EFFECT OF ESTRADIOL ON ENVIRONMENTAL STRESS RESPONSES IN *ARABIDOPSIS THALIANA*

A DISSERTATION

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I am submitting herewith a dissertation written by Pallavi Upadhyay, entitled Effect of Estradiol on Environmental Stress Responses in *Arabidopsis thaliana'*. I have examined the dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy with a major in Molecular Biology

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ABSTRACT
PALLAVI UPADHYAY

EFFECT OF ESTRADIOL ON ENVIRONMENTAL STRESS RESPONSES IN *ARABIDOPSIS THALIANA*

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Animal hormones enter into the water and soil systems *via* natural and anthropogenic sources. The goal of the study is to understand the effects of estradiol (ES) on *Arabidopsis thaliana* growth, metabolism and stress responses. Plants were treated with increasing estradiol concentrations. Morphological studies, biochemical and molecular analyses were performed on the control and treated plants. Plants treated with 10 and 100 nM ES showed enhanced root growth, shoot biomass and photosynthesis rate with a concomitantly higher carbohydrate and protein accumulation. However, a downregulation of phenylpropanoid (PP) pathway genes, *PAL1, PAL4, CHI, ANS*, and decrease in PP biosynthesis were observed. Phenylpropanoids have a wide range of functions in plant growth and development such as structural support and protection against environmental stresses. To study the effects of ES on stress responses, plants were exposed to drought conditions and pathogen stress. The effects of drought stress were studied by estimating proline, glutathione and hydrogen peroxide contents in the plants. Molecular analyses were also performed to study the effects of drought conditions on the stress-responsive genes *GER5, HSP101, HSP70b*. ES application enhanced the accumulation of above-mentioned antioxidants and induced a significantly higher gene

expression levels in Arabidopsis. To study biotic stress responses, *Pseudomonas syringae* pv. tomato DC3000 was used in basal resistance experiments. ES treatments rendered plants susceptible to bacterial pathogen. High ES concentration (10 μM) had a negative impact on primary and secondary metabolism, suggesting toxic effects. To analyze ES mode of action in PP gene downregulation, promoter analysis was performed, which suggested a role for an AP2/EREBP family transcription factor, ERF73, in controlling transcription of PP pathway genes. Arabidospsis PAL overexpression lines were constructed to establish the role of ES in the downregulation of PP pathway genes. Results from this study demonstrate that ES enhanced primary metabolism but reduced secondary metabolism in a dose-dependent manner. Although ES application decreased plant resistance to pathogen infection, it enhanced plant tolerance to drought stress. Therefore, it is concluded that ES can act as biostimulant at low concentrations. This study has practical agricultural applications and can serve as a primer for studying the effects of environmental estrogens and xenoestrogens on crop plants.

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CHAPTER I

INTRODUCTION

The sessile nature of plants renders them susceptible to a number of environmental stresses, both biotic and abiotic in nature. In recent years, the presence of mammalian sex hormones (MSH) *via* pharmaceutical and agricultural waste in the water supply has raised concerns about their activity as potential endocrine disruptors. The bulk of research has been focused on the effect of MSH on aquatic animals and little is known about their impact on plant life. Estrogen and progesterone, steroidal hormones derived from cholesterol, play important roles in sex determination, growth and development in vertebrates. The only steroidal hormones present in the plant kingdom are the brassinosteroids, which are involved in growth and development as well as plant stress responses. The presence of animal sex hormones, especially estrogen and its derivatives, in soil and irrigation water, have various effects on plants, ranging from sex determination to root and shoot developmental changes. Application of xenoestrogens to plants results in altered gene expression and downstream biochemical processes related to plant defense responses as well as to both primary and secondary metabolism.

1. Steroid Hormones

Steroid hormones belong to a class of biologically active compounds present in both plants and animals that are derived from cholesterol. Their basic structure of four fused rings, three cyclohexane (A, B, C) and one cyclopentane (D) rings, has variations in the side group at C17 (Fig. 1.1).

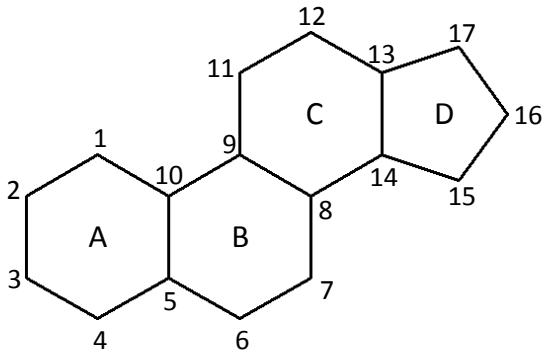

Figure 1.1 The steroid ring system with three cyclohexane (A, B and C) and one cyclopentane (D) rings. Numbering of carbons in the ring system is according to the IUPAC system.

Steroid hormones secreted by animal tissues, namely the adrenal cortex, gonads (ovaries and testis) and placenta, are classified into five broad categories: glucocorticoids, mineralocorticoids, progestogens, androgens and estrogens. Glucocorticoids, secreted by the adrenal cortex, are present in nearly all vertebrate cells and are involved in controlling glucose metabolism. Mineralocorticoids, as the name suggests, are required for the retention of the mineral sodium within the cells and help maintain the organism's salt and water balances. Progestogens are required for the maintenance of pregnancy and the menstrual cycle and are precursors for the biosynthesis of other steroid hormones. Estrogens are the predominant female sex hormones that are required for sex expression as well as growth and development. Androgens are the male hormones required for male sex expression and tissue regeneration (Ying et al., 2002). All animals easily excrete steroidal hormones in the environment, which are then introduced into the agricultural water supply and soil systems *via* the waste disposal and sewage treatment plants.

1.1. Steroid Hormones in Plants

Brassinosteroids (BRs) are a class of plant steroidal hormones with wide distribution in the plant kingdom. They are known to regulate several physiological and developmental functions in plants, such as cell division and elongation, floral development, senescence, circadian rhythm, as well as biosynthesis of other phytohormones. Apart from their impact on growth and developmental regulation, they also have a wide impact on various plant stress responses (Krishna, 2003). Brassinolide, the most common BR, is a polyhydroxylated form of the steroid precursor molecule 5α-cholestan, which is closely related to cholesterol. Among the known BRs in plants, the main variations exist in the A ring, particularly at C-2 and C-3. Besides these variations in the ring structure carbons, the side chains can display another level of variation by undergoing glycosylation, meristylation and laurylation (Bajguz, 2011).

1.2. Estrogens in the Environment

Excretion of the steroid hormones into the environment and the subsequent exposure of animals to these hormones can have far reaching effects on their physiology, reproduction, growth and development. Since these exogenous hormones alter the normal functioning of the cell, they are termed endocrine disruptors (Jobling *et al*., 1998). Among the steroid hormones, estrogens such as estrone, 17β-estradiol, estriol and estrogenic contraceptives such as ethinylestradiol and mestranol (Fig. 1.2) are well known endocrine disruptors.

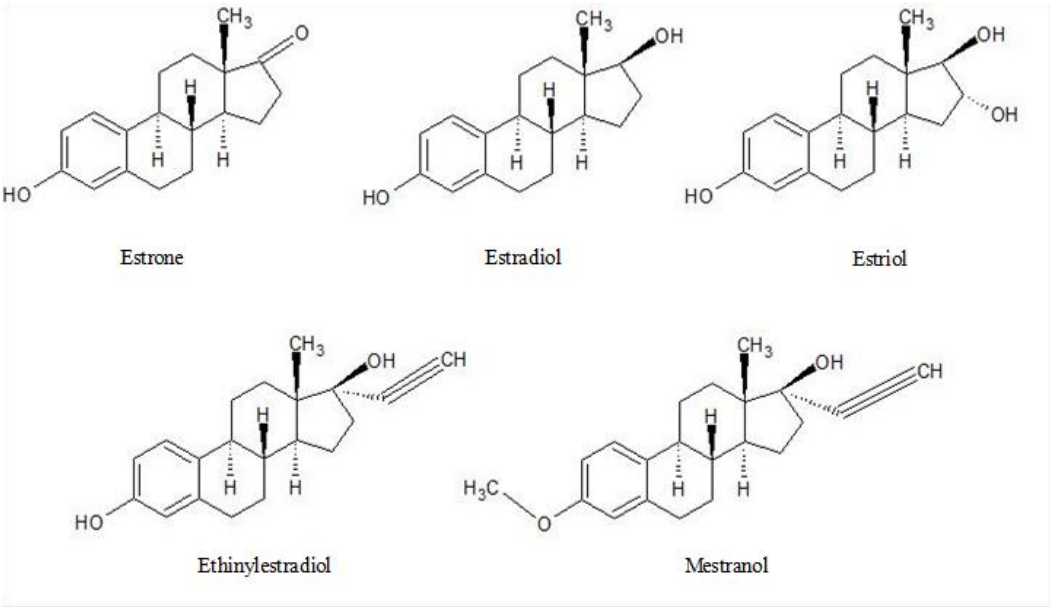

Figure 1.2 Structures of estrogen hormones and estrogenic contraceptives released in the environment.

Estrogens are excreted by both sexes but mostly by female vertebrates. A major source of estrogens is livestock animals such as cattle, pigs and poultry. The amount of 17β-estradiol in the excretion waste generated by poultry ranges from 14 to 533 ng/g dry waste (Shore *et al*., 1988; Shore *et al*., 1995).

In a comprehensive survey detailing the input of estrogens into activated sludge treatment plants, it was observed that male humans could excrete up to 1.6 µg/day of 17β-estradiol and 3.9 µg/day of estrone. Depending on the section of the female population (menstruating or post-menopausal), it was found that women excreted 2.4-3.5 µg/day of 17β-estradiol, 4-8 µg/day of estrone and 1-4.8 µg/day of estriol. Expectedly,

pregnant females excreted the highest amount of estrogens as 259 µg/day estradiol and 600 µg/day estrone and estriol. Based on these observations, it was estimated that the concentration of estrogens in the aqueous environment could reach ng/l levels (Johnson *et al*., 2000). Thus, the presence of estrogen hormones in the environment as potential endocrine disruptors is a major concern. It has been demonstrated that presence of as little as 1 ng/l 17β-estradiol led to the production of vitellogenin, the main egg yolk protein precursor, and feminization in male fish (Purdom *et al*., 1994; Hansen *et al*., 1998; Rodgers-Gray *et al*., 2000).

Significant levels of estrone (0.3 ng/l) were detected in The Netherlands in estuarine and freshwater samples (Belfroid *et al*., 1999) and estrone (1.5 ng/l), 17β-estradiol (0.11 ng/l) and estriol (0.33 ng/l) were detected in the waters of the Tiber River in Italy (Baronti *et al*., 2000). In a survey of surface waters, 17β-estradiol was found in 109 Japanese rivers with an average concentration of 1.8 ng/l depending on the weather conditions (Tabata *et al*., 2001). Estrogenic steroids ranging in concentrations from 0.35 ng/l to 0.7 ng/l have also been reported in drinking water samples in Germany (Kuch and Ballschmiter, 2001).

Amendment of agricultural soil with manure (poultry litter and cattle waste) and the resulting runoff has an impact on the ground water supply as well. Groundwater contamination was reported to be dependent on the quantity of applied animal manure on farmlands (Peterson *et al*., 2000). Application of 5 Mg/ha poultry litter to pastural land resulted in an average concentration of 3.5 µg/l 17β-estradiol in the runoff (Nichols *et al*., 1997). Water in Karst aquifers in northwest Arkansas was found to contain 6-66 ng/l 17β-

estradiol. A basal level of estrogen at 55 ng/kg in arable soil has been reported and this concentration reached up to 675 ng/kg upon the application of poultry litter to the soil (Finlay-Moore *et al*., 2000). It was reported that ethinylestradiol, the synthetic estrogenic contraceptive, was much more stable in soil as compared to natural estrogens like 17β-estradiol and estrone (Ying and Kookana, 2005; Czajka and Londry, 2006). Based on the above-mentioned findings, it can be concluded that estrogens are reaching the soil and water systems at a very high rate.

1.3. Effect of Applied Estrogens on Plant Growth and Development

In one of the earliest studies, it was demonstrated that estrone application had a positive impact on the root meristem cell division in a number or dioecious plants (Löve and Löve, 1945). Also estrone application stimulated growth of pea embryos (Helmkamp and Bonner, 1953) and seedlings (Kopcewicz, 1969). In a study involving the effect of mammalian steroidal hormones on sunflower (*Helianthus annuus*) plants, the application of 17β-estradiol promoted shoot growth but had an inhibitory effect on root growth of seedlings. In the same study, estradiol applied at 1 µg/plant resulted in the inhibition of axillary bud formation, whereas lower concentrations of the hormone promoted their development (Bhattacharya and Gupta, 1981). A comparative analysis of the effects of plant (24-epibrassinolide) and mammalian (estrone and estradiol) steroidal hormones on tomato showed that application of 0.01 µM 24-epibrassinolide inhibited the growth of cultured excised roots and at 0.1 µM concentration resulted in reduced root formation in shoot cuttings and decreased root biomass of intact seedlings. A similar negative effect on growth in excised roots, shoots and intact seedlings was observed after estradiol

application at a much higher concentration (1.0 µM) than that used for 24-epibrassinolide (Guan and Roddick, 1988). Irrigation of *Medicago sativa* plants with sewage water resulted in increased vegetative growth. The sewage water was found to contain 0.3 µg /l estrogen and it was speculated that the hormone might be responsible for the observed enhancement in growth. In the same study, application of 0.005-0.5 µg/l estradiol enhanced both root and shoot biomass of *M. sativa* plants but inhibited vegetative growth of the plants when applied at the higher concentration range of 50-500 µg/l (Shore *et al*., 1992). Application of 1 µM estrogen was also found to have a positive impact on the leaf and root growth in winter wheat seedlings (Janeczko, 2000).

Plants are able to metabolize some of the steroid hormones present in the environment although the actual biochemical processes are not well understood. It has been demonstrated that a nonspecific enzyme such as phenoloxidase from *Mucuna pruriens* cell cultures can hydroxylate 17β-estradiol to generate 4-hydroxyestradiol (Woerdenbag *et al*., 1990). The algal strain *Chlorella emersoni* C211-8H is able to convert progesterone to hydroprogesterone and dihydroxyprogesterone (Greca *et al*., 1996). The conversion of androstenedione, an intermediate of testosterone biosynthesis, to testosterone has been reported in *Pisum sativum* (Lin *et al*., 1979) and *Cucumis sativus* (Lin *et al*., 1983). Application of radioactive mevalonic acid to *Phaseolus vulgaris* seedlings led to its conversion to 17β-estradiol (Young *et al*., 1977). In addition, the interconversion of estrone and 17β-estradiol by *P. vulgaris* leaves has also been reported (Young *et al*., 1979). More recently, it was reported that maize seedlings could convert 17β-estradiol to estrone and *vice-versa*. In the same study, it was observed that maize

plants are able to oxidize the synthetic estrogen zeralanone to α-zeralanone (Card *et al*., 2013). Intact plants and excised stems of hybrid poplar (*Populous deltoids x nigra*) were able to transform 17β-estradiol to estrone and estriol and zeralanone to zeranol (Bircher *et al*., 2015).

In conclusion, estrogens affect the plant morphology, development and reproduction. However, no study thus far has published a molecular mechanism of estradiol action in plants.

1.4. Effect of Steroidal Hormone Application on Plant Stress Responses

Treatment of bean (*Phaseolus vulgaris*) seeds with various concentrations of MSH (progesterone, β-estradiol and androsterone) resulted in increased activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT). At the same time, a decrease of lipid peroxidation and H_2O_2 levels was also observed in the MSH-treated bean plants and was attributed to an increase in the levels of antioxidant enzymes, thus resulting in increased plant resistance to stress (Erdal, 2009). Similar results were seen in chickpea seeds treated with MSH (progesterone and β-estradiol at 10^{-6} M), which also showed elevated levels of α-amylase activity during germination. Since the MSH-treated seeds had a 100% germination rate, it was speculated that an increase in α-amylase activity induced by MSH contributed to the high germination rate (Erdal and Dumlupinar, 2010). In addition, exogenous application of MSH (progesterone, β-estradiol and androsterone) as a foliar spray to chickpea seedlings resulted in significantly higher root and leaf growth as compared to untreated plants (Erdal and Dumlupinar, 2011). Presence of inorganic elements within the cell affects a number of

metabolic processes by controlling the electrochemical status and several catalytic processes. It was reported that exogenous application of MSH (progesterone, β-estradiol and androsterone) in barley plants resulted in a significant increase in the inorganic element concentration of leaves. Both β-estradiol and androsterone (at 10 µM each) applications resulted in a significant increase of Cu, Mg, P, S, K, Fe, Zn, Al, Cl and Mn. However, a decrease in leaf Na concentration in MSH-treated plants was observed (Dumlupinar *et al*., 2011).

Another study showed that progesterone application in bean seedlings ameliorated the effects of salt stress by increasing the K^+/Na^+ ratio in the hormone-treated plants in comparison to untreated plants grown in artificial medium supplemented with 100mM NaCl (Erdal *et al*., 2012). The progesterone-treated bean plants under salt stress had higher activities of SOD, POX and CAT enzymes and displayed lower levels of lipid peroxidation as compared to the non MSH-treated plants (Erdal *et al*., 2012). Progesterone, β-estradiol and androsterone applications counteracted the effects of salinity stress by increasing germination rate and root and shoot length in maize (Erdal, 2012a) and wheat seedlings (Erdal, 2012b). The protective effect of MSH on plants has also been reported in the case of chilling stress (Genisel *et al*., 2013). Foliar application of progesterone to chickpea plants exposed to chilling stress resulted in enhanced antioxidant enzyme activity, and increased in chlorophyll content and relative leaf water content (Genisel *et al*., 2013).

In the presence of Cd or Cu, retardation of embryo germination and growth was observed in lentil (Chaoui and El Ferjani, 2013). However, the addition of 17β-estradiol

to the seed germination medium protected the lentil seedlings from the negative effects of heavy metal stress post germination. The estradiol-treated seedlings displayed significantly lower solute leakage in the presence of heavy metals as compared to untreated seedlings. However, this mitigating effect of estradiol did not prevent the accumulation of heavy metal ions in the cotyledons as both estradiol-treated and nontreated seedlings displayed no significant difference in the levels of accumulated Cd/Cu (Chaoui and El Ferjani, 2013).

A recent study from our laboratory demonstrated that application of 10 nM 17β-estradiol to Arabidopsis seedlings grown in liquid media resulted in the upregulation of stress responsive genes such as *HEAT SHOCK PROTEIN70* (*HSP70*) and *GLUTATHIONE S-TRANSFERASE23* (*GST23*). The same study showed an exclusive downregulation of genes involved in the biosynthesis of phenylpropanoids and glucosinolate, which are important classes of plant secondary metabolites involved in environmental stress responses (Adetunji, 2012).

1.5. Phenylpropanoid Secondary Metabolism in Plants

Phenylpropanoids (PPs) are plant secondary metabolites that play important roles in plant reproduction, growth and development, defense responses and structural support (Fraser and Chapple, 2011; Cheynier *et al.*, 2013). The PP pathway represents an important sink for the carbon fixed by primary metabolism in plants. The pathway utilizes skeletal molecules from primary metabolic pathways like glycolysis and the oxidative pentose phosphate pathway (Fig. 1.3). The first committed step of PP biosynthetic pathway is catalyzed by enzyme phenylalanine ammonia lyase (PAL) and

results in the deamination of phenylalanine generating cinnamic acid and ammonia. In higher plants, PAL activity is usually encoded by a gene family comprising of a number of genes as observed in the case of parsley (Appert *et al*., 1994), tomato (Lee *et al*., 1992) and Arabidopsis (Wanner *et al*., 1995; Fraser and Chapple, 2011). The *PAL* gene family in Arabidopsis is made up of 4 genes, *PAL1-PAL4*. Gene expression analysis and *in silico* promoter analysis of the *PAL* genes showed that PAL1 and PAL2 are the primary PAL enzymes in Arabidopsis (Raes *et al*., 2003). Physiological analysis of *pal* mutants in Arabidopsis bolsters the idea that PAL1 and PAL2 encode for the primary PAL activity in the plant. The *pal1pal2* double mutant plants, in comparison to the wild type, accumulated higher levels of phenylalanine, the enzyme substrate, and lower levels of lignin, a major end product of the PP biosynthetic pathway (Rohde *et al*., 2004). Similar observations were made by Huang and co-workers (2010) with respect to the *pal1pal2pal3pal4* quadruple mutant that was found to accumulate lower lignin levels.

The cinnamate molecule derived *via* PAL activity is further hydroxylated, to generate p-coumaric acid, by the enzyme cinnamate 4-hydroxylase (C4H) that belongs to the cytochrome P450 monooxygenase family of enzymes (Fig. 1.3). Unlike PAL, C4H activity is encoded by a single gene in Arabidopsis (Mizutani *et al*., 1997). Analysis of *c4h* mutant plants showed a marked decrease in lignin content as opposed to wild type plants (Schilmiller *et al*., 2009). In addition, plants lacking C4H activity also display aberrations in growth, such as dwarfism, and reproduction phenotypes such as male sterility (Schilmiller *et al*., 2009).

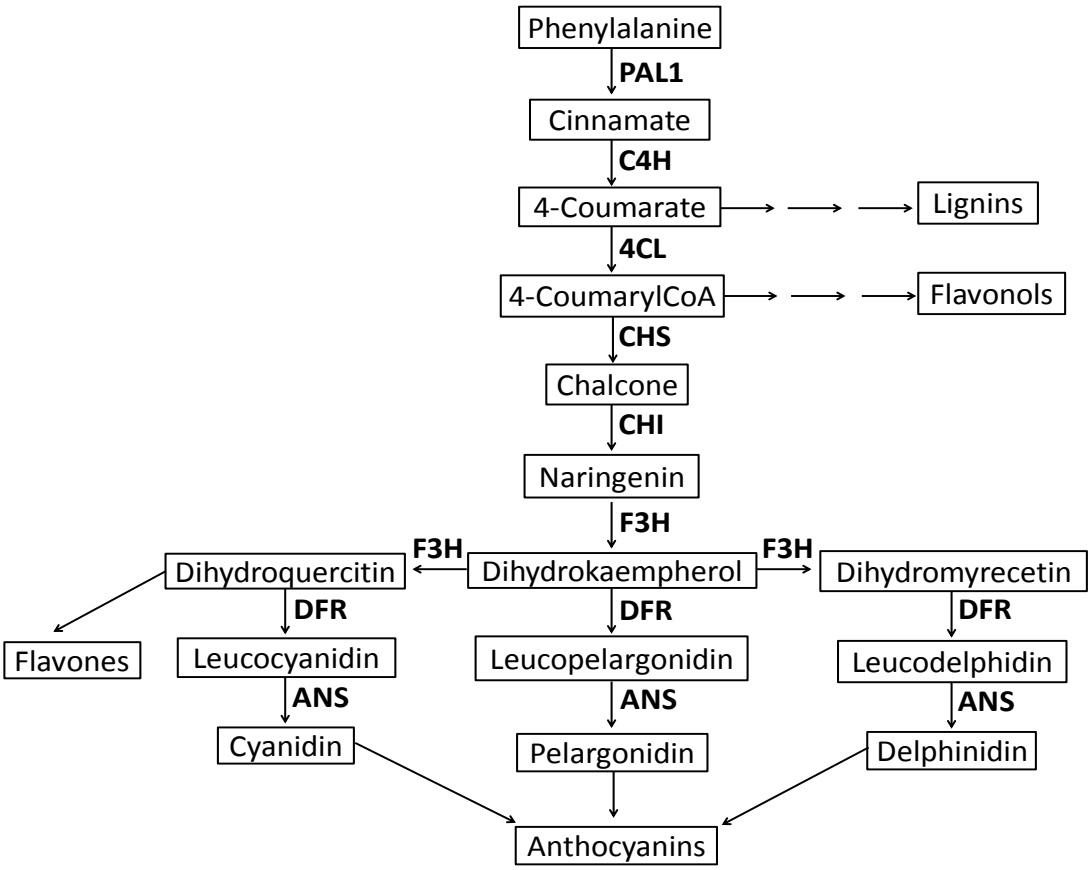

Figure 1.3 The phenylpropanoid biosynthesis pathway. Phenylalanine derived from the shikimate pathway is converted to cinnamic acid by PAL activity which marks the first committed step of the PP pathway. PAL=Phenylalanine Ammonia Lyase, C4H=Cinnamate 4 Hydroxylase, 4CL=4-coumarate-CoA ligase, CHS=Chalcone Synthase, CHI=Chalcone Isomerase, F3H=Flavanone 3-hydroxylase, DFR=Dihydroflvonol Reductase, ANS=Anthocyanidin Synthase.

In an ATP-dependent reaction, 4-coumarate:CoA ligase (4CL) catalyzes the formation of p-coumaroyl CoA from p-coumaric acid. In Arabidopsis, a family of 4 genes (*At4CL1-At4CL4*) encodes for this activity (Hamberger and Hahlbrock, 2004;

Costa *et al*., 2005). Formation of this thioester molecule marks an important branch point in the PP biosynthetic pathway that leads to the formation of flavonoids, anthocyanins, tannins and lignins (Vogt, 2010).

Synthesis of flavonoids in plants is initiated by building the flavonoid scaffold molecules that is subsequently worked upon by a number of enzymes to generate incredibly diverse classes of secondary metabolites. Flavonoid synthesis follows a series of condensation, isomerization, oxidation and reduction reactions. Chalcone synthase (CHS) represents the first committed step in the biosynthesis of flavonoids and catalyzes a Claisen condensation reaction between p-coumaroyl-CoA and 3 molecules of malonyl-CoA resulting in the formation of naringenin chalcone. The enzyme belongs to the type III polyketide synthases family (Austin and Noel, 2003).

Naringenin chalcone undergoes stereospecific cyclization to form the flavanone (2S)-naringenin by the action of chalcone isomerase (CHI). CHI activity in Arabidopsis is encoded by a family of five genes that include both catalytic and non-catalytic isoforms (Winkel-Shirley, 2001; Lepiniec *et al*., 2006; Appelhagen *et al*., 2014). The flavanone (2S)-naringenin is oxygenated at the C3 position by the enzyme flavanone 3-hydroxylase (F3H) to generate the flavonol dihydrokaempferol (Pelletier and Shirley, 1996). The next enzyme in the pathway, flavonoid 3´-hydroxylase (F3´H), is a cytochrome P450 monooxygenase member that hydroxylates the 3´ C of the B ring of dihydrokaempferol to generate dihydroquercitin. The enzyme can also accept kaempferol as a substrate to generate quercitin (Schoenbohm *et al*., 2000; Appelhagen *et al*., 2014).

Dihydroquercitin is reduced to leucocyanidin in a reaction catalyzed by the enzyme dihydroflavonol reductase (DFR). This step is deemed as the first committed step towards the production of anthocyanins and proanthocyanidins in plants (Shirley *et al*., 1992). Anthocyanins are natural pigment molecules that impart color to flowers and fruits. In fact, the name anthocyanin is composed of Greek terms that mean 'blue flower'. The first colored compound of the anthocyanin biosynthetic pathway is an anthocyanidin and it is synthesized by the oxidation of the C ring to form conjugated double bonds in leucocyanidin, reaction catalyzed by the enzyme anthocyanidin synthase (ANS) (Abrahams *et al*., 2003; Bowerman *et al*., 2012).

A number of phenylpropanoid derivatives, especially isoflavones, are very similar in structure and function to mammalian estrogens and are termed phytoestrogens (Patisaul and Jefferson, 2010). Phytoestrogens are able to induce estrogen-like activities in mice with surgically removed ovaries (Schmidt *et al*., 2006). It has been reported that consumption of isoflavone rich foods, like soy, by human females can result in the suppression of hormones such as estrogen, luteinizing hormone and follicle stimulating hormone, thus disrupting the ovulatory cycle (Wu *et al*., 2000). However, a number of effects beneficial to human health, such as relief from perimenopausal symptoms, prevention of osteoporosis and cardiovascular diseases, have also been attributed to phytoestrogens (Patisual and Jefferson, 2010).

1.6. Phenylpropanoids and Plant Stress Responses

Due to their sessile nature, plants respond differently than animals to environmental stress factors. Variations in temperature (extreme heat or cold), water availability (drought or flooding), salinity stress, UV-B radiation, etc., constitute the major abiotic stressors and herbivores, bacterial and fungal pathogens are some of the biotic stressors that plants have to deal with on a regular basis. It has been reported in a number of studies that plant secondary metabolism, especially the PP pathway derivatives, play important roles in growth and development, as well as in protecting plants against biotic and abiotic stresses (Bahler *et al*., 1991; Christie *et al*., 1994; Izaguirre *et al*., 2003; Zabala *et al*., 2006; Hawrylak-Nowak, 2008; Daneshmand *et al*., 2010; Verdan *et al*., 2011). Increased accumulation of PP compounds in response to biotic and abiotic stresses is a well-known plant adaptive feature (Dixon and Paiva, 1995). A common consequence of various plant stresses is the oxidative damage to cell membranes and biomolecules such as proteins and lipids by reactive oxygen species (ROS). Photooxidation and high energy electrons induced by UV radiation can be scavenged by phenylpropanoids such as anthocyanins (Grace and Logan, 2000). The loss of hydration resulting in oxidative stress has been observed as response to various abiotic stresses such as salinity, drought and temperature. Phenylpropanoids can ameliorate the effects of this oxidative stress by reducing ROS-like singlet oxygen ($^1O_2$), superoxide (O_2^-) and H_2O_2 (Korkina, 2007). Phenolic compounds derived from PP pathway act as feeding deterrents against phytophagous insects by either reducing the palatability of the plant tissue or by being toxic to insects (Lattanzio *et al*., 2006).

1.6.1. Salinity stress

Increased salt in the soil results in general cellular dehydration that in turn generates ionic and osmotic stresses in plant tissues. It was reported that salt sensitive wild potato varieties accumulated higher levels of anthocyanins in response to higher NaCl concentrations *in vitro* (Daneshmand *et al*., 2010). Irrigation of red pepper plants with moderate levels of NaCl resulted in increased accumulation of total phenolics and anthocyanins in the mature fruit (Navarro *et al*., 2006). Salinity stress in young barley seedlings resulted in increased accumulation of total phenolics and flavonoids (Ali and Abbas, 2003).

1.6.2. Cold stress

Plants display cold tolerance by modulating their secondary metabolism in response to low temperatures. One of the mechanisms, demonstrated by a number of plant species, is an increase in the lignin and suberin, products of PP pathway, as constituents of the cell walls during winter months (Moura *et al*., 2010). Transferring 7 day old maize seedlings to 10°C resulted in increased transcript accumulation for all major PP pathway genes including *PAL1*, *CHI* and *4CL*. This increased mRNA accumulation was accompanied by higher levels of anthocyanins in the cold stressed seedlings as compared to control plants (Christie *et al*., 1994). Effects of temperature variations on the production of PP pathway derivatives in callus cultures have been reported in a number of cases. Increased anthocyanin accumulation has been observed in callus cultures grown at low temperatures for strawberry (Zhang *et al*., 1997), Indian rhododendron (Chan *et al*., 2010), *Perilla frutescens* (Zhong and Yoshida, 1993) and carrot (Narayan *et al*., 2005).

1.6.3. Drought stress

Flavonoids are known to provide protection against drought stress in plants. Drought stress is defined as the low water availability in soil to such critical levels that, accompanied with atmospheric conditions, plant tissues continuously loose water. Plants that accumulate high levels of anthocyanins are more drought resistant (Chalker-Scott, 1999). Two well-known flavan-3-ol from tea, (-)-epicatechin (EC) and (-)-epigalloactechin gallate (EGCG) are known for their antioxidant properties. Both EC and EGCG levels were found to be enhanced by drought stress in tea plants (Hernández *et al*., 2006). The drought stress protective property of flavonoids is further supported by the fact that the purple cultivar of chili (*Capsicum frutescens*), with higher anthocyanin content, is more drought resistant than the green cultivar (Bahler *et al*., 1991).

1.6.4. Heavy metal stress

Heavy metal stress leads to increased reaction oxygen species production in plants leading to oxidative damage to lipids, proteins, nucleic acids and denaturation of cell membranes (Emamverdian *et al*., 2015). In general, increased levels of PPs protect the plant cellular structures and molecules by scavenging the high energy electrons generated by the reactive oxygen species (Viehweger, 2014). Studies show that PP biosynthetic pathway enzymes and PAL gene expression levels are enhanced due to the exposure of Lupine seedlings (*Lupinus luteus*) to cadmium, showing a protective effect of PPs during heavy metal exposure (Pawlak-Sprada *et al.*, 2011). The presence of heavy metals in soil is known to have a negative impact on the accumulation of PP pathway products, particularly anthocyanins. Presence of trace amounts of Ni in soil led to the inhibition of

PAL resulting in lower accumulation of anthocyanin in rye seedlings (Baranowska *et al*., 1996). Similar results due to Ni stress were observed in lettuce as well (Hawrylak *et al*., 2007). Recently, it was shown that presence of Se in growth media of hydroponically grown maize seedlings led to an increase in anthocyanin content (Hawrylak-Nowak, 2008).

1.6.5. UV radiation stress

Flavonoids are known to protect plant cells by absorbing UV radiations. The metal chelating ability by the hydroxyl group at the C3 position in the flavonoid molecule is responsible for scavenging free radical ions and ROS during UV-stress (Verdan *et al*., 2011). Recently, it was demonstrated that the expression of maize flavonol synthase gene *ZmFLS* expression is induced by UV-B radiation (Ferreyra *et al*., 2012). Increased flavonoid accumulation in response to UV-B radiation has been reported in a number of plant species such as Arabidopsis (Stracke et al., 2010; Kusano et al., 2011), petunia (Ryan *et al*., 2002), grapes (Berli *et al*., 2010), privet (Agati *et al*., 2011) and soybean (Mazza *et al*., 2000).

1.6.6. Herbivory stress

Plant defense against herbivores like grazing mammals and insects involve structural (lignin, trichomes and thorns) and chemical (phenylpropanoids and resin) adaptations to deter herbivory (Schardl and Chen, 2010). According to the co-evolution theory, the vast variety of flavonoid molecules has evolved to modulate plant-insect interactions and act as deterrent against insect herbivores (Kliebenstein, 2004). Expression of the PP biosynthesis pathway genes was found to be upregulated by both UV-B radiation and

Manduca sexta (tobacco hornworm) infestation in tobacco (Izaguirre *et al*., 2003). Presence of the C-glycosyl flavone maysin and its related compounds apimaysin and methoxymaysin within the corn silks provide resistance against *Helicoverpa zea* (corn earworm) (Snook *et al*., 1994). Overexpression of a Myb transcription factor responsible for maysin production in maize led to increased resistance against *H. zea* larvae (Johnson *et al*., 2007). The presence of tannins and phenolic compounds in the foliar tissue can shorten mammalian herbivores foraging by reducing the digestibility of the ingested plant tissue or by being toxic to the herbivores (Iason, 2005).

1.6.7. Pathogen stress

As opposed to the effect of *M. sexta* infestation in tobacco described above (Izaguirre *et al*., 2003), it was observed that infection by the oomycete *Phytophthora sojae* in parsley resulted in the inhibition of the PP pathway genes (Logemann and Hahlbrock, 2002). Interestingly, infection by *P. sojae* in soybean led to the induction of PP pathway genes in the resistant variety but not in the susceptible variety. This suggests that phenylpropanoids have a role in providing defense against fungal infections in soybean (Moy *et al*., 2004). Infection by *Fusarium solani* also induced PP pathway gene expression in soybean (Iqbal *et al*., 2005). In another study, RNAi-mediated downregulation of isoflavone synthase in soybean resulted in increased susceptibility to *P. sojae* infection as compared to wild type plants (Subramanian *et al*., 2005). Upregulation of PP metabolism genes has also been observed in *Medicago truncatula* in response to *Colletotrichum trifolli* (Torregrosa *et al*., 2004) and *Erysiphe pisi* (Foster-Hartnett et al., 2007) infections.

Different branches of the PP biosynthetic pathway are disparately affected by bacterial pathogens. Global gene expression analyses in soybean and *M. truncatula* demonstrated that, as a defense response, plants invest in the production of antioxidants as opposed to pigment production *via* the PP pathway (Samac and Graham, 2007). Microarray analysis of gene expression in soybean infected with *P. syringae* pv *glycenia* showed an upregulation in the expression of genes responsible for the synthesis of flavones and isoflavones. However, genes involved in the biosynthesis of anthocyanins were found to be exclusively downregulated (Zou *et al*., 2005). Another study confirmed the upregulation of flavones/isoflavone biosynthesis genes (with antioxidant and antimicrobial properties) in comparison to anthocyanin (pigment) biosynthesis genes in soybean plants infected with *P. syringae* (Zabala *et al*., 2006).

2. Objectives of the Study

The present study investigates the molecular mechanism underlying the effects of estrogen on plant physiological responses related to primary and secondary metabolism, biotic and abiotic stress responses and plant growth and development. Global analysis of gene expression in Arabidopsis seedlings treated with 17β-estradiol revealed a unique pattern of gene expression related to stress responsive and PP pathway genes. The stress responsive genes were upregulated and the key genes of the PP biosynthesis were exclusively downregulated (Adetunji, 2012). As shown by different studies (discussed above), both estradiol and flavonoids have ameliorative effects on plants under environmental stresses. Therefore, application of estradiol resulting in the downregulation of PP pathway genes appears counterintuitive as higher levels of PP

metabolites can potentially contribute towards better coping with stress. In this study, it was observed that estradiol application at the 10 and 100 nM concentration enhanced plant growth, photosynthesis rate and primary metabolite accumulation. The overall downregulation of PP pathway genes led to a reduction in the accumulation of PP metabolites in estradiol-treated plants. Exogenous application of 10 and 100 nM estradiol compromised Arabidopsis resistance against a bacterial pathogen. However, the same treatment rendered the plants more tolerant to drought stress. It is noteworthy that estradiol-treated plants were found to be resistant to abiotic stress (drought) but susceptible to biotic stressor (bacterial pathogen). To understand the molecular basis of this response, *PAL1* over-expressing plants were generated and were used as a paradigm to study the effects of estradiol application on PP pathway metabolism and plant stress responses. Constitutive expression of *PAL1* resulted in higher accumulation of PP products in the transgenic plants, which also displayed higher resistance to the bacterial pathogen.

REFERENCES

Abrahams, S., Lee, E., Walker, A.R., Tanner, G.J., Larkin, P.J., and Ashton, A.R. (2003). The Arabidopsis *TDS4* gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. Plant J. **35,** 624-636.

Adetunji, K. (2012). Microarray analysis of estradiol regulated gene expression in *Arabidopsis* seedlings. MS thesis. Texas Woman's University, TX, USA.

Agati, G., Biricolti, S., Guidi, L., Ferrini, F., Fini, A., and Tattini, M. (2011). The biosynthesis of flavonoids is enhanced similarly by UV radiation and root zone salinity in *L. vulgare* leaves. J. Plant Physiol. **168,** 204-212.

Ali, R. and Abbas, H. (2003). Response of salt stressed barley seedlings to phenylurea. Plant Soil Environ. **49,** 158-162.

Appelhagen, I., Thiedig, K., Nordholt, N., Schmidt, N., Huep, G., Sagasser, M., and Weisshaar, B. (2014). Update on transparent testa mutants from *Arabidopsis thaliana*: characterisation of new alleles from an isogenic collection. Planta **240,** 955-970.

Appert, C., Logemann, E., Hahlbrock, K., Schmid, J., and Amrhein, N. (1994). Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). Eur. J. Biochem. **225,** 491-499.

Austin, M.B. and Noel, J.P. (2003). The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. **20,** 79-110.

Bahler, B.D., Steffen, K.L., and Orzolek, M.D. (1991). Morphological and biochemical comparison of a purple-leafed and a green-leafed pepper cultivar. Hort. Sci. **26,** 736-736.

Bajguz, A. (2011). Brassinosteroids-occurrence and chemical structures in plants. In Brassinosteroids: A Class of Plant Hormones. S. Hayat and A. Ahmad, eds. (New York, Springer), pp. 1-27

Baranowska, M., Krupa, Z., and Orzol, D. (1996). Can anthocyanins be considered as heavy metal stress indicator in higher plants? Acta Physiol. Plant. **18,** 147-151.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., and Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. **34,** 5059-5066.

Belfroid, A., Van der Horst, A., Vethaak, A., Schäfer, A., Rijs, G., Wegener, J., and Cofino, W. (1999). Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands. Sci. Total Environ. **225,** 101-108.

Berli, F.J., Moreno, D., Piccoli, P., Hespanhol-Viana, L., Silva, M.F., Bressan-Smith, R., Cavagnaro, J.B., and Bottini, R. (2010). Abscisic acid is involved in the response of grape (*Vitis vinifera* L.) cv. Malbec leaf tissues to ultraviolet-B radiation by enhancing ultraviolet-absorbing compounds, antioxidant enzymes and membrane sterols. Plant Cell Environ. **33,** 1-10.

Bhattacharya, B. and Gupta, K. (1981). Steroid hormone effects on growth and apical dominance of sunflower. Phytochemistry **20,** 989-991.

Bircher, S., Card, M. L., Zhai, G., Chin, Y. P., and Schnoor, J. L. (2015). Sorption, uptake, and biotransformation of 17-β estradiol, 17-α ethinylestradiol, zeranol, and trenbolone acetate by hybrid poplar. Environ. Toxicol. Chem. **34,** 2906-2913.

Bowerman, P.A., Ramirez, M.V., Price, M.B., Helm, R.F., and Winkel, B.S. (2012). Analysis of T-DNA alleles of flavonoid biosynthesis genes in Arabidopsis ecotype Columbia. BMC Res. Notes **5.** doi: 10.1186/1756-0500-5-485.

Card, M. L., Schnoor, J. L., and Chin, Y. P. (2013). Transformation of natural and synthetic estrogens by maize seedlings. Environ. Sci. Tech. **47,** 5101-5108.

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. **70,** 1-9.

Chan, L., Koay, S., Boey, P., and Bhatt, A. (2010). Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. Biol. Res. **43,** 127-135.

Chaoui, A. and El Ferjani, E. (2013). β-Estradiol protects embryo growth from heavy-metal toxicity in germinating lentil seeds. J. Plant Growth Regul. **32,** 636-645.

Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V., and Martens, S. (2013). Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. Plant Physiol. Biochem.**72,** 1-20.

Christie, P.J., Alfenito, M.R., and Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta **194,** 541-549.

Costa, M.A., Bedgar, D.L., Moinuddin, S.G., Kim, K., Cardenas, C.L., Cochrane, F.C., Shockey, J.M., Helms, G.L., Amakura, Y., and Takahashi, H. (2005). Characterization *in vitro* and *in vivo* of the putative multigene 4-coumarate: CoA ligase network in Arabidopsis: syringyl lignin and sinapate/sinapyl alcohol derivative formation. Phytochemistry **66,** 2072-2091.

Czajka, C.P. and Londry, K.L. (2006). Anaerobic biotransformation of estrogens. Sci. Total Environ. **367,** 932-941.

Daneshmand, F., Arvin, M.J., and Kalantari, K.M. (2010). Physiological responses to NaCl stress in three wild species of potato in vitro. Acta Physiol. Plant. **32,** 91-101.

Dixon, R.A. and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell **7,** 1085-1097.

Dumlupinar, R., Genisel, M., Erdal, S., Korkut, T., Taspinar, M.S., and Taskin, M. (2011). Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. Biol. Trace Elem. Res. **143,** 1740-1745.

Emamverdian, A., Ding, Y., Mokhberdoran, F., and Xie, Y. (2015). Heavy metal stress and some mechanisms of plant defense response. Sci. World J. doi: 10.1155/2015/756120.

Erdal, S. (2009). Effects of mammalian sex hormones on antioxidant enzyme activities, H_2O_2 content and lipid peroxidation in germinating bean seeds. Ataturk Univ. Ziraat Fak. Derg. **40,** 79-85.

Erdal, S. (2012a). Exogenous mammalian sex hormones mitigate inhibition in growth by enhancing antioxidant activity and synthesis reactions in germinating maize seeds under salt stress. J. Sci. Food Agric. **92,** 839-843.

Erdal, S. (2012b). Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J. Sci. Food Agric. **92,** 1411-1416.

Erdal, S. and Dumlupinar, R. (2011). Mammalian sex hormones stimulate antioxidant system and enhance growth of chickpea plants. Acta Physiol. Plant. **33,** 1011-1017.

Erdal, S. and Dumlupinar, R. (2010). Progesterone and β-estradiol stimulate seed germination in chickpea by causing important changes in biochemical parameters. Zeitschrift für Naturforschung **65,** 239-244.

Erdal, S., Genisel, M., Turk, H., and Gorcek, Z. (2012). Effects of progesterone application on antioxidant enzyme activities and K+/Na+ ratio in bean seeds exposed to salt stress. Toxicol. Ind. Health **28,** 942-946.

Ferreyra, M.L.F., Casas, M.I., Questa, J.I., Herrera, A.L., DeBlasio, S., Wang, J., Jackson, D., Grotewold, E., and Casati, P. (2012). Evolution and expression of tandem duplicated maize flavonol synthase genes. Front. Plant Sci. **3,** 101. http://dx.doi.org/10.3389/fpls.2012.00101

Finlay-Moore, O., Hartel, P., and Cabrera, M. (2000). 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. **29,** 1604-1611.

Foster-Hartnett, D., Danesh, D., Penuela, S., Sharopova, N., Endre, G., Vandenbosch, K.A., Young, N.D., and Samac, D.A. (2007). Molecular and cytological responses of *Medicago truncatula* to *Erysiphe pisi*. Mol. Plant Pathol. **8,** 307-319.

Fraser, C.M. and Chapple, C. (2011). The phenylpropanoid pathway in Arabidopsis. Arabidopsis Book **9,** e0152.

Genisel, M., Turk, H., and Erdal, S. (2013). Exogenous progesterone application protects chickpea seedlings against chilling-induced oxidative stress. Acta Physiol. Plant. **35,** 241-251.

Grace, S.C. and Logan, B.A. (2000). Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **355,** 1499-1510.

Greca, M.D., Florentino, A., Pinto, G., Pollio, A. Previtera, L. (1996). Biotransformation of progesterone by the green alga *Chlorella emersonii* C211-8H. Phytochemistry **41**, 1527-1529.

Guan, M. and Roddick, J.G. (1988). Epibrassinolide-inhibition of development of excised, adventitious and intact roots of tomato (*Lycopersicon esculentum*): comparison with the effects of steroidal estrogens. Physiol. Plant. **74,** 720-726.

Hamberger, B. and Hahlbrock, K. (2004). The 4-coumarate:CoA ligase gene family in *Arabidopsis thaliana* comprises one rare, sinapate-activating and three commonly occurring isoenzymes. Proc. Natl. Acad. Sci. U. S. A. **101,** 2209-2214.

Hansen, P., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., and Blaise, C. (1998). Vitellogenin–a biomarker for endocrine disruptors. Trends Anal. Chem. **17,** 448-451.

Hawrylak, B., Matraszek, R., and Szymańska, M. (2007). Response of lettuce (*Lactuca sativa* L.) to selenium in nutrient solution contaminated with nickel. Veg. Crop. Res. **67,** 63-70.

Hawrylak-Nowak, B. (2008). Changes in anthocyanin content as indicator of maize sensitivity to selenium. J. Plant Nutr. **31,** 1232-1242.

Helmkamp, G. and Bonner, J. (1953). Some relationships of sterols to plant growth. Plant Physiol. **28,** 428-436.

Hernández, I., Alegre, L., and Munné-Bosch, S. (2006). Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants. Phytochemistry **67,** 1120-1126.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.H., Yu, J.Q., and Chen, Z. (2010). Functional analysis of the Arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. Plant Physiol. **153,** 1526-1538.

Iason, G. (2005). The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proc. Nutr. Soc. **64,** 123-131.

Iqbal, M., Yaegashi, S., Ahsan, R., Shopinski, K.L., and Lightfoot, D.A. (2005). Root response to *Fusarium solani* f. sp. *glycines*: temporal accumulation of transcripts in partially resistant and susceptible soybean. Theor. Appl. Genet. **110,** 1429-1438.

Izaguirre, M.M., Scopel, A.L., Baldwin, I.T., and Ballare, C.L. (2003). Convergent responses to stress. Solar ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. Plant Physiol. **132,** 1755-1767.

Janeczko, A. (2000). Influence of selected steroids on plant physiological processes-especially flowering induction. PhD Thesis, Agriculture University, Krakow, Poland.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., and Sumpter, J.P. (1998). Widespread sexual disruption in wild fish. Environ. Sci. Technol. **32,** 2498-2506.

Johnson, A., Belfroid, A., and Di Corcia, A. (2000). Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci. Total Environ. **256,** 163-173.

Johnson, E.T., Berhow, M.A., and Dowd, P.F. (2007). Expression of a maize Myb transcription factor driven by a putative silk-specific promoter significantly enhances resistance to *Helicoverpa zea* in transgenic maize. J. Agric. Food Chem. **55,** 2998-3003.

Kliebenstein, D. (2004). Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. Plant Cell Environ. **27,** 675-684.

Kopcewicz, J. (1969). Influence of estrone on growth and endogenous gibberellins content in dwarf pea. Bull. Acad. Pol. Sci. Biol. **17,** 727-731.

Korkina, L. (2007). Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. Cell Mol. Biol. **53,** 15-25.

Krishna, P. (2003). Brassinosteroid-mediated stress responses. J. Plant Growth Regul. **22,** 289-297.

Kuch, H.M. and Ballschmiter, K. (2001). Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environ. Sci. Technol. **35,** 3201-3206.

Kusano, M., Tohge, T., Fukushima, A., Kobayashi, M., Hayashi, N., Otsuki, H., Kondou, Y., Goto, H., Kawashima, M., and Matsuda, F. (2011). Metabolomics reveals comprehensive reprogramming involving two independent metabolic responses of Arabidopsis to UV-B light. Plant J. **67,** 354-369.

Lattanzio, V., Lattanzio, V.M., and Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In Phytochemistry Advances in Research, Vol. 661. F. Imperato, ed. (India, Research Singnapost) pp. 23-67.

Lee, S.W., Robb, J., and Nazar, R.N. (1992). Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J. Biol. Chem. **267,** 11824-11830.

Lepiniec, L., Debeaujon, I., Routaboul, J., Baudry, A., Pourcel, L., Nesi, N., and Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. Annu. Rev. Plant Biol. **57,** 405-430.

Lin, J., Palevitch, D., and Heftmann, E. (1983). Reduction of 4-andro-stene-3, 17-dione by growing cucumber plants. Phytochemistry **22,** 1149-1154

Lin, J., Proebsting, W.M. and Heftmann, E. (1979). Conversion of 4-androstene-3, 17-dione to testosterone by *Pisum sativum*. Phytochemistry **18,** 1667-1669

Logemann, E. and Hahlbrock, K. (2002). Crosstalk among stress responses in plants: pathogen defense overrides UV protection through an inversely regulated ACE/ACE type of light-responsive gene promoter unit. Proc. Natl. Acad. Sci. U. S. A. **99,** 2428-2432.

Löve, Á and Löve, D. (1945). Experiments on the effects of animal sex hormones on dioecious plants. Ark. Botanik. **32,** 1-60.

Mazza, C.A., Boccalandro, H.E., Giordano, C.V., Battista, D., Scopel, A.L., and Ballare, C.L. (2000). Functional significance and induction by solar radiation of ultraviolet-absorbing sunscreens in field-grown soybean crops. Plant Physiol. **122,** 117-126.

Mizutani, M., Ohta, D., and Sato, R. (1997). Isolation of a cDNA and a genomic clone encoding cinnamate 4-hydroxylase from Arabidopsis and its expression manner *in planta*. Plant Physiol. **113,** 755-763.

Moura, J. C. M. S., Bonine, C. A. V., De Oliveira Fernandes Viana, J., Dornelas, M. C., and Mazzafera, P. (2010). Abiotic and biotic stresses and changes in the lignin content and composition in plants. J. Integr. Plant Biol. **52,** 360-376.

Moy, P., Qutob, D., Chapman, B.P., Atkinson, I., and Gijzen, M. (2004). Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. Mol. Plant-Microbe Interact. **17,** 1051-1062.

Narayan, M., Thimmaraju, R., and Bhagyalakshmi, N. (2005). Interplay of growth regulators during solid-state and liquid-state batch cultivation of anthocyanin producing cell line of *Daucus carota*. Process Biochem. **40,** 351-358.

Navarro, J.M., Flores, P., Garrido, C., and Martinez, V. (2006). Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chem. **96,** 66-73.

Nichols, D., Daniel, T., Moore, P., Edwards, D., and Pote, D. (1997). Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. J. Environ. Qual. **26,** 1002-1006.

Patisaul, H.B., and Jefferson, W. (2010). The pros and cons of phytoestrogens. Front. Neuroendocrinol. **31,** 400-419.

Pawlak-Sprada, S., Arasimowicz-Jelonek, M., Podgorska, M. and Deckert, J. (2011). Activation of phenylpropanoid pathway in legume plants exposed to heavy metals. Part 1. Effects of cadmium and lead on phenylalanine ammonia lyase gene expression, enyzme activity and lignin content. Act. Biochim. Polon. **58**, 211-216.

Pelletier, M.K. and Shirley, B.W. (1996). Analysis of flavanone 3-hydroxylase in Arabidopsis seedlings. Coordinate regulation with chalcone synthase and chalcone isomerase. Plant Physiol. **111,** 339-345.

Peterson, E., Davis, R., and Orndorff, H. (2000). 17 β-Estradiol as an indicator of animal waste contamination in mantled karst aquifers. J. Environ. Qual. **29,** 826-834.

Purdom, C., Hardiman, P., Bye, V., Eno, N., Tyler, C., and Sumpter, J. (1994). Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. **8,** 275-285.

Raes, J., Rohde, A., Christensen, J.H., Van de Peer, Y., and Boerjan, W. (2003). Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol. **133,** 1051-1071.

Rodgers-Gray, T.P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J.E., Waldock, M.J., Sumpter, J.P., and Tyler, C.R. (2000). Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environ. Sci. Technol. **34,** 1521-1528.

Rohde, A., Morreel, K., Ralph, J., Goeminne, G., Hostyn, V., De Rycke, R., Kushnir, S., Van Doorsselaere, J., Joseleau, J.P., Vuylsteke, M., Van Driessche, G., Van Beeumen, J., Messens, E., and Boerjan, W. (2004). Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell **16,** 2749-2771.

Ryan, K.G., Swinny, E.E., Markham, K.R., and Winefield, C. (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. Phytochemistry **59,** 23-32.

Samac, D.A. and Graham, M.A. (2007). Recent advances in legume-microbe interactions: recognition, defense response, and symbiosis from a genomic perspective. Plant Physiol. **144,** 582-587.

Schardl, C. L., and Chen, F. (2010). Plant defences against herbivore attack. *eLS*. doi: 10.1002/9780470015902.a0001324.pub2

Schilmiller, A.L., Stout, J., Weng, J., Humphreys, J., Ruegger, M.O., and Chapple, C. (2009). Mutations in the cinnamate 4-hydroxylase gene impact metabolism, growth and development in Arabidopsis. Plant J. **60,** 771-782.

Schmidt, S., Degen, G.H., Seibel, J., Hertrampf, T., Vollmer, G., and Diel, P. (2006). Hormonal activity of combinations of genistein, bisphenol A and 17-beta-estradiol in the female Wistar rat. Arch. Toxicol. **80,** 839–845.

Schoenbohm, C., Martens, S., Eder, C., Forkmann, G., and Weisshaar, B. (2000). Identification of the *Arabidopsis thaliana* flavonoid 3'-hydroxylase gene and functional expression of the encoded P450 enzyme. Biol. Chem. **381,** 749-753.

Shirley, B.W., Hanley, S., and Goodman, H.M. (1992). Effects of ionizing radiation on a plant genome: analysis of two Arabidopsis transparent testa mutations. Plant Cell **4,** 333-347.

Shore, L., Correll, D., and Chakraborty, P. (1995). Relationship of fertilization with chicken manure and concentrations of estrogens in small streams. In Animal Waste and the Land-Water Interface. K. Steele, ed. (New York, Lewis Publishers) pp. 155-162.

Shore, L.S., Kapulnik, Y., Ben-Dor, B., Fridman, Y., Wininger, S., and Shemesh, M. (1992). Effects of estrone and 17 β-estradiol on vegetative growth of *Medicago sativa.* Physiol. Plant. **84,** 217-222.

Shore, L.S., Shemesh, M., and Cohen, R. (1988). The role of oestradiol and oestrone in chicken manure silage in hyperoestrogenism in cattle. Aust. Vet. J. **65,** 68-68.

Sieveking, D.P., Lim P., Chow R.W.Y., Dunn, L.L., Bao, S., McGrath, K.C.Y., Heather, A.K., Handelsman, D.J., Celermajer, D.S., Ng, M.K.C. (2010) A sex-specific role for androgens in angiogenesis. J. Exp. Med. **207**, 345-357

Snook, M.E., Widstrom, N.W., Wiseman, B.R., Gueldner, R.C., Wilson, R.L., Himmelsbach, D.S., Harwood, J.S., and Costello, C.E. (1994). New flavone C-glycosides from corn (*Zea mays* L) for the control of the corn earworm (*Helicoverpa zea*). In Bioregulators for Crop Protection and Pest Control. P. A. Hedin, ed. (Washington DC, ACS Symposium Series 557) pp. 122-135.

Subramanian, S., Graham, M.Y., Yu, O., and Graham, T.L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. Plant Physiol. **137,** 1345-1353.

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M., and Magara, Y. (2001). Estrogenic influences of estradiol-17b, p-nonylphenol and bis-phenol-A on Japanese Medaka (*Oryzias latipes*) at detected environmental concentrations. Water Sci. Technol. **43,** 109-116.

Torregrosa, C., Cluzet, S., Fournier, J., Huguet, T., Gamas, P., Prospéri, J., Esquerré-Tugayé, M., Dumas, B., and Jacquet, C. (2004). Cytological, genetic, and molecular analysis to characterize compatible and incompatible interactions between *Medicago truncatula* and *Colletotrichum trifolii*. Mol. Plant-Microbe Interact. **17,** 909-920.

Verdan, A.M., Wang, H.C., García, C.R., Henry, W.P., and Brumaghim, J.L. (2011). Iron binding of 3-hydroxychromone, 5-hydroxychromone, and sulfonated morin: implications for the antioxidant activity of flavonols with competing metal binding sites. J. Inorg. Biochem. **105,** 1314-1322.

Viehweger, K. (2014). How plants cope with heavy metals. Bot. Studies. **55,** 1–12.

Vogt, T. (2010). Phenylpropanoid biosynthesis. Mol. Plant **3,** 2-20.

Wanner, L.A., Li, G., Ware, D., Somssich, I.E., and Davis, K.R. (1995). The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. **27,** 327-338.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. **126,** 485-493.

Woerdenbag, H.J., Pras, N., Frijlink, H.W., Lerk, C.F., and Malingré, T.M. (1990). Cyclodextrin-facilitated bioconversion of 17β-estradiol by a phenoloxidase from *Mucuna pruriens* cell cultures. Phytochemistry **29,** 1551-1554.

Wu, A.H., Stanczyk, F.Z., Hendrich, S., Murphy, P.A., Zhang, C., Wan, P., and Pike, M.C. (2000). Effects of soy foods on ovarian function in premenopausal women. Br. J. Cancer. **82,** 1879–1886.

Ying, G. and Kookana, R.S. (2005). Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. Environ. Toxicol. Chem. **24,** 2640-2645.

Young, I.J., Knights, B.A. and Hillman, J.R. (1977). Oestradiol and its biosynthesis in *Phaseolus vulgaris* L. Nature **267,** 429.

Young, I.J., Knights, B.A. and Hillman, J.R. (1979). The metabolism of estrogens in vivo and in vitro by *Phaseolus vulgaris*. Z. Pflanzenphysiol. **94,** 307-316.

Zabala, G., Zou, J., Tuteja, J., Gonzalez, D.O., Clough, S.J., and Vodkin, L.O. (2006). Transcriptome changes in the phenylpropanoid pathway of *Glycine max* in response to *Pseudomonas syringae* infection. BMC Plant. Biol. **6,** 26. doi: 10.1186/1471-2229-6-26.

Zhang, W., Seki, M., and Furusaki, S. (1997). Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Sci. **127,** 207-214.

Zhong, J. and Yoshida, T. (1993). Effects of temperature on cell growth and anthocyanin production in suspension cultures of *Perilla frutescens*. J. Ferment. Bioeng. **76,** 530-531.

Zou, J., Rodriguez-Zas, S., Aldea, M., Li, M., Zhu, J., Gonzalez, D.O., Vodkin, L.O., DeLucia, E., and Clough, S.J. (2005). Expression profiling soybean response to *Pseudomonas syringae* reveals new defense-related genes and rapid HR-specific downregulation of photosynthesis. Mol. Plant-Microbe Interact. **18,** 1161-1174.

CHAPTER II

EFFECTS OF 17B-ESTRADIOL ON GROWTH, PHOTOSYNTHESIS RATE AND PRIMARY METABOLITES OF *ARABIDOPSIS THALIANA*

A Chapter Submitted for Publication in the

Journal *Biologia Plantarum*

Pallavi Upadhyay

Abstract

Mammalian sex hormones are spread in the environment from natural and anthropogenic sources. In the present study, the effect of estradiol on *Arabidopsis thaliana* growth and physiological characteristics were investigated. Treatments of Arabidopsis plants with 10 and 100 nM 17 β-estradiol resulted in enhanced root growth and shoot biomass. In addition, treated plants had an increased rate of photosynthesis with a concomitant increase in carbohydrate and protein accumulation. Plants exposed to higher concentrations of 17 β-estradiol (10 µM) had significantly lower root growth, biomass, photosynthesis rate and accumulated primary metabolites indicating a toxic effect of estradiol. These results indicate that at low concentrations, estradiol functions as a biostimulant on the growth, yield and primary metabolism of Arabidopsis.

Additional key words: biostimulant, photosynthesis rate, root, total carbohydrate, total chlorophyll, total protein, silique

Abbreviations: ANOVA – Analysis of Variance, d – day, EE - environmental estrogens, ES – estradiol, EtOH – ethanol, F. W. - fresh weight, MS - Moorashige and Skoog medium, SD – standard deviation

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In vertebrates, estrogen and androgen steroidal hormones have important functions in development and reproduction. In plants, brassinosteroids are the only class of naturally occurring steroidal hormones and play an important role in the plant growth, development and stress responses (Schaller, 2003). A number of studies, some as early as 1920s, demonstrated that application of mammalian sex hormones affect the growth and development of plants, from cell divisions to flowering, sex expression, embryo growth and modulation of stress responses, concluding that mammalian sex hormones act as potential plant growth regulators (Geuns 1978, Janeczko and Skoczowski 2005; Janeczko *et al.* 2012; Chaoui and El Ferjani 2013).

In one of the earliest studies, it was shown that estrone application had a stimulatory effect on the root meristem cell division in a number or dioecious plants (Löve and Löve, 1945). In a study involving the effect of mammalian steroidal hormones on sunflower (*Helianthus annuus*) seedlings, the application of 17β-estradiol (ES) promoted shoot growth but had an inhibitory effect on root growth (Bhattacharya and Gupta, 1981).

Application of 1 µM estrogen to winter wheat seedlings promoted leaf and root growth (Janeczko, 2000). It was observed that irrigation of *Medicago sativa* plants with sewage water, which contained 0.3 µg L^{-1} estrogen, resulted in increased vegetative growth (Shore et al., 1992). Estrogens originating from human and livestock wastes lead to the contamination of water and soil systems all over the world (Finlay-Moore *et al.* 2000; Ying and Kookana 2005; Jiang *et al.* 2012; Schultz *et al.* 2013). Although significant progress has been made in establishing the biological and ecological consequences of animal exposure to environmental estrogens (EE) (Schultz *et al.* 2013; Sumpter and Jobling 2013), there is still a big gap of knowledge in regard to the effects of EE on plants, especially crop plants as sources of food for an exponentially growing human population. In the present study we describe the effects of ES application on the growth, yield, photosynthesis and primary metabolism of Arabidopsis in a study design that simulates plant exposure to EE. For that purpose, Arabidopsis seeds were germinated on agar medium containing ES to simulate soil exposure to EE and, during vegetative development, plants were sprayed with ES solutions to simulate irrigation conditions.

Impact of ES on root growth was studied by germinating Arabidopsis wild type seeds on Moorashige and Skoog (MS) medium (Murashige and Skoog, 1962) plates containing increasing concentrations of ES (10 nM, 100 nM, 10 µM). Arabidopsis seedlings germinated on MS plates without ES or supplemented with 0.01% ethanol were used as control plants. Root length and lateral root numbers were recorded using ImageJ software (rsweb.nih.gov/ij) at 14 d post germination after which plants were transferred from MS plates to sterilized soil and maintained in a Percival growth chamber at long-day

conditions, 22°C, 50% humidity, and 200 μmol/m^2/s light intensity to complete their life cycle. Foliar spray treatments with above-mentioned ES concentrations were applied to plants two more times, on a weekly basis, until maturity. Fresh and dry shoot biomass (mg) and number of siliques was recorded at 35 days post germination.

To determine the impact of ES treatments (MS medium with ES for germination and one foliar ES application) on the rate of photosynthesis, total leaf chlorophyll content was determined in 21 days old plants by the method of (Lichtenthaler and Buschmann, 2001) and expressed as μg g^{-1} fresh weight (F. W.). Photosynthesis rate was estimated on 21-day-old Arabidopsis plants by using the LI-6400 portable photosynthesis system (LI-COR, Lincoln, NE, USA) and calculated based on leaf biomass according to the manufacturer's instructions.

Total proteins and total carbohydrates in leaf tissues from ES-treated and untreated 21-day-old Arabidopsis plants were estimated. Total soluble protein content was estimated according to the Bradford method (Bradford, 1976) and protein concentration was expressed as mg g^{-1} F. W. For carbohydrate analysis, 0.1 g leaf tissue was ground with 80% acetone and extracted with 80% ethanol. The extracts were mixed with 5 ml anthrone reagent, heated at 100°C (10 min) and allowed to cool on ice. Absorbances were read at 630 nm and total carbohydrate content was expressed as mg g^{-1} F. W. based on a glucose standard curve (Hansen and Møller, 1975). All the experiments were conducted three times in triplicates. Statistical analysis was performed using one way ANOVA ($P < 0.05$ was considered as significant).

Significantly increased root length was observed in 14 days old seedlings exposed to low ES concentrations of 10 nM (7.43±0.41cm) and 100 nM (7.51±0.4 cm) as compared to control plants (4.14±0.29 cm) (Fig. 2.1 A, C). However, treatments with higher concentration of ES (10 µM) resulted in a significant decrease in root length (2.4±0.22 cm) (Fig. 2.1 A, C). A similar trend was observed with respect to lateral root growth in that seedlings exposed to 10 and 100 nM ES displayed a significantly higher number of lateral roots (6.8±0.91-7.1±0.56) as compared to the control treatments (4.2±0.91) and plants treated with 10 µM ES which displayed the lowest lateral root number (3.2±0.63) (Fig. 2.1 B, C). These results are consistent with similar results obtained with chickpea seedlings in that exogenous application of 10^{-4}-10^{-12} M ES as a foliar spray resulted in enhanced root and shoot length growth (Erdal and Dumlupinar, 2011).

The 10 and 100 nM ES treatments significantly increased the fresh and dry weight biomass of Arabidopsis plants (Table 2.1). The 10 nM ES treatments increased the plant F.W. by 26% and dry weight by 42%. Plants treated with 100 nM ES increased their F.W. by 17% and dry weight by 33%. In addition, treatments with low concentrations of estradiol stimulated the reproductive development of Arabidopsis, reflected in increased silique yield (Table 2.1). The 10 and 100 nM ES-treated plants generated a significantly higher number of siliques, 87.68±13.53 and 81.79±13.52, respectively, than control plants with no ES treatment, which yielded 71.11±12.71 siliques. When compared to the controls and the other ES treatments, the application of 10 µM ES resulted in the lowest biomass accumulation and silique number (Table. 2.1). The effect of mammalian sex hormones on plant reproductive development was studied before. Application of selected

estrogens on Arabidopsis plants grown on artificial medium, winter wheat, sage (*Salvia splendens*) and chicory (*Cichorium intybus*) was found to stimulate flowering (Janeczko et al., 2003). More recently, it was reported that *in vitro* culture of unpollinated Arabidopsis pistils on media supplemented with estrone and progesterone resulted in the development of autonomous endosperm (Rojek et al., 2015).

Chlorophyll content was not significantly affected by 10 and 100 nM ES applications (Fig. 2.2 B). However, a significantly higher photosynthesis rate was observed in these plants when compared to the controls or the 10 µM ES-treated plants (Fig. 2 A). An increased rate of photosynthesis for the 10 and 100 nM ES-treated plants could be due to enhanced shoot biomass (Table 2.1). The increased biomass was accompanied by an enhanced rate of photosynthesis, which resulted in the higher accumulation of both carbohydrates and protein levels in the 10 and 100 nM ES-treated plants when compared to the control and 10 µM ES-treated plants. The negative trend in growth and development of 10 µM ES-treated plants as compared to control and 10 and 100 nM ES-treated plants was also reflected in the total chlorophyll content and photosynthesis rate. The 10 µM ES-treated plants had a 38% decrease in chlorophyll content and 40% decrease in photosynthetic rate (Fig. 2.2 A, B).

Total carbohydrate and protein increase in 10 and 100 nM ES-treated plants correlated with their biomass increase. Plants treated with 10 and 100 nM ES accumulated significantly higher levels of both total carbohydrates (6.87±0.5mg/g and 6.86±0.29mg/g F. W., respectively, representing a 43% increase) and proteins (7.51±1.41mg/g, a 30% increase and 6.88±0.7mg/g F. W., a 17% increase, respectively)

when compared to control plants (Fig. 2.3 A, B). High concentration ES (10 µM ES) applications resulted in a significant decrease in total carbohydrates (3.03±0.4mg/g F. W.) and proteins (3.6±1.1mg/g F. W.) (Fig. 2.3 A, B).

It is evident from these results that application of low ES concentrations (10 and 100 nM) has a positive impact on the vegetative growth and fruit yield of Arabidopsis plants. The highest concentration of ES (10 µM) applied to plants was found to have a negative effect on the growth and physiology parameters studied, indicating a toxic effect. Similar results were obtained with *M. sativa* plants for which lower concentrations of ES had a positive impact on plant growth whereas higher concentrations of the hormone resulted in growth inhibition (Shore et al., 1992). Janeczko (2000) reported that 10^{-6} M ES stimulated while 10^{-5} M ES inhibited winter wheat seedling growth.

Our study indicates that estradiol can stimulate photosynthesis, resulting in enhanced primary metabolism, growth and yield in Arabidopsis. Based on these results, we suggest that estradiol acts as a biostimulant for plant growth at lowers concentrations, similar to concentrations of EE in contaminated environments. Low concentrations of EE induce adverse effects in animals, especially aquatic animals (Sumpter and Jobling 2013), but it may be that same concentrations have positive effects on plant growth, yield and protections against abiotic stresses and therefore may be beneficial for increasing agricultural productivity. More studies should be performed on the effects of estrogens, individually and in mixtures, on other plant species for elucidating their mechanism of action and their beneficial involvement in attenuating the effects of environmental stresses. It is suggested that mammalian sex hormones mediate their effects in plants at

the level of gene transcription by means of specific receptors as in animal cells as well as through non-genomic pathways. Yang *et al.* (2005) characterized a putative Membrane Steroid Binding Protein (MSBP1) that binds progesterone with high affinity and functions in regulating growth in Arabidopsis. Estrogen binding proteins were reported in *Solanaceae* (Milanesi *et al.* 2004) and steroid binding proteins specific for progesterone and 17β-estradiol were reported in *Triticum aestivum* (Janeczko *et al.* 2008). Other research implies that the stimulating effects of human steroid hormone application on plants are due to increase in antioxidant activities in plant cells which enhance the plant resistance to environmental stresses (Erdal and Dumlupinar 2011, Erdal 2012).

Our results demonstrate that estradiol can influence growth and yield of Arabidopsis plants in a dose-dependent manner. The biostimulant activity of estradiol could be used in practice, especially in small-scale cultures of crop and horticultural plants.

Table 2.1: Effect of 17 β-estradiol application on biomass accumulation and silique generation in Arabidopsis plants. Different superscript symbols represent statistically significant differences ($P<0.05$, ANOVA). The results are means ± SD (n = 30).

Treatment	Fresh weight (g)	Dry weight (g)	Silique #
Control	2.89±0.71*	0.33±0.12^{a}	71.11±12.71$^{\psi}$
EtOH	2.76±0.81*	0.31±0.18^{a}	68.20±15.04$^{\psi}$
10 nM	3.65±0.96**	0.47±0.16^{b}	87.68±13.53$^{\Delta}$
100 nM	3.40±0.89**	0.44±0.20^{b}	81.79±13.52$^{\Delta}$
10 µM	1.98±0.63***	0.20±0.14^{c}	38.77±12.97$^{\delta}$

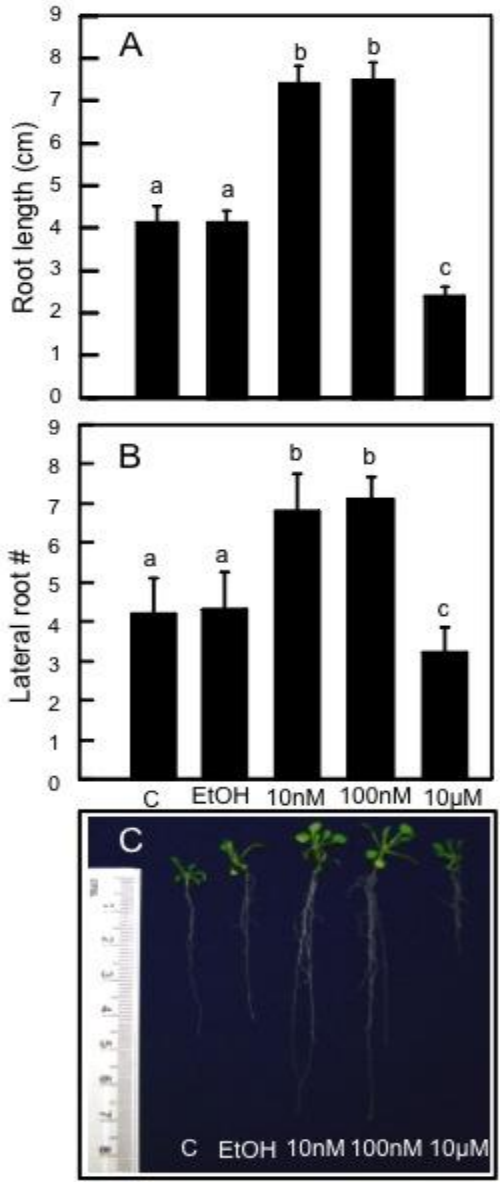

Fig. 2.1. Effect of 17 β-estradiol applications on root growth in Arabidopsis. (A) Root length of control and estradiol-treated Arabidopsis seedlings. (B) Number of lateral roots in 14 days old, control and estradiol-treated Arabidopsis seedlings. (C) Representative image of the roots in 14-day-old, control and ES-treated Arabidopsis seedlings. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences ($P < 0.05$, ANOVA). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 μM = applied ES treatments.

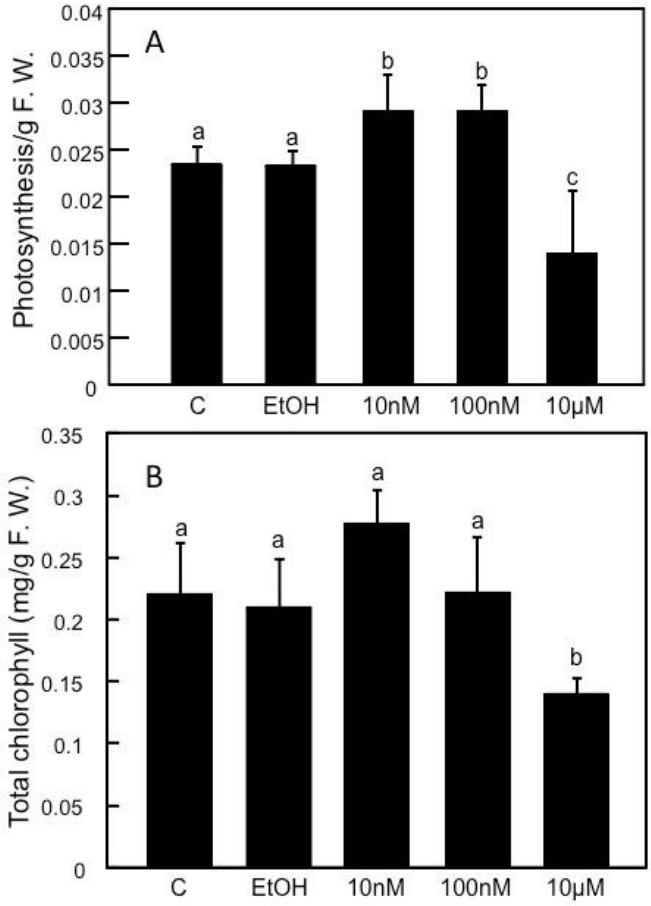

Fig. 2.2. Effect of 17 β-estradiol applications on photosynthesis rate and chlorophyll content of Arabidopsis. (A) Photosynthesis rate and (B) Total chlorophyll content of 21 days old, control and estradiol-treated Arabidopsis leaves. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences.

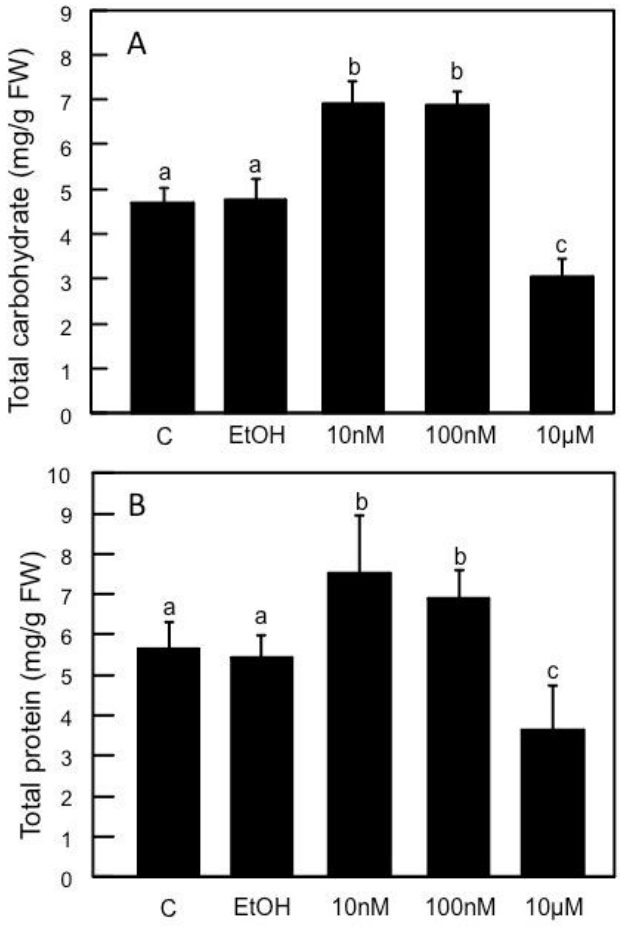

Fig. 2.3. Effect of 17 β-estradiol applications on carbohydrate and protein levels in Arabidopsis. (A) Total carbohydrate content and (B) Total protein content of 21 days old, control and ES-treated Arabidopsis plants. Results are means ± SD (n = 9). Different alphabets represent statistically significant differences.

References

Bhattacharya, B., Gupta, K.: Steroid hormone effects on growth and apical dominance of sunflower. - Phytochemistry. **20**: 989-991, 1981.

Bradford, M.M.: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. - Anal. Biochem. **72**: 248-254, 1976.

Chaoui, A., El Ferjani, E.: β-estradiol protects embryo growth from heavy-metal toxicity in germinating lentil seeds. - J. Plant Growth Regul. **32**: 636-645, 2013.

Clouse, S.D.: A history of brassinosteroid research from 1970 through 2005: Thirty-five years of phytochemistry, physiology, genes and mutants. - J. Plant Growth Regul. **34**: 828-844, 2015.

Erdal, S.: Exogenous mammalian sex hormones mitigate inhibition in growth by enhancing antioxidant activity and synthesis reactions in germinating maize seeds under salt stress. - J. Sci. Food Agric. **92**: 839-843, 2012.

Erdal, S., Dumlupinar, R.: Mammalian sex hormones stimulate antioxidant system and enhance growth of chickpea plants. - Acta Physiol. Plant. **33**: 1011-1017, 2011.

Finlay-Moore, O., Hartel, P., Cabrera, M.: 17 β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. - J. Environ. Qual. **20**:1604-161, 2000.

Fridman, Y., Savaldi-Goldstein, S.: Brassinosteroids in growth control: how, when and where. - Plant Sci. **209**: 24-31, 2013.

Geuns, J.: Steroid hormones and plant growth and development. - Phytochemistry **17**: 1-14, 1978.

Hansen, J., Møller, I.: Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. - Anal. Biochem. **68**: 87-94, 1975.

Janeczko, A.: Influence of selected steroids on plant physiological processes - especially flowering induction. - Doctor Theses, Agricultural University, Krakow, Poland., 2000.

Janeczko, A., Budziszewska, B., Skoczowski, A., Dybała, M.: Specific binding sites for progesterone and 17 β-estradiol in cells of *Triticum aestivum* L. - Acta Biochim. Pol. **55**:707-711, 2008.

Janeczko, A., Filek, W.: Stimulation of generative development in partly vernalized winter wheat by animal sex hormones. - Acta Physiol. Plant. **24**: 291-295, 2002.

Janeczko, A., Filek, W., Biesaga-Kościelniak, J., Marcińska, I., Janeczko, Z.: The influence of animal sex hormones on the induction of flowering in *Arabidopsis thaliana*: comparison with the effect of 24-epibrassinolide. - Plant Cell Tissue Organ Culture. **72**: 147-151, 2003.

Janeczko, A., Korucek, M., Marcinska, I.: Mammalian androgen stimulates photosynthesis in drought-stressed soybean. - Cent. Eur. J. Biol. **7**: 902-909, 2012.

Jeneczko, A., Skoczowski, A.: Mammalian sex hormones in plants. -Folia Histochem Cytobiol. **43**: 71-79, 2005.

Jiang, W., Yan, Y., Ma, M., Wang, D., Lou, Q., Wang, Z., Satyanarayanan, S.K.: Assessment of source water contamination by estrogenic disrupting compounds in China. - J. Environ. Sci. **24**: 320-328, 2012.

Kopcewicz, J.: Influence of estrogens on flower formation in *Cichorium intybus* L. - Naturwissenschaften **57**: 136-136, 1970.

Kopcewicz, J., Porazinski, Z.: Effects of growth regulators, steroids and estrogen fraction from sage plants on flowering of a long day plant, *Salvia splendens*, grown under non-inductive light conditions. - Biol. Plant. **16**: 132-135, 1974.

Lichtenthaler, H.K., Buschmann, C.: Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. - Curr. Prot. Food Anal. Chem. 2001. doi: 10.1002/0471142913.faf0403s01

Löve, Á., Löve, D.: Experiments on the effects of animal sex hormones on dioecious plants. - Ark. Botanik **32**: 1-60, 1945.

Milanesi, L., Boland, R.: Presence of estrogen receptor (ER)-like proteins and endogenous ligands for ER in *Solanaceae*. - Plant Sci. **166**: 397-404, 2004.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bio assays with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Rojek, J., Pawełko, Ł, Kapusta, M., Naczk, A., Bohdanowicz, J.: Exogenous steroid hormones stimulate full development of autonomous endosperm in *Arabidopsis thaliana*. - Acta Soc. Bot. Pol. **84**: 287-301, 2015.

Schultz, M.M., Minarik, T.A., Martinovic-Weigelt, D., Curran, E.M., Bartell, S.E., Schoenfuss, H.L.: Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. - Environ. Int. **61**:138-149, 2013.

Shore, L.S., Kapulnik, Y., Ben-Dor, B., Fridman, Y., Wininger, S., Shemesh, M.: Effects of estrone and 17 β-estradiol on vegetative growth of *Medicago sativa*. - Physiol. Plant. **84**: 217-222, 1992.

Sumpter, J.P., Jobling, S.: The occurrence, causes, and consequesnces of estrogens in the aquatic environment. - Environ. Toxicol. Chem. **32**: 249-251, 2013.

Yang, X.H., Xu, Z.H., Xue, H.W.: Arabidopsis membrane steroid binding protein 1 is involved in inhibition of cell elongation. - Plant Cell **17**: 116–131, 2005.

Ying, G., Kookana, R.S.: Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. - Environ. Toxicol. Chem. **24**: 2640-2645, 2005.

CHAPTER III

EFFECT OF 17B-ESTRADIOL ON *ARABIDOPSIS* SECONDARY METABOLISM AND BIOTIC STRESS RESPONSE

A Chapter to Be Submitted for Publication in the

Acta Physiologiae Plantarum

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Abstract

Increased pharmaceutical and agricultural activities have led to the introduction of animal steroidal hormones, including estrogens, into the agricultural soil. The effects of estradiol on Arabidopsis metabolism and stress responses were studied on plants treated with increasing estradiol concentrations (10 nM, 100 nM and 10 µM), which resulted in the downregulation of phenylpropanoid pathway genes (*PAL1*, *PAL4*, *CHI* and *ANS*) and subsequent decreased accumulation of phenolics, anthocyanins and flavonoids. Phenypropanoids play an important role in plant development and environmental stress responses. Estradiol-treated plants were inoculated with *Pseudomonas syringae* pv. tomato DC3000 and basal resistance was determined. Estradiol treatments rendered plants susceptible to the pathogen. In addition, the expression of *PR1*, a marker for pathogen defense response, in estradiol-treated leaves was decreased. Results illustrate the effects of estradiol, a xenobiont, on plant secondary metabolism and biotic stress

response and could have practical agricultural applications regarding the effect of environmental estrogens and xenoestrogens on the crop plants.

Keywords: Arabidopsis, Estradiol, *PAL1*, Phenylpropanoids, *Pseudomonas syringae*, Secondary metabolism

Introduction

Estrogens are a class of steroidal hormones synthesized in all vertebrates, known to regulate growth, metabolism, developmental processes and reproduction. Estrogens and estrogen-like compounds (xenoestrogens) from livestock manure, animal waste, and human waste (especially pharmaceutical waste), are being disposed of and excreted into the agricultural soil and ground water at a very high rate (Shore and Shemesh 2003, Stumpe and Marschner 2009). Estrogen concentrations in the soil can range from 0.5 ng/L to 70 ng/L depending on the soil type and pollutant source (Hanselman et al., 2003).

Estrogen-like compounds have been isolated from various plant species (Janeczko and Skoczowski 2005). Studies have also reported that application of animal steroids, such as estrogens, progesterones and androsterones, to plants influence plant growth and stress responses. Application of estradiol (ES) increased the growth rate of *Medicago sativa* (Shore et al. 1992), promoted shoot growth in sunflower (Bhattacharya and Gupta 1981) and induced flowering in several plant species, such as *Cichorium intybus* (Kopcewicz 1970) and *Arabidopsis thaliana* (Janeczko et al. 2003). Ylstra and coworkers (1995) found that treatment of tobacco plants with animal hormones increased pollen

germination. Androsterone (1 nM) treatment of maize seedlings alleviated chilling stress-induced oxidative damage by elevating the level of antioxidants in the plant (Erdal 2012a). Similarly, ES application (10 nM) enhanced the level of salt stress tolerance in wheat seedlings (Erdal 2012b).

The only class of naturally occurring steroidal phytohormones are the brassinosteroids (BRs). Apart from their role in plant growth and developmental regulation, BRs are also involved in various environmental stress responses (Kagale et al. 2007; Fridman and Savaldi-Goldstein 2013). Owing to structural and chemical similarities between BRs and estrogens, it can be speculated that estrogens, mammalian hormones, are able to induce similar effects in plants with respect to growth, development and stress responses.

Other plant chemicals, such as secondary metabolites are known to affect both growth and development, as well as defense responses (Vogt 2010). Phenylpropanoids (PPs) such as flavonoids, lignins and tannins belong to the largest and most diverse group of secondary metabolites. It has been reported, that during plant-microbe interaction, the process of lignification is upregulated (Bhuiyan et al. 2009). Similarly, many PP compounds are shown to have antimicrobial properties and are known to accumulate during pathogen and herbivore attacks (Kliebenstein, 2004). Moreover, PP compounds are involved in ameliorating abiotic stresses such as heat and cold stress (Rivero et al. 2001; Stefanowska et al. 2002). In recent years, due to their protective nature, there is a growing interest in researching the PP antioxidant properties in scavenging UV-generated free radicles during oxidative stress in plants (Jansen et al. 2001; Tattini et al. 2004).

One of the key steps in defense during a biotic stress is the induction of PP pathway genes which leads to an increase in PP production, hypersensitive reactions, localized tissue death (chlorosis and necrosis), and several signaling pathways to aid the plant defense mechanisms (e.g., production of lignins, phytoalexins, phenolics, etc.) (Galis et al. 2010). Since Phenylalanine Ammonia Lyase (PAL) is the first committed enzyme of the PP biosynthetic pathway and is needed for the activation of PP biosynthesis, it has been noticed that tobacco and Arabidopsis plants lacking PAL activity were more susceptible to pathogen attack than the corresponding wild type plants (Maher et al. 1994; Huang et al. 2010).

Plant secondary metabolism, specifically accumulation of PPs, is negatively affected by xenobiotic compounds such as pesticides (Lydon and Duke 1989). Relatively less is known about the effects of exogenous estrogens on plant secondary metabolism and biotic stress responses.

In the present study, PP accumulation in ES-treated and untreated Arabidopsis plants was evaluated by employing biochemical studies and gene expression analyses. The effect of the xenoestrogen 17β-estradiol on the plant biotic stress responses against a bacterial pathogen was also observed.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia wild type (WT) seeds (Lehle Seeds, TX, USA) were surface sterilized according to Kagale et al. (2007) and grown in Murashige and

Skoog (1962) nutrient medium solidified with 1% agar and containing increasing ES concentrations (10 nM, 100 nM, 10 μM). Arabidopsis plants germinated on MS plates with 0.01% ethanol were used as control. At day 14 post germination, the seedlings were transferred from MS plates into pots in a Percival growth chamber under long-day conditions at 22°C, 50% humidity, and 200 μmol/m2/s. Foliar spray treatments with above-mentioned ES concentrations were applied to plants once a week until maturity. Tissues were collected at day 21 for biochemical assays and qPCR. All experiments were conducted in triplicates and repeated thrice.

Phenolics estimation

Total phenolics estimation was performed according to Velioglu et al. (1998) with some modifications. Leaf tissues (0.2 g) were extracted in 2 ml acidic methanol (80% methanol with 1% HCl) at room temperature for 2 h. Extracts were centrifuged at 1000 X *g* for 15 min. Supernatants (100 μl) were mixed with Folin-Ciocalteu reagent (0.75 ml) and incubated at 22°C for 5 min. Sodium bicarbonate (0.1 M, 0.75 ml) was added to the reaction mixture, which was allowed to stand at 22°C for another 90 min. The absorbance was measured at 725 nm and total phenolics content was estimated using a *p*-coumaric acid standard curve and expressed as μg mg^{-1} fresh weight.

Flavonoid estimation

Flavonoid estimation was performed according to Chang et al. (2002). Flavonoid extraction from 0.5 g leaf tissues was carried out overnight in 95% ethanol. Flavone and

flavonol contents were estimated by the $AlCl_3$ colorimetric method against a quercetin standard at 415 nm and flavanones were measured by the 2, 4-diphenylhydrazine colorimetric method against a naringenin standard at 495 nm. Flavonoid content was expressed as $\mu g\ mg^{-1}$ fresh weight.

Anthocyanin estimation

Total anthocyanin estimation was performed according to Laxmi et al. (2004). Anthocyanins were extracted from 0.2 g leaves with 3 ml 1% acidic methanol overnight. Phase separation was performed next day by adding 3 ml chloroform and 2 ml water. Absorbance of the aqueous phase was measured at 530 nm and 657 nm. Anthocyanin content was calculated using the formula $\lambda_{530} - \lambda_{657}$ and expressed as per gram fresh weight.

Pathogen inoculation

Pathogen inoculations were performed by infiltration of leaves of six 21-day-old plants for each ES treatments and controls with a suspension (OD_{600} = 0.0002) of the virulent *Pseudomonas syringae* pv. tomato DC3000 (*Pst*) strain in 10 mM $MgCl_2$. To determine bacterial growth, inoculated leaves were harvested at 3-days-post inoculation and 1cm leaf discs were homogenized in 10 mM $MgCl_2$. Diluted leaf extracts were plated on King's B medium supplemented with kanamycin (100 mg/ml) and incubated at 28°C for 2 days before counting the colony-forming units (cfu) as previously described (Xu et al. 2006).

RNA isolation and qPCR

RNA was isolated from leaves of 21 days old ES-treated and untreated plants using the Plant RNA isolation reagent (Life Technologies, CA, USA) following the manufacturer's protocol. RT-PCR was performed using the RETROscript reverse transcription kit (ThermoFisher, USA). Primers were designed using the Primer-3 software. The following primer sequences were used for the PCR analysis: PAL1F: 5´CGGTGTCGCACTTCAGAAGGAA3´, PAL1R: 5´GGATACCGGAAAATCCTTGGAGGAG3´, PAL4F: 5´CCGAGGAACGGACAGTTATGGAG3´, PAL4R: 5´GGGCCAAATATTCCGGCATTCAAG3´, CHIF: 5´CGGCCTCCTCCAATCCATTATTCC3´, CHIR: 5´GCTCCTCCGTAGTTTTTCCCTTCCA3´, ANSF: 5´TGGGTCACTGCAAAATGTGT3´, ANSR: 5´TCACAAAACACAGCCCAAGA3´, PR1F: 5´GGTAGCGGTGACTTGTCTGG3´, PR1R: 5´ACTTTGGCACATCCGAGTCT3´, EF1αF: 5´TTCACCCTTGGTGTCAAGCAGATG3´, EF1αR: 5´TCAGGGTTGTATCCGACCTTCTTCA3´. The real-time PCR experiments were carried out using the iQ SYBR® Green supermix and the BioRAD CFX96 RT-PCR detection system (BioRad, USA) according to the manufacturers' instructions. Reaction parameters were set as follows: initial denaturation at 95°C for 5 mins followed by 35 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C. The relative RNA levels in each

sample were calibrated and normalized against *EF1α* expression that was used as an internal control.

Statistical Analysis

Data are the means ± SD of three independent replicates. Data were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared using Tukey's test. Comparisons with $P < 0.05$ were considered significantly different.

Results

Estradiol application results in decreased accumulation of phenylpropanoids

To determine the effect of ES on secondary metabolite production, total leaf phenolics, flavonoids and anthocyanins in Arabidopsis leaves were estimated (Fig. 3.1a, b, c). A decline in PP synthesis in response to increasing ES treatments was observed. Total PP contents were found to be the lowest in 10 µM ES-treated plants in comparison with the 10 and 100 nM ES-treated and control Arabidopsis plants (Fig. 3.1a). The low accumulation of total phenolics in response to ES treatment is reflected in the total flavonoid and anthocyanin contents of ES-treated and untreated Arabidopsis plants (Fig. 3.1b, c).

Decreased expression of the phenylpropanoid pathway genes by estradiol application is transcriptionally controlled

The decrease in PP accumulation as a result of ES treatments suggests that ES may have a negative impact on the transcription of key genes in the PP biosynthesis pathway in *Arabidopsis*. This was found to be the case, as the expression of *PAL4*, *CHI*, *ANS*, and *PAL1*, was downregulated in ES-treated plants (Fig. 3.2). The expression of *PAL1*, *PAL4* and *ANS* was found to be nearly two-fold higher in the control plants than that observed in the ES-treated plants. The expression of *CHI* was found to be significantly lower in 10µM ES-treated plants only. qPCR analysis of the RNA isolated from ES-treated and control plants demonstrated that the ES induced downregulation in PP pathway genes transcription is dose-dependent for *PAL1*, *PAL4* and *ANS* (Fig. 3.2).

Estradiol application results in compromised resistance against bacterial pathogen

The growth of a virulent bacterial pathogen, *Pseudomonas syringae* pv. tomato DC3000 (*Pst*), was studied to see the effects of ES-induced low PP levels on plant defense responses. At a low dose of inoculation (OD600 = 0.0002), the ES-treated plants were significantly more susceptible to the pathogen and displayed both enhanced disease symptoms and bacterial growth than control plants (Fig. 3.3 a, b). The compromised resistance to *Pst* correlated with the reduced expression level of *PATHOGENESIS RELATED1* (*PR1*) in the ES-treated plants (Fig. 3.4). In addition, the ES-treated plants accumulated the *PAL1* and *PAL4* transcripts at a significantly lower level than that in the controls in response to *Pst* infection (Fig. 3.5a, b).

Discussion

In this study, it was observed that ES treatment of Arabidopsis plants resulted in the downregulation of PP pathway genes, which was accompanied by a lowered accumulation of PPs. Estimations of PPs as total flavonoids, phenolics and anthocyanins revealed significantly lower concentrations of these compounds in the ES-treated plants compared to controls as a result of the downregulation of PP pathway genes.

Phenolic compounds are required for normal plant growth and development and also play an important role in protecting the plants against stress and pathogen attacks (Bhattacharya et al. 2010). Plant secondary metabolism is both enhanced and repressed in response to exposure to various chemicals. Inhibition of PP pathway enzymes by exogenous application of chemicals results in lowered accumulation of PP products in plants. Studies on the effect of xenobionts revealed that addition of 2-aminoindane-2-phosphonic acid, a chemical that inhibits PAL activity, to tissue culture media lead to significant decrease in the accumulation of flavonoids and total phenolics in *Ulmus americana* (Jones et al. 2012), *Artemisia annua* and *Acer saccharum* (Jones and Saxena 2013). Conversion of the PAL product trans-cinnamic acid to p-coumaric acid by C4H was inhibited by the addition of piperonylic acid (PIP) to *Nicotiana tabaccum* culture cells (Schoch et al. 2002) leading to decreased accumulation of PP pathway products. The inhibitory effects of PIP were reversed by the exogenous application of caffeic acid (hydroxycinnamic acid) in soybean. However, the addition of methylene dioxocinnamic acid, inhibitor of 4CL (enzyme responsible for the conversion of p-coumaric acid to

coumaryl-CoA) even in the presence of caffeic acid led to the downregulation of the PP pathway genes (Bubna et al. 2011). Similarly, inhibition of PP pathway biosynthetic enzymes by herbicides such as glyphosate and alachlor led to decreased accumulation of PP pathway derived flavonoids and phenolic compounds (Lydon and Duke 1989). Based on above-mentioned studies, it is evident that inhibition or reduction of early PP pathway biosynthetic enzyme activities has a drastic impact on the accumulation of the downstream PP pathway products.

The effect of xenoestrogens on plant secondary metabolism has not been studied before. Brassinosteriods are the only plant steroid hormones and they are structurally similar to ES. It has been seen that ES application has a protective effect on plants under oxidative stress (Erdal 2012b), which is similar to the effect of BRs on plant stress responses (Kagale et al. 2007). It has been reported that exogenous application of BRs resulted in the stimulation of PP biosynthetic pathway, resulting in increased accumulation of PPs in plants (Ahammed et al. 2013; Koca and Karaman 2015). Whereas in the present study, which is the first report of the impact of exogenous ES on plant secondary metabolism, it was observed that ES application resulted in decreased of the total phenolics, anthocyanin and flavonoid accumulation in *Arabidopsis*. These observations suggest that lower concentrations of ES inhibit the accumulation of PPs and higher concentrations (10 µM ES) is even toxic to Arabidopsis.

The observed reduced accumulation of PPs induced by ES application in Arabidopsis may be a xenobiotic effect and/or ES may act as a transcriptional regulator of the PP pathway genes in similar mechanistic way as in mammals. A downregulation in the

expression of *PAL1*, *PAL4*, *CHI* and *ANS*, whose products control the flux through the PP biosynthesis pathway, was observed in the ES-treated Arabidopsis seedlings. PAL1 is the first committed enzyme of the PP pathway and CHI and ANS represent important branch-points within the pathway with respect to the production of PPs (Dixon et al. 2002). However, further experimentation would be needed to establish if ES could act as transcriptional regulator in plants.

It has been reported that *PAL1* expression is required for basal resistance against the virulent pathogen *Pseudomonas syringae* DC3000 (Huang et al. 2010). Overexpression of pepper *PAL1* gene in Arabidopsis resulted in increased resistance against *Pst* DC3000 and *Hyalopernospora arabidopsidis* infections. In addition, a positive correlation was found between *PAL1* expression and *PR1* (*PATHOGENESIS RELATED1*) expression and SA accumulation in the infected leaf tissue (Kim and Hwang 2014). Treatment of tobacco plants with the polyphenolic flavonol fisetin hydrate resulted in significantly lower disease severity induced by bacterial pathogen *Pectobacterium carotovorum* (Song et al. 2013). Transgenic expression of tryptophan decarboxylase gene in potato led to a decrease in the accumulation of PPs and the transgenic plants were more susceptible to the fungal pathogen *Phytophthora infestans* (Yao et al. 1995). In another study, mutant Arabidopsis plants accumulating low levels of PP were found to be susceptible to *Botrytis cinerea* (Kliebenstein et al. 2005). Plants are known to induce or suppress the levels of flavonoids in response to pathogens (Dixon 2001). Both PAL activity and PP accumulation were stimulated by *Pseudomonas fluorescens* infection in tomato plants (Kandan et al. 2002). In our study, ES application resulted in reduced *PAL1* expression

levels and PP accumulation, rendering Arabidopsis plants susceptible to a virulent pathogen. ES-treated Arabidopsis plants showed a more severe phenotype in response to *Pst* inoculation and the bacterial growth 3 DPI was found to be significantly higher in ES-treated plants than the controls. The overall PP levels in the infected plants appear to be negatively correlated with *Pst* growth and it was observed that 10µM ES-treated plants showing lowest total phenolics, flavonoids and anthocyanins, are the most severely impacted by *Pst* inoculation. This observation in conjunction with literature reported results on plant-pathogen interactions (Huang et al. 2010, Song et al. 2013, Kim and Hwang 2014) suggests an important role for the PPs, whose flux is controlled by *PAL1*, in providing resistance against bacterial pathogens in plants. Another important observation from our study was the upregulation of *PAL1* and *PAL4* expressions in response to pathogen inoculation. However, there were no significant differences found in *PAL1* and *PAL4* transcript levels between the mock-treated and ES-treated pathogen inoculated plants. Lowered expression of *PAL1* and *PAL4* was associated with *PR1* downregulation in ES-treated pathogen inoculated plants. Increased *PR1* expression in response to pathogen infection is an important indicator of mounting plant defense responses in addition to SA accumulation (Kim and Hwang 2014). As reported previously by (Kim and Hwang 2014), it was observed in the current study that there is a direct correlation between the levels of *PAL1*and *PR1* expression. These results suggest that compromised resistance against *Pst* in ES-treated Arabidopsis plants may be due to compromised plant defense responses as well as lowered accumulation of PPs.

In conclusion, our results demonstrate a unique effect of ES on plant PP metabolism. ES application in Arabidopsis resulted in compromised resistance against a bacterial pathogen. This decreased resistance may be a direct result of lowered expression of PP biosynthesis pathway genes which in turn led to a reduction in production of PPs. Based on these findings, we suggest that ES is acting as a xenobiont that is regulating the PP pathway at a transcriptional level and impacting PP biosynthetic pathway in *Arabidopsis*. However, further studies are required to better understand the underlying molecular mechanism of ES effects on secondary metabolism and stress responses.

The impact of mammalian sex hormones in the plant secondary metabolism and associated stress responses has not been studied in great detail. These are novel findings that contribute to our current knowledge of plant responses to xenoestrogens. Thus, these results will serve as a primer to study the effects of animal steroidal hormones and xenobionts on crop plants for the purpose of improving agricultural yield and productivity.

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References

Ahammed GJ, Zhou YH, Xia XJ, Mao WH, Shi K, Yu JQ (2013) Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene. Biol Plant 57: 154-158.

Bhattacharya B, Gupta K (1981) Steroid hormone effects on growth and apical dominance of sunflower. Phytochemistry 20: 989-999.

Bhattacharya A, Sood P, Citovsky V (2010) The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. Mol Plant Pathol 11: 705–719.

Bhuiyan NH, Selvaraj G, Wei Y, King J (2009) Role of lignification in plant defense. Plant Signal Behav 4: 158-159.

Bubna GA, Lima RB, Zanardo DY, Dos Santos WD, Ferrarese MD, Ferrarese-Filho O (2011) Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (*Glycine max*). J Plant Physiol 168: 1627-1633.

Chang C, Yang M, Wen H, Chern J (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 10: 178–182.

Dixon RA (2001) Natural products and plant disease resistance. Nature 411: 843-847.

Dixon RA, Achnine L, Kota P, Liu CJ, Reddy MS, Wang L (2002) The phenylpropanoid pathway and plant defense: A genomics perspective. Mol Plant Pathol 3: 371–390.

Erdal S (2012a) Androsterone-induced molecular and physiological changes in maize seedlings in response to chilling stress. Plant Physiol Biochem 57: 1–7.

Erdal S (2012b) Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J Sci Food Agric 92: 1411–1416.

Fridman Y, Savaldi-Goldstein S (2013) Brassinosteroids in growth control: how, when and where. Plant Sci 209: 24-31.

Galis I, Onkokesung N, Baldwin IT (2010) New insights into mechanisms regulating differential accumulation of phenylpropanoid-polyamine conjugates (PPCs) in herbivore-attacked *Nicotiana attenuata* plants. Plant Signal Behav 5: 610–613.

Hanselman TA, Graetz DA, Wilkie AC (2003) Manure-borne estrogens as potential environmental contaminants: a review. Environ Sci Technol 37: 5471-5478.

Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou Y-H, Yu J-Q, Chen Z (2010) Functional analysis of the Arabidopsis PAL gene family in plant growth, development, and response to environmental stress. Plant Physiol 153: 1526-1538.

Janeczko A, Filek W, Biesaga-Koscielniak J, Marcinska I, Janeczko Z (2003) The influence of animal sex hormones on the induction of flowering in Arabidopsis *thaliana*: comparison with the effect of 24-epibrassinolide. Plant Cell Tissue Organ Cult 72: 147–151.

Janeczko A, Skoczowski A (2005) Mammalian sex hormones in plants. Folia Histochem Cytobiol 43: 71–79.

Jansen MAK, Van den Noort RE, Tan MYA, Prinsen E, Largimini LM, Thorneley RNF (2001) Phenol-oxidizing peroxidases contribute to the protection of plants from Inhibition ultraviolet radiation stress. Plant Physiol 126**:** 1012–1023.

Jones AM, Chattopadhyay A, Shukla M, Zoń J, Saxena PK (2012) Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in American elm (*Ulmus americana*). BMC Plant Biol 12: 1. doi: 10.1186/1471-2229-12-75.

Jones AM, Saxena PK (2013) to reduce oxidative browning in plant tissue culture. PLoS One 8(10):e76802. doi: 10.1371/journal.pone.0076802

Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P (2007) Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. Planta 225: 353–364.

Kandan A, Commare RR, Nandakumar R, Ramaih M, Raguchander T, Samiyappan R (2002) Induction of phenylpropanoid metabolism by *Pseudomonas fluorescens* against tomato spotted wilt virus in tomato. Folia Microbiol 47: 121-129.

Kim DS, Hwang BK (2014) An important role of the pepper phenylalanine ammonia-lyase gene (PAL1) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J Exp Bot 65: 2295-306.

Kliebenstein D, Rowe HC, Denby KJ (2005) Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J 44: 25-36**.**

Koca N, Karaman S (2015) The effects of plant growth regulators and L-phenylalanine on phenolic compounds of sweet basil. Food Chem 166: 515-521.

Kopcewicz J (1970) Influence of estrogen on the flower formation in *Cichorium intybus*. L Naturwissenschaften 57: 136–137.

Laxmi A, Paul LK, Peters JL, Khurana JP (2004) *Arabidopsis* constitutive photomorphogenic mutant, *bls1*, displays altered brassinosteroid response and sugar sensitivity. Plant Mol Biol 56: 185–201.

Lydon J, Duke SO (1989) Pesticide effects on secondary metabolism of higher plants. Pest Sci 25: 361-73.

Maher EA, Bate NJ, Ni W, Elklnd Y, Dixon RA, Lamb CJ (1994) Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proc Natl Acad Sci USA 91: 7802-7806.

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol Plant 15: 473–497.

Rivero RM, Ruiz JM, Garcia PC, López-Lefebre LR, Sánchez E, Romero L (2001) Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. Plant Sci 160: 315–321.

Schoch GA, Nikov GN, Alworth WL, Werck-Reichhart D (2002) Chemical inactivation of the cinnamate 4-hydroxylase allows for the accumulation of salicylic acid in elicited cells. Plant Physiol 130: 1022-1031.

Shore LS, Kapulnik Y, Ben-Dor B, Fridman Y, Wininger Y, Shemesh M (1992) Effects of estrone and 17-β-estradiol on vegetative growth of *Medicago sativa*. Physiol Plant 84: 217–222.

Shore LS, Shemesh M (2003) Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. 75: 1859–1871.

Song GC, Ryu SY, Kim YS, Lee JY, Choi JS, Ryu CM (2013) Elicitation of induced resistance against *Pectobacterium carotovorum* and *Pseudomonas syringae* by specific individual compounds derived from native Korean plant species. Molecules 18: 12877-12895.

Stefanowska M, Kuras M, Kacperska A (2002) Low temperature-induced modifications in cell ultrastructure and localization of phenolics in winter oil seed rape (*Brassica napus* L. var. *oleifera* L.) leaves. Ann Bot 90: 637–645.

Stumpe B, Marschner B (2009) Factors controlling the biodegradation of 17beta-estradiol, estrone and 17alpha-ethinylestradiol in different natural soils. Chemosphere 74: 556–562.

Tattini M, Galardi C, Pinelli P, Massai R, Remorini D, Agati G (2004) Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol 163: 547–561.

Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 46: 4113–4117.

Vogt T (2010) Phenylpropanoid biosynthesis. Mol Plant 3: 2-20.

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell 18: 1310–1326.

Yao K, De Luca V, Brisson N (1995) Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. Plant Cell 7: 1787-1799.

Ylstra B, Touraev A, Brinkmann AO, Heberle-Bors E, Tunen A (1995) Steroid hormones stimulate germination and tube growth of in vitro matured Tobacco pollen. Plant Physiol 107: 639-643.

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Fig. 3.1 Estradiol negatively impacts production of phenolics in *Arabidopsis*. (a) Total phenolic content of 21 days old, control and estradiol-treated Arabidopsis leaves. (b) Estradiol application results in reduced flavonoid biosynthesis in 21 days old, non-treated and estradiol-treated Arabidopsis. Flavonoid content is presented as sum of naringenin and quercitin equivalents. (c) Estradiol application results in reduced anthocyanin production in 21 days old, control and estradiol-treated Arabidopsis leaves. Different alphabets represent statistically significant difference (means ± SD; $P<0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments.

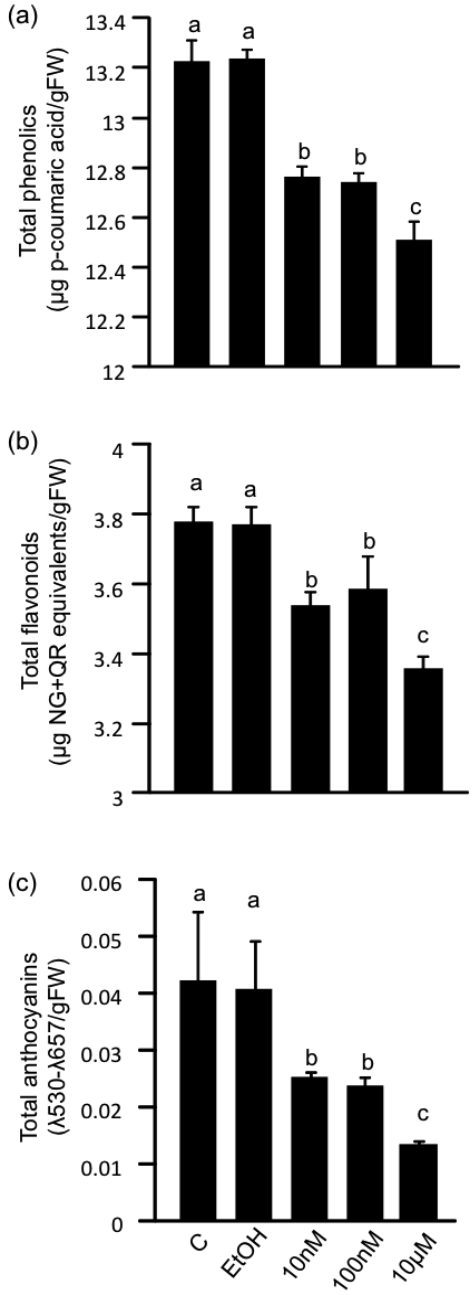
Fig. 3.2 Reduced expression level of the phenylpropanoid pathway genes (a) *PAL1*, (b) *PAL4*, (c) *CHI*, and (d) *ANS* due to estradiol application is transcriptionally regulated.

RNA was isolated from 21-day-old, control and estradiol-treated Arabidopsis leaves. Different alphabets represent statistically significant differences (means ± SD; $P<0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments.

Fig. 3.3 Estradiol application results in increased susceptibility to *P. syringae* pv. DC3000 in Arabidopsis. (a) Bacterial growth in control and estradiol-treated Arabidopsis plants at 0 and 3 DPI. (b) Representative image of pathogen inoculated Arabidopsis leaves at 3 DPI showing pathogen induced chlorosis. Results are means ± SD ($P<0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments.

Fig. 3.4 *PR1* expression in estradiol-treated and control *Arabidopsis* plants inoculated with *P. syringae* pv. DC300 or $MgCl_2$ as mock. Asterisk represents statistically significant difference (means ± SD; $P<0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments.

Fig. 3.5. *PAL1* (a) and *PAL4* (b) expression in estradiol-treated and control Arabidopsis plants inoculated with *P. syringae* pv. DC300 or $MgCl_2$ mock. Results are cumulative of three independent experiments. Asterisk represents statistically significant difference (means ± SD; $P<0.05$, ANOVA, $n = 9$). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol treatments.



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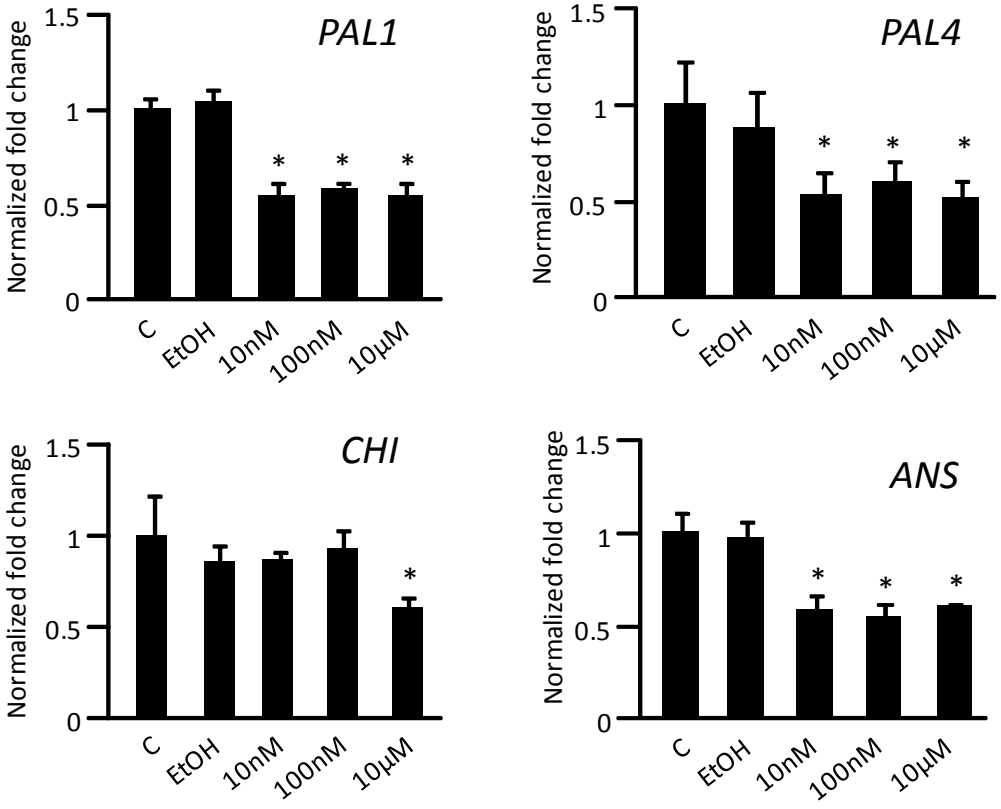

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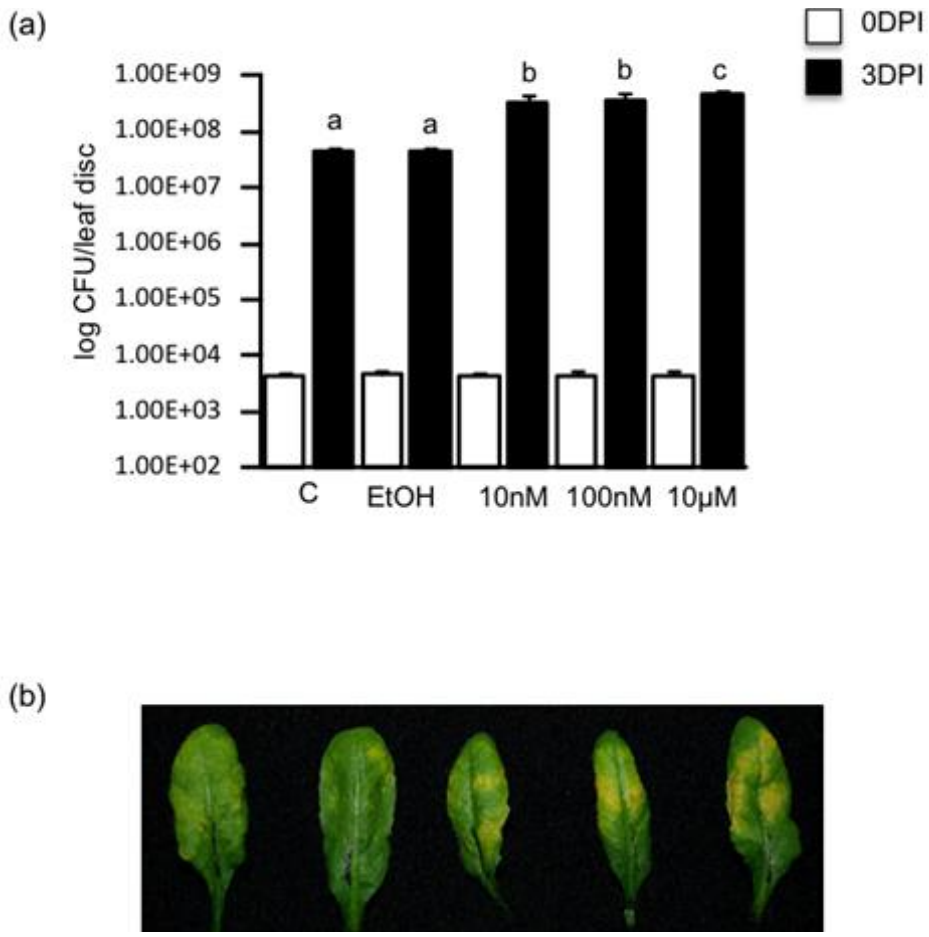

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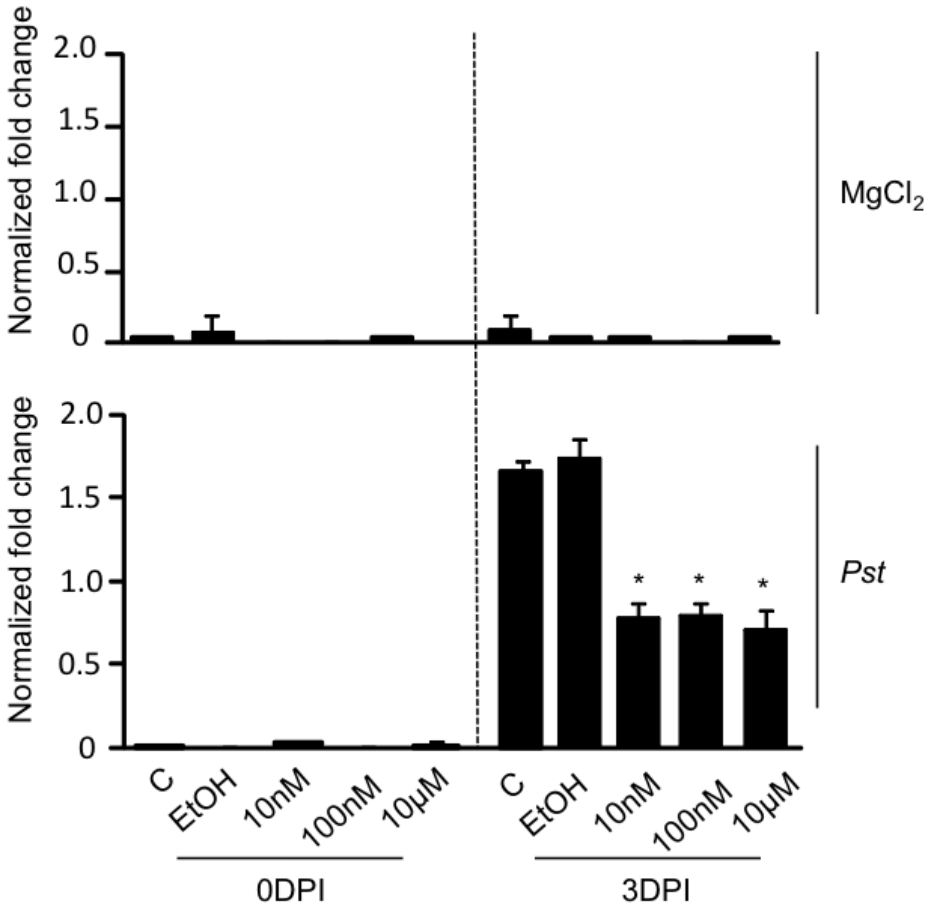

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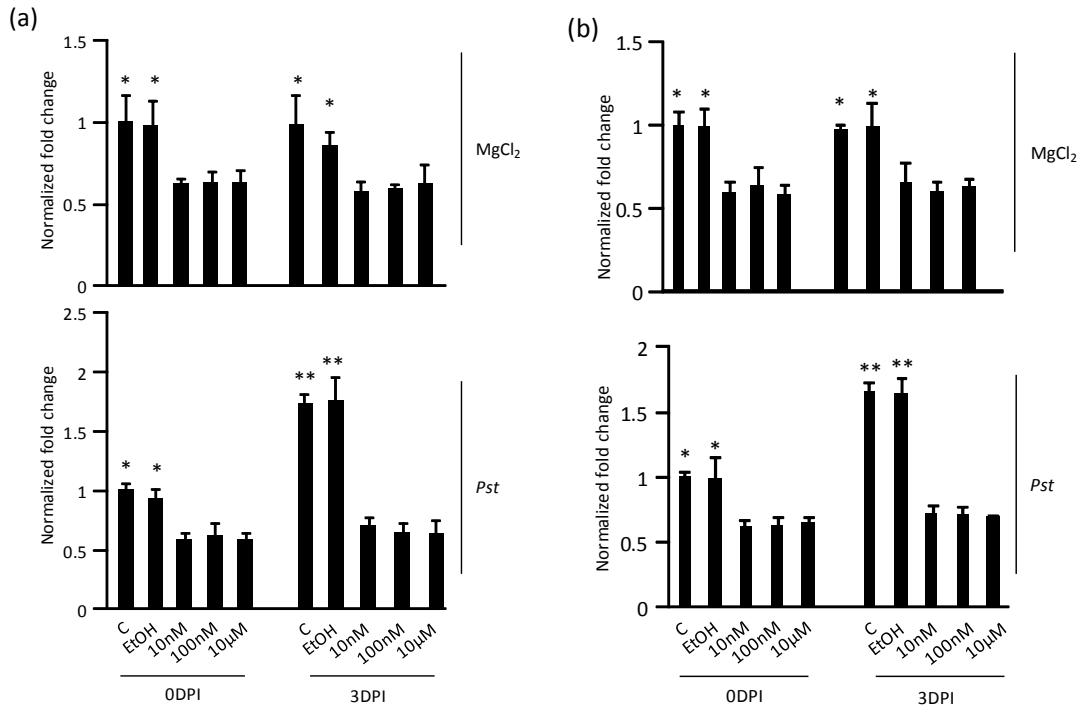

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CHAPTER IV

APPLICATION OF 17β-ESTRADIOL CONFERS TOLERANCE AGAINST DROUGHT STRESS IN *ARABIDOPSIS THALIANA*

A Paper to be Submitted for Publication in the

International Journal of Plant Biology and Research

Pallavi Upadhyay

Abstract

Animal steroidal hormones, including estrogens, are being introduced into the agricultural soil and water supply from increased pharmaceutical and farm waste. The goal of the study was to understand the effect of estradiol (ES) application on Arabidopsis drought stress responses. Plants treated with increasing estradiol concentrations (10 nM, 100 nM) and subjected to drought stress conditions by withholding water for 7 days resulted in the upregulation of stress reponsive genes and increased tolerance to drought stress. Higher concentrations of ES (10 µM) rendered the plants susceptible to drought stress. These plants had the lowest survival rate among all ES-treated plants. Results from the study will have practical agricultural applications regarding the effect of environmental estrogens and xenoestrogens on crop plants.

Keywords: Xenoestrogens, *Arabidopsis*, Drought stress, Glutathione, H_2O_2, Proline

Introduction

Environmental contamination with mammalian sex hormones (MSH) is an ecological concern. Increasing levels of MSH are being introduced into the agricultural soil and water supply through farm and pharmaceutical wastes. Despite the fact that MSH act as endocrine disruptors in aquatic animals, a number of studies have demonstrated the beneficial role of MSH in plants, which help coping with abiotic stress in plants (1-7).

Drought is an important manifestation of abiotic stresses in plants, which tends to be a primary production-limiting factor in crop plants. Drought aggravates the effect of the other biotic and abiotic plant stress responses. In addition, common consequence of various abiotic stresses (e.g., drought stress, salt stress, heavy metal stress, etc.) is the oxidative damage to the cell and production of reactive oxygen species (ROS). Studies have a shown a dircect correlation between the stimulated levels of the antioxidant system and drought tolerance in the plant species (8, 9).

Treatment of bean, *Phaseolus vulgaris* seeds with varying concentrations of MSH (progesterone, β-estradiol and androsterone) resulted in increased activity of antioxidant enzymes such as superoxide dismutase (SOD), peroxidase (POX) and catalase (CAT), which are known to be active in drought stress. At the same time, a decreased lipid peroxidation and H_2O_2 levels was observed in the MSH treated bean plants, attributed to an increase in antioxidant enzyme activity which correlated to increased resistance to stress in bean plants (1).

MSH application can impact the inorganic element constitution in plant cells was also evident from the study that showed progesterone application in bean seedlings

ameliorated the effects of salt stress by increasing the K^+/Na^+ ratio in the hormone treated plants in comparison to untreated plants grown in artificial medium supplemented with 100mM NaCl (3). In the same study, it was reported that progesterone treated bean plants under salt stress had higher activity of SOD, POX and CAT enzymes and displayed lower levels of lipid peroxidation as compared to the non MSH treated plants (3). MSH (progesterone, β-estradiol and androsterone) application mitigated the effects of salinity stress, such as lower germination rate, decreased root and shoot length, in maize (4) and wheat seedlings (5). The protective effect of MSH on plants has also been reported in the case of chilling stress (6). Foliar application of progesterone to chickpea plants exposed to chilling stress resulted in enhanced antioxidant enzyme activity, chlorophyll content and relative leaf water content in comparison to untreated plants exposed to chilling stress (6).

In the presence of Cd or Cu, retardation in the embryo germination and growth was observed in lentil. However, the addition of 17β-estradiol (ES) to the seed germination medium protected the lentil seedlings from the negative effects of heavy metal stress post germination. The ES-treated seedlings displayed significantly lower solute leakage in the presence of heavy metals as compared to non-treated seedlings thus protecting the plants against nutrient loss (7).

Plants employ a number of strategies to cope with drought stress. One of the most important antioxidant molecules used by plant cells is glutathione (GSH), a tripeptide composed of the amino acids glutamic acid, cystine and glycine (γ-L-Glutamyl-L-cystineglycine). GSH is the reduced form of the molecule, which during abiotic stresses,

upon reaction with oxidizing agents is converted to GSSG (10)(Zagorchev et al., 2013). Plant cells usually maintain a higher concentration of GSH as compared to GSSG and it has been reported in a number of cases that glutathione levels (GSH/GSSG ratio) rapidly changes in response to water deficit and help maintain the redox homeostasis of the cell (11).

Generation of reactive oxygen species (ROS) by the plant cell is a response common to a number of stress conditions. Hydrogen peroxide (H_2O_2) is a unique ROS species that plays a dual role within the plant cells (10). High levels of H_2O_2 generated during abiotic stress (including drought stress) can damage cellular structures and biomolecules but a low concentration of H_2O_2 is maintained within the cells to act as a signaling molecule that controls plant growth and development (13, 14).

Accumulation of proline in response to stress has been reported in both plants and animals. Under stress conditions that cause water deficit within the cell, high concentrations of proline can protect membrane structures, stabilize the quaternary structure of proteins and even scavenge the hydroxyl radical (15).

In the present study, we demonstrate that exogenous application of ES to Arabidopsis plants results in enhanced tolerance to drought stress which correlates with increase in the levels of glutathione, H_2O_2 and proline. However, higher concentrations of ES (10 µM) proved to be phytotoxic as these plants were more susceptible to drought stress as compared to the controls.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia wild type (WT) seeds (Lehle Seeds, TX, USA) were surface sterilized according to the procedure described previously (16; 17) and grown in Murashige and Skoog (18) nutrient medium solidified with 1% agar and amended with either increasing concentrations of ES (10 nM, 100 nM, 10 μM). Arabidopsis seedlings germinated on MS plates without ES or with 0.01% ethanol (solvent for ES) were used as controls. At day 14, the seedlings were transferred from MS plates into pots in a Percival growth chamber under long-day conditions at 22°C, 50% humidity, and 200 μmol/m^2/s and were sprayed with ES once a week untill maturity.

Drought stress

Drought stress studies were performed according to the procedure employed by Kagale et al. (17) with slight modifications. ES-treated and untreated, non-stressed plants were watered every third day by subirrigation. Drought conditions in the 21-day-old plants were introduced by withholding water for 7 days. At the end of the treatment, leaf tissue was collected for biochemical and gene expression analysis. Following the drought stress, watering was resumed for a week. Plants that survived were counted, and the percentage survival rate for each treatment was calculated. These experiments were repeated thrice, each time with 30 plants.

Proline estimation

Estimation of proline was conducted according to a previously published protocol by Bates *et al.* (19) in Arabidopsis leaves and expressed as µmoles/g fresh weight.

Glutathione estimation

Total glutathione in Arabidopsis leaves was estimated using the Glutathione Assay Kit (Sigma Aldrich, USA) following the manufacturers specifications and expressed as µmoles/g fresh weight.

Hydrogen peroxide estimation

Hydrogen peroxide in Arabidopsis leaves was estimated using the Amplex® Red Hydrogen Peroxide/Peroxidase Kit (Thermofischer Sci, USA) and expressed as µmoles/g fresh weight.

RNA isolation and qRT-PCR

RNA was isolated from leaves of ES-treated and untreated drought stressed and unstressed 21-day-old plants using the Plant RNA isolation reagent (Life Technologies, USA) following the manufacturer's protocol. RT-PCR from the total RNA extracted was performed using the RETROscript reverse transcription kit (Thermo Fisher Sci, USA). Primers were designed using the Primer-3 software. The following primer sequences were used for the PCR analysis: (*GSTU*, Glutathione S Transferase) GSTU3F: 5´CAATGGCCGAGAAAGAAGAG3´, GSTU3R:

5´AAGTAGCAACGGGCTCTTGA3´, (*GEr5*, Gem-related 5) GER5F: 5´CATCGGAATGTTCCATACCTGGAGT3´, GER5R: 5´TTGGCTCTGTTCCGAAAATCTGTCT3´, (*HSP70*, Heat Shock Protein 70) HSP70bF: 5´AGGATAAAACCGCTGGTGTG3´, HSP70bR: 5´ATTCTTGGCCTCCACCTTCT3´, (*HSP101*, Heat Shock Protein 101) HSP101F: 5´GCCAAGTGTGCCTGACACCATTAGT3´, HSP101R: 5´GCTTTATCCGGTAAATGCCGACCA3´, (*EF1α*, Eukaryotic Elongation Factor α) EF1αF: 5´TTCACCCTTGGTGTCAAGCAGATG3´, EF1αR: 5´TCAGGGTTGTATCCGACCTTCTTCA3´. The real-time PCR experiments were carried out using the iQ SYBR® Green supermix and the BioRAD CFX96 RT-PCR detection system (BioRad, USA) according to the manufacturers' protocol. Reaction parameters were set as follows: initial denaturation at 95°C for 5 mins followed by 35 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C. The relative RNA levels in each sample were calibrated and normalized against *EF1α* expression that was used as an internal control.

Statistical Analysis

Data are the means ± SD of three independent replicates. Data were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared using Tukey's test. Comparisons with $P < 0.05$ were considered significantly different.

Results

Estradiol application enhances drought stress tolerance in *Arabidopsis*

When plants were 21 days old, drought stress conditions were generated by withholding irrigation for 7 days, after which ES-treated and control plants developed the drought characteristic stress symptoms of wilting, chlorosis and tissue senescence. However, 10 and 100 nM ES-treated plants coped better than controls and 10 µM ES-treated plants in terms of tissue senescence and chlorosis development (Fig. 4.1a). Following the drought stress period, watering was resumed and the percentage of surviving plants was determined. Significantly higher tolerance to drought stress was observed in 10 and 100 nM ES-treated plants with 74% and 78% of the plants surviving drought stress, respectively, as compared to 40% regularly watered control plants and to 36% EtOH control plants. The lowest tolerance to drought stress was recorded for 10µM ES-treated plants with an 18% survival rate (Fig. 4.1b).

Estradiol application enhances glutathione levels in *Arabidopsis* during drought stress

Under simulated drought conditions, an increase in glutathione levels was observed in all Arabidopsis plants irrespective of the treatment. However, significantly higher levels of glutathione were accumulated by Arabidopsis plants treated with 10 nM and 100 nM ES than the control plants. The lowest levels of glutathione were estimated in leaves of plants treated with 10µM ES under drought stress and even under non-stress conditions (Fig. 4.2).

Estradiol treatment enhances hydrogen peroxide levels in *Arabidopsis* leaves during drought stress

H_2O_2 can scavenge high energy electrons generated during abiotic stresses and thus protect plant cells, however high levels of H_2O_2 can contribute towards oxidative stress. It was observed that, under non-stress conditions, the Arabidopsis plants treated with 10 µM ES accumulated significantly higher levels of H_2O_2, as compared to the controls and other ES treatments, indicating increased levels of drought-induced oxidative stress in these plants. The levels of H_2O_2 in control and 10 and 100 nM ES-treated plants under non-stress were not significantly differents (Fig. 4.3). Plants treated with 10 and 100 nM ES accumulated the highest levels of H_2O_2 under drought conditions (Fig. 4.3).

Estradiol application enhances proline accumulation in *Arabidopsis* during drought stress

Proline, an essential amino acid, is a compatible osmolyte, which protects cellular structures and biomolecules during drought stress. Leaves of both 10 and 100 nM ES-treated Arabidopsis plants accumulated significantly higher levels of proline under drought stressed than the unstressed plants. As observed with glutathione levels, the lowest proline levels were accumulated by plants treated with 10 µM ES (Fig. 4.4).

Estradiol application upregulates stress-related gene expression

Real time-PCR was performed to assess the expression levels of four stress responsive genes known to play an important role in providing the plant cell with

protection during drought conditions. Enhanced expression of all the studied genes, *GLUTATHIONE S-TRANSFERASEU3* (*GSTU3*), *GEm-RELATED5* (*GER5*), *HEAT SHOCK PROTEIN101* (*HSP101*) and *HSP70b* was observed 7 days post drought stress (Fig. 4.5 a, b). Under drought stress, the expression of *GSTU3* was 3.5 fold higher in the drought stressed 10 and 100 nM ES-treated plants than the non-stress control leaves. Expression levels of *GER5*, in drought stressed 10 and 100 nM ES-treated plants, was determined to be nearly four folds higher than in the control leaves. Expression of both *HSP101* and *HSP70b* was induced by drought stress and the highest expression was observed in 10 and 100 nM ES-treated drought stressed leaves at 2.5 and 2-fold higher than the control leaves, respectively. The application of 10 and 100 nM ES, under non-drought conditions, also resulted in enhanced expression of *GSTU3* and *GER5* (Fig. 4.5a, b).

Discussion

In this paper the effect of 17β-estradiol application on *Arabidopsis* response to drought stress is demonstrated. To the best of our knowledge this is the first study reporting on the changes in stress related gene expression in ES-treated plants. A previous study employing microarray analysis revealed that expression of stress responsive genes was upregulated by ES application in Arabidopsis seedlings (20). As a validation of those results, a quantitative PCR analysis of *GSTU3*, *GER5*, *HSP70b* and *HSP101* expression in ES-treated and untreated plants in this study showed that the expression of these stress responsive genes is elevated in response to drought stress.

The glutathione S-transferase enzymes play an important role in plant stress resistance as they facilitate the binding of GSH to xenobiotic substrates (21, 22). Transgenic expression of a tomato GST, *LeGSTU2*, resulted in enhanced drought stress tolerance in Arabidopsis (23). *GSTU3* displays increased expression in response to abiotic stress conditions (24). Similarly, the expression of *GER5* is induced by ABA and abiotic stress and is required for growth and development in Arabidopsis (25). Significantly higher expression of both *GSTU3* and *GER5* was observed in drought treated 10 and 100 nM ES-treated plants which may have contributed to their enhanced tolerance towards drought stress. The 10 and 100 nM ES treatments may have primed the plants for increased tolerance as the non-stressed plants displayed elevated expression of *GSTU3* when compared to the other treatments.

Heat Shock Proteins (HSPs) belong to a specialized class of chaperone proteins whose expression is induced in response to a number of abiotic stresses (26). In *Arabidopsis*, both *HSP70b* and *HSP101* are required for developing thermotolerance against heat stress (27; 28). Even in the absence of heat stress, expression of both *HSP70b* and *HSP101* was elevated in response to drought stress and was found to be significantly higher in 10 and 100 nM ES-treated plants. In a manner similar to the expression of *GSTU3*, the expression of *HSP101* was elevated in non-stressed 10 and 100 nM ES-treated plants. These results are similar to the effect of brassinosteroid application on Arabidopsis plants where treatment with the hormone resulted in increased tolerance to drought stress and elevated expression of *HSP* genes (17). Since HSPs are required for proper protein folding and function in response to abiotic stresses it can be suggested that

application of ES can potentially enhance the ability of plants to cope with other forms of abiotic stress.

In response to drought stress, significantly higher accumulation of GSH, H_2O_2 and proline was observed in 10 and 100 nM ES-treated plants as compared to the control plants, which could explain the increased stress tolerance of these plants. Rice (*Oryza sativa* var. *indica*) seedlings grown under water deficit conditions showed a consistent decline in GSH concentrations for the drought sensitive lines as opposed to the drought resistant varieties (29). However, in case of wheat (*Triticum* sp.) plants grown under drought conditions, GSH concentration increased in the flag leaves of both drought resistant and sensitive varieties (30). Generation of drought stress by the application of PEG-6000 to the seedlings of *Brassica campestris* and *Brassica juncea* resulted in increased GSSG levels (21, 31 and the references within). *B. juncea* seedlings, in the presence of PEG-6000, displayed elevated levels of GSH as well (21). A similar increase in proline levels was observed in plants that were exposed to drought conditions (32). Studies involving Arabidopsis and rice demonstrate that accumulation of proline as a compatible osmolyte is an essential response of the plant cell to drought stress (33, 34). The beneficial effect of ES treatment in coping with drought stress is evident from the observation that 10 and 100 nM ES-treated plants accumulated the highest levels of both GSH and proline when subjected to drought stress. The abiotic stress resistance afforded by GSH, proline and H_2O_2 becomes apparent in the high survival rate of 10 and 100 nM ES-treated plants when recovering from drought stress. High levels of H_2O_2 can by itself result in oxidative damage to the cells (10) but it has been demonstrated in a number of

plants species, including maize (35), mustard (36), tall fescue and rye grass (37), that an increase in H_2O_2 levels primes the plants towards enhanced drought stress resistance. Significant increases in H_2O_2 levels from those in plants under non-drought conditons were observed in drought stressed Arabidopsis plants treated with 10 and 100 nM ES. This increase was in response to drought stress and not to the ES treatment and it can be speculated that H_2O_2 contributed to the enhanced drought stress tolerance of these plants.

The most anomalistic responses to ES application were observed for plants treated with 10 µM ES. These plants displayed the lowest survival rate after the induced drought stress. The levels of GSH and proline accumulated by these plants in response to drought stress were the lowest among all the treatments, which can explain their poor performance in coping with the stress conditions. However, the 10 µM ES-treated plants accumulated significantly higher levels of H_2O_2 even under non-stress conditions, which remain unaltered in response to drought stress suggesting that 10 µM ES-treated Arabidopsis plants are producing high levels of ROS and are under constant oxidative stress like conditions.

This study looks into the impact of ES treatment on Arabidopsis responses against drought stress both at the gene expression and biochemical level. The results presented here indicate that ES treatment at the 10 and 100 nM concentrations can be beneficial for plant survival under drought stress; however, at higher concentrations (10 µM), ES can be detrimental to plant growth and survival. These observations open new avenues for understanding the impact of environment ES in plant stress responses and its potential application in agriculture. However, further studies using different hormone

concentrations and stress responsive genes are required for a more comprehensive understanding of these effects.

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References

1. Erdal S. Effects of mammalian sex hormones on antioxidant enzyme activities, H_2O_2 content and lipid peroxidation in germinating bean seeds. J Facul Agric. 2009;40(2):79-85.
2. Dumlupinar R, Genisel M, Erdal S, Korkut T, Taspinar MS, Taskin M. Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. Biol Trace Elem Res. 2011;143(3):1740-1745.
3. Erdal S, Genisel M, Turk H, Gorcek Z. Effects of progesterone application on antioxidant enzyme activities and K+/Na+ ratio in bean seeds exposed to salt stress. Toxicol Ind Health. 2012;28(10):942-946.
4. Erdal S. Exogenous mammalian sex hormones mitigate inhibition in growth by enhancing antioxidant activity and synthesis reactions in germinating maize seeds under salt stress. J Sci Food Agric. 2012;92(4):839-843.
5. Erdal S. Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J Sci Food Agric. 2012;92(7):1411-1416.

6. Genisel M, Turk H, Erdal S. Exogenous progesterone application protects chickpea seedlings against chilling-induced oxidative stress. Acta Physiol Plant. 2013;35(1):241-251.

7. Chaoui A, El Ferjani E. β-Estradiol protects embryo growth from heavy-metal toxicity in germinating lentil seeds. J Plant Growth Regul. 2013;32(3):636-645.

8. Pastori GM, Trippi VS. Oxidative stress induces high rate of glutathione reductase synthesis in a drought resistant maize strain. Plant Cell Physiol. 1992;33:957–961.

9. Contour-Ansel D, Torres-Franklin ML, Cruz de Carvalho MH, D'Arcy-Lameta A, Zuily-Fodil Y. Glutathione reductase in leaves of cowpea: Cloning of two cDNAs, expression and enzymatic activity under progressive drought stress, desiccation and abscisic acid treatment. Ann Bot. 2006;98:1279–1287.

10. Zagorchev L, Seal CE, Kranner I, Odjakova M. A central role for thiols in plant tolerance to abiotic stress. Intern J Molec Sci. 2013;14(4):7405-7432.

11. Anjum NA, Ahmad I, Mohmood I, Pacheco M, Duarte AC, Pereira E, Khan NA, Iqbal M, Prasad MNV. Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. Environ Exp Bot. 2012;75:307-324.

12. Das K, Roychoudhury A. Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front Environ Sci. 2014;2:53. http://dx.doi.org/10.3389/fenvs.2014.00053

13. Bhattacharjee S. An inductive pulse of hydrogen peroxide pretreatment restores redox-homeostasis and oxidative membrane damage under extremes of temperature in two rice cultivars. Plant Growth Regul. 2012;68(3):395-410.

14. Petrov VD, Van Breusegem F. Hydrogen peroxide-a central hub for information flow in plant cells. AoB Plants. 2012;2012:pls014. doi: 10.1093/aobpla/pls014

15. Verbruggen N, Hermans C. Proline accumulation in plants: a review. Amino Acids. 2008;35(4):753-759.

16. Abrahám E, Rigó G, Székely G, Nagy R, Koncz C, Szabados L. Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in *Arabidopsis*. Plant Mol Biol. 2003;51(3):363-372.

17. Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. Planta. 2007;225(2):353-364.

18. Murashige T. Skoog F. A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant. 1962; 15: 473-497.

19. Bates L, Waldren R, Teare I. Rapid determination of free proline for water-stress studies. Plant Soil. 1973;39(1):205-207.

20. Adetunji K. Microarray analysis of estradiol regulated gene expression in *Arabidopsis* seedlings. 2012. MS Thesis. Texas Woman's University, Denton, Texas, USA.

21. Alam MM, Hasanuzzaman M, Nahar K, Fujita M. Exogenous salicylic acid ameliorates short-term drought stress in mustard (*Brassica juncea* L.) seedlings by up-regulating the antioxidant defense and glyoxalase system. Austr J Crop Sci. 2013;7(7):1053-1063.

22. Edwards R, Dixon DP, Walbot V. Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. 2000;5(5):193-198.

23. Xu J, Xing X, Tian Y, Peng R, Xue Y, Zhao W, Yao Q-H. Transgenic *Arabidopsis* plants expressing tomato glutathione S-transferase showed enhanced resistance to salt and drought stress. PLoS One. 2015;10(9):e0136960. http://dx.doi.org/10.1371/journal.pone.0136960

24. Sappl PG, Carroll AJ, Clifton R, Lister R, Whelan J, Harvey Millar A, Singh KB. The *Arabidopsis* glutathione transferase gene family displays complex stress regulation and co-silencing multiple genes results in altered metabolic sensitivity to oxidative stress. Plant J. 2009;58(1):53-68.

25. Baron KN, Schroeder DF, Stasolla C. GEm-Related 5 (GER5), an ABA and stress-responsive GRAM domain protein regulating seed development and inflorescence architecture. Plant Sci. 2014;223:153-166.

26. Yu A, Li P, Tang T, Wang J, Chen Y, Liu L. Roles of hsp70s in stress responses of microorganisms, plants, and animals. BioMed Res Intern. 2015;2015. doi: org/10.1155/2015/510319

27. Sung DY, Vierling E, Guy CL. Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family. Plant Physiol. 2001;126(2):789-800.

28. Hong S, Vierling E. Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J. 2001;27(1):25-35.

29. Pyngrope S, Bhoomika K, Dubey R. Reactive oxygen species, ascorbate–glutathione pool, and enzymes of their metabolism in drought-sensitive and tolerant indica rice (*Oryza sativa* L.) seedlings subjected to progressing levels of water deficit. Protoplasma. 2013;250(2):585-600.

30. Herbinger K, Tausz M, Wonisch A, Soja G, Sorger A, Grill D. Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. Plant Physiol Biochem. 2002;40(6):691-696.

31. Hossain MA, Mostofa MG, Fujita M. Heat-shock positively modulates oxidative protection of salt and drought-stressed mustard (*Brassica campestris* L.) seedlings. J Plant Sci Molec Breed. 2013;2(1):2. doi: 10.7243/2050-2389-2-2

32. Bhaskara GB, Yang T, Verslues PE. Dynamic proline metabolism: importance and regulation in water limited environments. Front Plant Sci. 2015;6. doi: 10.3389/fpls.2015.00484

33. Rejeb KB, Jdey A, Abdelly C, Savoure A. Hydrogen peroxide produced by NADPH oxidases increases proline accumulation during salt or mannitol stress in *Arabidopsis thaliana*. New Phytol. 2015;208:1138-1148.

34. Todaka D, Shinozaki K, Yamaguchi-Shinozaki K. Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. Front Plant Sci. 2015;6. doi: 10.3389/fpls.2015.00084

35. Gondim FA, Miranda RdS, Gomes-Filho E, Prisco JT. Enhanced salt tolerance in maize plants induced by H_2O_2 leaf spraying is associated with improved gas exchange rather than with non-enzymatic antioxidant system. Theor Exp Plant Physiol. 2013;25(4):251-260.

36. Hossain MA, Fujita M. Hydrogen peroxide priming stimulates drought tolerance in mustard (*Brassica juncea* L.) seedlings. Plant Gene Trait. 2013;4(1). doi: 10.5376/pgt.2013.04.0020

37. Wang Y, Zhang J, Li J, Ma X. Exogenous hydrogen peroxide enhanced the thermotolerance of *Festuca arundinacea* and *Lolium perenne* by increasing the antioxidative capacity. Acta Physiol Plant. 2014;36(11):2915-2924.

List of Figures

Figure 4.5: Quantitative PCR analysis of gene expression for stress related genes in Arabidopsis in response to drought stress in control and estradiol-treated plants. Estradiol application enhances stress related gene expression. Asterisks represent statistically significant differences ($P<0.05$, ANOVA). The results are means ± SD (n = 9). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol concentrations. White bars (control), Black bars (drought).

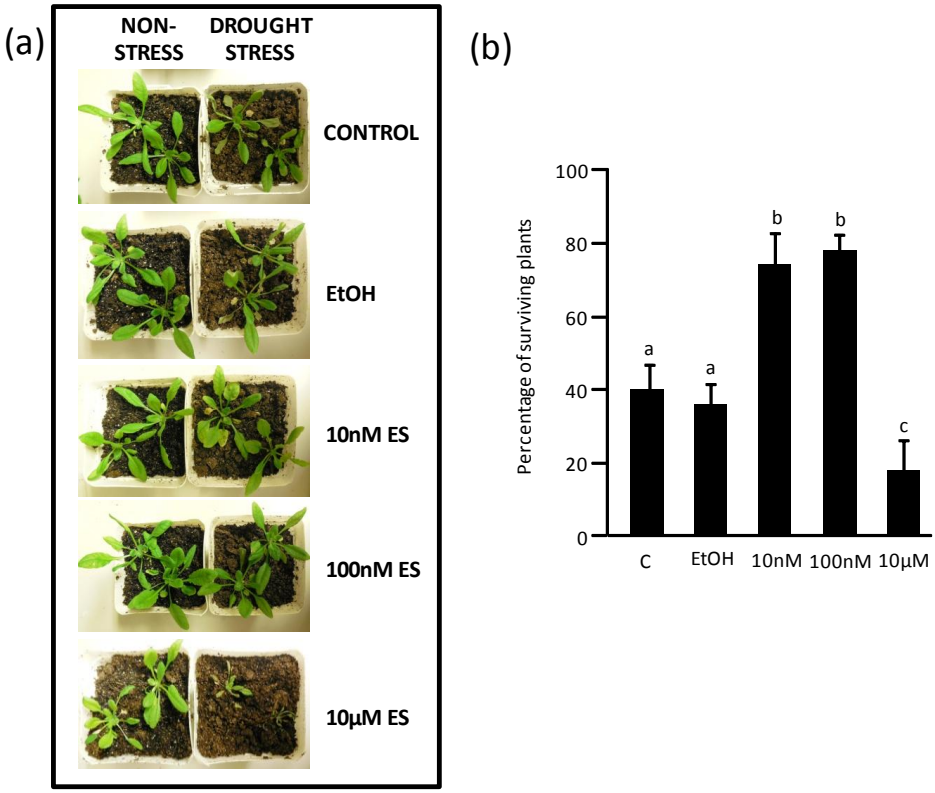

Figure 4.1: Estradiol application enhances drought stress tolerance in Arabidopsis. (a) Representative image of estradiol-treated and untreated plants following 7 days of drought stress. (b) Plant survival after drought stress. The results are means ± SD (n = 30). Different alphabets represent statistically significant difference (P<0.05, ANOVA). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol concentrations.

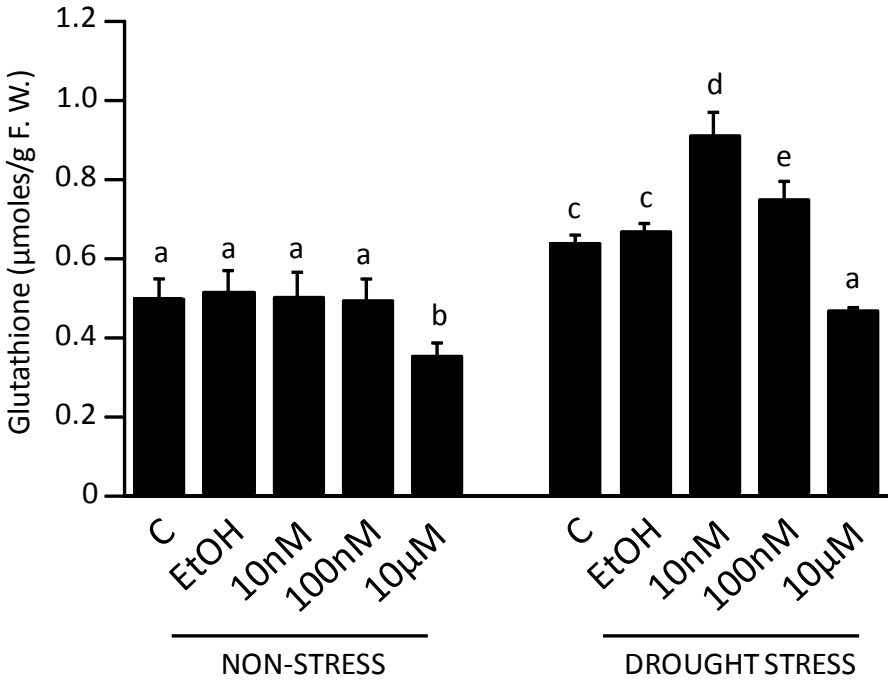

Figure 4.2: Estradiol application results in increased glutathione accumulation in Arabidopsis leaves. Different alphabets represent statistically significant difference =(P<0.05, ANOVA). The results are means ± SD (n = 9). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol concentrations.

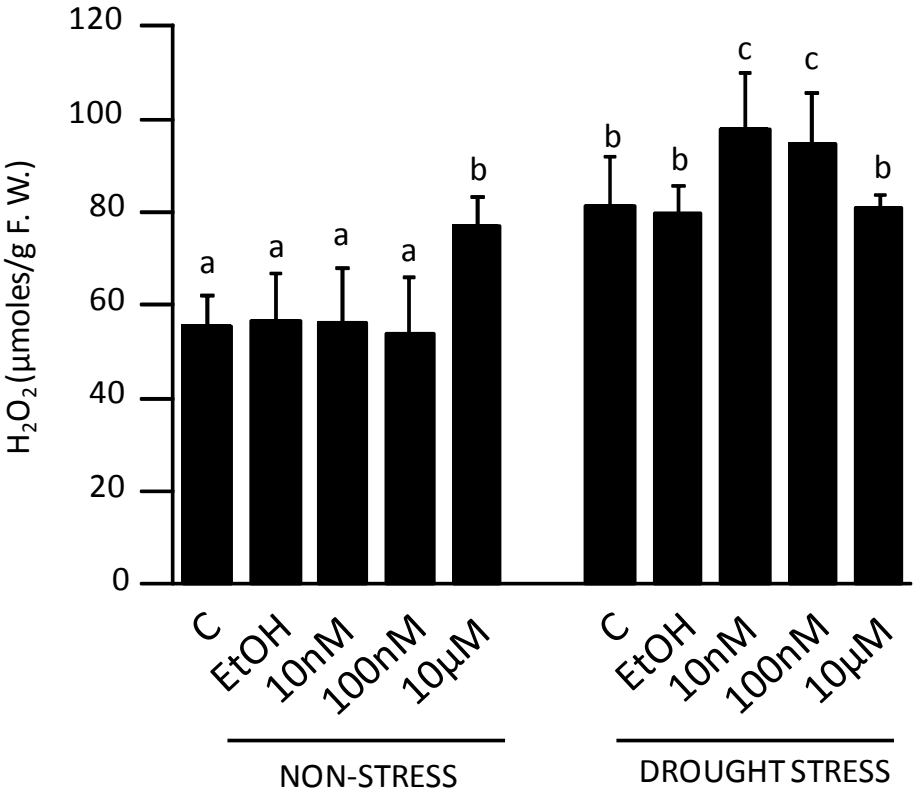

Figure 4.3: H_2O_2 accumulation is enhanced by estradiol treatment in Arabidopsis leaves during drought stress. Different alphabets represent statistically significant difference (P<0.05, ANOVA). The results are means ± SD (n = 9). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol concentrations.

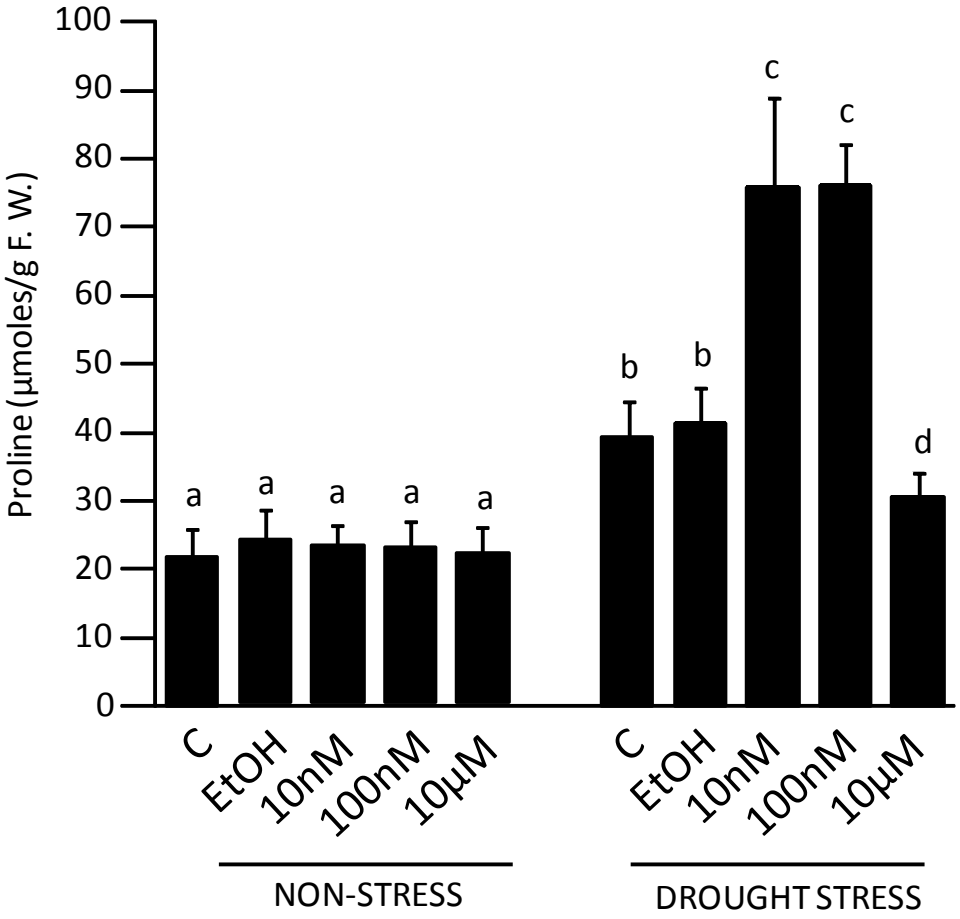

Figure 4.4: Estradiol application enhances proline accumulation in Arabidopsis during drought stress. Different alphabets represent statistically significant difference ($P<0.05$, ANOVA). The results are means ± SD (n = 9). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol concentrations.

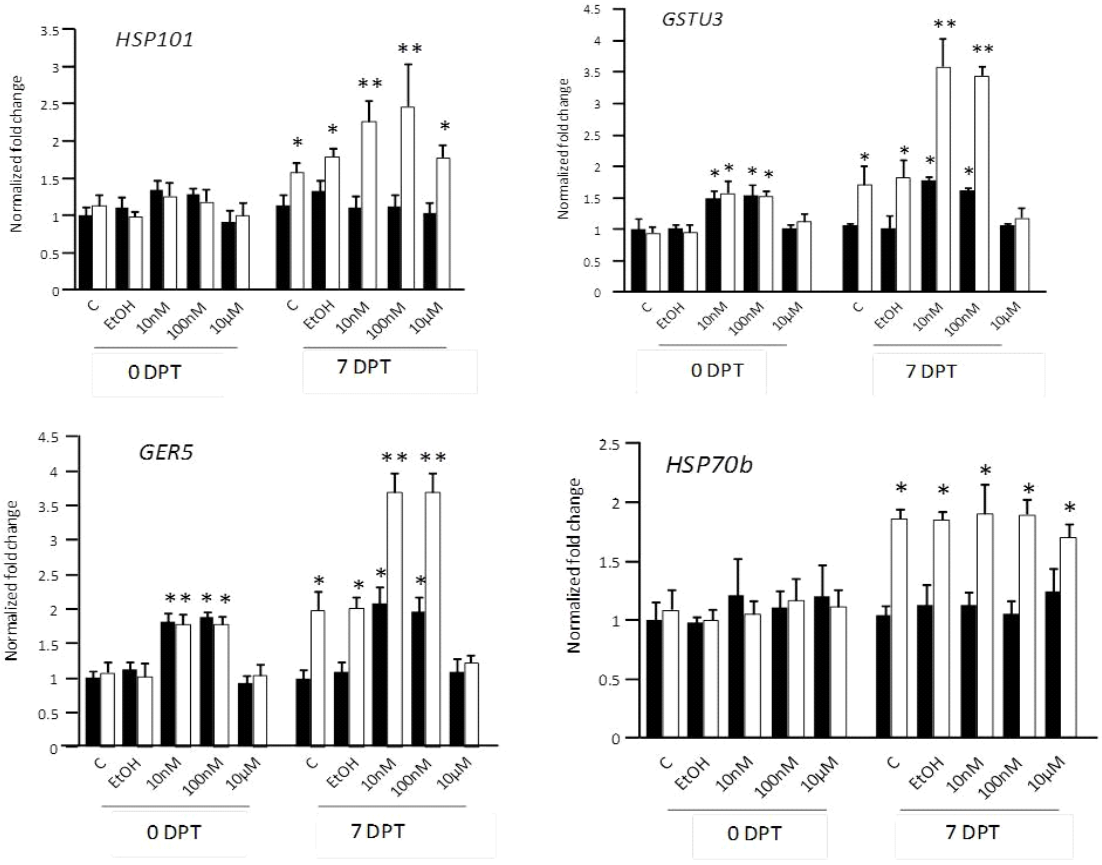

Figure 4.5: Quantitative PCR analysis of gene expression for stress related genes in Arabidopsis in response to drought stress in control and ES-treated plants. Estradiol application enhances stress related gene expression. Asterisks represent statistically significant differences (P<0.05, ANOVA). The results are means ± SD (n = 9). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM, 100 nM, 10 µM = applied estradiol

concentrations. White bars (no stress), Black bars (drought stress). DPT = Days Post Treatment.

CHAPTER V

AN INQUIRY INTO THE MECHANISM OF ACTION OF 17β-ESTRADIOL APPLICATION ON *ARABIDOPSIS THALIANA*

A Paper to be Submitted for Publication in the

Journal *Physiology and Molecular Biology of Plants*

Pallavi Upadhyay

Abstract

Increased pharmaceutical and agricultural activities have led to the introduction of animal steroidal hormones, including estrogens, into the agricultural soil. The goal of the study is to understand the molecular basis of the phenotypes displayed by estradiol-treated Arabidopsis plants. Plants treated with increasing estradiol concentrations (10nM, 100 nM and 10 μM) were found to have low *PAL1* expression level and subsequently reduced phenypropanoid biosynthesis. The estradiol-treated plants also displayed compromised resistance against *Pseudomonas syringae* pv. tomato DC3000. Overexpression of *PAL1* under the control of *CaMV* 35S alleviated the effects of estradiol application suggesting that the downregulation of the PP pathway by estradiol may be transcriptional in nature. The potential role of *ERF73*, an AP2/EREBP transcription factor, in modulating the estradiol-treated phenotype was also investigated. However, the phenotype of *erf73* mutants and wild type plants upon estradiol application suggested that *ERF73* may not play a role in controlling *PAL1* expression.

Keywords: Arabidopsis, 17β-estradiol, phenylpropanoids, *PAL1*, *ERF73*

Introduction

Estrogens are female sex hormones synthesized in all vertebrates known to regulate growth, metabolism, developmental processes and reproduction (Mauvais-Jarvis et al., 2013). There is an ever increasing influx of estrogens and estrogen-like compounds (xenoestrogens), from livestock and human waste, being introduced into the agricultural soil and ground water (Stumpe and Marschner, 2010). A number of studies have demonstrated that exogenous application of 17β-estradiol (ES) provides a protective effect against environmental stress in plants by increasing the activity of stress responsive enzymes like superoxide dismutase and peroxidase that can scavenge high energy electron carrying reactive oxygen species (Erdal and Dumlupinar, 2011; Dumlupinar et al., 2011; Erdal, 2012). Phenylpropanoid (PP) pathway is an important secondary metabolic pathway whose products are known to provide defense against biotic stresses and relieved abiotic stresses (Dixon and Paiva, 1995). The products derived *via* this pathway show a high diversity (e.g., flavonoids, anthocyanins, lignins) and their levels increase in response to both biotic and abiotic stresses (Ferrer et al., 2008; Payyavula et al., 2012).

The first committed step of PP pathway is catalyzed by the enzyme phenylalanine ammonia lyase (PAL) that results in the deamination of phenylalanine generating cinnamic acid and ammonia. The *PAL* gene family in Arabidopsis is made up of 4 genes

(*PAL1-PAL4*). Gene expression analysis and *in silico* promoter analysis of the *PAL* genes showed that PAL1 and PAL2 are the primary PAL enzymes in Arabidopsis (Raes et al., 2003). The *pal1pal2* double mutant plants, in comparison to the wild type, accumulated higher levels of phenylalanine, the enzyme substrate. In the same study it was shown that the double mutant plants accumulated lower levels of lignin, a major end product of the PP pathway (Rohde et al., 2004). Similar observations were made with respect to the *pal1pal2pal3pal4* quadruple mutant that was found to accumulate lower lignin levels and had compromised resistance against the bacterial pathogen *Pseudomonas syringae* (Huang et al., 2010).

Previous work in our laboratory has shown that ES application to Arabidopsis seedlings results in altered expression of PP pathway genes with most of the key genes of the pathway being downregulated by the hormone (Adetunji, 2012). In our study, exogenous application of ES to Arabidopsis plants resulted in decreased *PAL1* mRNA accumulation, which in turn was responsible for lowered accumulation of PP pathway products such as phenolics, anthocyanins and flavonoids. *PAL1* overexpressing plants were generated to explain the ES-treated phenotype of Arabidopsis plants, i.e. lowered accumulation of PPs and enhanced biomass. The observed phenotype is the result of the transcriptional control of *PAL1* expression by ES. The role of an AP2 (Apetala2)/EREBP (Ethylene Response Element Binding Protein) transcription factor, ERF73 (ETHYLENE RESPONSE FACTOR73), as a potential mediator of ES action in Arabidopsis was also studied.

Material and Methods

Plant material and growth conditions

Arabidopsis thaliana ecotype Columbia wild type (WT) seeds were purchased from Lehle Seeds, TX, USA and seeds for mutants *pal1* (Salk_022804), *erf73-1* (Salk_039484) and *erf73-2* (Salk_023445) were obtained from the Arabidopsis Biological Resource Center at The Ohio State University, Ohio, USA. Wild type and mutant seeds were surface sterilized according to the procedure described previously (Kagale *et al.* 2007) and grown in Murashige and Skoog (1962) nutrient medium solidified with 1% agar and amended with either increasing concentrations of estradiol (10 nM, 100 nM, 10 μM). Arabidopsis seedlings germinated on MS plates without ES or with 0.01% ethanol were used as control plants. At day 14, the seedlings were transferred from MS plates into pots in a Percival growth chamber under long-day conditions at 22°C, 50% humidity, and 200 $\mu mol/m^2/s$ until day 21, at which time tissues were collected for biochemical assays and RT-PCR.

erf73 mutant validation

The homozygous nature of T-DNA insertion in the *erf73* mutant plants was determined using PCR. The following primers were used; ERF73-G-F: 5´TCATTACAGACAGTGGCGAAA3´, ERF73-G-R: 5´CCTGAAGCATGATGTTTGTGA3´ and LBa1: 5´TGGTTCACGTAGTGGGCCATCG3´. Amplification of 0.5kb genomic *ERF73* portion was performed according to the following conditions: initial denaturation at 95°C

for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds each, annealing at 55°C for 30 seconds and extension at 72°C for 30 seconds. A final extension at 72°C for 7 minutes was performed. Amplification of 0.6 kb *ERF73* genomic/T-DNA left border portion was performed according to the following conditions: initial denaturation at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C for 30 seconds each, annealing at 55°C for 30 seconds and extension at 72°C for 35 seconds. A final extension at 72°C for 7 minutes was performed. The PCR products were resolved electrophoretically on 1.5% agarose gel and the absence of genomic amplicon with the presence of the T-DNA amplicon in the same sample indicated homozygosity of the mutants.

***PAL1*-overexpression transgenic plants**

cDNA generated from Arabidopsis leaves (as described in the RNA isolation and RT-PCR section) was used to amplify the PAL1 coding sequence using the following primers: PAL1-Start-F: 5´ATGGAGATTAACGGGGCACA3´ and PAL1-Stop-R: 5´TTAACATATTGGAATGGGAGCTCCG3´. The 2.7 kb sequence was cloned into the pCR8®/GW/TOPO® entry vector (ThermoFisher, USA). The pCR8-PAL1 clones were subcloned into the destination vector pMDC32 (Curtis and Grossniklaus, 2003) using the LR clonase reaction (ThermoFisher, USA). The pMDC32-PAL1 vector was transformed into the *Agrobacterium tumefaciens* strain GV3101, which was subsequently used to transform *pal1* plants using the floral dip method (Zhang et al., 2006).

Total phenolics estimation

Total phenolics estimation in Arabidopsis leaves was performed according to Velioglu *et al*. (1998) with some modifications. The leaf tissues (0.2 g) were extracted in 2 ml acidic methanol (80% methanol with 1% HCl) at room temperature for 2 h. Extracts were centrifuged at 1000 X *g* for 15 min. The supernatant (100 µl) was mixed with the Folin-Ciocalteu reagent (0.75 ml) and incubated at 22°C for 5 min. Sodium bicarbonate (0.75 ml) was further added to the reaction mixture which was allowed to stand at 22°C for an additional 90 min. The absorbance was measured at 725 nm and total phenolics content was estimated using a *p*-coumaric acid standard curve and expressed as µg mg^{-1} fresh weight.

Flavonoid estimation

Flavonoid estimation was performed according to Chang *et al*. (2002). Flavonoid extraction from 0.5 g leaf tissues was carried out overnight in 95% ethanol. Flavones and flavonols were measured by $AlCl_3$ colorimetric method against a quercetin standard at 415 nm, whereas flavanones were measured by the 2,4-diphenylhydrazine colorimetric method against a naringenin standard at 495 nm. Flavonoid content was expressed as µg mg^{-1} fresh weight.

Total anthocyanin estimation

Total anthocyanin estimation was performed according to Laxmi *et al*. (2004). Total anthocyanins were extracted from 0.2 g leaves with 3 ml 1% acidic methanol overnight.

Phase separation was performed by adding 3 ml chloroform and 2 ml water. Absorbance of the aqueous phase was measured at 530 nm and 657 nm. By subtracting the absorbance at 657 nm from 530 nm, the anthocyanin content was calculated and expressed as λ530–λ657/g fresh weight.

Pathogen inoculation

Pathogen inoculations were performed by infiltrating leaves of six 21-day-old plants for each treatment with a suspension (OD_{600} = 0.0002) of the virulent *Pseudomonas syringae* pv. tomato DC3000 (*Pst*) strain in 10 mM $MgCl_2$. To determine bacterial growth, inoculated leaves were harvested at 3-days-post inoculation (DPI) and homogenized in 10 mM $MgCl_2$. Diluted leaf extracts were plated on King's B medium supplemented with kanamycin (100 mg/ml) and incubated at 28°C for 2 d before counting the colony-forming units (cfu) as previously described (Xu *et al.* 2006).

RNA isolation and RT-PCR

RNA was isolated from leaves of 21-day old ES-treated or untreated plants using the Plant RNA isolation reagent (Life Technologies, USA) following the manufacturer's protocols. RT-PCR from the total RNA extracted was performed using the RETROscript reverse transcription kit (ThermoFisher, USA). Primers were designed using the Primer-3 software. The following primer sequences were used for the PCR analysis: PAL1F: 5´CGGTGTCGCACTTCAGAAGGAA3´, PAL1R: 5´GGATACCGGAAAATCCTTGGAGGAG3´, EF1αF:

5´TTCACCCTTGGTGTCAAGCAGATG3´, EF1αR: 5´TCAGGGTTGTATCCGACCTTCTTCA3´. The real-time PCR experiments were carried out using the iQ SYBR® Green supermix and the BioRAD CFX96 RT-PCR detection system (BioRad, USA) according to the manufacturers' suggestions. Reaction parameters were set as follows: initial denaturation at 95°C for 5 mins followed by 35 cycles of 30s at 95°C, 30s at 55°C and 30s at 72°C. The relative RNA levels in each sample were calibrated and normalized against *EF1α* expression, which was used as an internal control. The experiments were performed in triplicates and repeated thrice.

In silico promoter analysis

The 110 Arabidopsis genes downregulated by 10 nM ES application, determined in a previous microarray analysis (Adetunji, 2012) were used for promoter analysis using the Athamap (www.athamap.de) program. Starting from the ATG start site, 1kb portion of the promoter regions of the downregulated genes was analysed. To determine the transcription factor binding sites the default settings of Athamap program was used.

Statistical Analysis

Data are the means ± SD of three independent replicates. Data were subjected to a one-way analysis of variance (ANOVA) and the mean differences were compared using Tukey's test. Comparisons with $P < 0.05$ were considered significantly different.

Results

ES downregulates *PAL1* expression level

Transgenic Arabidopsis plants constitutively expressing *PAL1* under the control of the 35S promoter were treated with increasing concentrations of ES. In case of the wild type (WT) plants, *PAL1* expression was found to be downregulated by all the applied ES concentrations (10 nM, 100 nM and 10 µM). When compared to the WT plants, the *PAL1* overexpression (*PAL1*-OE) plants displayed nearly two-fold higher expression of *PAL1* that remained unaltered upon ES application (Fig. 5.1).

ES induced downregulation of secondary metabolism is not observed in *PAL1* overexpression plants

Since *PAL1* encodes for the first committed enzyme of the phenylpropanoid (PP) pathway which also acts as the rate limiting step for the pathway, it was expected that its constitutive expression will result in enhanced accumulation of secondary metabolism products in *Arabidopsis*. In the ES-treated WT leaves, the lowered expression of *PAL1* resulted in the decreased levels of the PP pathway products such as anthocyanins, flavonoids and phenolics. However, *PAL1*-OE line accumulated significantly higher levels of anthocyanins, phenolics and flavonoids as compared to the control and ES-treated WT plants (Fig. 5.2). In addition, it was observed that ES treatment had no effect on the secondary metabolism in *PAL1*-OE plants as the levels of the PP pathway derivatives showed no significant differences between the ES-treated and untreated transgenic plants (Fig. 5.2).

PAL1 overexpression enhances resistance against *Pseudomonas*

Secondary metabolism, especially the PP pathway, has an important role in plant responses against pathogen atacks. It was hypothesized that the *PAL1*-OE plants, synthesizing higher levels of PP products, would display increased resistance against a pathogen attack. Therefore, the *PAL1*-OE plants were subjected to bacterial pathogen infection. As opposed to the ES-treated WT plants, which displayed enhanced susceptibility towards *P. syringae* pv. DC3000, the *PAL1* overexpressing plants had significantly increased resistance towards the pathogen, resulting in reduced bacterial growth at 3 DPI. The increased resistance was found to be independent of ES application as no significant difference in bacterial growth was observed between control (untreated) and 10 nM ES-treated *PAL1*-OE plants (Fig. 5.3).

In silico promoter analysis of Arabidopsis genes downregulated by ES application reveals potential AP2/EREBP transcription factor binding sites

The objective of the promoter analysis was to identify a putative transcription factor that interacts with ES and thus controls the downregulation of important secondary metabolism genes (especially of PP pathway) in *Arabidopsis.* Athamap program was used to identify the transcription factor(s) that could bind to the promoter (1-kb region upstream of transcription start site) for all 110 downregulated genes identified in the previous microarray analysis of ES-treated Arabidopsis seedlings (Adetunji, 2012). The analysis revealed that members of the AP2 (APETALA2)/EREBP (Ethylene Responsive Element Binding Protein) transcription factor family can potentially bind to the promoter

regions of all analyzed genes (Table 5.1). An AP2/EREBP transcription factor gene *ERF73* (*At1g72360*) was found to be downregulated by ES application (Adetunji, 2012). Thus, it was speculated that *ERF73* regulate the transcriptional downregulation of the PP pathway by interacting with ES. Based on this analysis, it was decided to study the role of *ERF73* by employing the *erf73* mutant plants.

PAL1 expression may be independent of *ERF73* function

ES application at 10 and 100 nM was found to enhance the fresh and dry biomass of Arabidopsis leaves and total silique numbers. Application of 10µM ES resulted in significantly lower leaf biomass and siliques (Table 5.2). The fresh and dry weights of *erf73* plants in addition to the silique numbers were found to be similar to those of the WT (Table 5.2). It has been shown that ES application results in lowered expression of PAL1 in Arabidopsis WT plants (Fig. 5.1). Quantitative RT-PCR analysis of 10 nM ES-treated *erf73* mutant plants displayed lowered *PAL1* expression that was not significantly different from that of 10 nM ES-treated WT plants (Fig. 5.4). Taken together these results suggest that *PAL1* expression and the related phenotypes in response to ES treatment may be independent of *ERF73* function.

Discussion

The present study is the first report that looks into the molecular mechanisms that are responsible for the observed decrease in the expression of PAL1 and other PP pathway biosynthesis genes (Adetunji, 2012) in response to ES application to *Arabidopsis*.

Regulation of PAL activity in plants is influenced by a number of factors (Zhang and Liu, 2015). The transcriptional regulation of *PAL* and a number of other PP pathway genes has been demonstrated to be under the control of MYB transcription factors. Analysis of the proximal promoter of *PAL1*, *CHS* and *4CL* reveals the presence of a number of AC-rich motifs, which are known binding sites for MYB transcription factors (Yang et al., 2001; Zhao and Dixon, 2011). Heterologous expression of AmMYB305 from snapdragon is able to transactivate the *PAL* promoter in tobacco protoplasts (Sablowski et al., 1994). The Lim-domain containing proteins that are zinc-finger type of transcription factors can also bind the AC-rich motifs of the *AtPAL* promoter and positively regulate its expression (Kawaoka and Ebinuma, 2001). However, the KNOX family of transcription factors negatively regulates the expression of PP pathway genes including the *PAL* family, *4CL* and *4CH* (Mele et al., 2003; Zhang et al., 2015).

It is evident from our results that ES application results in the downregulation of *PAL1* transcript accumulation. The consequence of decreased *PAL1* expression is seen in the form of lowered accumulation of PPs in the ES-treated Arabidopsis leaves. The ES regulation of *PAL1* expression is transcriptional in nature is bolstered by the observation that constitutive expression of *PAL1* under the viral 35S promoter, in the *pal1* mutant background, is unaffected by ES application. Our results suggest that the lowered expression of *PAL1* in ES-treated WT plants may be due to a direct or indirect influence of ES on the transcription of *PAL1* that is lost when *PAL1* is transcribed from a viral promoter. As to how ES is able to downregulate *PAL1* expression is not clear and further work is needed to decipher the molecular basis of this observation. ES application may be

able to lower the expression of *PAL1* and other PP pathway genes by inhibiting positive regulators like MYB transcription factor (Zhao and Dixon, 2011) or by enhancing the binding of a negative regulator such as the KNOX transcription factor (Zhang et al., 2015). Either possibility would result in the lowered expression of the PP pathway genes upon ES application.

Transgenic Arabidopsis plants overexpressing *PAL1* displayed increased resistance to *Pst* infection that remained unchanged upon ES application. In comparison, ES application to WT Arabidopsis plants resulted in compromised resistance against the bacterial pathogen. Induction of PAL activity in response to bacterial pathogens has been reported in pepper (Kim and Hwang, 2014) and tomato (Vanitha et al., 2009). Recently, it was demonstrated that overexpression of pepper (*Capsicum annum*) *CaPAL1* in Arabidopsis plants resulted in increased resistance against *Pst* infection (Kim and Hwang, 2014). The transgenic *PAL1*-OE plants generated in our study also show similar results when confronted with the same bacterial pathogen.

An important aspect of plant defense against bacterial pathogen is the salicylic acid (SA) mediated defense signaling (Vlot et al., 2009). It has been demonstrated that plants with suppressed PAL activity have decreased SA content and display compromised resistance against pathogens (Pallas et al., 1996; Kim and Hwang, 2014). Since the ES-treated plants show decreased expression of *PAL1*, it is possible that these plants may be compromised in the PAL mediated SA accumulation and signaling induced in response to *Pst*. It has been shown that increased accumulation of PP products in response to *Pst* infection in tomato provides antioxidative effect against the oxidative damage induced by

the bacterial pathogen (López-Gresa et al., 2011). Thus the lowered expression of *PAL1* and other PP pathway genes, in response to ES application, resulting in the decreased accumulation of PP compounds can also contribute towards the compromised resistance of ES-treated plants.

The AP2/EREBP family of transcription factors in Arabidopsis is composed of 25 genes that control the expression of genes involved in carbohydrate, hormone and stress signaling (Riechmann and Meyerowitz, 1998). Since the promoter analysis of genes downregulated by ES application (Adetunji, 2012) revealed the presence of an AP2/EREBP binding site present in the promoter region of each gene it was decided to look into the role of the AP2/EREBP family of transcription factors in mediating the effects of ES treatment. *ERF73* was a prominent member of this family whose expression was also decreased in the ES-treated plants as opposed to the controls (Adetunji, 2012). The putative role of *ERF73* in controlling the transcription of PAL1 can be demonstrated with three possible modes of actions: (a) *ERF73* does not interact with *PAL1*: if *ERF73* has no role in controlling *PAL1* expression then in that case the *PAL1* expression levels of *erf73* plants would be similar to those found in WT plants. (b) *ERF73* is a negative regulator of *PAL1*: in case *ERF73* negatively regulates *PAL1* expression, *erf73* plants will display increased *PAL1* expression as compared to the WT plants. (c) *ERF73* is a positive regulator of *PAL1*: if *ERF73* is a positive regulator of *PAL1* then *erf73* plants should display lower *PAL1* expression as compared to the WT plants (Fig. 5.5).

However, the *erf73* mutants were found to behave like the WT plants with respect to biomass accumulation and *PAL1* expression when treated with ES. Based on the

observations related to the *erf73* mutant plants it may be suggested that *ERF73* does not mediate the effect of ES at the transcriptional level. Another possibility is that *ERF73* belongs to a subfamily of related transcription factors (within the AP2/EREBP family) with similar functions that may compensate for the transcriptional role of *ERF 73* thus rendering its function redundant.

The study presented in this paper looks into the mechanism by which ES controls the downregulation of the PP pathway in *Arabidopsis*. It is our assertion that ES, by an as of yet unknown mechanism, is able to transcriptionally downregulate the expression of *PAL1*. *ERF7*3 expression was found to be downregulated, along with *PAL1*, upon ES application. The mutant analysis of *erf73* plants revealed that *ERF73* may not mediate the downregulation of *PAL1* expression upon ES application. Further work is required to ascertain the role of a transcription factor or protein that may modulate this effect.

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References

Adetunji K (2012) Microarray analysis of estradiol regulated gene expression in *Arabidopsis* seedlings. MS thesis. Texas Woman's University, Denton, Texas USA.

Chang C, Yang M, Wen H, Chern J (2002) Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal 10: 178–182

Curtis MD, Grossniklaus U (2003) A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol 133: 462-469

Dixon RA, Paiva NL (1995) Stress-induced phenylpropanoid metabolism. Plant Cell 7: 1085-1097

Dumlupinar R, Genisel M, Erdal S, Korkut T, Taspinar MS, Taskin M (2011) Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. Biol Trace Elem Res 143: 1740-1745

Erdal S, Dumlupinar R (2011) Mammalian sex hormones stimulate antioxidant system and enhance growth of chickpea plants. Acta Physiol Plant 33: 1011-1017

Erdal S (2012) Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J Sci Food Agric 92: 1411-1416

Ferrer J, Austin M, Stewart C, Noel J (2008) Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol Biochem 46: 356-370

Huang J, Gu M, Lai Z, Fan B, Shi K, Zhou YH, Yu JQ, Chen Z (2010) Functional analysis of the Arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. Plant Physiol 153: 1526-1538

Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P (2007) Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. Planta 225: 353-364

Kawaoka A, Ebinuma H (2001) Transcriptional control of lignin biosynthesis by tobacco LIM protein. Phytochemistry 57: 1149-1157

Kim DS, Hwang BK (2014) An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J Exp Bot 65: 2295-2306

Laxmi A, Paul LK, Peters JL, Khurana JP (2004) *Arabidopsis* constitutive photomorphogenic mutant, *bls1*, displays altered brassinosteroid response and sugar sensitivity. Plant Mol Biol 56: 185–201

López-Gresa MP, Torres C, Campos L, Lisón P, Rodrigo I, Bellés JM, Conejero V (2011) Identification of defence metabolites in tomato plants infected by the bacterial pathogen *Pseudomonas syringae*. Environ Exp Bot 74: 216-228

Mauvais-Jarvis F, Clegg DJ, Hevener AL (2013) The role of estrogens in control of energy balance and glucose homeostasis. Endocr Rev 34: 309-338

Mele G, Ori N, Sato Y, Hake S (2003) The knotted1-like homeobox gene *BREVIPEDICELLUS* regulates cell differentiation by modulating metabolic pathways. Genes Dev 17: 2088-2093

Murashige T, Skoog F (1962) A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol Plant 15: 473-497

Pallas JA, Paiva NL, Lamb C, Dixon RA (1996) Tobacco plants epigenetically suppressed in phenylalanine ammonia lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J 10: 281-293

Payyavula RS, Navarre DA, Kuhl JC, Pantoja A, Pillai SS (2012) Differential effects of environment on potato phenylpropanoid and carotenoid expression. BMC Plant Biol 12. doi: 39-2229-12-39

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in *Arabidopsis*. Plant Physiol 133: 1051-1071

Riechmann JL, Meyerowitz EM (1998) The AP2/EREBP family of transcription factors. Biol Chem 379: 633-646

Rohde A, Morreel K, Ralph J, Goeminne G, Hostyn V, De Rycke R, Kushnir S, Van Doorsselaere J, Joseleau JP, Vuylsteke M, Van Driessche G, Van Beeumen J, Messens E, Boerjan W (2004) Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell 16: 2749-2771

Sablowski RW, Moyano E, Culianez-Macia FA, Schuch W, Martin C, Bevan M (1994) A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J 13: 128-137.

Stumpe B, Marschner B (2010) Dissolved organic carbon from sewage sludge and manure can affect estrogen sorption and mineralization in soils. Environ Pollut 158: 148-154

Vanitha SC, Niranjana SR, Umesha S (2009) Role of phenylalanine ammonia lyase and polyphenol oxidase in host resistance to bacterial wilt of tomato. J Phytopathol 157: 552-557.

Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agric Food Chem 46: 4113–4117

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic acid, a multifaceted hormone to combat disease. Annu Rev Phytopathol 47: 177-206

Xu X, Chen C, Fan B, Chen Z (2006) Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell 18: 1310–1326

Yang S, Sweetman, JP, Amirsadeghi S, Barghchi M, Huttly AK, Chung WI, Twell D (2001) Novel anther-specific myb genes from tobacco as putative regulators of phenylalanine ammonia-lyase expression. Plant Physiol 126: 1738-1753.

Zhang X, Henriques R, Lin S, Niu Q, Chua N (2006) Agrobacterium-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Nature Protocols 1: 641-646.

Zhang X, Liu C (2015) Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol Plant 8: 17-27

Zhang X, Gou M, Guo C, Yang H, Liu CJ (2015) Down-regulation of Kelch domain-containing F-box protein in Arabidopsis enhances the production of (poly)phenols and tolerance to ultraviolet radiation. Plant Physiol 167: 337-350

Zhao Q, Dixon RA (2011) Transcriptional networks for lignin biosynthesis: more complex than we thought? Trends Plant Sci 16: 227-233

Table 5.1: Results generated by Athamap (www.athamap.de) for the proximal promoter analysis of Arabidopsis genes whose expressions were downregulated by ES application (Adetunji, 2012). The table shows top five putative transcription factors that can potentially bind to the promoters of the analyzed genes. TFBS = Transcription Factor Binding Site.

Transcription Factor	Number of Genes	Family	Sum of TFBSs in Total	Theoretical Number of TFBSs	Occurrence/ Theoretical Ratio
TEIL	110	AP2/EREBP	597	583.49	1.02
MYB	109	MYB	463	367.56	1.26
MYB	109	MYB	463	367.56	1.26
ZmHOX2A	109	HD-HOX	505	731.73	0.69
ALFIN1	104	HD-PHD	377	529.02	0.71

Table 5.2: Effect of estradiol application on biomass accumulation and silique generation in ES-treated and untreated Arabidopsis plants. Different superscript symbols represent statistically significant differences ($P<0.05$, ANOVA). The results are means ± SD (n = 15). C = no treatment; EtOH = 0.01% ethanol treated; 10 nM = applied ES concentration; *erf73 = ERF73* knockout mutant line.

Treatment	**Fresh weight (g)**	**Dry weight (g)**	**Silique #**
Control	2.87±0.51*	0.36±0.18^{a}	69.41±13.61$^{\Psi}$
EtOH	2.74±0.32*	0.32±0.14^{a}	70.18±17.29$^{\Psi}$
10nM	3.41±0.56**	0.44±0.18^{b}	86.48±16.38$^{\Delta}$
100nM	3.59±0.68**	0.46±0.21^{b}	83.39±14.29$^{\Delta}$
10µM	1.84±0.72***	0.21±0.12^{c}	40.54±16.25$^{\delta}$
erf73	2.82±0.45*	0.36±0.23^{a}	68.14±14.35 $^{\Psi}$
erf73(EtOH)	2.91±0.62*	0.32±0.14^{a}	73.74±16.31 $^{\Psi}$
erf73(10nM)	3.82±0.42**	0.46±0.16^{b}	84.39±14.54 $^{\Delta}$
erf73(100nM)	3.51±0.30**	0.42±0.25^{b}	86.71±12.38 $^{\Delta}$
erf73(10µM)	1.99±0.46***	0.22±0.13^{c}	41.35±13.58 $^{\delta}$

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treatment; EtOH = 0.01% ethanol treated; 10 nM = applied ES concentration; *erf73* = *ERF73* knockout mutant line.

Figure 5.5: Model of the role of putative transcription factor (TF) in downregulation of *PAL1* expression upon ES application. (a) TF and *PAL1* do not interact, (b) TF is a negative regulator of *PAL1* transcription and (c) TF is a positive regulator of *PAL1* transcription.

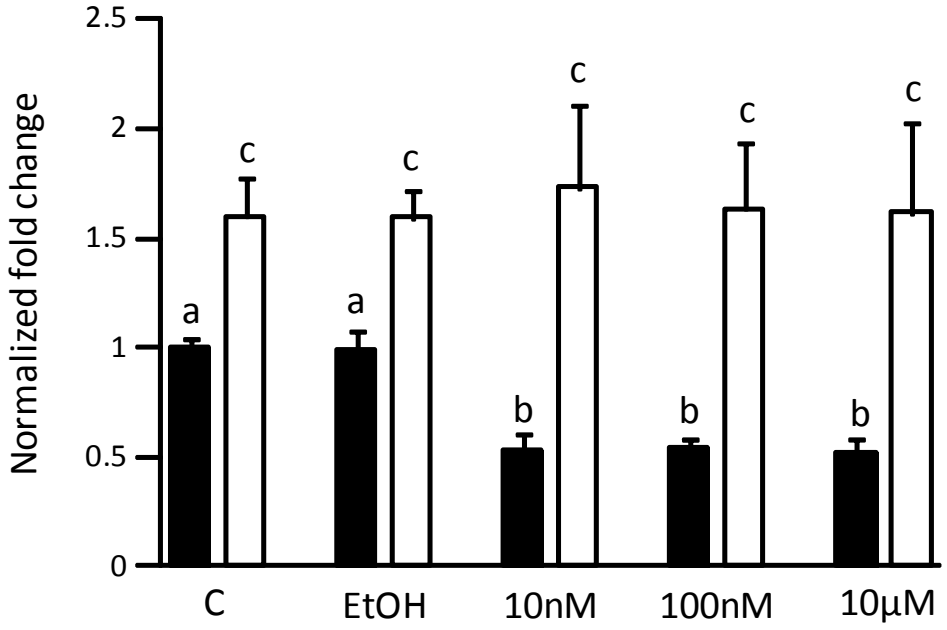

Figure 5.1: Quantitative RT-PCR analysis of *PAL1* expression in estradiol-treated and control WT and *PAL1*-overexpression line (*PAL1*-OE) Arabidopsis plants. Results are cumulative of three independent experiments (means ± SD, n = 9). Letters represent statistically significant differences ($P<0.05$, ANOVA). C = no treatment; EtOH = 0.01% ethanol treated; 10nM, 100nM, 10µM = applied ES concentrations. Black bars = WT, White bars = *PAL1*-OE.

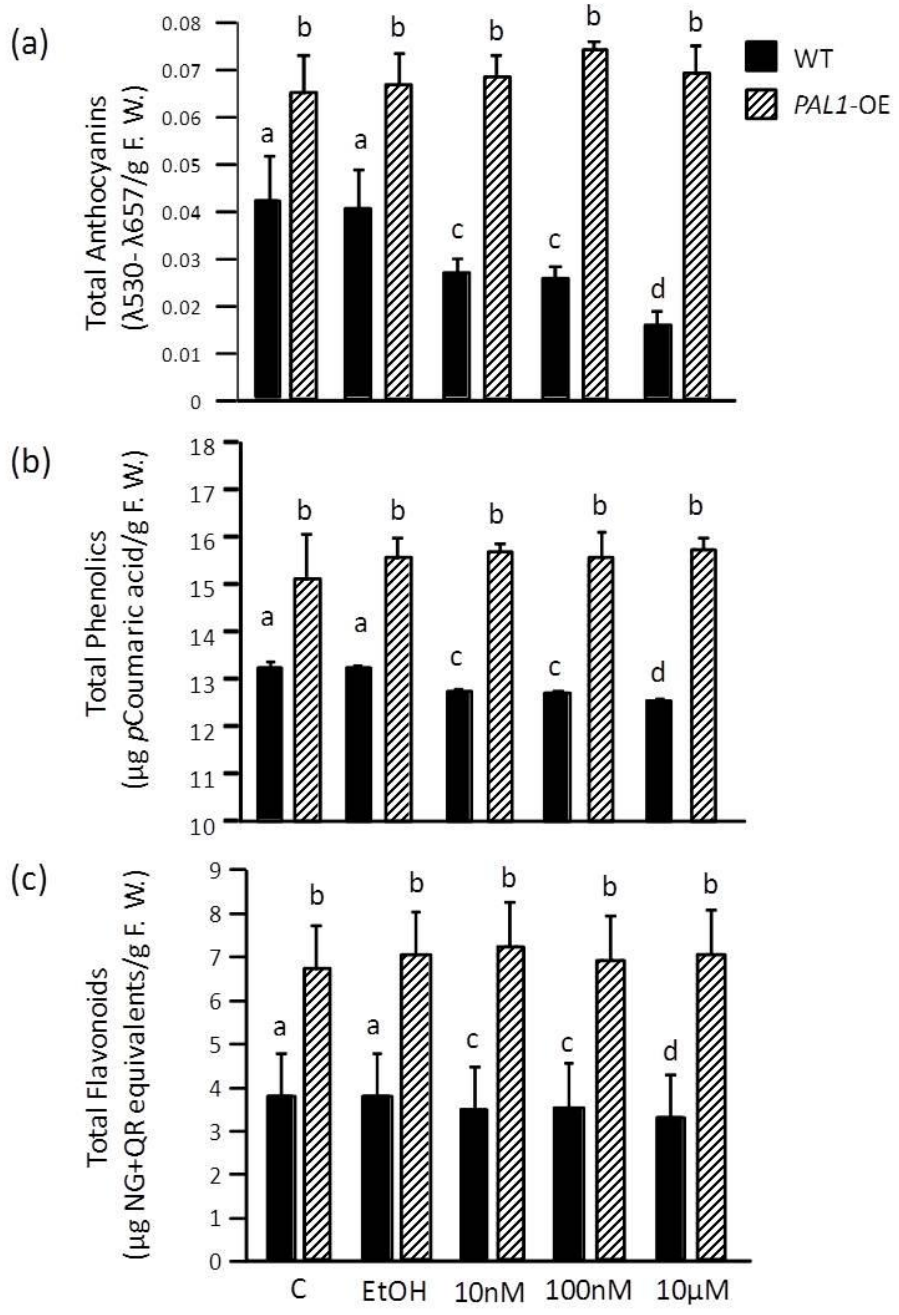

Figure 5.2: *PAL1* overexpression results in enhanced accumulation of the secondary metabolites (a) anthocyanins, (b) phenolics and (c) flavonoids. Results are cumulative of

three independent experiments (means ± SD, n = 9). Letters represent statistically significant differences ($P<0.05$, ANOVA). C = no treatment; EtOH = 0.01% ethanol treated; 10nM, 100nM, 10µM = applied ES concentrations, NG = Naringenin, QR = Quercitin, *PAL1*-OE = *PAL1* overexpression line.

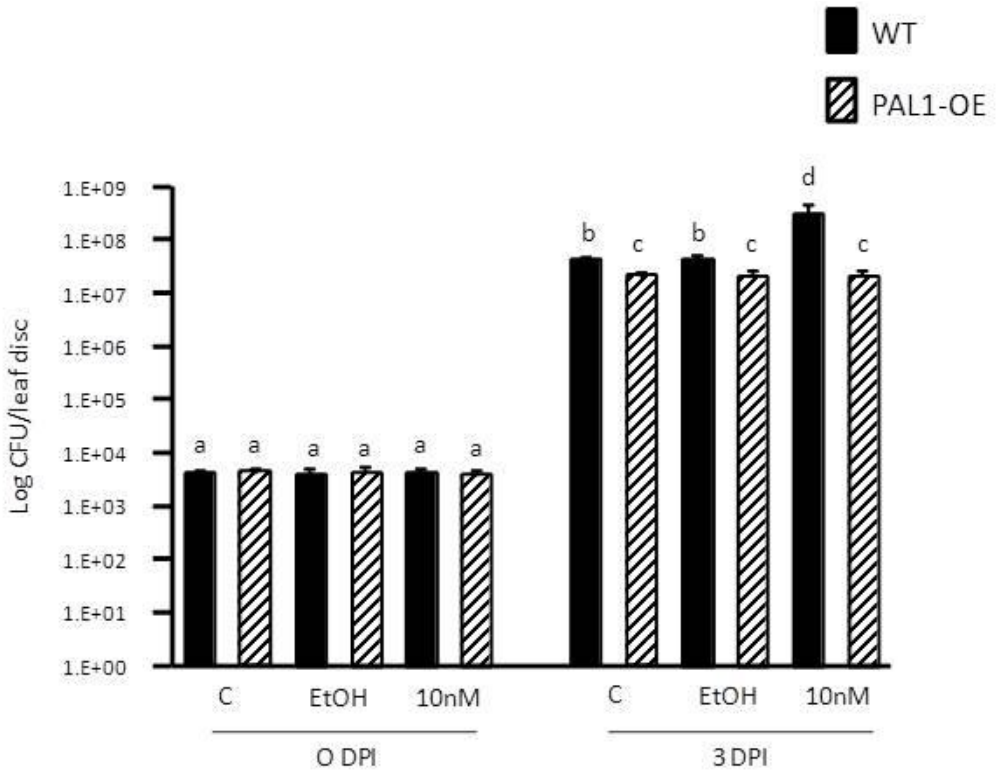

Figure 5.3: Overexpression of *PAL1* reduces Arabidopsis susceptibility to *P. syringae* pv. DC3000. Bacterial growth in control and ES-treated WT and *PAL1*-OE plants at 0 and 3 DPI. Results are means ± SD (n = 15). C = no treatment, EtOH = 0.01% ethanol treated, 10 nM = applied ES concentration, *PAL1*-OE = *PAL1* overexpression line.

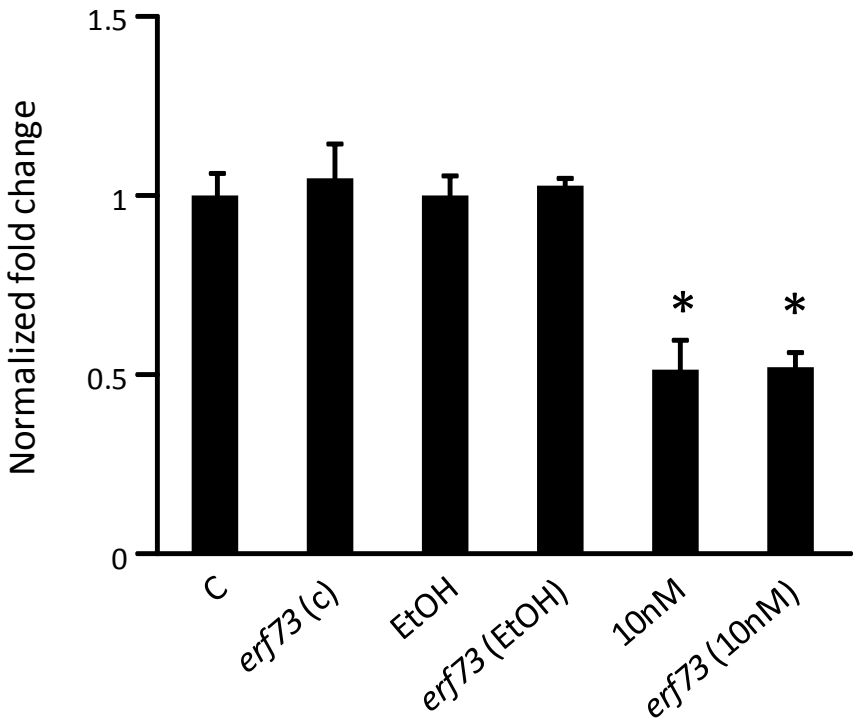

Figure 5.4: Quantitative RT-PCR analysis of *PAL1* expression in ES-treated and untreated Arabidopsis WT and *erf 73* mutant plants. Asterisk represents statistically significant differences ($P<0.05$, ANOVA). Results are means ± SD (n = 9). C = no treatment; EtOH = 0.01% ethanol treated; 10 nM = applied ES concentration; *erf73* = *ERF73* knockout mutant line.

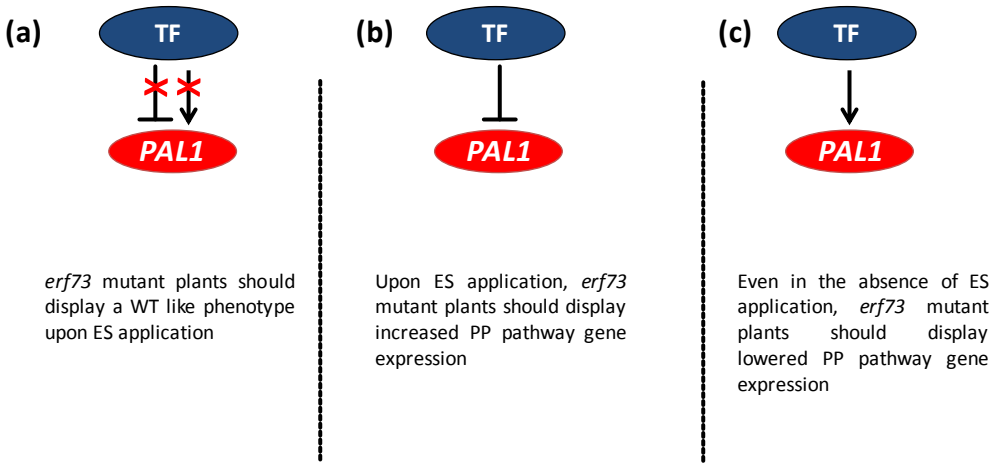

Figure 5.5: Model of the role of putative transcription factor (TF) in downregulation of *PAL1* expression upon ES application. (a) TF and *PAL1* do not interact, (b) TF is a negative regulator of *PAL1* transcription and (c) TF is a positive regulator of *PAL1* transcription.

CHAPTER VI

DISCUSSION

The research presented in this dissertation aims to understand the Arabidopsis biochemical and molecular responses to ES application. The increasing presence of MSH in the environment, especially in the agricultural water and soil, could have long standing effects on plants. The presence of estrogens in the open environment is of special concern as they can act as endocrine disruptors for animals. But their effect on plant fitness is not well understood. One of the main aspects of plant fitness is maintaining a balance between growth and defense responses. The simple fact that plants are sessile in nature makes this a unique choice for them in the face of environmental stress when limited resources have to be allocated to an expensive endeavor like mounting a defense response instead of providing for the maximum growth of young tissue, flowers and fruits (Herms and Mattson, 1992; Huot et al., 2014).

Application of 10 and 100 nM ES to Arabidopsis seedlings resulted in enhanced root growth and higher leaf biomass as compared to the control plants. ES-treated plants displayed an increased photosynthesis rate as well as higher accumulation of carbohydrates and proteins, which could contribute to the increased biomass of these plants. The same hormone treatment resulted in an enhanced ability for the Arabidopsis plants to cope with drought stress and increased accumulation of antioxidant molecules like proline and GSH. In addition, higher levels of H_2O_2 were also observed in the ES -

treated plants as compared to the controls. Elevated levels of proline, GSH and H_2O_2 provide protection during the loss of hydration triggered by drought. ES appears to be acting similar to the plant steroidal hormone BR in terms of its effects on growth, development and response to abiotic stress. It has been observed that exogenous application of BRs not only enhances growth but has also an ameliorative effect against abiotic stress in plants (Vardhini and Rao, 2003; Kagale et al., 2007).

An opposite effect was observed with respect to the synthesis of secondary metabolites upon ES application. ES application to Arabidopsis seedlings resulted in the downregulation of genes involved in the biosynthesis of PPs, which had a negative impact on the accumulation of phenolics, anthocyanins and flavonoids. Compounds derived from the PP pathway are secondary metabolites that play an important role in providing defense against environmental stresses (Huang et al., 2010; Kim and Hwang, 2014). In this study, it was observed that lowered accumulation of PP pathway compounds in Arabidopsis leaves, due to ES application, compromised the plants' defense against the bacterial pathogen *Pst*. Salicyic acid is the plant hormone partially derived from the PP pathway within the plant cell and is required for plant defense responses against bacterial pathogens (Divi et al., 2010; Kim and Hwang, 2014; Zhang et al., 2015). It is possible that the lowering of *PAL1, CHS, CHI* and other key PP pathway biosynthesis gene expression due to ES application results in the reduced SA levels in *Arabidopsis,* which undermines its defense against the bacterial pathogen. Further work on determining SA levels in response to ES treatments would help establish this point.

Exogenous application of ES at 10 and 100 nM resulted in increased levels of carbohydrates and proteins accompanied by enhanced root growth and leaf biomass. Concurrently decreased accumulation of PP pathway products is also observed. It appears that ES application results in the increase of primary metabolism and growth by compromising secondary metabolism and defense against a biotic stressor. Thus it may be construed that in the presence of a chemical (e.g., 17β-estradiol), Arabidopsis plants favor plant growth over defense against a pathogen (Herms and Mattson, 1992; Bolton, 2009; Huot et al., 2014). Since it is still not clear as to why the ES-treated plants are able to cope better with an abiotic stress as compared to a biotic one, future work should include an inquiry into the molecular basis of this response by examining the expression of genes involved in plant defense and metabolism, in greater detail, in response to ES treatment.

Increasing the concentration of applied ES did not have an additive effect on the observed phenotype of 10 and 100 nM ES-treated Arabidopsis plants. The application of 10 µM ES was found to be phytotoxic as the plants displayed the lowest root growth and leaf biomass as compared to the controls and other ES treatments. These plants were also found to have the lowest accumulation of PP pathway products and the lowest survival rate in response to drought stress. The 10 µM ES-treated plants accumulate higher levels of H_2O_2, which remained unchanged in response to drought stress. The constantly elevated levels of a ROS such as H_2O_2 can contribute to stress-like conditions in ES-treated plants, which resulted in their small size and compromised resistance to environmental stresses. The lower levels of flavonoids in these plants could also

contribute towards the constant stress like conditions as the flavonoid-peroxidase system is an important antioxidant system in plants that accepts high-energy electrons from H_2O_2 (Yamasaki et al., 1997).

Previous studies have demonstrated that estrogen application has an ameliorative effect on plant stress responses (Erdal, 2009; Dumlupinar et al., 2011; Erdal, 2012). This study is the first to investigate the underlying molecular mechanism of estrogen action on plants. It can be speculated that ES application results in the downregulation of PP pathway genes by either acting as a negative regulator of transcription or by preventing the binding of a positive transcriptional regulator to the promoters of these genes (Fig. 6.1). The phenotypes of the ES-treated plants are a result of the transcriptional control of PP pathway genes and not a secondary effect of ES application as shown by studies performed with transgenic plants overexpressing *PAL1*. With *PAL1* under the transcriptional control of a constitutive promoter, the *PAL1*-OE plants were found to be independent of ES effects. However, further studies are required to determine the possible identity of the transcription factor or the promoter elements that can contribute to the effects of ES on plant growth, development and stress responses.

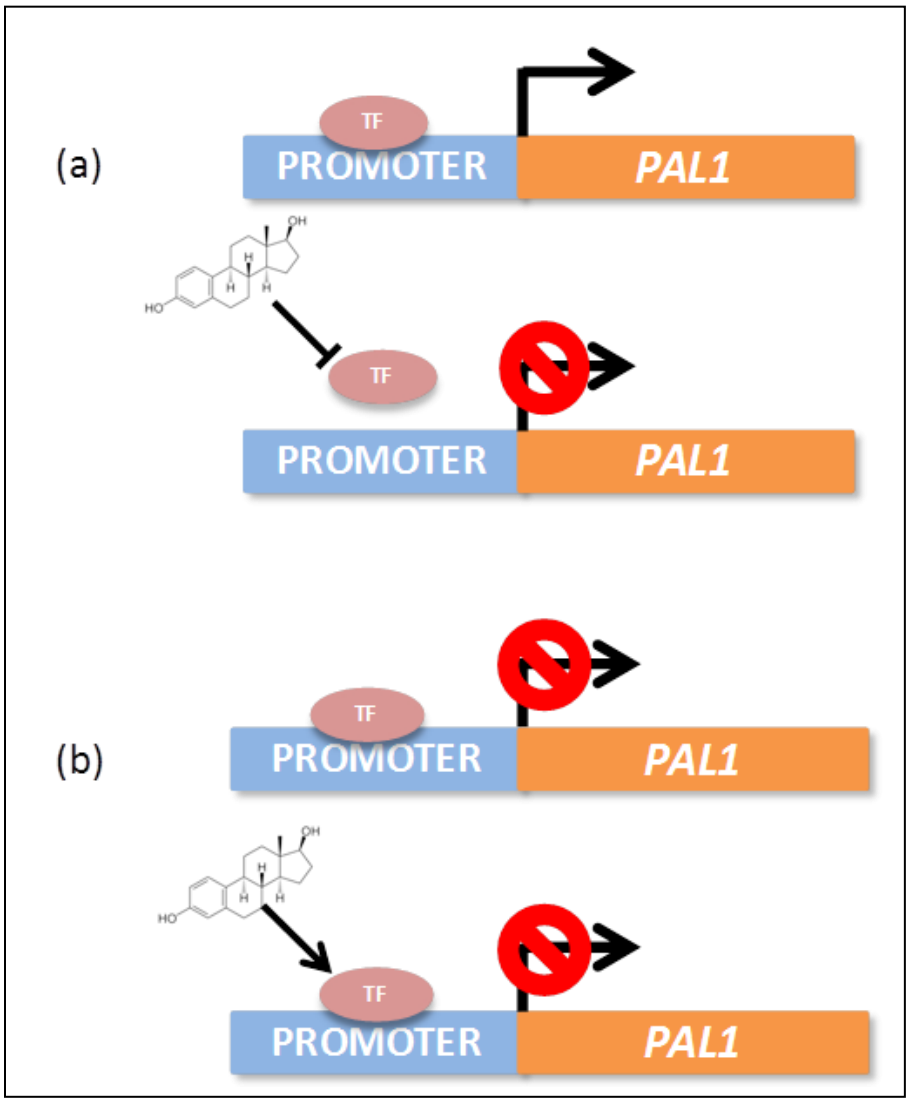

Figure 6.1 Possible mode of estradiol action in Arabidopsis. (a) Estradiol downregulates the expression of PP pathway genes by preventing the binding of a positive transcriptional regulator. (b) Estradiol downregulates the expression of PP pathway genes by enhancing the binding of a negative regulator to the promoter. TF=Transcription Factor.

As a paradigm for such studies, *ERF73*, an AP2/EREBP transcription factor family member, was identified from the list of genes downregulated by ES and the possible phenotypic outcomes of the experiments were speculated (Fig. 6.2). However, the *erf73* mutant plants were found to have a phenotype similar to that of the wild type plants in the terms of growth and development and in the presence of ES.

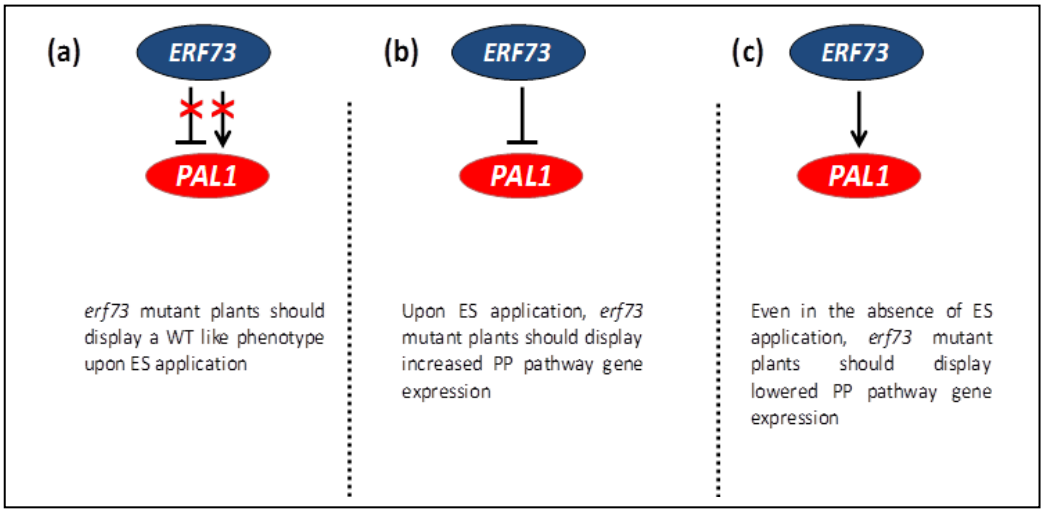

Figure 6.2 Model of the role of *ERF73* in downregulation of *PAL1* expression upon estradiol (ES) application. (a) *ERF73* and *PAL1* do not interact, (b) *ERF73* is a negative regulator of *PAL1* transcription and (c) *ERF73* is a positive regulator of *PAL1* transcription.

A possible explanation could be that *ERF73* does not play a role in mediating the ES response in *Arabidopsis*. Another possible explanation is that other AP2/EREBP members could compensate for the *ERF73* mutation. A detailed analysis of mutants for

the transcription factors that can control the PP pathway would help identify the molecular partner that mediates the transcriptional response to ES treatment in *Arabidopsis*. Based on the results presented in this dissertation, a model for ES action on plants is proposed (Fig. 6.3).

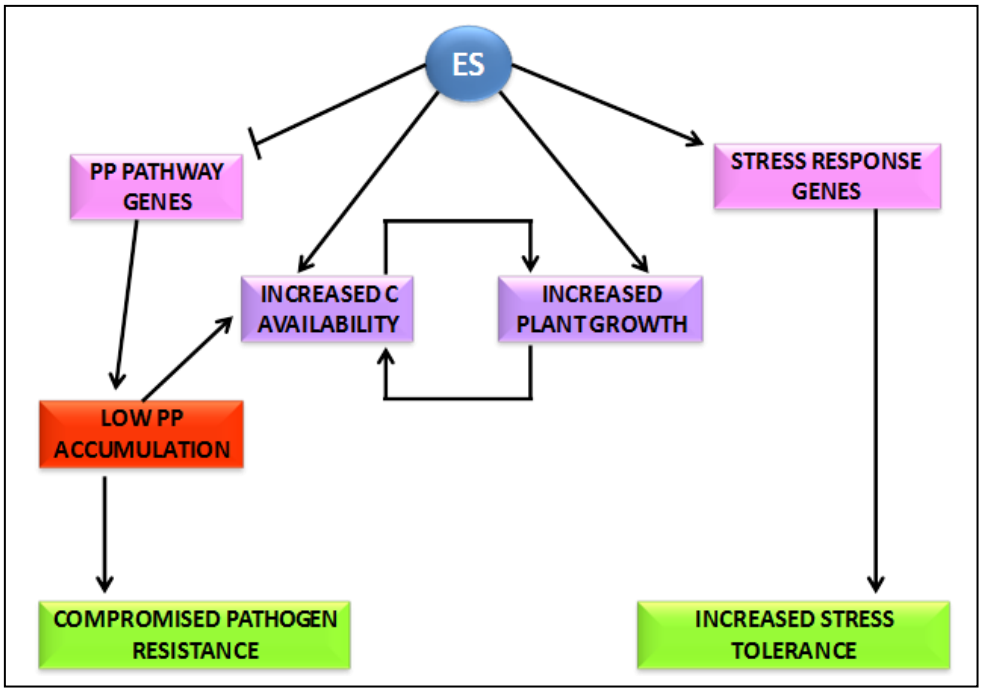

Figure 6.3 Summary of estradiol (ES) application effects on Arabidopsis growth, metabolism, gene expression, and stress responses.

Exogenous ES is able to alter the expression of several genes related to plant stress responses and secondary metabolism. The upregulation of abiotic stress responsive genes results in increased tolerance to drought stress whereas the downregulation of PP biosynthesis genes results in the lower accumulation of PP pathway products. A

concurrent effect of ES application is increased plant growth and photosynthesis. This is reflected in the increased availability of carbon as evidenced by the higher accumulation of primary metabolic products like carbohydrates and proteins. As compared to the WT, higher carbohydrates and proteins in ES-treated plants can contribute directly to plant growth. In the same instance, lowering of PP pathway products, which are a significant carbon sink, can potentially contribute to the available carbon pool. However low levels of PPs result in compromised resistance to a bacterial pathogen in ES-treated plants. Thus, ES may be acting as a metabolic switch that compromises secondary metabolism and biotic stress responses in favor of primary metabolism, growth and abiotic stress response.

The results presented here are a significant step towards our understanding of the effects of xenoestrogens on plants. This study demonstrates that ES is able to stimulate photosynthesis, primary metabolism, growth and development in *Arabidopsis thaliana* and may be used as a model for other agriculturally important plant species to enhance their productivity. Further studies are needed to establish the impact of ES on plant stress responses in other plants for its possible commercial application.

REFERENCES

Bolton, M.D. (2009). Primary metabolism and plant defense-fuel for the fire. Mol. Plant-Microbe Interact. **22,** 487-497.

Divi, U., Rahman, T., and Krishna, P. (2010). Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. BMC Plant Biol. **10,** 151.

Dumlupinar, R., Genisel, M., Erdal, S., Korkut, T., Taspinar, M.S., and Taskin, M. (2011). Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. Biol. Trace Elem. Res. **143,** 1740-1745.

Erdal, S. (2009). Effects of mammalian sex hormones on antioxidant enzyme activities, H_2O_2 content and lipid peroxidation in germinating bean seeds. Ataturk Univ. Ziraat Fak. Derg. **40,** 79-85.

Erdal, S. (2012). Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J. Sci. Food Agric. **92,** 1411-1416.

Herms, D.A. and Mattson, W.J. (1992). The dilemma of plants: to grow or defend. Quart. Rev. Biol.**67,** 283-335.

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.H., Yu, J.Q., and Chen, Z. (2010). Functional analysis of the *Arabidopsis PAL* gene family in plant growth, development, and response to environmental stress. Plant Physiol. **153,** 1526-1538.

Huot, B., Yao, J., Montgomery, B.L., and He, S.Y. (2014). Growth–defense tradeoffs in plants: a balancing act to optimize fitness. Molecular Plant **7,** 1267-1287.

Kagale, S., Divi, U.K., Krochko, J.E., Keller, W.A., and Krishna, P. (2007). Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. Planta **225,** 353-364.

Kim, D.S. and Hwang, B.K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J. Exp. Bot. **65,** 2295-2306.

Sablowski, R.W., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C., and Bevan, M. (1994). A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J. **13,** 128-137.

Vardhini, B.V. and Rao, S.S.R. (2003). Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. Plant Growth Regul. **41,** 25-31.

Yamasaki, H., Sakihama, Y., and Ikehara, N. (1997). Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiol. **115,** 1405-1412.

Yang, S., Sweetman, J.P., Amirsadeghi, S., Barghchi, M., Huttly, A.K., Chung, W.I., and Twell, D. (2001). Novel anther-specific myb genes from tobacco as putative regulators of phenylalanine ammonia-lyase expression. Plant Physiol. **126,** 1738-1753.

Zhang, Y., Fu, X., Hao, X., Zhang, L., Wang, L., Qian, H., and Zhao, J. (2015). Molecular cloning and promoter analysis of the specific salicylic acid biosynthetic

pathway gene phenylalanine ammonia-lyase (AaPAL1) from *Artemisia annua*. Biotechnol. Appl. Biochem. doi: 10.1002/bab.1403

COMPREHENSIVE REFERENCE LIST

Abrahám, E., Rigó, G., Székely, G., Nagy, R., Koncz, C., and Szabados, L. (2003). Light-dependent induction of proline biosynthesis by abscisic acid and salt stress is inhibited by brassinosteroid in Arabidopsis. Plant Mol. Biol. **51,** 363-372.

Abrahams, S., Lee, E., Walker, A.R., Tanner, G.J., Larkin, P.J., and Ashton, A.R. (2003). The Arabidopsis *TDS4* gene encodes leucoanthocyanidin dioxygenase (LDOX) and is essential for proanthocyanidin synthesis and vacuole development. Plant J. **35,** 624-636.

Adetunji, K. (2012). Microarray analysis of estradiol regulated gene expression in *Arabidopsis* seedlings. MS thesis. Texas Woman's University, TX, USA.

Agati, G., Biricolti, S., Guidi, L., Ferrini, F., Fini, A., and Tattini, M. (2011). The biosynthesis of flavonoids is enhanced similarly by UV radiation and root zone salinity in *L. vulgare* leaves. J. Plant Physiol. **168,** 204-212.

Ahammed, G.J., Zhou, Y.H., Xia, X.J., Mao, W.H., Shi, K., and Yu, J.Q. (2013). Brassinosteroid regulates secondary metabolism in tomato towards enhanced tolerance to phenanthrene. Biol. Plant. **57,** 154-158.

Alam, M.M., Hasanuzzaman, M., Nahar, K., and Fujita, M. (2013). Exogenous salicylic acid ameliorates short-term drought stress in mustard (*Brassica juncea* L.) seedlings by up-regulating the antioxidant defense and glyoxalase system. Aust. J. Crop Sci. **7,** 1053-1063.

Ali, R. and Abbas, H. (2003). Response of salt stressed barley seedlings to phenylurea. Plant Soil Environ. **49,** 158-162.

Anjum, N.A., Ahmad, I., Mohmood, I., Pacheco, M., Duarte, A.C., Pereira, E., Umar, S., Ahmad, A., Khan, N.A., and Iqbal, M. (2012). Modulation of glutathione and its related enzymes in plants' responses to toxic metals and metalloids—a review. Environ. Exp. Bot. **75,** 307-324.

Appelhagen, I., Thiedig, K., Nordholt, N., Schmidt, N., Huep, G., Sagasser, M., and Weisshaar, B. (2014). Update on transparent testa mutants from *Arabidopsis thaliana*: characterisation of new alleles from an isogenic collection. Planta **240,** 955-970.

Appert, C., Logemann, E., Hahlbrock, K., Schmid, J., and Amrhein, N. (1994). Structural and catalytic properties of the four phenylalanine ammonia-lyase isoenzymes from parsley (*Petroselinum crispum* Nym.). Eur. J. Biochem. **225,** 491-499.

Austin, M.B. and Noel, J.P. (2003). The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. **20,** 79-110.

Bahler, B.D., Steffen, K.L., and Orzolek, M.D. (1991). Morphological and biochemical comparison of a purple-leafed and a green-leafed pepper cultivar. Hort. Sci. **26,** 736-736.

Bajguz, A. (2011). Brassinosteroids-occurrence and chemical structures in plants. In Brassinosteroids: a Class of Plant Hormones, S. Hayat and A. Ahmad, eds. (New York, Springer), pp. 1-27.

Baranowska, M., Krupa, Z., and Orzol, D. (1996). Can anthocyanins be considered as heavy metal stress indicator in higher plants? Acta Physiol. Plant. **18,** 147-151.

Baron, K.N., Schroeder, D.F., and Stasolla, C. (2014). GEm-Related 5 (GER5), an ABA and stress-responsive GRAM domain protein regulating seed development and inflorescence architecture. Plant Sci. **223,** 153-166.

Baronti, C., Curini, R., D'Ascenzo, G., Di Corcia, A., Gentili, A., and Samperi, R. (2000). Monitoring natural and synthetic estrogens at activated sludge sewage treatment plants and in a receiving river water. Environ. Sci. Technol. **34,** 5059-5066.

Bates, L., Waldren, R., and Teare, I. (1973). Rapid determination of free proline for water-stress studies. Plant Soil **39,** 205-207.

Belfroid, A., Van der Horst, A., Vethaak, A., Schäfer, A., Rijs, G., Wegener, J., and Cofino, W. (1999). Analysis and occurrence of estrogenic hormones and their glucuronides in surface water and waste water in The Netherlands Sci. Total Environ. **225,** 101-108.

Berli, F.J., Moreno, D., Piccoli, P., Hespanhol-Viana, L., Silva, M.F., Bressan-Smith, R., Cavagnaro, J.B., and Bottini, R. (2010). Abscisic acid is involved in the response of grape (*Vitis vinifera* L.) cv. Malbec leaf tissues to ultraviolet-B radiation by enhancing ultraviolet-absorbing compounds, antioxidant enzymes and membrane sterols. Plant Cell Environ. **33,** 1-10.

Bhaskara, G.B., Yang, T., and Verslues, P.E. (2015). Dynamic proline metabolism: importance and regulation in water limited environments. Front. Plant Sci. **6.**

Bhattacharjee, S. (2012). An inductive pulse of hydrogen peroxide pretreatment restores redox-homeostasis and oxidative membrane damage under extremes of temperature in two rice cultivars. Plant Growth Regul. **68,** 395-410.

Bhattacharya, A., Sood, P., and Citovsky, V. (2010). The roles of plant phenolics in defence and communication during *Agrobacterium* and *Rhizobium* infection. Molec. Plant Pathol. **11,** 705–719

Bhattacharya, B. and Gupta, K. (1981). Steroid hormone effects on growth and apical dominance of sunflower. Phytochemistry **20,** 989-991.

Bhuiyan, N.H., Selvaraj, G., Wei, Y., and King, J. (2009). Role of lignification in plant defense. Plant Signal. Behav. **4,** 158-159.

Bircher, S., Card, M. L., Zhai, G., Chin, Y. P., and Schnoor, J. L. (2015). Sorption, uptake, and biotransformation of 17-β estradiol, 17-α ethinylestradiol, zeranol, and trenbolone acetate by hybrid poplar. Environ. Toxicol. Chem. **34,** 2906-2913.

Bolton, M.D. (2009). Primary metabolism and plant defense-fuel for the fire. Mol. Plant-Microbe Interact. **22,** 487-497.

Bowerman, P.A., Ramirez, M.V., Price, M.B., Helm, R.F., and Winkel, B.S. (2012). Analysis of T-DNA alleles of flavonoid biosynthesis genes in Arabidopsis ecotype Columbia. BMC Res. Notes **5.** doi: 10.1186/1756-0500-5-485.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72,** 248-254.

Bubna, G.A., Lima, R.B., Zanardo, D.Y., Dos Santos, W.D., Ferrarese, M.D., and Ferrarese-Filho, O. (2011). Exogenous caffeic acid inhibits the growth and enhances the lignification of the roots of soybean (*Glycine max*). J Plant Physiol. **168,** 1627-1633.

Card, M. L., Schnoor, J. L., and Chin, Y. P. (2013). Transformation of natural and synthetic estrogens by maize seedlings. Environ. Sci. Tech. **47,** 5101-5108.

Chalker-Scott, L. (1999). Environmental significance of anthocyanins in plant stress responses. Photochem. Photobiol. **70,** 1-9.

Chan, L., Koay, S., Boey, P., and Bhatt, A. (2010). Effects of abiotic stress on biomass and anthocyanin production in cell cultures of *Melastoma malabathricum*. Biol. Res. **43,** 127-135.

Chang, C., Yang, M., Wen, H., and Chern, J. (2002). Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. **10,** 178–182.

Chaoui, A. and El Ferjani, E. (2013). β-estradiol protects embryo growth from heavy-metal toxicity in germinating lentil seeds. J. Plant Growth Regul. **32,** 636-645.

Cheynier, V., Comte, G., Davies, K.M., Lattanzio, V., and Martens, S. (2013). Plant phenolics: recent advances on their biosynthesis, genetics, and ecophysiology. Plant Physiol. Biochem.**72,** 1-20.

Christie, P.J., Alfenito, M.R., and Walbot, V. (1994). Impact of low-temperature stress on general phenylpropanoid and anthocyanin pathways: enhancement of transcript abundance and anthocyanin pigmentation in maize seedlings. Planta **194,** 541-549.

Clouse, S.D. (2015). A history of brassinosteroid research from 1970 through 2005: Thirty-five years of phytochemistry, physiology, genes and mutants. J. Plant Growth Regul. **34,** 828-844.

Contour-Ansel, D., Torres-Franklin, M.L., Cruz de Carvalho, M.H., D'Arcy-Lameta, A., Zuily-Fodil, Y. (2006). Glutathione reductase in leaves of cowpea: Cloning of two cDNAs, expression and enzymatic activity under progressive drought stress, desiccation and abscisic acid treatment. Ann. Bot. **98,** 1279–1287.

Costa, M.A., Bedgar, D.L., Moinuddin, S.G., Kim, K., Cardenas, C.L., Cochrane, F.C., Shockey, J.M., Helms, G.L., Amakura, Y., and Takahashi, H. (2005). Characterization *in vitro* and *in vivo* of the putative multigene 4-coumarate: CoA ligase network in *Arabidopsis*: syringyl lignin and sinapate/sinapyl alcohol derivative formation. Phytochemistry **66,** 2072-2091.

Curtis, M.D. and Grossniklaus, U. (2003). A gateway cloning vector set for high-throughput functional analysis of genes in planta. Plant Physiol. **133,** 462-469.

Czajka, C.P. and Londry, K.L. (2006). Anaerobic biotransformation of estrogens. Sci. Total Environ. **367,** 932-941.

Daneshmand, F., Arvin, M.J., and Kalantari, K.M. (2010). Physiological responses to NaCl stress in three wild species of potato *in vitro*. Acta Physiol. Plant. **32,** 91-101.

Das, K. and Roychoudhury, A. (2014). Reactive oxygen species (ROS) and response of antioxidants as ROS-scavengers during environmental stress in plants. Front. Environ. Sci. **2,** 53. doi.org/10.3389/fenvs.2014.00053

Divi, U., Rahman, T., and Krishna, P. (2010). Brassinosteroid-mediated stress tolerance in Arabidopsis shows interactions with abscisic acid, ethylene and salicylic acid pathways. BMC Plant Biol. **10,** 151. doi:10.1186/1471-2229-10-151

Dixon, R.A. and Paiva, N.L. (1995). Stress-induced phenylpropanoid metabolism. Plant Cell **7,** 1085-1097.

Dixon, R.A. (2001). Natural products and plant disease resistance. Nature **411,** 843-847.

Dixon, R.A., Achnine, L., Kota, P., Liu, C.J., Reddy, M.S., and Wang, L. (2002). The phenylpropanoid pathway and plant defense: a genomics perspective. Mol Plant Pathol **3,** 371–390.

Dumlupinar, R., Genisel, M., Erdal, S., Korkut, T., Taspinar, M.S., and Taskin, M. (2011). Effects of progesterone, β-estradiol, and androsterone on the changes of inorganic element content in barley leaves. Biol. Trace Elem. Res. **143,** 1740-1745.

Edwards, R., Dixon, D.P., and Walbot, V. (2000). Plant glutathione S-transferases: enzymes with multiple functions in sickness and in health. Trends Plant Sci. **5,** 193-198.

Emamverdian, A., Ding, Y., Mokhberdoran, F., and Xie, Y. (2015). Heavy metal stress and some mechanisms of plant defense response. Sci. World J. doi: 10.1155/2015/756120.

Erdal, S. (2009). Effects of mammalian sex hormones on antioxidant enzyme activities, H_2O_2 content and lipid peroxidation in germinating bean seeds. Ataturk Univ. Ziraat Fak. Derg. **40,** 79-85.

Erdal, S. (2012a). Exogenous mammalian sex hormones mitigate inhibition in growth by enhancing antioxidant activity and synthesis reactions in germinating maize seeds under salt stress. J. Sci. Food Agric. **92,** 839-843.

Erdal, S. (2012b). Alleviation of salt stress in wheat seedlings by mammalian sex hormones. J. Sci. Food Agric. **92,** 1411-1416.

Erdal, S. (2012c). Androsterone-induced molecular and physiological changes in maize seedlings in response to chilling stress. Plant Physiol. Biochem. **57,** 1–7.

Erdal, S. and Dumlupinar, R. (2010). Progesterone and β-estradiol stimulate seed germination in chickpea by causing important changes in biochemical parameters. Zeitschrift für Naturforschung **65,** 239-244.

Erdal, S. and Dumlupinar, R. (2011). Mammalian sex hormones stimulate antioxidant system and enhance growth of chickpea plants. Acta Physiol. Plant. **33,** 1011-1017.

Erdal, S., Genisel, M., Turk, H., and Gorcek, Z. (2012). Effects of progesterone application on antioxidant enzyme activities and K+/Na+ ratio in bean seeds exposed to salt stress. Toxicol. Ind. Health **28,** 942-946.

Ferrer, J., Austin, M., Stewart, C., and Noel, J. (2008). Structure and function of enzymes involved in the biosynthesis of phenylpropanoids. Plant Physiol. Biochem. **46,** 356-370.

Ferreyra, M.L.F., Casas, M.I., Questa, J.I., Herrera, A.L., DeBlasio, S., Wang, J., Jackson, D., Grotewold, E., and Casati, P. (2012). Evolution and expression of tandem duplicated maize flavonol synthase genes. Front. Plant Sci. **3,** 101. doi: 10.3389/fpls.2012.00101

Finlay-Moore, O., Hartel, P., and Cabrera, M. (2000). 17β-estradiol and testosterone in soil and runoff from grasslands amended with broiler litter. J. Environ. Qual. **29,** 1604-1611.

Foster-Hartnett, D., Danesh, D., Penuela, S., Sharopova, N., Endre, G., Vandenbosch, K.A., Young, N.D., and Samac, D.A. (2007). Molecular and cytological responses of *Medicago truncatula* to *Erysiphe pisi*. Mol. Plant Pathol.**8,** 307-319.

Fraser, C.M., and Chapple, C. (2011). The phenylpropanoid pathway in *Arabidopsis*. Arabidopsis Book **9,** e0152. doi: 10.1199/tab.0152

Fridman, Y., and Savaldi-Goldstein, S. (2013). Brassinosteroids in growth control: how, when and where. Plant Sci. **209,** 24-31.

Galis, I., Onkokesung, N., and Baldwin, I.T. (2010). New insights into mechanisms regulating differential accumulation of phenylpropanoid-polyamine conjugates (PPCs) in herbivore-attacked *Nicotiana attenuata* plants. Plant Signal Behav **5,** 610–613.

Genisel, M., Turk, H., and Erdal, S. (2013). Exogenous progesterone application protects chickpea seedlings against chilling-induced oxidative stress. Acta Physiol. Plant. **35,** 241-251.

Geuns, J. (1978). Steroid hormones and plant growth and development. Phytochemistry **17,** 1-14.

Gondim, F.A., Miranda, R.d.S., Gomes-Filho, E., and Prisco, J.T. (2013). Enhanced salt tolerance in maize plants induced by H_2O_2 leaf spraying is associated with improved gas exchange rather than with non-enzymatic antioxidant system. Theor. Exp. Plant Physiol. **25,** 251-260.

Goeppert, N., Dror, I., and Berkowitz, B. (2014). Detection, fate and transport of estrogen family hormones in soil. Chemosphere **95,** 336-345.

Grace, S.C. and Logan, B.A. (2000). Energy dissipation and radical scavenging by the plant phenylpropanoid pathway. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **355,** 1499-1510.

Greca, M.D., Florentino, A., Pinto, G., Pollio, A. Previtera, L. (1996). Biotransformation of progesterone by the green alga *Chlorella emersonii* C211-8H. Phytochemistry **41**, 1527-1529.

Guan, M. and Roddick, J.G. (1988). Epibrassinolide-inhibition of development of excised, adventitious and intact roots of tomato (*Lycopersicon esculentum*): comparison with the effects of steroidal estrogens. Physiol. Plant **74,** 720-726.

Hamberger, B. and Hahlbrock, K. (2004). The 4-coumarate:CoA ligase gene family in *Arabidopsis thaliana* comprises one rare, sinapate-activating and three commonly occurring isoenzymes. Proc. Natl. Acad. Sci. U. S. A. **101,** 2209-2214.

Hanselman, T.A., Graetz, D.A., and Wilkie, A.C. (2003). Manure-borne estrogens as potential environmental contaminants: a review. Environ. Sci. Technol. **37,** 5471-5478.

Hansen, J. and Møller, I. (1975). Percolation of starch and soluble carbohydrates from plant tissue for quantitative determination with anthrone. Anal. Biochem. **68,** 87-94.

Hansen, P., Dizer, H., Hock, B., Marx, A., Sherry, J., McMaster, M., and Blaise, C. (1998). Vitellogenin–a biomarker for endocrine disruptors. Trends Anal. Chem. **17,** 448-451.

Hawrylak, B., Matraszek, R., and Szymańska, M. (2007). Response of lettuce (*Lactuca sativa* L.) to selenium in nutrient solution contaminated with nickel. Veg. Crop. Res. **67,** 63-70.

Hawrylak-Nowak, B. (2008). Changes in anthocyanin content as indicator of maize sensitivity to selenium. J. Plant Nutr. **31,** 1232-1242.

Helmkamp, G. and Bonner, J. (1953). Some Relationships of Sterols to Plant Growth. Plant Physiol. **28,** 428-436.

Herbinger, K., Tausz, M., Wonisch, A., Soja, G., Sorger, A., and Grill, D. (2002). Complex interactive effects of drought and ozone stress on the antioxidant defence systems of two wheat cultivars. Plant Physiol. Biochem. **40,** 691-696.

Herms, D.A. and Mattson, W.J. (1992). The dilemma of plants: to grow or defend. Quart. Rev. Biol. **67,** 283-335.

Hernández, I., Alegre, L., and Munné-Bosch, S. (2006). Enhanced oxidation of flavan-3-ols and proanthocyanidin accumulation in water-stressed tea plants. Phytochemistry **67,** 1120-1126.

Hong, S. and Vierling, E. (2001). Hsp101 is necessary for heat tolerance but dispensable for development and germination in the absence of stress. Plant J. **27,** 25-35.

Hossain, M.A. and Fujita, M. (2013). Hydrogen Peroxide priming stimulates drought tolerance in mustard (*Brassica juncea* L.) seedlings. Plant Gene Trait **4,** 1. doi: 10.5376/pgt.2013.04.0020

Hossain, M.A., Mostofa, M.G., and Fujita, M. (2013). Heat-shock positively modulates oxidative protection of salt and drought-stressed mustard (*Brassica campestris* L.) seedlings. J. Plant Sci. Mol. Breed 2**,** 2. doi: 10.7243/2050-2389-2-2

Huang, J., Gu, M., Lai, Z., Fan, B., Shi, K., Zhou, Y.H., Yu, J.Q., and Chen, Z. (2010). Functional analysis of the Arabidopsis *PAL* gene family in plant growth, development, and response to environmental stress. Plant Physiol. **153,** 1526-1538.

Huot, B., Yao, J., Montgomery, B.L., and He, S.Y. (2014). Growth–defense tradeoffs in plants: a balancing act to optimize fitness. Molecular Plant **7,** 1267-1287.

Iason, G. (2005). The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. Proc. Nutr. Soc. **64,** 123-131.

Iqbal, M., Yaegashi, S., Ahsan, R., Shopinski, K.L., and Lightfoot, D.A. (2005). Root response to *Fusarium solani* f. sp. *glycines*: temporal accumulation of transcripts in partially resistant and susceptible soybean. Theor. Appl. Genet. **110,** 1429-1438.

Izaguirre, M.M., Scopel, A.L., Baldwin, I.T., and Ballare, C.L. (2003). Convergent responses to stress. Solar ultraviolet-B radiation and *Manduca sexta* herbivory elicit overlapping transcriptional responses in field-grown plants of *Nicotiana longiflora*. Plant Physiol. **132,** 1755-1767.

Jansen, M.A.K., Van den Noort, R.E., Tan, M.Y.A., Prinsen, E., Largimini, L.M., and Thorneley, R.N.F. (2001). Phenol-oxidizing peroxidases contribute to the protection of plants from ultraviolet radiation stress. Plant Physiol. **126,** 1012–1023

Janeczko, A. (2000). Influence of selected steroids on plant physiological processes-especially flowering induction. PhD Thesis, Agriculture University, Krakow, Poland.

Janeczko, A., Budziszewska, B., Skoczowski, A., Dybała, M. (2008). Specific binding sites for progesterone and 17 β-estradiol in cells of *Triticum aestivum* L. Acta Biochim. Pol. **55,** 707-711.

Janeczko, A., and Filek, W. (2002). Stimulation of generative development in partly vernalized winter wheat by animal sex hormones. Acta Physiol. Plant. **24,** 291-295.

Janeczko, A., Filek, W., Biesaga-Kościelniak, J., Marcińska, I., and Janeczko, Z. (2003). The influence of animal sex hormones on the induction of flowering in *Arabidopsis thaliana*: comparison with the effect of 24-epibrassinolide. Plant Cell Tissue Org. **72,** 147-151.

Janeczko, A., Korucek, M., and Marcinska, I. (2012). Mammalian androgen stimulates photosynthesis in drought-stressed soybean. Cent. Eur. J. Biol. **7,** 902-909.

Janeczko, A., and Skoczowski, A. (2005). Mammalian sex hormones in plants. Folia Histochem. Cytobiol. **43,** 71-79.

Jansen, M.A.K., Van den Noort, R.E., Tan, M.Y.A., Prinsen. E., Largimini, L.M., and Thorneley, R.N.F. (2001). Phenol-oxidizing peroxidases contribute to the protection of plants from Inhibition ultraviolet radiation stress. Plant Physiol. **126,** 1012–1023.

Jiang, W., Yan, Y., Ma, M., Wang, D., Lou, Q., Wang, Z., and Satyanarayanan, S.K. (2012). Assessment of source water contamination by estrogenic disrupting compounds in China. J. Environ. Sci. **24,** 320-328.

Jobling, S., Nolan, M., Tyler, C.R., Brighty, G., and Sumpter, J.P. (1998). Widespread sexual disruption in wild fish. Environ. Sci. Technol. **32,** 2498-2506.

Johnson, A., Belfroid, A., and Di Corcia, A. (2000). Estimating steroid oestrogen inputs into activated sludge treatment works and observations on their removal from the effluent. Sci. Total Environ. **256,** 163-173.

Johnson, E.T., Berhow, M.A., and Dowd, P.F. (2007). Expression of a maize Myb transcription factor driven by a putative silk-specific promoter significantly enhances resistance to Helicoverpa zea in transgenic maize. J. Agric. Food Chem. **55,** 2998-3003.

Jones, A.M., Chattopadhyay, A., Shukla, M., Zoń, J., and Saxena, P.K. (2012). Inhibition of phenylpropanoid biosynthesis increases cell wall digestibility, protoplast isolation, and facilitates sustained cell division in American elm (*Ulmus americana*). BMC Plant Biol. **12**, 1. doi: 10.1186/1471-2229-12-75

Jones, A.M., and Saxena, P.K. (2013). Inhibition of phenylpropanoid biosynthesis in *Artemisia annua* L.: a novel approach to reduce oxidative browning in plant tissue culture. PLoS One. **8,** e76802. doi: 10.1371/journal.pone.0076802

Kagale, S., Divi, U.K., Krochko, J.E., Keller, W.A., and Krishna, P. (2007). Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. Planta **225,** 353-364.

Kandan, A., Commare, R.R., Nandakumar, R., Ramaih, M., Raguchander, T., and Samiyappan, R. (2002). Induction of phenylpropanoid metabolism by *Pseudomonas fluorescens* against tomato spotted wilt virus in tomato. Folia Microbiol. **47,** 121-129.

Kawaoka, A. and Ebinuma, H. (2001). Transcriptional control of lignin biosynthesis by tobacco LIM protein. Phytochemistry **57,** 1149-1157.

Kim, D.S. and Hwang, B.K. (2014). An important role of the pepper phenylalanine ammonia-lyase gene (*PAL1*) in salicylic acid-dependent signalling of the defence response to microbial pathogens. J. Exp. Bot. **65,** 2295-2306.

Kliebenstein, D. (2004). Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. Plant, Cell Environ. **27,** 675-684.

Kliebenstein, D., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J. **44,** 25-36.

Koca, N., and Karaman, S. (2015). The effects of plant growth regulators and L-phenylalanine on phenolic compounds of sweet basil. Food Chem. **166,** 515-521.

Kopcewicz, J. (1969). Influence of estrone on growth and endogenous gibberellins content in dwarf pea. Bull. Acad. Pol. Sci. Biol. **17,** 727-731.

Kopcewicz, J. (1970). Influence of estrogens on flower formation in *Cichorium intybus* L. Naturwissenschaften **57,** 136-136.

Kopcewicz, J., and Porazinski, Z. (1974). Effects of growth regulators, steroids and estrogen fraction from sage plants on flowering of a long day plant, *Salvia splendens*, grown under non-inductive light conditions. Biol. Plant. **16,** 132-135.

Korkina, L. (2007). Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. Cell Mol. Biol. **53,** 15-25.

Krishna, P. (2003). Brassinosteroid-mediated stress responses. J. Plant Growth Regul. **22,** 289-297.

Kuch, H.M. and Ballschmiter, K. (2001). Determination of endocrine-disrupting phenolic compounds and estrogens in surface and drinking water by HRGC-(NCI)-MS in the picogram per liter range. Environ. Sci. Technol. **35,** 3201-3206.

Kusano, M., Tohge, T., Fukushima, A., Kobayashi, M., Hayashi, N., Otsuki, H., Kondou, Y., Goto, H., Kawashima, M., and Matsuda, F. (2011). Metabolomics reveals comprehensive reprogramming involving two independent metabolic responses of *Arabidopsis* to UV-B light. Plant J. **67,** 354-369.

Lattanzio, V., Lattanzio, V.M., and Cardinali, A. (2006). Role of phenolics in the resistance mechanisms of plants against fungal pathogens and insects. In Phytochemistry Advances in Research, Vol. 661. F. Imperato, ed. (India, Reseaech Signapost) pp. 23-67.

Laxmi, A., Paul, L.K., Peters, J.L., and Khurana, J.P. (2004). *Arabidopsis* constitutive photomorphogenic mutant, *bls1*, displays altered brassinosteroid response and sugar sensitivity. Plant Mol. Biol. **56,** 185–201.

Lee, S.W., Robb, J., and Nazar, R.N. (1992). Truncated phenylalanine ammonia-lyase expression in tomato (*Lycopersicon esculentum*). J. Biol. Chem. **267,** 11824-11830.

Lepiniec, L., Debeaujon, I., Routaboul, J., Baudry, A., Pourcel, L., Nesi, N., and Caboche, M. (2006). Genetics and biochemistry of seed flavonoids. Annu. Rev. Plant Biol. **57,** 405-430.

Lichtenthaler, H.K., and Buschmann, C. (2001). Chlorophylls and carotenoids: Measurement and characterization by UV-VIS spectroscopy. Curr. Prot. Food Anal. Chem. doi: 10. 1002/0471142913.faf0403s01.

Lin, J., Palevitch, D., and Heftmann, E. (1983). Reduction of 4-andro-stene-3, 17-dione by growing cucumber plants. Phytochemistry **22,** 1149-1154.

Lin, J., Proebsting, W.M. and Heftmann, E. (1979). Conversion of 4-androstene-3, 17-dione to testosterone by *Pisum sativum*. Phytochemistry **18,** 1667-1669.

Logemann, E. and Hahlbrock, K. (2002). Crosstalk among stress responses in plants: pathogen defense overrides UV protection through an inversely regulated ACE/ACE type of light-responsive gene promoter unit. Proc. Natl. Acad. Sci. U. S. A. **99,** 2428-2432.

López-Gresa, M.P., Torres, C., Campos, L., Lisón, P., Rodrigo, I., Bellés, J.M., and Conejero, V. (2011). Identification of defence metabolites in tomato plants infected by the bacterial pathogen *Pseudomonas syringae*. Environ. Exp. Bot. **74,** 216-228.

Löve, Á and Löve, D. (1945). Experiments on the effects of animal sex hormones on dioecious plants. Ark. Botanik. **32,** 1-60.

Lydon, J., and Duke, S.O. (1989) Pesticide effects on secondary metabolism of higher plants. Pest. Sci. **25,** 361-73.

Maher, E.A., Bate, N.J., Ni, W., Elklnd, Y., Dixon, R.A., and Lamb, C.J. (1994). Increased disease susceptibility of transgenic tobacco plants with suppressed levels of preformed phenylpropanoid products. Proc. Natl. Acad. Sci. USA **91,** 7802-7806.

Mauvais-Jarvis, F., Clegg, D.J., and Hevener, A.L. (2013). The role of estrogens in control of energy balance and glucose homeostasis. Endocr. Rev. **34,** 309-338.

Mazza, C.A., Boccalandro, H.E., Giordano, C.V., Battista, D., Scopel, A.L., and Ballare, C.L. (2000). Functional significance and induction by solar radiation of

ultraviolet-absorbing sunscreens in field-grown soybean crops. Plant Physiol. **122,** 117-126.

Mele, G., Ori, N., Sato, Y., and Hake, S. (2003). The knotted1-like homeobox gene *BREVIPEDICELLUS* regulates cell differentiation by modulating metabolic pathways. Genes Dev. **17,** 2088-2093.

Milanesi, L., Boland, R. (2004). Presence of estrogen receptor (ER)-like proteins and endogenous ligands for ER in *Solanaceae*. Plant Sci. **166,** 397-404.

Mizutani, M., Ohta, D., and Sato, R. (1997). Isolation of a cDNA and a genomic clone encoding cinnamate 4-hydroxylase from *Arabidopsis* and its expression manner *in planta*. Plant Physiol. **113,** 755-763.

Moura, J. C. M. S., Bonine, C. A. V., De Oliveira Fernandes Viana, J., Dornelas, M. C., and Mazzafera, P. (2010). Abiotic and biotic stresses and changes in the lignin content and composition in plants. J. Integr. Plant Biol. **52,** 360-376.

Moy, P., Qutob, D., Chapman, B.P., Atkinson, I., and Gijzen, M. (2004). Patterns of gene expression upon infection of soybean plants by *Phytophthora sojae*. Mol. Plant-Microbe Interact. **17,** 1051-1062.

Murashige, T. and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiol. Plant. **15,** 473-497.

Narayan, M., Thimmaraju, R., and Bhagyalakshmi, N. (2005). Interplay of growth regulators during solid-state and liquid-state batch cultivation of anthocyanin producing cell line of *Daucus carota*. Process Biochem. **40,** 351-358.

Navarro, J.M., Flores, P., Garrido, C., and Martinez, V. (2006). Changes in the contents of antioxidant compounds in pepper fruits at different ripening stages, as affected by salinity. Food Chem. **96,** 66-73.

Nichols, D., Daniel, T., Moore, P., Edwards, D., and Pote, D. (1997). Runoff of estrogen hormone 17β-estradiol from poultry litter applied to pasture. J. Environ. Qual. **26,** 1002-1006.

Pallas, J.A., Paiva, N.L., Lamb, C., and Dixon, R.A. (1996). Tobacco plants epigenetically suppressed in phenylalanine ammonia-lyase expression do not develop systemic acquired resistance in response to infection by tobacco mosaic virus. Plant J. **10,** 281-293.

Patisaul, H.B., and Jefferson, W. (2010). The pros and cons of phytoestrogens. Front. Neuroendocrinol. **31,** 400-419.

Pastori, G.M., Trippi, and V.S. (1992) Oxidative stress induces high rate of glutathione reductase synthesis in a drought resistant maize strain. Plant Cell Physiol. **33,** 957–961.

Pawlak-Sprada, S., Arasimowicz-Jelonek, M., Podgorska, M. and Deckert, J. (2011). Activation of phenylpropanoid pathway in legume plants exposed to heavy metals. Part 1. Effects of cadmium and lead on phenylalanine ammonia lyase gene expression, enyzme activity and lignin content. Act. Biochim. Polon. **58**, 211-216.

Payyavula, R.S., Navarre, D.A., Kuhl, J.C., Pantoja, A., and Pillai, S.S. (2012). Differential effects of environment on potato phenylpropanoid and carotenoid expression. BMC Plant. Biol. **12**. doi: 39-2229-12-39.

Pelletier, M.K. and Shirley, B.W. (1996). Analysis of flavanone 3-hydroxylase in *Arabidopsis* seedlings. Coordinate regulation with chalcone synthase and chalcone isomerase. Plant Physiol. **111,** 339-345.

Peterson, E., Davis, R., and Orndorff, H. (2000). 17 β-Estradiol as an indicator of animal waste contamination in mantled karst aquifers. J. Environ. Qual. **29,** 826-834.

Petrov, V.D. and Van Breusegem, F. (2012). Hydrogen peroxide-a central hub for information flow in plant cells. AoB Plants **2012,** pls014. doi: 10.1093/aobpla/pls014

Purdom, C., Hardiman, P., Bye, V., Eno, N., Tyler, C., and Sumpter, J. (1994). Estrogenic effects of effluents from sewage treatment works. Chem. Ecol. **8,** 275-285.

Pyngrope, S., Bhoomika, K., and Dubey, R. (2013). Reactive oxygen species, ascorbate–glutathione pool, and enzymes of their metabolism in drought-sensitive and tolerant indica rice (*Oryza sativa* L.) seedlings subjected to progressing levels of water deficit. Protoplasma **250,** 585-600.

Raes, J., Rohde, A., Christensen, J.H., Van de Peer, Y., and Boerjan, W. (2003). Genome-wide characterization of the lignification toolbox in *Arabidopsis*. Plant Physiol. **133,** 1051-1071.

Rejeb, K.B., Jdey, A., Abdelly, C., and Savoure, A. (2015). Hydrogen peroxide produced by NADPH oxidases increases proline accumulation during salt or mannitol stress in *Arabidopsis thaliana*. New Phytol. **208,** 1138-1148.

Riechmann, J.L., and Meyerowitz, E.M. (1998). The AP2/EREBP family of transcription factors. Biol. Chem. **379,** 633-646.

Rivero, R.M., Ruiz, J.M., Garcia, P.C., López-Lefebre, L.R., Sánchez, E., and Romero, L. (2001). Resistance to cold and heat stress: accumulation of phenolic compounds in tomato and watermelon plants. Plant Sci. **160,** 315–321.

Rodgers-Gray, T.P., Jobling, S., Morris, S., Kelly, C., Kirby, S., Janbakhsh, A., Harries, J.E., Waldock, M.J., Sumpter, J.P., and Tyler, C.R. (2000). Long-term temporal changes in the estrogenic composition of treated sewage effluent and its biological effects on fish. Environ. Sci. Technol. **34,** 1521-1528.

Rohde, A., Morreel, K., Ralph, J., Goeminne, G., Hostyn, V., De Rycke, R., Kushnir, S., Van Doorsselaere, J., Joseleau, J.P., Vuylsteke, M., Van Driessche, G., Van Beeumen, J., Messens, E., and Boerjan, W. (2004). Molecular phenotyping of the *pal1* and *pal2* mutants of *Arabidopsis thaliana* reveals far-reaching consequences on phenylpropanoid, amino acid, and carbohydrate metabolism. Plant Cell **16,** 2749-2771.

Rojek, J., Pawełko, Ł, Kapusta, M., Naczk, A., and Bohdanowicz, J. (2015). Exogenous steroid hormones stimulate full development of autonomous endosperm in *Arabidopsis thaliana*. Acta Soc. Bot. Pol. **84,** 287-301.

Ryan, K.G., Swinny, E.E., Markham, K.R., and Winefield, C. (2002). Flavonoid gene expression and UV photoprotection in transgenic and mutant Petunia leaves. Phytochemistry **59,** 23-32.

Sablowski, R.W., Moyano, E., Culianez-Macia, F.A., Schuch, W., Martin, C., and Bevan, M. (1994). A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J. **13,** 128-137.

Samac, D.A. and Graham, M.A. (2007). Recent advances in legume-microbe interactions: recognition, defense response, and symbiosis from a genomic perspective. Plant Physiol. **144,** 582-587.

Sappl, P.G., Carroll, A.J., Clifton, R., Lister, R., Whelan, J., Harvey Millar, A., and Singh, K.B. (2009). The *Arabidopsis* glutathione transferase gene family displays complex stress regulation and co-silencing multiple genes results in altered metabolic sensitivity to oxidative stress. Plant J. **58,** 53-68.

Schaller, H. (2003). The role of sterols in plant growth and development. Prog. Lipid Res. **42,** 163-175.

Schardl, C. L., and Chen, F. (2010). Plant defences against herbivore attack. *eLS*. doi: 10.1002/9780470015902.a0001324.pub2

Schilmiller, A.L., Stout, J., Weng, J., Humphreys, J., Ruegger, M.O., and Chapple, C. (2009). Mutations in the cinnamate 4-hydroxylase gene impact metabolism, growth and development in Arabidopsis. Plant J. **60,** 771-782.

Schmidt, S., Degen, G.H., Seibel, J., Hertrampf, T., Vollmer, G., and Diel, P. (2006). Hormonal activity of combinations of genistein, bisphenol A and 17beta-estradiol in the female Wistar rat. Arch. Toxicol. **80,** 839–845.

Schoch, G.A., Nikov, G.N., Alworth, W.L., and Werck-Reichhart, D. (2002). Chemical inactivation of the cinnamate 4-hydroxylase allows for the accumulation of salicylic acid in elicited cells. Plant Physiol. **130,** 1022-1031.

Schoenbohm, C., Martens, S., Eder, C., Forkmann, G., and Weisshaar, B. (2000). Identification of the *Arabidopsis thaliana* flavonoid 3'-hydroxylase gene and functional expression of the encoded P450 enzyme. Biol. Chem. **381,** 749-753.

Schultz, M.M., Minarik, T.A., Martinovic-Weigelt, D., Curran, E.M., Bartell, S.E., Schoenfuss, H.L. (2013). Environmental estrogens in an urban aquatic ecosystem: II. Biological effects. Environ. Int. **61,** 138-149.

Shirley, B.W., Hanley, S., and Goodman, H.M. (1992). Effects of ionizing radiation on a plant genome: analysis of two *Arabidopsis* transparent testa mutations. Plant Cell **4,** 333-347.

Shore, L., Correll, D., and Chakraborty, P. (1995). Relationship of fertilization with chicken manure and concentrations of estrogens in small streams. In Animal Waste and the Land-Water Interface. K. Steele, ed. (New York, Lewis Publishers) pp. 155-162.

Shore, L.S., Kapulnik, Y., Ben-Dor, B., Fridman, Y., Wininger, S., and Shemesh, M. (1992). Effects of estrone and 17 β-estradiol on vegetative growth of *Medicago sativa.* Physiol. Plant. **84,** 217-222.

Shore, L.S., and Shemesh, M. (2003). Naturally produced steroid hormones and their release into the environment. Pure Appl. Chem. **75,** 1859–1871.

Shore, L., Shemesh, M., and Cohen, R. (1988). The role of oestradiol and oestrone in chicken manure silage in hyperoestrogenism in cattle. Aust. Vet. J. **65,** 68-68.

Sieveking, D.P., Lim P., Chow R.W.Y., Dunn, L.L., Bao, S., McGrath, K.C.Y., Heather, A.K., Handelsman, D.J., Celermajer, D.S., Ng, M.K.C. (2010) A sex-specific role for androgens in angiogenesis. J Exp. Med. **207**, 345-357

Snook, M.E., Widstrom, N.W., Wiseman, B.R., Gueldner, R.C., Wilson, R.L., Himmelsbach, D.S., Harwood, J.S., and Costello, C.E. (1994). New flavone C-glycosides from corn (*Zea mays* L) for the control of the corn earworm (*Helicoverpa zea*). In Bioregulators for Crop Protection and Pest Control. P. A. Hedin, ed. (Washington DC, ACS Symposium Series 557) pp. 122-135.

Song, G.C., Ryu, S.Y., Kim, Y.S., Lee. J.Y., Choi, J.S., and Ryu, C.M. (2013). Elicitation of induced resistance against *Pectobacterium carotovorum* and *Pseudomonas syringae* by specific individual compounds derived from native Korean plant species. Molecules **18**, 12877-12895.

Stefanowska, M., Kuras, M., and Kacperska, A. (2002). Low temperature-induced modifications in cell ultrastructure and localization of phenolics in winter oil seed rape (*Brassica napus* L. var. *oleifera* L.) leaves. Ann. Bot. **90,** 637–645.

Stumpe, B., and Marschner, B. (2009). Factors controlling the biodegradation of 17 beta-estradiol, estrone and 17 alpha-ethinylestradiol in different natural soils. Chemosphere **74,** 556–562

Stumpe, B. and Marschner, B. (2010). Dissolved organic carbon from sewage sludge and manure can affect estrogen sorption and mineralization in soils. Environ. Pollut. **158,** 148-154.

Subramanian, S., Graham, M.Y., Yu, O., and Graham, T.L. (2005). RNA interference of soybean isoflavone synthase genes leads to silencing in tissues distal to the transformation site and to enhanced susceptibility to *Phytophthora sojae*. Plant Physiol. **137,** 1345-1353.

Sung, D.Y., Vierling, E., and Guy, C.L. (2001). Comprehensive expression profile analysis of the *Arabidopsis* Hsp70 gene family. Plant Physiol. **126,** 789-800.

Sumpter, J.P., Jobling, S. (2013). The occurrence, causes, and consequesnces of estrogens in the aquatic environment. Environ. Toxicol. Chem. **32,** 249-251.

Tabata, A., Kashiwada, S., Ohnishi, Y., Ishikawa, H., Miyamoto, N., Itoh, M., and Magara, Y. (2001). Estrogenic influences of estradiol-17b, p-nonylphenol and bis-phenol-A on Japanese Medaka (*Oryzias latipes*) at detected environmental concentrations. Water Sci. Technol. **43,** 109-116.

Tattini, M., Galardi, C., Pinelli, P., Massai, R., Remorini, D., and Agati, G. (2004). Differential accumulation of flavonoids and hydroxycinnamates in leaves of *Ligustrum vulgare* under excess light and drought stress. New Phytol. **163,** 547–561.

Todaka, D., Shinozaki, K., and Yamaguchi-Shinozaki, K. (2015). Recent advances in the dissection of drought-stress regulatory networks and strategies for development of drought-tolerant transgenic rice plants. Front. Plant Sci. **6,** 2015. doi: 10.3389/fpls.2015.00084

Torregrosa, C., Cluzet, S., Fournier, J., Huguet, T., Gamas, P., Prospéri, J., Esquerré-Tugayé, M., Dumas, B., and Jacquet, C. (2004). Cytological, genetic, and molecular analysis to characterize compatible and incompatible interactions between *Medicago truncatula* and *Colletotrichum trifolii*. Mol. Plant-Microbe Interact. **17,** 909-920.

Vanitha, S.C., Niranjana, S.R., and Umesha, S. (2009). Role of phenylalanine ammonia lyase and polyphenol oxidase in host resistance to bacterial wilt of tomato. J. Phytopathol. **157,** 552-557.

Vardhini, B.V. and Rao, S.S.R. (2003). Amelioration of osmotic stress by brassinosteroids on seed germination and seedling growth of three varieties of sorghum. Plant Growth Regul. **41,** 25-31.

Velioglu, Y.S., Mazza, G., Gao, L., and Oomah, B.D. (1998). Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J. Agric. Food Chem. **46,** 4113–4117

Verbruggen, N. and Hermans, C. (2008). Proline accumulation in plants: a review. Amino Acids **35,** 753-759.

Verdan, A.M., Wang, H.C., García, C.R., Henry, W.P., and Brumaghim, J.L. (2011). Iron binding of 3-hydroxychromone, 5-hydroxychromone, and sulfonated morin: implications for the antioxidant activity of flavonols with competing metal binding sites. J. Inorg. Biochem. **105,** 1314-1322.

Viehweger, K. (2014). How plants cope with heavy metals. Bot. Studies. **55,** 1–12.

Vlot, A.C., Dempsey, D.A., and Klessig, D.F. (2009). Salicylic acid, a multifaceted hormone to combat disease. Annu. Rev. Phytopathol. **47,** 177-206.

Vogt, T. (2010). Phenylpropanoid biosynthesis. Mol. Plant **3,** 2-20.

Wang, Y., Zhang, J., Li, J., and Ma, X. (2014). Exogenous hydrogen peroxide enhanced the thermotolerance of *Festuca arundinacea* and *Lolium perenne* by increasing the antioxidative capacity. Acta Physiol. Plant. **36,** 2915-2924.

Wanner, L.A., Li, G., Ware, D., Somssich, I.E., and Davis, K.R. (1995). The phenylalanine ammonia-lyase gene family in *Arabidopsis thaliana*. Plant Mol. Biol. **27,** 327-338.

Winkel-Shirley, B. (2001). Flavonoid biosynthesis. A colorful model for genetics, biochemistry, cell biology, and biotechnology. Plant Physiol. **126,** 485-493.

Woerdenbag, H.J., Pras, N., Frijlink, H.W., Lerk, C.F., and Malingré, T.M. (1990). Cyclodextrin-facilitated bioconversion of 17 β-estradiol by a phenoloxidase from *Mucuna pruriens* cell cultures. Phytochemistry **29,** 1551-1554.

Wu, A.H., Stanczyk, F.Z., Hendrich, S., Murphy, P.A., Zhang, C., Wan, P., and Pike, M.C. (2000). Effects of soy foods on ovarian function in premenopausal women. Br. J. Cancer. **82,** 1879–1886.

Xu, J., Xing, X., Tian, Y., Peng, R., Xue, Y., Zhao, W., and Yao, Q. (2015). Transgenic *Arabidopsis* plants expressing tomato glutathione S-transferase showed enhanced resistance to salt and drought stress. PLoS One **10,** 9. http://dx.doi.org/10.1371/journal.pone.0136960

Xu, X., Chen, C., Fan, B., and Chen, Z. (2006). Physical and functional interactions between pathogen-induced *Arabidopsis* WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell **18,** 1310–1326.

Yamasaki, H., Sakihama, Y., and Ikehara, N. (1997). Flavonoid-peroxidase reaction as a detoxification mechanism of plant cells against H_2O_2. Plant Physiol. **115,** 1405-1412.

Yang, S., Sweetman, J.P., Amirsadeghi, S., Barghchi, M., Huttly, A.K., Chung, W.I., and Twell, D. (2001). Novel anther-specific myb genes from tobacco as putative regulators of phenylalanine ammonia-lyase expression. Plant Physiol. **126,** 1738-1753.

Yang, X.H., Xu, Z.H., and Xue, H.W. (2005). *Arabidopsis* membrane steroid binding protein 1 is involved in inhibition of cell elongation. Plant Cell **17,** 116–131.

Yao, K., De Luca, V., and Brisson, N. (1995). Creation of a metabolic sink for tryptophan alters the phenylpropanoid pathway and the susceptibility of potato to *Phytophthora infestans*. Plant Cell **7,** 1787-1799.

Ying, G. and Kookana, R.S. (2005). Sorption and degradation of estrogen-like-endocrine disrupting chemicals in soil. Environ. Toxicol. Chem. **24,** 2640-2645.

Ying, G., Kookana, R.S., and Ru, Y. (2002). Occurrence and fate of hormone steroids in the environment. Environ. Intl. **28,** 545-551.

Ylstra, B., Touraev, A., Brinkmann, A.O., Heberle-Bors, E., and Tunen, A. (1995). Steroid hormones stimulate germination and tube growth of in vitro matured Tobacco pollen. Plant Physiol. **107,** 639-643.

Young, I.J., Knights, B.A. and Hillman, J.R. (1977). Oestradiol and its biosynthesis in *Phaseolus vulgaris* L. Nature **267,** 429.

Young, I.J., Knights, B.A. and Hillman, J.R. (1979). The metabolism of estrogens in vivo and in vitro by *Phaseolus vulgaris*. Z. Pflanzenphysiol. **94,** 307-316.

Yu, A., Li, P., Tang, T., Wang, J., Chen, Y., and Liu, L. (2015). Roles of Hsp70s in stress responses of microorganisms, plants, and animals. BioMed Res. Intl. doi: org/10.1155/2015/510319.

Zabala, G., Zou, J., Tuteja, J., Gonzalez, D.O., Clough, S.J., and Vodkin, L.O. (2006). Transcriptome changes in the phenylpropanoid pathway of *Glycine max* in response to *Pseudomonas syringae* infection. BMC Plant. Biol. **6,** 26. doi: 10.1186/1471-2229-6-26.

Zagorchev, L., Seal, C.E., Kranner, I., and Odjakova, M. (2013). A central role for thiols in plant tolerance to abiotic stress. Intl. J. Mol. Sci. **14,** 7405-7432.

Zhang, W., Seki, M., and Furusaki, S. (1997). Effect of temperature and its shift on growth and anthocyanin production in suspension cultures of strawberry cells. Plant Sci. **127,** 207-214.

Zhang, X., Henriques, R., Lin, S., Niu, Q., and Chua, N. (2006). *Agrobacterium*-mediated transformation of *Arabidopsis thaliana* using the floral dip method. Mol. Plant **1,** 641-646.

Zhang, X. and Liu, C. (2015). Multifaceted regulations of gateway enzyme phenylalanine ammonia-lyase in the biosynthesis of phenylpropanoids. Mol. Plant **8,** 17-27.

Zhang, X., Gou, M., Guo, C., Yang, H., and Liu, C.J. (2015). Down-regulation of Kelch domain-containing F-box protein in Arabidopsis enhances the production of (poly)phenols and tolerance to ultraviolet radiation. Plant Physiol. **167,** 337-350.

Zhang, Y., Fu, X., Hao, X., Zhang, L., Wang, L., Qian, H., and Zhao, J. (2015). Molecular cloning and promoter analysis of the specific salicylic acid biosynthetic pathway gene phenylalanine ammonia-lyase (AaPAL1) from *Artemisia annua*. Biotechnol. Appl. Biochem. doi: 10.1002/bab.1403

Zhao, Q. and Dixon, R.A. (2011). Transcriptional networks for lignin biosynthesis: more complex than we thought? Trends Plant Sci. **16,** 227-233.

Zhong, J. and Yoshida, T. (1993). Effects of temperature on cell growth and anthocyanin production in suspension cultures of *Perilla frutescens*. J. Ferment. Bioeng. **76,** 530-531.

Zou, J., Rodriguez-Zas, S., Aldea, M., Li, M., Zhu, J., Gonzalez, D.O., Vodkin, L.O., DeLucia, E., and Clough, S.J. (2005). Expression profiling soybean response to *Pseudomonas syringae* reveals new defense-related genes and rapid HR-specific downregulation of photosynthesis. Mol. Plant-Microbe Interact. **18,** 1161-1174.