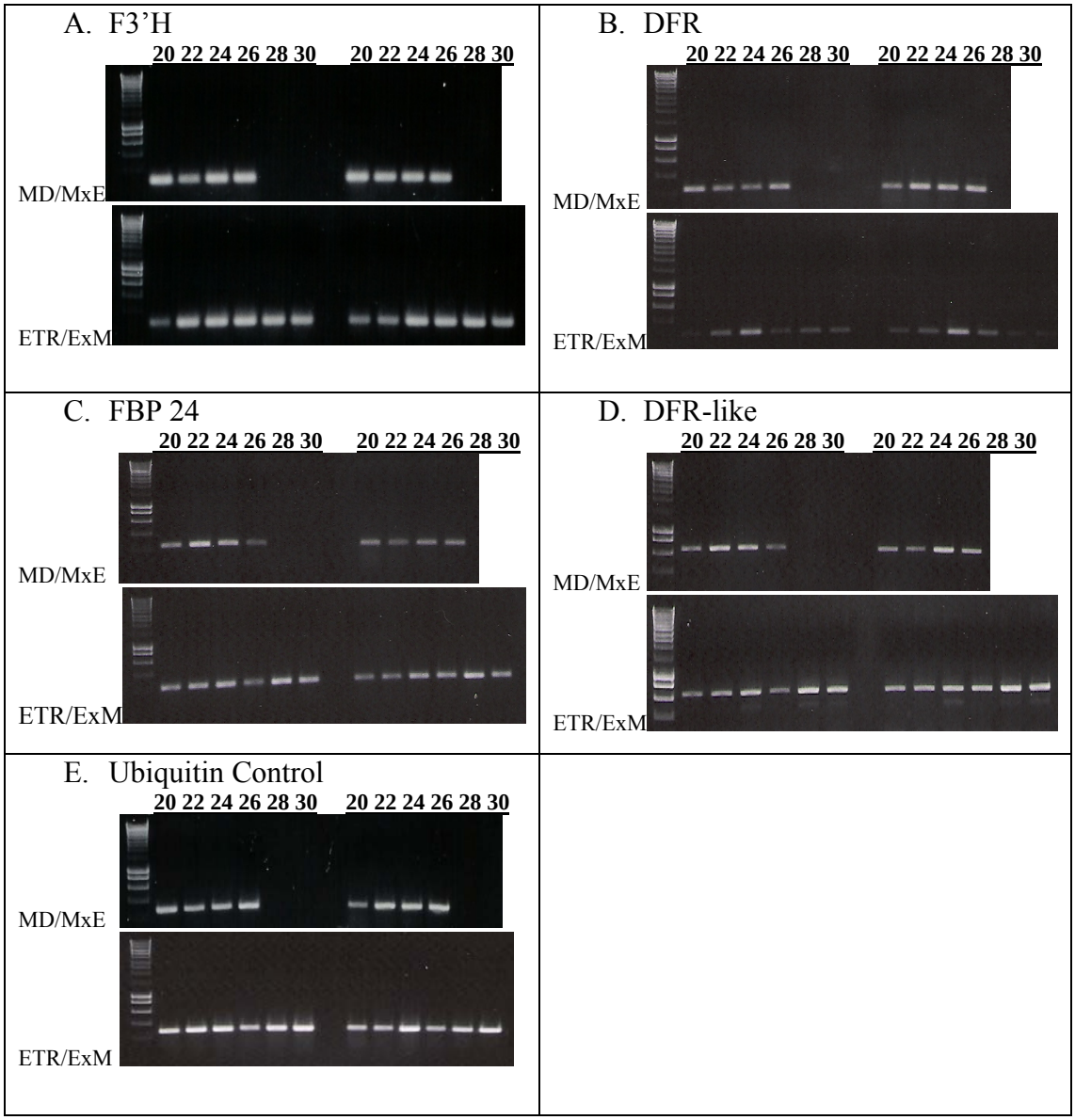


Figure 4-4 RT-PCR mRNA expression analysis of genes involved in the proanthocyanidin synthesis pathway. Seed tissue was analyzed at 20, 22, 24, and 26 days after pollination (mature seeds) in MD and MxE and 20, 22, 24, 26, 28, and 30 days after pollination (mature seeds) in ETR and ExM.

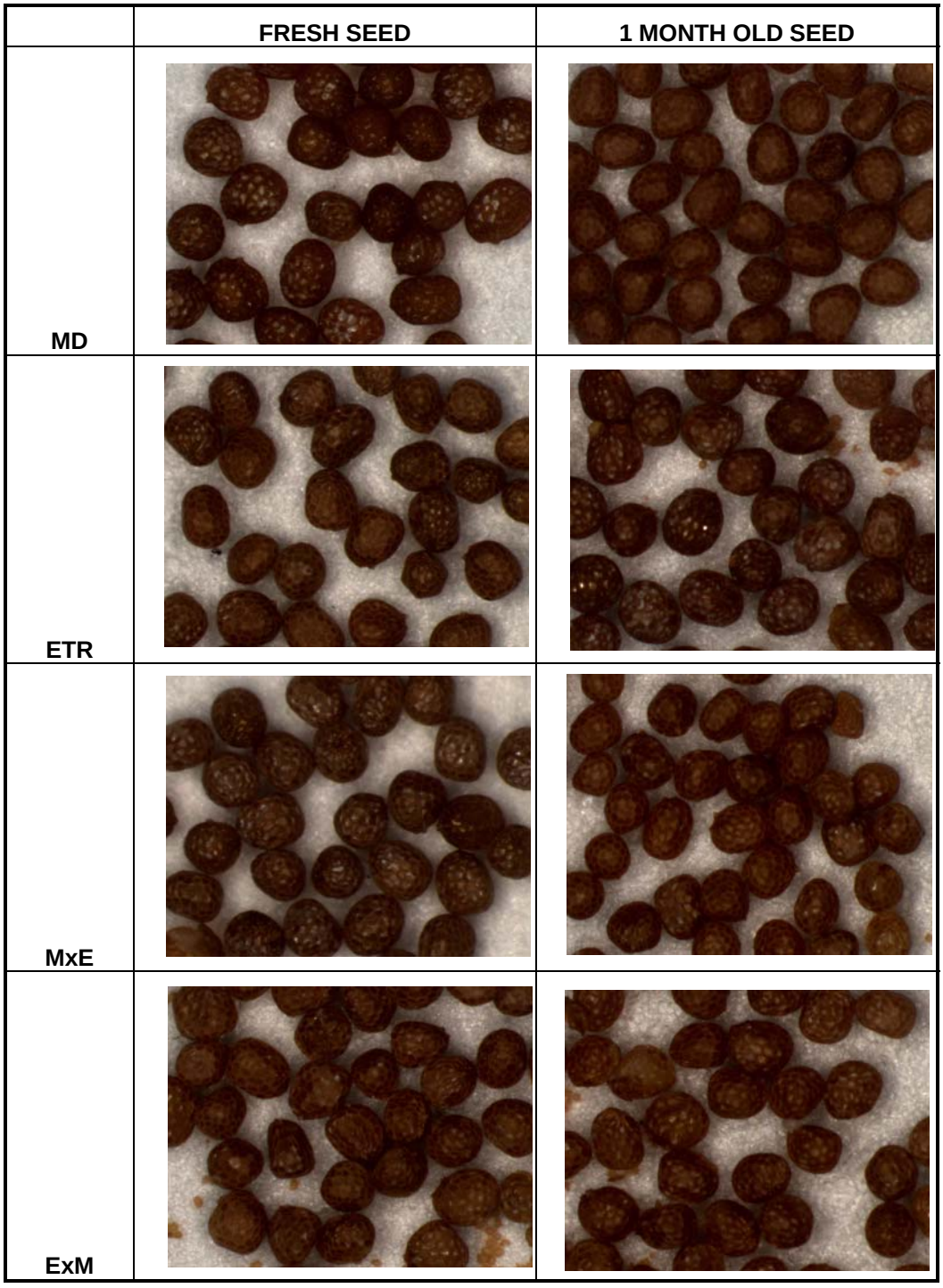


Figure 4-5 Freshly harvested and 1 month old seeds of all genotypes stained with 1% vanillin to detect presence of flavan-3,4-diols (leucoanthocyanidins) and flavan-3-ol (catechins).

A gene that was more highly expressed in MD tissue than in 44568 at 25 DAP in the array experiments encodes a seed maturation protein, known as a late embryonic abundant protein (LEA). Accumulation of these proteins occurs at late stages of seed development in Arabidopsis (Baker et al., 1988). These genes are known to be expressed during the desiccation phase of seed development and are thought to help protect the seed from extreme dehydration, though their exact mechanism of action is not known (Dure et al., 1989; Koorneef et al., 2002; Brocard et al., 2003). One explanation for this gene being expressed more in MD tissue than 44568 tissue at 25 DAP is that the MD seeds are at the last phase of development when desiccation is occurring, and the LEA proteins are thought to protect the seeds from further damage from the dehydration. 44568 seeds are not at this point in development at 25 DAP, so expression of these genes is at significantly lower levels than MD (Figure 4-1). The expression was similar in 44568 and ExM (♀x ♂) genotypes which are the two genotypes with delayed seed development. This expression difference further confirms the developmental delay of the fruit and seed tissue in 44568 and ExM genotypes (Chapter 3).

Another gene with differential regulation discovered in the microarray results was a gene that showed homology to beta-xylosidase. mRNA expression was confirmed to be higher in MD fruit tissue at 25 days after pollination compared to 44568 tissue through RT-PCR analysis (Table 4-3). Beta-xylosidase is involved in the metabolism of xyloglucans within the secondary cell wall in plant tissue (Goujon et al., 2003). MD fruit tissue is completely ripe at this time-point in development; therefore, expression of this gene may be higher because of breakdown of fruit tissue during the ripening process.

Expression would be expected to be lower in 44568 fruit tissue since the ripening processes have not begun at this time-point in development.

A seed imbibition gene was also more highly expressed in MD whole fruit tissue than 44568 at 25 days after pollination in the array results. The Arabidopsis seed imbibition gene is known as *din10* (dark-induced 10) and was given the name seed imbibition because expression was found in imbibed seeds (Fujiki et al., 2001). *din10* mRNA is up-regulated in the dark, but this dark-induced expression is suppressed by sucrose application. These genes have some identity (37%) to genes that encode enzymes involved in the metabolism of the raffinose family oligosaccharides, which play a role in protecting plant tissue during desiccation and cold tolerance (Fujiki et al., 2001). Although this gene has only been minimally studied in seed development, it is possible that, since it shows homology to the enzymes involved in the metabolism of raffinose oligosaccharides, it is induced during the latter stages of development to help protect the seeds from stress during the desiccation process. The fact that MD seeds begin to desiccate before 44568 seeds would explain the differential regulation of this gene at 25 days after pollination.

The last gene that was studied in the detailed mRNA expression analysis was a gene that showed homology to a dehydration induced protein. When expression was examined in the 44568, MD and reciprocal cross late seed tissue series, the results determined that mRNA expression decreased in 44568 and ExM seeds at 30 DAP (Figure 4-1). This decrease in expression was not seen at seed maturity, 26 days after pollination, in MD and MxE seed tissue. The dehydration induced gene was initially isolated in Arabidopsis as a result of observations of increased expression in drought conditions

(Yamaguchi-Shinozaki and Shinozaki, 1993). If this gene was discovered due to induction of expression in tissues in dry conditions, it may be induced during dessication phases of seed development. Expression may be affected in 44568 and ExM seeds if the seeds of these two genotypes do not dry down properly. Improper dessication of these seeds may be affecting the severity of the dormancy induction within these seeds, which would help explain the reduced germination seen in freshly harvested 44568 seeds (Chapter 3).

Several other genes involved in various plant processes were discovered to have differential regulation of expression between 44568 and MD fruit tissue from the microarray experiment results. A gene that showed homology to an ent-kaurene oxidase gene, involved in the first committed step of gibberellin biosynthesis (Hedden and Kamiya, 1997), was differentially regulated in maternal tissue experiments. Expression was checked in whole fruit tissue, and ent-kaurene oxidase mRNA was shown to be more predominant in the 44568 tissue at 25 DAP (Table 4-3). This gene has been shown to be involved in an important step in the production of active gibberellins (Sun and Kamiya, 1994; Yamaguchi et al., 1998). In experiments with *etr1-2* seeds in Arabidopsis elevated levels of gibberellins were observed, possibly to compensate for the increased sensitivity and/or elevated levels of ABA (Chiwocha et al., 2005). Therefore, it is likely that levels of gibberellins are also increased in petunia *etr1-1* seeds, which would account for the higher expression in the 44568 tissue.

Condensed Tannin Analysis

Two genes that exhibited differential regulation in the microarray experiments were both involved in a secondary metabolism pathway involving anthocyanins. The first gene discovered in the microarray experiments showed homology to a myb transcription

factor that has been shown to activate transcription of a gene encoding phenylalanine lyase, an enzyme involved in the phenylpropanoid metabolism (Sablowski et al., 1994, 1995). mRNA expression was more highly expressed in 44568 tissue at 25 days after pollination (Table 4-3). The other gene with differential expression in the array results was FBP24, a transcription factor involved in proanthocyanidin synthesis (Nesi et al., 2002). FBP24 had higher expression in 44568 whole fruit tissue compared to MD whole fruit tissue. Additionally, seed color was altered in seeds freshly harvested from the 44568 and ExM genotypes. This visual difference and the discovery of expression differences from the microarray results, it was thought that the presence of the *etr1-1* transgene may be affecting the levels of condensed tannins in these genes.

RT-PCR analysis of genes encoding enzymes involved in the condensed tannin synthesis pathway produced various results. Expression of genes involved in the upstream portions of the synthesis pathway exhibited more dramatic differences than genes more downstream in the synthesis pathway. Expression of dihydroflavonol reductase exhibited the most dramatic differences between the genotypes. Expression of DFR was reduced in the genotypes produced on the 44568 maternal plants but stayed constant in MD and MxE. A delay of induction of mRNA expression of flavonol 3'hydroxylase was also observed in the genotypes produced on the 44568 maternal parent. A delay in induction may be attributed to the delay in seed development of these two genotypes. Expression of the genes involved in the latter portions of the condensed tannin synthesis pathway, dihydroflavonol reductase-like and FBP24, did not exhibit considerable differences in expression between any of the genotypes. These expression differences observed in genes involved in the proanthocyanin synthesis pathway illustrate

that it is possible that the reduction in ethylene sensitivity of seeds produced on a 44568 maternal parent may have impact on proanthocyanidin levels in the seeds, especially since color differences are seen in these seeds, although it is difficult to determine whether levels are increased or decreased in these seeds without quantitative measurements.

Another approach was taken in order to determine if condensed tannin levels were altered in any of the genotypes containing the *etr1-1* transgene. A vanillin stain was used due to the fact that the vanillin turns red upon binding to flavan-3,4-diols (leucoanthocyanidins) and flavan-3-ol (catechins). This assay is used commonly with *transparent testa* mutants of Arabidopsis, which are reduced in levels of condensed tannins (Debeaujon et al., 2000, 2001; Nesi et al., 2002). Red staining was not observed in any of the genotypes of seeds at fresh harvest or after one month of storage. The dark brown nature of petunia seeds may not make it possible to stain with vanillin, since this stain is typically used on colorless mutant seeds (Debeaujon et al., 2000, 2001).

Another possible explanation of the color differences seen in the 44568 seeds compared to MD seeds is that the levels of condensed tannins are not altered but the rate of oxidation of the tannins is affected due to the reduction in ethylene sensitivity. The oxidation of the tannins contributes to the brown color in Arabidopsis seeds (Debeaujon et al., 2001); therefore, this could account for the color differences seen in 44568 and ExM seeds if levels of condensed tannin are not different from MD and MxE. 44568 seeds are lighter in color at fresh harvest compared to MD seeds, but they do become darker in color through storage. Additionally, seeds at fresh harvest have reduced germination, whereas the darker seeds germinate at higher rates after increased storage

periods (Chapter 3). The oxidation of tannins may help increase the permeability of the seed coat and allow for imbibition of water, initiating the germination processes. If 44568 seeds are slow to oxidize the tannins, the permeability of the seed coat may be decreased for a longer period of time, contributing to the delayed germination capability.

Conclusion

Several of the differentially regulated genes isolated through microarray analysis exhibited mRNA expression pattern differences between 44568 and MD due to the developmental delay of 44568 seeds. Genes involved in cell structure and expansion, such as expansins were expressed more in 44568 tissue over MD at 25 DAP. Genes involved in seed storage protein accumulation, such as LEA proteins, were more abundantly expressed in MD tissue than 44568 tissue at 25 DAP. These gene expression differences further confirm that 44568 fruit and seed tissue are developmentally delayed compared to MD at 25 DAP.

Also, some potentially interesting genes, such as the gene that encodes a dehydration induced protein, were identified which may further explain ethylene's role in the late maturation and desiccation phase of seed development and the subsequent initiation of seed dormancy. The putative dehydration-induced gene had reduced expression in 44568 seeds at maturity compared to MD seeds, which could lead to the conclusion that the 44568 seeds do not dry down properly. This might affect the switch from developmental processes to germination signals; therefore, affecting the strength of dormancy that is induced in the 44568 seeds.

The experiments that were developed around condensed tannins, due to FBP24 and a myb transcription factor being expressed more in 44568 fruit than MD fruit in the microarray experiments, did not definitively confirm that tannin content was altered in

the seed coats of 44568 compared to MD seeds. One gene involved in the synthesis pathway, dihydrol-flavonol reductase (DFR) showed a reduction in expression in the 44568 and ExM compared to MD and MxE. A reduction in seed coat tannins would parallel the lighter seed coat tissue seen in 44568 seeds at fresh harvest, but would not explain the stronger induction of dormancy in these seeds since condensed tannins are thought to contribute to increased dormancy. The rate of oxidation of the condensed tannins in these seeds could be affected due to the fact the seeds become darker in color over time. Further investigation into this pathway in petunia, such as identifying the genes involved in the oxidation process, and the resulting quantitative levels of tannins would need to be conducted in order to come to any final conclusions on whether tannin content is affected in the phenotypes reduced in ethylene sensitivity.

APPENDIX
ABI3 ANALYSIS AND MICROARRAY DATA

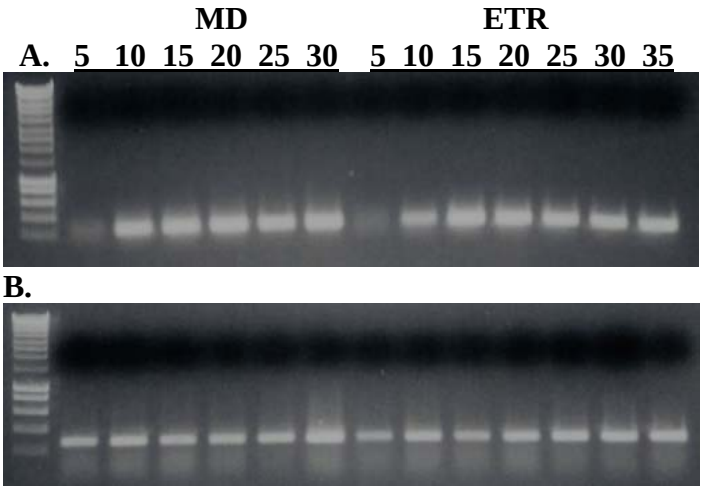


Figure A-1. RT-PCR analysis of PhABI3. Lanes are following: Ladder, MD 5, MD 10, MD 15, MD 20, MD 25, MD 30, ETR 5, ETR 10, ETR 15, ETR 20, ETR 25, ETR 30, ETR 35 Whole Fruit tissue. **A**. Expression of PhABI3 **B**. Ubiquitin-Control

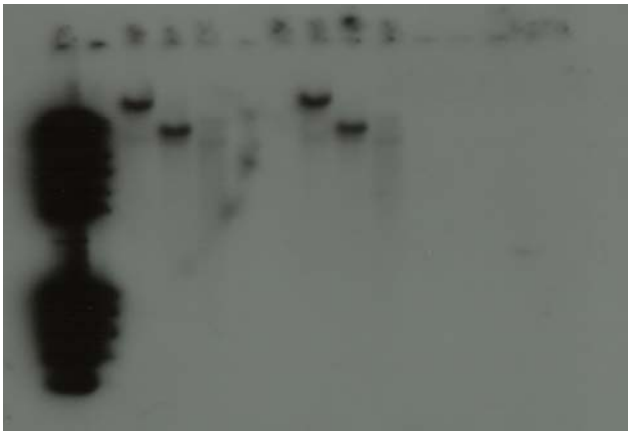


Figure A-2. ABI3 Southern Analysis. Single distinct banding pattern of genomic DNA probed with PhABI3 illustrates that ABI3 is a single copy gene in *Petunia x hybrida*. Lanes are in following order: ladder, BamHI, EcoRI, HindIV, empty lane, BamHI, EcoRI, HindIV (repeat of first three enzyme lanes).

Table A-1. cDNA library clones included on microarray chip experiments. YF= Young Fruit library, RF= Ripe Fruit library, PP= Post Pollination library, C2H4= Ethylene Treated Flowers library.

Petunia Plate	Description	Accession #
YF-1RCA		
A07	polymorphic antigen p450	CV299386
A08	aquaporin TIP7	CV299387
B04	F-actin binding protein	CV299394
B09	NEC1	CV299399
C10	MAR-binding protein	CV299411
D09	40S ribosomal protein	CV299421
D10	60S ribosomal protein	CV299422
D11	oxygen evolving enhancer protein	CV299423
E01	probable phenylalanyl tRNAs	CV299424
E11	glutathione S-transferase	CV299425
G10	glucose-6-phosphate	CV299452
H02	probable lipoxygenase	CV299456
YF-2		
A10	myb-related protein 305	CV299475
C10	glucose acyltransferase	CV299498
D01	S locus F box S2 ligase	CV299500
D03	putative glucosyltransferase	CV299502
D05	beta-alanine synthase	CV299504
D12	annexin	CV299511
E03	Superoxide dismutase	CV299514
E09	cytosolic aconitase	CV299520
F02	ripening related protein	CV299524
F12	eIF4E	CV299533
G07	Gip1-like protein	CV299540
H04	ferritin	CV299547
H11	NT4	CV299554
H12	Myb oncoprotein homolog	CV299555
YF-3R		
A02	glutamine synthetase	CV299557
B07	betaine-aldehyde dehydrogenase	CV299574
B11	extensin-tomato	CV299578
C05	PKF1	CV299583
E01	Glyceradlehyde 3-phosphate	CV299602
E06	MADS box transcription factor	CV299607
F02	Bax inhibitor 1	CV299614
F07	ent-kaurene oxidase	CV299619
G01	NTGP1	CV299624
H03	MADS box transcription factor	CV299636
YF-4R		
A05	GDSL-motif lipase/hydroxylase	CV299727
B01	Glyceradlehyde 3-phosphate	CV299734
B06	MADS box transcription factor	CV299739
B10	cysteine protease	CV299743

Table A-1. Continued

B12	polygalacturonase inhibitor	CV299744
C06	Ca2+ dependent lipid binding proteins	CV299750
C12	cell division cycle protein	CV299756
G10	caffeoyl CoA O methyltransferase	CV299800
H05	ABC transporter	CV299805
H06	ferritin	CV299806
YF-5		
A06	alcohol dehydrogenase class	CV299649
B01	receptor histidine kinase	CV299654
B04	Pyruvate kinase isozyme	CV299657
C05	IAA amidohydrolase	CV299666
C10	lipid transfer protein	CV299671
D04	1,4 benzoquinone reductase	CV299675
D06	ACC carboxylase	CV299677
E05	putative isoamylase	CV299687
E11	J1P	CV299691
H03	lycopene cyclase	CV299715
H06	alcohol dehydrogenase like	CV299718
YF-5A		
A11	beta cyanoalanine synthase	CV299821
C12	HR7	CV299841
F06	carotenoid 9,10- 9', 10' cleavage dioxygenase	CV299869
G03	putative transcription factor	CV299877
H03	transcription factor like	CV299889
H07	putative lipoxygenase	CV299892
YF-6		
C06	ubiquitin conjugating enzyme	CV299925
D01	14-3-3 protein	CV299929
E03	AER- Nicotiana	CV299942
E05	bifunctional dihydrofolate	CV299944
E09	glutamate decarboxylase	CV299947
F02	peroxidase	CV299952
F03	floral defensin like protein	CV299953
H10	14-3-3 protein	CV299983
B11	putative UDP-glucose	CV299918
YF-7		
D04	Aminoacylase-1	CV300021
E08	putative beta alanine pyruvate	CV300036
F11	putative cytochrome p450	CV300051
G04	putative bHLH transcription factor	CV300055
G08	14-3-3 isoform	CV300059
YF-8		
A08	ethylene response factor 3	CV300081
D10	transcription factor B3 family	CV300118
D11	light inducible protein	CV300119
D12	UTP: alpha-D-glucose-1-phosphate	CV300120
E03	glycine hydroxymethyltransferase	CV300123
F01	bZIP transcription factor	CV300132

Table A-1. Continued

G01	TAF-3	CV300144
G09	TAF-2	CV300152
G11	beta-amylase	CV300154
G12	glucose-6-phosphate 1-dehydrogenase	CV300155
YF-9		
E12	cationic peroxidase	CV300222
D05	putative F-box	CV300204
D09	TIR-NBS disease resistance	CV300208
D12	putative AP2 domain	CV300211
F05	GH3-like protein	CV300227
G06	putative two component histidine kinase	CV300240
G09	floral binding protein number	CV300243
H11	Putative 60S ribosomal protein	CV300256
H12	Putative ATP synthase	CV300257
RF-1		
A02	lipid body associated membrane protein	CV300498
A10	7S globulin	CV300504
B04	11S globulin like precursor	CV300509
B05	11S globulin precursor	CV300510
B06	2S albumin	CV300511
B08	NADPH cytochrome p450 oxidoreductase	CV300513
C03	putative cytochrome p450	CV300520
D03	albumin seed storage protein	CV300531
E02	transcription factor JERF1	CV300541
E04	2S seed albumin-1 large	CV300543
G01	geraniol 10-hydroxylase	CV300560
G02	Non-specific lipid	CV300561
G04	putative seed maturation	CV300562
G11	S-adenosylmethionine decarboxylase	CV300569
H08	LEA	CV300578
H09	maturase	CV300579
RF-2		
B03	lipid body associated membrane protein	CV300596
C07	PGPS/NH21	CV300612
C09	dehydration induced protein	CV300614
C11	oleosin-like protein salt	CV300616
D01	similar to dehydrogenases	CV300618
D11	embryogenic potential marker	CV300628
D12	similar to caltrin like protein	CV300629
H02	cyc07	CV300662
H05	caleosin	CV300665
RF-3		
C12	probable isocitrate dehydrogenase	CV300787
E05	maturation protein PM3	CV300801
E10	2S albumin- 1 large	CV300806
F12	malate dehydrogenase	CV300817
G02	putative t-SNARE SED5	CV300818
H04	malate dehydrogenase	CV300829
RF-4		

Table A-1. Continued

A07	11S globulin seed storage	CV300678
B01	2S albumin	CV300684
D05	glutamate synthase	CV300706
E08	glutamine synthetase	CV300719
E10	sugar transporter like protein	CV300721
F10	steroleosin	CV300731
G11	P21	CV300742
H06	dehydrin homolog	CV300748
H08	Cu2+ transporting ATPase	CV300750
H09	pectinesterase like protein	CV300751
RF-5		
A10	ethylene responsive element	CV300847
B04	PGPS/NH21	CV300853
B08	Gigantea like protein	CV300857
C02	seed maturation protein	CV300863
C05	ent-kaurene oxidase	CV300866
C06	LON protease homologue	CV300867
F01	putative (1,4) beta mannase	CV300897
G04	translation initiation factor	CV300911
G12	senescense associated cysteine protease	CV300918
H01	expansin related protein 1	CV300919
H05	vicilin like protein precursor	CV300923
RF-6		
A07	LEA	CV300935
B02	seed storage protein Lec2SA	CV301284
B10	cinnamoyl-CoA reductase family	CV300948
C02	seed storage protein Lec2SA	CV300952
E12	Glutamate dehydrogenase B	CV300984
F03	S-adenosylmetionine: 2-demethy	CV300986
F07	seed maturation protein PM3	CV300990
G01	ovate protein	CV300996
G07	senescense associated protein	CV301002
RF-7		
B01	desiccation-related protein	CV301027
C09	oleosin	CV301046
E01	SUMO protein	CV301061
D05	maturase	CV301053
D07	ent-kaurenoic acid oxidase	CV301055
E11	2-oxoglutarate-dependent dioxygen.	CV301070
F09	ent-kaurenoic acid oxidase	CV301079
H02	coenzyme Q	CV301095
RF-8		
A01	gamma response 1 protein	CV301106
A03	napin	CV301108
B11	isopentenyle diphosphate isomerase	CV301126
C01	secretory peroxidase	CV301128
G09	Nectarin 1 precursor	CV301180
H01	transcription factor JERF1	CV301184
H04	MtN30	CV301187

Table A-1. Continued

H09	delta-8 sphingolipid desaturase	CV301192
RF-9		
B09	adenylate kinase related protein	CV301214
C08	dessication related protein	CV301223
D07	coat protein 3	CV301234
E07	cell division cycle protein	CV301245
F08	glyoxalase II	CV301256
F12	2-oxoglutarate-dependent dioxygenase	CV301260
H02	hydrolase	CV301274
H03	glyoxylase family protein	CV301275
H10	beta carotene hydroxylase	CV301281
H11	putative argininosuccinate	CV301282
C2H4-1		
B08	jasmonic acid 2	CV292654
B09	late embryogenesis protein 1	CV292655
C10	giberellin 2-oxidase	CV292668
E06	dioxygenase	CV292683
F01	ethylene forming enzyme	CV292689
C2H4-3		
A03	cellulose synthase	CV292807
B03	nectarin	CV292818
B08	nodulin	CV292822
C04	P18 protein	CV292831
E08	glutaredoxin	CV292858
H09	calmodulin 7	CV292894
C2H4-5		
A02	alcohol dehydrogenase	CV292993
B08	phosphoenolpyruvate	CV293009
B11	putative arabinose	CV293012
C10	MRP-like ABC transporter	CV293022
E07	dioxygenase	CV293043
F02	sterol-C5(6) desaturase	CV293050
F09	Bax inhibitor 1	CV293057
C2H4-10		
A04	S-adenosylmethionine synthetase	CV293274
B02	lipase/hydrolase	CV293282
B04	NAD-malate dehydrogenase	CV293283
C2H4-10D		
C11	Brassinosteroid regulated protein	CV293982
D12	ethylene receptor	CV293993
C2H4-11		
B08	late embryogenesis protein 1	CV293786
D05	late embryogenesis protein 1	CV293804
C2H4-14G50		
A06	ABA inducible protein	CV293342
C12	EEF53	CV293368
D07	myb related protein	CV293375
D11	beta-glucosidase	CV293379
H12	ABA inducible protein	CV293420

Table A-1. Continued

C2H2-15		
B03	bZip DNA binding protein	CV293869
C2H4-25		
B10	prohibitin	CV294866
C09	seed imbitition	CV294877
G10	dehydration induced protein	CV294
G12	cytochrome p450	CV294923
H10	ORF270/2	CV294933
C2H4-26		
A09	drought induced protein	CV294943
D07	alcohol dehydrogenase	CV294975
H07	floral homeotic protein	CV295020
C2H4-31		
E05	heat shock cognate protein	CV295366
E12	RAD23 protein	CV295370
F12	beta keto acyl reductase	CV295380
C2H4-32		
B11	sucrose transport protein	CV295407
D07	alpha glucan phosphorylase	CV295421
PP-3		
A11	AP2 domain containing	CV298007
C08	F box protein	CV298027
C11	MADS box FBP23	CV298029
C12	Ser/Thr specific phosphatase	CV298030
PP-4		
B10	4-hydroxyphenylpyruvate dioxygen.	CV298108
C06	sucrose phosphate	CV298115
E12	oxygen evolving enhancer protein	CV298142
F01	pectin methyl esterase	CV298143
G12	floral homeotic protein FBP	CV298165
PP-5		
B06	JAB	CV298193
C10	glucan 1,3 beta gucosidase	CV298209
C11	transcription factor	CV298210
H06	probable isocitrate dehydrogenase	CV298263
PP-6		
A03	hydroxymethyltransferase	CV298271
C10	malate dehydrogenase	CV298301
D03	AIM1 protein	CV298305
D09	PGPS/D4	CV298310
F08	beta fructofuranosidase	CV298332
F12	putative beta galactosidase	CV298335
PP-7		
A06	malate dehydrogenase	CV298363
A08	myb related tf	CV298365
E05	caffeic acid O-	CV298408
F05	RPT2	CV298420
G09	Initiation factor 5A	CV298436
H06	P18 protein	CV298445

Table A-1. Continued

H11	myb related tf	CV298450
PP-8		
A09	squalene monoxygenase	CV298460
A11	seed imbitition	CV298461
D06	myb related tf	CV298490
F09	beta mannosidase	CV298517
G02	P18 protein	CV298522
PP-9		
B11	Initiation factor 5A	CV298562
E07	thiazole biosynthetic enzyme	CV298585
H01	DEAD box	CV298606
H02	4-nitrophenylphosphate	CV298607
PP-10		
A12	beta 1,3 glucanase	CV298623
C04	FUSCA6	CV298631
B04	cationic peroxidase	CV298625
C05	glutamate decarboxylase	CV298632
C07	PGPS/D3	CV298634
E10	putative beta alanine synthase	CV298651
PP-11		
D07	isoflavone reductase	CV298714
D12	phenylalanine ammonia lyase	CV298719
E09	expansin-tomato	CV298725
F11	malate dehydrogenase- mitochondria	CV298737
H11	delta-12 fatty acid	CV298757
PP-12		
A03	alcohol dehydrogenase	CV298761
C04	MADS box tf FBP23	CV298782
PP-13		
B07	putative ripening	CV298843
B12	beta-xylosidase	CV298846
F11	early light induced protein	CV298878
PP-14		
A02	pollen specific protein NTP3	CV298895
B01	cytokinin binding protein	CV298900
B10	lipoxygenase	CV298907
D07	MADs box FBP5	CV298919
PP-15		
D09	myb related tf	CV298996
G05	3-glucanase	CV299020
PP-16		
A09	carotenoid 9,10	CV299044
C05	fatty acid hydroxylase	CV299063
G08	sucrose synthase	CV299110
G10	anther specific LAT52 protein	CV299112
PP-17		
A07	P21- petunia	CV299130
C07	myb tf	CV299154
D07	P70	CV299165

Table A-1. Continued

D09	putative MAP kinase	CV299167
G08	floral homeotic protein	CV299202
G09	invertase	CV299203
G10	invertase	CV299204
H09	P18	CV299213
PP-18		
A05	nucloid DNA binding protein	CV299218
A06	12-oxophytodienoate	CV299219
A07	DNA binding protein 2	CV299220
A10	2,3 bisphosphoglycerate-independent phosphoglycerate mutase	CV299223
A11	6,7 dimethyl-8-ribityllumazine synthase precursor	CV299224
B05	serine hydroxymethyletransferase	CV299228
C11	shaggy protein kinase	CV299244
D10	senescense related protein	CV299251
D12	fibrillin	CV299253
E12	negative transcription regulator	CV299263
G08	invertase	CV299279
H07	sucrose synthase	CV299288
PP-19		
F05	water stress inducible protein	CV299354
F12	phi-1	CV299361
G12	ascorbate peroxidase	CV299371
H12	sucrose synthase	CV299380
UNKNOWNS		
YF-1RCA		
A04	unknown	CV299383
A10	unknown	CV299389
E07	unknown	CV299430
G11	unknown	CV299453
H09	unknown	CV299462
YF-2		
A01	unknown	CV299466
A04	unknown	CV299469
A05	unknown	CV299470
B06	unknown	CV299482
C01	unknown	CV299489
E10	unknown	CV299521
E11	unknown	CV299522
F02	ripening related protein	CV299524
F08	unknown	CV299529
F12	eIF4E	CV299533
PP-16-A09	carotenoid 9,10	CV299044
H12	unknown	CV299125
YF-3R		
B03	unknown	CV299570
B04	unknown	CV299571
B05	unknown	CV299572
C10	unknown	CV299588

Table A-1. Continued

D09	lipid transfer protein	CV299598
D10	unknown	CV299599
E06	MADS box gene	CV299607
F02	Bax inhibitor 1	CV299614
F04	unknown	CV299616
G08	unknown	CV299630
G10	unknown	CV299632
H03	MADS box gene	CV299636
H10	unknown	CV299642
H11	unknown	CV299643
H02	unknown	CV299635
G06	unknown	CV299628

Whole Fruit 25 DAP ETR vs MD		Average Spot Ratio	Description	Is it same in:	
Ethylene down-regulated		**5-6 Spots**		**EIN2 25**	**Maternal**
RF-5-H01	expansin related protein	4.54	Cell Wall	yes	yes
YF-1RCA-D11	oxygen evolving enhancer protein	3	Photosynthesis	yes	yes
YF-6-F03	floral defensin like protein	2.87	Stress response	yes	no
YF-5-C10	lipid transfer protein/seed storage/protease inhibitor	2.77	Seed Storage	no	no
YF-1RCA-E11	glutathione S-transferase	2.23	Stress response	yes	yes
YF-3R-H03	FBP24 MADS box transcription factor	2.2	Seed Coat Tannins	no	no
YF-1RCA-G10	glucose-6 phosphate	1.82	Seed Storage	no	no
YF-3R-B07	betaine-aldehyde dehyrogenase	1.42	Stress response	yes	yes
YF-1RCA-H02	probable lipoxygenase	1.3	JA	yes	yes
RF-4-H06	dehydrin homolog	0.97	Stress response	yes	yes
RF-4-E10	sugar transporter like protein	0.93	Seed Storage	yes	yes
YF-5-H03	lycopene cyclase	1	Carotenoids	yes	no
YF-6-F03	floral defensin like protein	2.87	Stress response	yes	no
Ethylene Up-regulated					
C2H2-1-E06	dioxygenase (GA)	2.47	GA	no	yes
C2H2-11-D05	late embryogenesis protein1	2.1	Seed Storage	no	yes
PP-8-A11	seed imbibition-like protein	1.96	Stress Response	yes	yes
PP-16-G08	sucrose synthase	1.42	Seed Storage	yes	no
PP-14-B10	lipoxygenase	1.38	JA	no	yes
PP-4-B10	4-hydroxyphenylpyruvate dioxygenase	1.38	Photosynthesis	yes	yes
C2H4-5-A02	alcohol dehydrogenase	1.25	Misc.	no	no
PP-13-B12	beta-xylosidase	0.87	Cell Wall	yes	yes
PP-17-G10	invertase	0.65	Seed Storage	yes	no

Figure A-3. Complete list of differentially regulated clones in 44568 and MD whole fruit microarray experiment at 25 DAP.

MD 25 Maternal vs ETR 25 Maternal		Avg spot ratio	Description	Is it the same in:	
Ethylene Down-Regulated		**5-6 spots**		**Whole Fruit?**	**EIN2?**
RF-5-H01	expansin	6.07	Cell Wall	yes	yes
YF-1RCA-E01	probably phenylalnyl tRNAs	2.64	Protein synthesis	no	no
YF-1RCA-H02	probable lipoxygenase	2.62	JA	yes	yes
RF-4-H06	dehydrin homolog	2.51	Stress Response	yes	yes
YF-1RCA-E11	glutathione S-transferase	2.27	Stress Response	yes	yes
PP-9-E07	thiazole biosynthetic enzyme	2.13	Stress Response	no	no
YF-1RCA-D11	oxygen evolving enhancer protein	2.1	Photosynthesis	yes	yes
YF-3R-F07	ent-kaurene oxidase	2.04	GA	no	yes
YF-3R-B07	betaine aldehyde dehydrogenase	1.94	Stress Response	yes	yes
C2H2-26-A09	drought induced protein	1.8	Stress Response	no	no
RF-7-D07	ent-kaurenoic acid oxidase	1.53	GA	no	yes
RF-1-B04	11S globulin like precursor	1.46	storage protein	no	yes
YF-7-E08	putative beta alanine pyruvate	1.38	Stress Response	no	no
RF-5-B08	gigantea like protein	1.34	Photosynthesis/Light	no	yes
C2H4-31-E05	heat shock cognate protein	1.29	Stress Response	yes	yes
RF-4-E10	sugar transporter like protein	1.23	Seed Storage	yes	yes
RF-7-E11	2-oxoglutarate-dependent dioxygenase	1.12	Flavonols	no	yes
		Avg spot ratio	**Description**	**Is it the same in:**	
Ethylene Up-Regulated		**5-6 spots**		**Whole Fruit?**	**EIN2?**
RF-8-G09	nectarin 1- precursor	3.78	Nectary/Defense	no	no
PP-14-B10	lipoxygenase	2.5	JA	yes	no
PP-13-B12	beta xylosidase	2.38	cell wall	yes	yes
C2H2-1-B09	late embryogenesis protein 1-LEA5	2.28	Seed Storage	yes	yes
PP-18-H07	sucrose synthase	2.08	Seed Storage	yes	yes
PP-19-F12	phi-1	2.01	Stress Response	no	no
C2H2-1-B7	dioxygenase	1.97	Misc.	yes	no
PP-18-G08	invertase	1.93	Seed Storage	yes	yes
PP-4-B10	4-hydroxypheylpyruvate dioxygenase	1.88	Photosynthesis	yes	yes
C2H2-1-B08	jasmonic acid 2	1.7	JA	no	no
C2H2-1-B03	nectarin	1.62	Nectary/Defense	no	yes
C2H4-25-C05	seed imbibition	1.6	Stress Response	yes	yes
RF-1-G02	non specific lipid	1.34	Seed Storage	no	no
RF-5-C02	seed maturation protein- LEA4	1.23	Seed Storage	yes	yes

Figure A-4. Complete list of differentially regulated clones in 44568 and MD maternal tissue microarray experiment at 25 DAP.

MD 25 vs EIN2 25 Whole Fruit		Average Spot Ratio	Description	Is is the same in:	
Ethylene Down-Regulated		**2-3 spots**		**ETR?**	**ETR Maternal?**
YF-1RCA-H02	probable lipoxygenase	2.95	JA	yes	yes
RF-7-E11	2-oxoglutarate-dependent dioxygenase	2.9	Flavonols	no	yes
YF-6-F03	floral defensin like protein	2.87	Stress Response	yes	yes
YF-3R-F07	ent-kaurene oxidase	2.27	GA	no	yes
RF-5-B08	gigantea like protein	2.15	Photosynthesis/Light	no	yes
YF-1RCA-B09	NEC1	2.1	Nectary	no	no
RF-4-H06	dehydrin homolog	1.93	Stress Response	yes	yes
PP-8-A09	Squalene monoxygenase	1.73	Isoprenoids/Stress	no	no
YF-1RCA-D11	oxygen evolving enhancer protein	1.6	Photosynthesis/Light	yes	yes
RF-7-D07	ent-kaurenoic acid oxidase	1.53	GA	no	yes
YF-3R-B07	betaine-aldehyde dehydrogenase	1.5	Stress Response	yes	yes
YF-5-H03	lycopene cyclase	1.4	Carotenoids	yes	no
RF-6-C02	seed storage protein Lec2Sa	1.33	Seed Storage	no	no
RF-1-A04	2S albumin	1.3	Seed Storage	no	no
YF-8-D11	light inducible protein	1.25	Photosynthesis/Light	no	no
RF-8-C01	secretory peroxidase	1.25	Stress Response	no	no
YF-2-C10	glucose acyltransferase	1.25	Metabolism	no	no
RF-4-E10	sugar transporter like protein	1.25	Metabolism	yes	yes
RF-1-B05	11S globulin	1.23	Seed Storage	no	yes
YF-1RCA-E11	glutathione S-transferase	1.13	Stress Response	yes	yes
RF-9-F08	glyoxalase II	1	Stress Response	no	no
PP-17-D07	P70	1	Cell Structural	no	no
Ethylene Up-Regulated					
C2H2-1-B09	late embryogenesis protein	2.3	Seed Storage	yes	yes
PP-8-A11	seed imbibition	2.17	Stress Response	yes	yes
YF-2-D12	annexin	2	Stress Response	no	no
PP-19-H07	sucrose synthase	1.8	Metabolism	yes	yes
C2H2-14G50-H12	ABA inducible protein	1.47	ABA induced	no	
C2H4-3-B03	nectarin	1.45	Nectary	no	yes
PP-4-B10	4-hydroxypheylpyruvate dioxygenase	1.4	Photosynthesis	yes	yes
PP-16-G08	sucrose synthase	1.35	Metabolism	yes	yes
YF-5A-C12	HR7	1.35	Stress Response	no	no
YF-9-F05	GH3 like protein	1.35	Misc	no	no
PP-13-B12	beta-xylosidase	1.33	Cell Wall	yes	yes
YF-1RCA-A08	aquaporin TIP7	1.3	Cell Wall	no	no
C2H4-5-B08	phosphoenolpyruvate	1.3	Misc	no	
PP-17-G10	invertase	1.1	Metabolism	yes	yes
RF-5-C02	seed maturation protein-LEA4	1.07	Seed Storage	yes	yes

Figure A-5. Complete list of differentially regulated clones in *ein2* and MD whole fruit tissue microarray experiment at 25 DAP.

Whole Fruit EIN 30 DAP vs MD 25 DAP		Average Spot Ratio	Description	Is is the same in:
Ethylene Down-Regulated		**5-6 Spots**		**ETR 25?**
PP-8-A11	seed imbibition	2.26	Stress Response	no
PP-7-F05	RPT2	2.15	Photosynthesis/Light	no
RF-9-H10	beta carotene hydroxylase	1.05	Carotenoids	no
Ethylene Up-Regulated				
RF-2-C09	dehydration induced protein	1.82	Stress Response	no
RF-4-H09	pectinerase like protein	1.3	Ripening	no
RF-2-D01	similar to dehydrogenases	1.25	Misc	no
RF-2-D12	unknown protein	1.14	Unknown	no

Figure A-6. Complete list of differentially regulated clones in *ein2* and MD whole fruit tissue microarray experiment at 30 DAP.

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BIOGRAPHICAL SKETCH

Jennifer Davis was born in Denver, Colorado. Her family moved to New Port Richey, Florida, when she was six, and she lived there until the beginning of her undergraduate studies at the University of Florida in the fall of 1997. During her undergraduate work she completed a research project with Drs. Harry Klee and Joe Ciardi through the Undergraduate Research Scholars Program. She also completed an undergraduate internship in flower breeding with Goldsmith Seeds. Jennifer graduated with a Bachelor of Science degree with honors from the University of Florida in spring of 2001. Her major was environmental horticulture with a minor in plant molecular and cellular biology. She will graduate in December of 2005, with a Doctor of Philosophy in plant molecular and cellular biology. Jennifer is married to Keith Davis; their first child, Kyle Michael, was born in October 2005.