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RNA-SEQ ANALYSIS OF TRANSGENIC ARABIDOPSIS THALIANA WITH CONFERRED OVERPRODUCTION OF PUTRESCENE BY A MOUSE ORNITHINE DECARBOXYLASE GENE

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THESIS

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Thank you to my family and friends whose steadfast support made all the difference.

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ABSTRACT

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Polyamines are a class of low molecular weight, nitrogenous bases that participate in many important functions in plants, from germination to senescence and many steps in between. These molecules have been shown to play key roles in various abiotic and biotic stress responses which makes their biosynthetic pathway a focal point for engineering plants to better adapt to rapidly changing local environments and global climate change. Previous work with plants capable of producing high polyamine titers shows that they have superior stress responses as compared to their wild type counterparts. This study investigated what broader impacts a genetic manipulations to a basic metabolic pathway may have on the overall profile of gene expression of young plants of *Arabidopsis thaliana*. We investigated the effects of these transgenic changes, in model system *A. thaliana, in* plants with the conferred trait of polyamine overproduction, specifically putrescene, by way of transgenic manipulation using a mouse (*Mus musculus*) ornithine-decarboxylase (*ODC*) gene, which has been used extensively over the years for this purpose. Employing Next Generation Sequencing (NGS) technology, we compared the transcriptomic differences between wild type plants and those genetically engineered to live with high putrescine either constitutively or in response to short-term induction. Our results show that polyamine overproduction has wide-ranging impacts on not only the neighboring pathways of amino acids and their closely related sub-pathways but also plant growth regulator biosynthetic

pathways (e.g., the abscisic acid metabolic pathway), specifically in relation to stimulating a stress response – even in the absence of a traditional stressor. This enhancement of polyamine biosynthesis and accumulation shows the promise of metabolic genetic engineering as a way to produce stress-tolerance in plants, and potentially increased nitrogen and carbon assimilation leading to higher biomass accumulation.

INTRODUCTION

The study of polyamines (PAs) in plants has drawn much excitement as genetic techniques and analyses have enabled the manipulation of the plants' ultimate genotype and the phenotype; and, the entire metabolism (Farr et al., 2014; Seifi & Shelp, 2019; Stitt & Sonnewald, 1995). Polyamines are organic polycations found in all living organisms and are known to participate in many physiological and developmental processes. Certain PAs have been found to be essential for all life (Chattopadhyay et al., 2002; Hamasaki-katagiri et al., 1997). In higher plants, for instance, the accumulation of PAs is associated with not only regular growth and development, but also engendering tolerance to extreme growing conditions such as increased salinity or drought (Handa & Mattoo, 2010; Hasan et al., 2021). Using RNA-seq analysis techniques, whole families of genes have been discovered to participate in concert when confronted with stress and many differentially expressed genes (DEGs), such as those involved in stomatal regulation, oxidation responses, and ion channel regulation, involve PAs (Gill & Tuteja, 2010; Mohanta et al., 2017; Shi & Gu, 2020). The present study was aimed at investigating the influence PAs had on the regulation of genes using RNAseq technologies during a timed experiment in *Arabidopsis thaliana*. The major objective was to study differences between long-term (constitutive) and short-term (inducible) increase in PA accumulation in young *A. thaliana* plants.

Polyamines are aliphatic nitrogenous bases that exist primarily in their free form in higher plants, but also in other forms; e.g., covalently conjugated or non-covalently conjugated (Chen et al., 2019). The highly conserved PA biosynthetic pathway starts with the decarboxylation of either ornithine (Orn), in most eukaryotes, or arginine (Arg), in bacteria and

most plants. Putrescine (Put), spermidine (Spd), and spermine (Spm), shown in Figure 1, are known for being the most common PAs in all eukaryotes (Michael, 2016; Minguet et al., 2008).

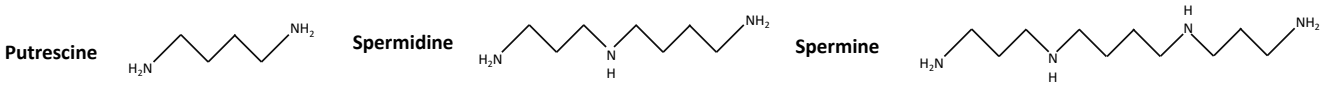


Figure 1. *Putrescine is a di-amine. Synthesis of spermidine (triamine) and spermine (tetramine), require one and two additional amine groups, respectively (Kusano et al, 2008).*

Polyamines are cationic molecules that play numerous critical roles in plant survival. They are involved in cell membrane and macromolecule stabilization, stress response modulation, protein synthesis and function, ion channel and stoma regulation, cell differentiation and proliferation regulation, nucleotide synthesis, and they also have antioxidant properties (Chen et al., 2019; Gill & Tuteja, 2010; Imai et al., 2004; Minocha et al., 2014; Pegg, 2014). A group of molecules at the crossroads of many critical pathways, PAs have the flexibility to facilitate multiple events during a response to stress.

Hyper-ionic and hyperosmotic stress are brought on when plants are exposed to higher-than-normal salt concentrations. A plant's reaction to such stimuli may be the production of hormones, metabolites and/or specialized proteins that play a role in a complex response to stressors (Mohanta et al., 2017). Not only does the accumulation of Na^+ ions in the cytosol suppress enzyme activity, the resulting osmotic imbalance leads to oxidative stress as well. Limiting water causes stoma to close, reducing CO_2 intake; the over-reduction of O_2 giving way to reactive oxidizing molecules, such as H_2O_2, occurs which ultimately leads to cellular membrane damage and leaf senescence (Jithesh et al., 2006; Verslues & Juenger, 2011; Xin et al., 2018).

Polyamines, produced in reaction to this kind of stress, not only sweep up free radicals themselves but they also stimulate the production of yet other antioxidative molecules. In fact,

the catabolism of PAs, itself, produces oxidizing molecules which, like a positive feedback loop, stimulates more antioxidant production (Saha et al., 2015; Taie et al., 2019; Verma & Mishra, 2005). Polyamines have antioxidative properties, but will also work to stabilize lipid membrane constituents and intermembrane transport proteins attempting to contradict ion toxicity (Saha et al., 2015). Maintaining membrane and protein stability is a primary function of PAs in the case of heat stress – Spd has been shown to directly influence the stability of thylakoid membrane proteins (Wang et al., 2018).

Found ubiquitously throughout all plant tissues, Put is irreversibly made from Orn and/or Arg (Figure 2). In animals, bacteria, fungi and many higher plants, Put is synthesized from Orn by way of ornithine decarboxylase (ODC – E.C. 4.1.1.17). A pathway common to all plants, Put is also synthetized from Arg by arginine decarboxylase (ADC – E.C. 4.1.1.19), followed by two additional reactions. Glutamate is an important constituent in the proline and γ-aminobutyric acid (GABA) pathways (Figure 2), but as Orn is both a product of glutamate and a precursor to Put, its concentration not only directly regulates Put synthesis, but also indirectly regulates the partitioning of glutamate, proline, and GABA into different amino acid biosynthetic pathways (Majumdar et al., 2016).

The well-characterized ethylene biosynthetic pathway employs an enzyme known as S-adenosylmethionine (SAM) decarboxylase (SAMDC – E.C. 4.1.1.50), which converts SAM into decarboxylated SAM (dcSAM), also a vital substrate for biosynthesis of higher PAs. The enzymes SPDS and SPMS use dcSAM as a co-substrate to make Spd and Spm from Put and Spd, respectively (Kusano et al., 2008, 2011). Thus, PA biosynthesis competes with the ethylene biosynthetic pathway by needing the same substrate, i.e. dcSAM (Figure 2). Given that ethylene and PAs have somewhat opposite physiological effects, it is not surprising that PAs play a

significant role in reducing ethylene production and its effects on senescence (Agudelo-Romero et al., 2013; Mohanta et al., 2017; Seifi & Shelp, 2019). However, this is not a universal occurrence in all plants (Lasanajak et al., 2014; Quan et al., 2002). When Put production was upregulated via genetic engineering, there was no significant impact on ethylene biosynthesis, largely because the accumulation of Spd and Spm remained largely unchanged (Lasanajak et al., 2014; Quan et al., 2002). These results show that the biosynthesis of higher PAs is more tightly regulated in plants than that of Put.

Gamma-aminobutyric acid is well known to have a pivotal role in a plant's ability to manage oxidative stress and both glutamate and Put are precursors of GABA (Majumdar et al., 2016; Podlesakova et al., 2019; Salvatierra et al., 2016; Shelp et al., 2012; Yang et al., 2011). The drive of the Put→GABA pathway is greater than that of the Put→SPD→SPM pathways (Podlesakova et al., 2019; Shelp et al., 2012). Further, oxidative catabolism of Put generates H_2O_2 which, itself, stimulates a signal transduction process necessary to the plant's stress response (Soares et al., 2018). The Put metabolic pathway is complex, and due to its pleiotropic roles, the homeostasis of Put naturally includes several points of control that can require feedback from other pathways (Figure 2). The regulation of ADC, ODC, and SAMDC production is one of those homeostatic mechanisms (Mattoo et al., 2010). Much work has been done to tune the dials of the enzymes working in these pathways, but PAs are, themselves, throughputs in other metabolic processes.

Polyamines are found in all plant tissue types and positive correlations have been often observed between the accumulation of PAs and the heightened vigor of plant growth (D. Chen et al., 2019). Polyamine variants accumulate differentially and serve specific functions depending on tissue type. The individual impacts of Spd, Spm, and Put are diverse and though these PAs

share utility in the same biosynthetic pathways, their functions are often unique (Handa & Mattoo, 2010). Spermine has broad spectrum prophylactic effects in the face of stress by inducing appropriate hormones and modulating oxidative response pathways (Hasan et al., 2021) and it is uniquely necessary to regulate tonoplast membrane channels and maintaining signal transduction pathways during long term salt exposure (Alet et al., 2012; Seifi & Shelp, 2019). A yeast study revealed that its cell cycle grinds to a halt at the cell division phase in the absence of Spd (Chattopadhyay et al., 2002). Considering distribution of individual PAs, Put is found to accumulate in leaves whereas Spd and Spm are often found in greater quantities in other organs; and even within individual cells, different PAs have different localization patterns (Takahashi et al., 2018). It could be reasoned that though many of the roles that PAs play in stress response may overlap, they must take place in different tissues, at different times during a response event.

It is hard not to notice how interrelated the PA biosynthetic pathway is to the metabolism of many other amino acids (Figure 2). Importantly, the anabolism of these compounds requires ready access to nitrogen. Nitrate, which is converted to ammonia, and ammonia itself are typical nitrogen inputs in the soil. Nitrogen assimilation and detoxification are controlled by glutamate synthase or glutamate dehydrogenase, respectively (Paschalidis et al., 2019). This makes glutamate a pivotal hub in nitrogen dissemination among many amino acid and PA biosynthetic pathways. A majority of nitrogen mass in plants is built into and utilized by photosynthetic cycles processing water and CO_2 into sugars, linking the intake rates of carbon and nitrogen for smooth metabolic functioning (Agren et al., 2012; Shan et al., 2016; Zheng, 2009). Meanwhile, nitrogen assimilation from nitrate requires energy, carbohydrate scaffolding, and reducing agents all provided by photosynthesis, further cementing the tight relationship of carbon and nitrogen (Du et al., 2016).

During periods of stress, plants have a variety of responses, but some like closing stomata in response to drought or salt stress can directly impact photosynthesis throughputs and create severe imbalances. In response to periods of high heat, plants promote high concentrations of soluble sugars. This changes the regulatory climate of certain carbohydrate metabolism pathways which indirectly impacts nitrogen pathways (Ruan et al., 2010). Spermidine added to heat stressed plants has been shown to both restore carbohydrate metabolism and upregulate the metabolism of ammonia (Shan et al., 2016). The synthesis of PAs, itself, is a reaction to excess nitrogen, mitigating cytotoxic side effects of NH_3 (Serapiglia et al., 2008). Polyamines both incorporate nitrogen in their own metabolism and actively regulate the broader nitrogen metabolism in plants.

Managing nitrogen throughputs is one of many ways that PAs influence the wider metabolic landscape of a plant throughout its development. Whether it is flower development, fruit maturation, organogenesis, senescence, or stress response, PAs have been shown to play an important role in each (Chen et al., 2019). The genes along PA metabolic pathways have been common targets for genetic manipulation, with the assumption that upregulating the PA pathway may lead to many downstream benefits (Gupta et al., 2019; Pandey et al., 2015) There may be some costs, however. The ability to engineer the plant genome is not new, but new RNA-seq analysis techniques can be used to look at everything being transcribed at a particular moment – to see how one change (e.g. in PAs) causes a cascade of metabolic consequences.

Next Generation Sequencing (NGS) analysis technology is beautifully equipped to widen the scope of metabolic studies to the entire transcriptome. With as much nuance living things exert

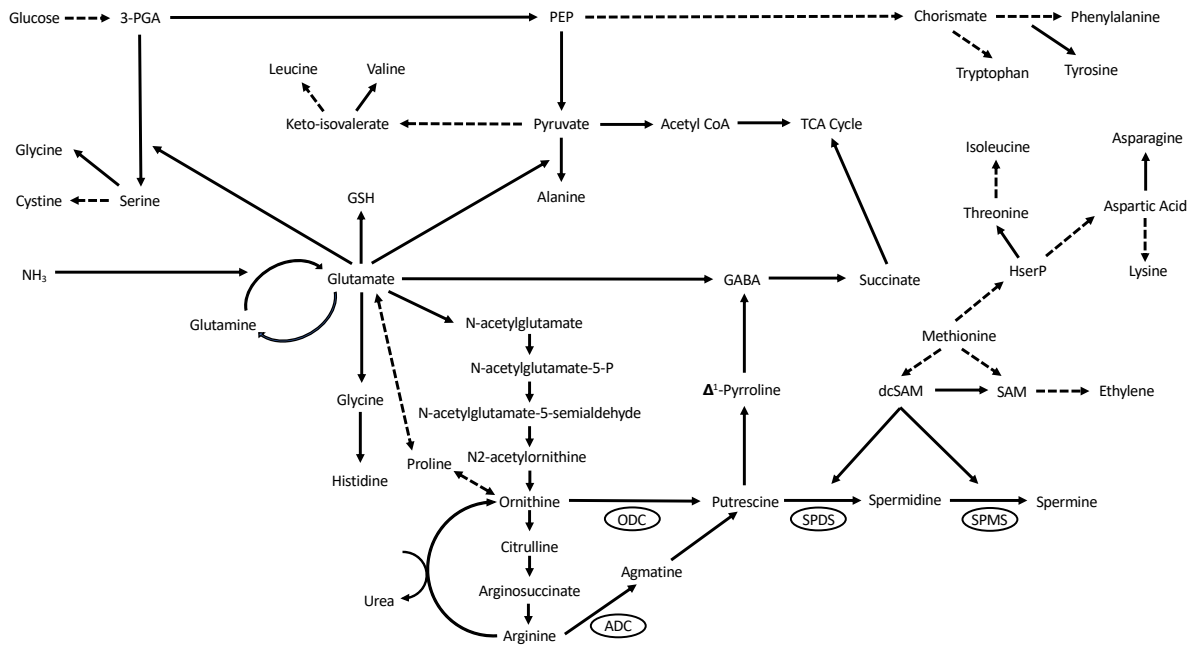


Figure 2. *The typical biosynthetic network for the synthesis of amino acids and PAs starting with base metabolic inputs: glucose and ammonia. Abbreviations: PEP= phosphoenolpyruvate; 3-PGA= 3-phosphoglyceric acid; TCA= tricarboxylic acid cycle; GSH= glutathione; GABA= ϒ-aminobutyric acid; ODC= ornithine decarboxylase; ADC= arginine decarboxylase; SPDS= spermidine synthase (E.C. 2.5.1.16); SPMS=spermine synthase (E.C. 2.5.1.22); SAM= S-adenosylmethionine; dcSAM= decarboxylated S-adenosylmethionine* (Forde & Lea, 2020; Quan et al., 2002).

when managing biotic and abiotic stresses, it can be difficult to fully comprehend the interconnectivity of every pathway involved. Our theme here is that PAs support the stress response in plants, but they do not work alone – RNA-seq is the best approach to see other molecular mechanisms at play at the same time under the same conditions. The following are some examples of how RNA-seq technologies are being leveraged for progress in understanding metabolic pathways and interactions.

Drought stress studies commonly look at the activity of bZIP transcription factors due to their importance in the abscisic acid (ABA) metabolic pathway. Abscisic acid is known for its utility in several developmental stages as well as its role in stress response (Tuteja, 2007). Researchers from the University of Suwon in Korea used microarray and PCR to determine the function of one bZIP transcription factor, *OsABF2* (*Oryza sativa* ABA-responsive element

binding factor 2) in rice, which is a transcriptional regulator along abiotic stress response pathways (Hossain et al., 2010). Work of this specificity is necessary for the foundation of databases of gene ontology – in fact, these databases are the reason RNA-seq is worth doing in the first place. In another plant drought stress study, researchers used RNA-seq techniques to analyze their drought stress experiments and identified several significant families of transcription factors, including the bZIP family, in the broader response pathway. The study went further to identify differentially regulated genes along several key pathways related to methyltransferase, transferase, and superoxide metabolic activity (Zhou et al., 2021). Where there is oxidative stress, there may be activity along the PA pathway as well. With gene-level precision and transcriptome-wide scope, NGS technology and RNA-seq analysis methods are powerful tools for investigating the variety of roles PAs play in the wider metabolism.

The use of transgenic plants to demonstrate the impact of over or under production of PAs has generated a lot of information about the importance of PA biosynthesis, accumulation, and their use by cells and tissues. In living systems, no one metabolic pathway stands alone. It is surmised that the metabolic impact of PA over-production in transgenic cell lines is likely to produce consequences throughout other regions of the metabolome, and the means by which tissues synchronize PA accrual is not as well characterized (Handa & Mattoo, 2010; Majumdar et al., 2017; Page et al., 2016).

Though PA studies carried out at UNH have shown that these important metabolites derive several positive attributes in plant development and health, the major research goal is to use this approach to generate plants that produce more biomass from the increased sequestration of nitrogen (N) as well as carbon (C) from the environment. Manipulating the PA pathway through genetic engineering was postulated to be an effective yet simple way to demonstrate the

proof-of-principle for this approach in the model plant *A. thaliana*. There was both an increase in N accumulation and accompanying C accumulation in this plant in response to genetic manipulation of Put biosynthesis using a mouse *ODC* gene under the control of a constitutive promoter (Majumdar, 2011). The next step being worked on is demonstrating if similar results could be obtained with a fast-growing tree like poplar. Several species and hybrid clones of poplar are grown all over the world for their fast growth and short harvest cycle for fiber, timber and biomass for energy production (Cho et al., 2021; Townsend et al., 2019). This study is focused on understanding the broad impact of changing the cellular contents of a single PA (i.e. Put) for either a short time (using an inducible promoter to control the transgene) or long term (using a constitutive promoter) on the spectrum of changes in gene expression in *A. thaliana*.

The initial experimental design for this approach began with the work of Dr. Rajtilak Majumdar in the Minocha Lab at UNH. The Minocha lab has a long history of the genetic manipulation to the PA pathway using a mouse *ODC* gene in several plant species (Andersen et al., 1998; Bastola et al., 1995; Bhatnagar et al., 2001; DeScenzo & Minocha, 1993; Lasanajak et al., 2014; Majumdar, 2011; Mohapatra et al., 2010). A parallel study to the *mODC* transgene was initiated later for studying the manipulation of Spd synthase (*AtSPDS*) or S-adenosylmethionine decarboxylase (*AtSAMDC*) gene, which produces a precursor for the production of Spd and Spm. Upregulating either Put or Spd/Spm production could stimulate the entire pathway to further enhance the need for N and C for increased Glu production; Glu is the primary source of Orn, which is the immediate substrate of *ODC*. The parallel study of the *mODC* and *AtSPDS* genes yielded a significant amount of knowledge and data about the regulation of PA metabolism and its regulation (Majumdar, 2011; Minocha et al., 2014; Mohapatra et al., 2010).

In the present transgenic work, the amplified *mODC* cDNA sequence was cloned into plasmids using either of two types of promoters: estradiol-inducible (IND) or constitutive (CON – 2x35S promoter). The activities of these promoters for regulation of the *mODC* gene were verified by Majumdar (2011). With plasmid accuracy verified, *Agrobacterium tumefaciens* were used to transform *A. thaliana* by the floral dip method (Clough & Bent, 1998). Though *A. thaliana* was used due to its well-studied model plant designation, a study of transgenic poplar clone NM-6, transformed with either the *mODC* or *AtSPDS* gene was started later. Using the second or third generation transgenic A. thaliana plants for mODC, several physiological and biochemical studies have been conducted in the past few years. In the present study, sixth generation (T_6) seeds of the *mODC* transgenic Arabidopsis line (that was produced by Dr. Majumdar) were used.

The exhaustive investigation into the metabolic phenotype was conducted with several generations of the two types of mODC-transgenic plants to better understand the interaction of C, N, and other metabolic pathways with a focus on the nitrogen metabolic pathways. Some of the major findings in the study of PA-related pathways in cell cultures of poplar (*Populus nigra* x *maximowiczii* – clone NM6) and *A. thaliana* have been published over the years. Genetic techniques combined with rigorous metabolic analysis have yielded some important understandings about PAs and the impact of this genetic manipulation.

The plants used in this study were transformed with either constitutive or inducible constructs of mODC. They were then verified for overproduction and high accumulation of Put. It was also shown that high Put production is accompanied by high degree of Put catalysis, which generates H_2O_2 and stimulates the production of stress enzymes associated with oxidative stress response. The differential expression of several genes responsible for this oxidative stress

response in low and high Put genotypes are reported here. Previous work also shows that in addition to an increase in the cellular contents of GABA and many amino acids, high Put plants have shown an increase in total C and N content. Nitrogen is necessary to build the enzymes that operate the photosynthetic biochemical machine, and make up the other structural proteins; C is the throughput of photosynthesis, and also makes up a great deal of structural biomass (Tang et al., 2018). Some of the metabolomic analysis of a poplar NM-6 cell culture line has shown that high Put plants show higher expression of genes associated with increasing carbohydrates, organic acids, and other amines (Page et al., 2016). Genes associated with C and N metabolism also showed changes in expression. Through elegant and meticulous biochemical studies, it was found that Orn is a regulatory molecule in the PA pathway, and it drives an increase in N by way of Glu. This work continues with studies currently underway by other lab members looking at the response of the transgenic plants to various forms and applications of N fertilizers and to study the effects of this manipulation on salt and heavy metal stress.

This present study is focused on temporal transcriptomic changes that arise as a result of Put accumulation. We focused on the genetic differences and similarities in gene expression between plants showing constitutive expression and short-lived induced expression of the transgene *mODC* conferring the ability for increased Put production. There is much data to support the idea that this transgene would be a valuable improvement to economically important plants if the hypothesized increase in PAs is accompanied by increased C and N accumulation and stress tolerance. It is vital to know the effects of such central metabolic changes on the profile of changes in other metabolic pathways using transcriptomic analysis. This RNA-seq study embarks on this effort. Though this is an exploratory endeavor, there were some specific

aims to support the findings from previous studies looking at N utilization as well as the negative feedback associated with PA overproduction.

The specific objectives of my study were as follows. (1) To determine a best method for facilitating the highest degree of expression change in the transgene *mODC*. (2) To identify differentially expressed genes in the major pathways important to our PA story, such as nitrogen metabolism and arginine synthesis. (3) To filter out the genes with the greatest change in expression and investigate their function to bring detailed resolution to the question: what are the physiological implications of the *mODC* transgene in plants with either constitutive or inducible constructs?

MATERIALS AND METHODS

Transgenic materials

Originally, wild type *Arabidopsis thaliana* (ecotype Colombia-0) was transformed with a plasmid containing a mouse *ODC* gene (cDNA) which is responsible for the production of Put from Orn (Majumdar, 2011; Majumdar et al., 2013). Two different promoters were employed to ultimately create two functionally different transgenic lines of *A. thaliana* (Majumdar, 2011). The Cauliflower Mosaic Virus promoter (CaMV 35S) (plasmid pMDC32) was used for constitutive transgene expression and the human Z_3EV promoter (estradiol inducible – IND – Ohira et al., 2017) – plasmid pMDC7 for expression in the presence of estradiol (Figure 3). The original plasmids also carry hygromycin (70 μg/mL) resistance gene. The transformed plants were cultivated over several generations, each generation used in experimentation confirming its conferred genetic ability of Put overproduction. The gene, herein, is referred to as *mODC* and further specified as either CON for the constitutive promoter or IND for the inducible promoter. Both lines of *mODC* transgenic plants used in the experiments described here are of the same sixth generation (T_6) seed lots.

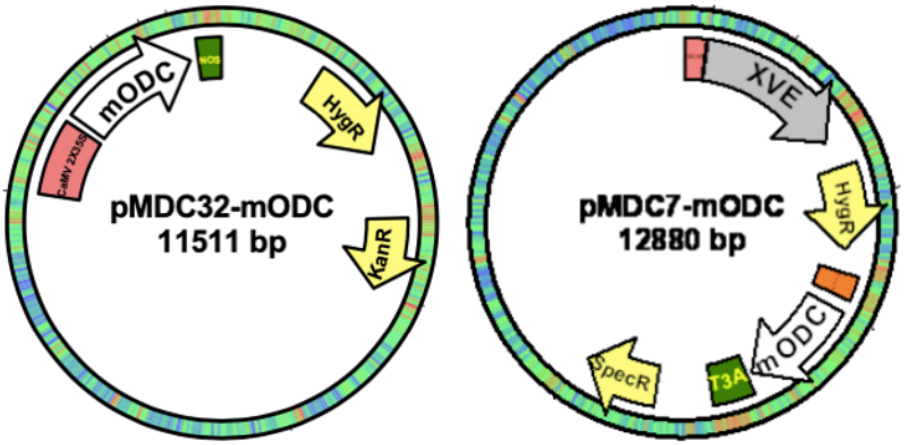


Figure 3. *The mODC open reading frame is 1190bp in length, initially cloned into pCR.80/GW/TOPO and pENTR™/D-TOPO® plasmids. The mODC gene was then transferred to the pMDC32 and pMDC7 plasmids, for CON and IND expression, respectively, for plant transformation* (Majumdar et al., 2011). *Hygromycin and kanamycin or spectinomycin resistance*

genes were also included in the cassette for transformed plant and bacterial selection functions, respectively.

Plant growth conditions and the production of subsequent generations

Unsterilized *mODC* (CON and IND) and wild type (WT) seeds were sown in a sterilized soil mixture comprised of potting soil (Sun Gro Horticulture, Agawam MA), in 9 cm square plastic pots. The seeded pots were placed in plastic trays containing water (53 cm L x 28 cm W x 10 cm H), covered in foil, and kept at 4° C for 36-48 hours. Following this cold period, the foil was removed and replaced with a spacious clear plastic tray lid, and the trays were moved to a growth room at ~20° C with a 16/8-hour photoperiod under standard grow lights (80 +/- 10 μE m^{-2} s^{-1}). Seedlings were thinned once secondary leaves had fully emerged so that there were approximately 15 seedlings per pot. The remaining plants grew to maturity and bolts were staked using thin wooden 30 cm long dowels using twist ties. After staking the flowering plants, different genotypes were kept in separate trays, yet within the same growth room. All experiments involving plants grown in soil were maintained in this growth room.

Plants were watered from below on a regular schedule of every three to four days, where every other watering included 0.3 g/L Miracle-Gro fertilizer (N24-P8-K16). Upon the first signs of browning in the siliques, an indicator of fruit maturity, watering was discontinued. Individual foil catchment trays were built around each pot. After several weeks, the plants in each pot were dried out and well on their way to complete desiccation. Siliques were plucked or the entire plants were crumpled by hand into the catchment foil. The fully dried biomass was incrementally sieved until mostly seeds remained, and the seeds were stored in microfuge tubes at 4° C.

In-vitro growth conditions

Wild type and *mODC* seeds were sterilized using two independent washes of ethanol solution within the confines of a laminar flow hood. Twenty milligrams of seeds were gently

agitated for five minutes in a solution of 70% ethanol and 1% Triton-X100 detergent in a sterile microfuge tube. This solution was removed by pipette once seeds settled and then replaced with a wash of 95% ethanol and gently agitated for seven minutes. Seeds were drawn up by pipette and spread out on sterile Whatman #1 filter paper discs in a sterile glass 100 mm Petri dish (Corning Inc., Corning NY) without a lid; all under a laminar flow hood. Seeds were allowed to dry in this shallow vessel until no visible trace of ethanol remained. Seeds were then spread onto the solid growth medium prepared in plastic Petri dishes by gently tapping to avoid over-crowding.

Growth medium used to culture the seeds was Murashige and Skoog (1962) basal salt and vitamin powder at 50% strength (2.15 g/L concentration – PhytoTech Lab, Lenexa KS), a 0.5 g/L concentration of 2-(N-Morpholino)-ethanesulfonic acid (MES) (Sigma, Burlington MA), 1% laboratory-grade sucrose (Fisher Scientific, Fairlawn NJ), and 0.8% agar (Midsci, St. Louis MO). The pH of the medium was adjusted to 5.5 using 1 M potassium hydroxide dropwise. The solution was subdivided into 100 mL aliquots and poured into 250 mL Erlenmeyer flasks. Agar was added to each flask at this stage. All flasks were capped with foil and autoclaved for 20-minutes using the liquid cycle. Molten medium was allowed to cool to 55° C before pouring into plates. Hygromycin, at a final concentration of 30 μg/mL, was added to the medium intended to cultivate transgenic seeds for selection of transgenic plants after the medium had cooled to about 50-55° C.

The plates were sealed with 3M Millipore tape around their circumference where the lid meets the plate and remained sealed until use. The plates were wrapped with aluminum foil in a stack and placed in a 4° C cold room for 24-36 hours. Upon removal, the plates were uncovered, and spread out in a walk-in growth chamber that was maintained at 25 +/- 1° C with fluorescent

grow lights (80 +/- 10 µE m^{-2} s^{-1}) on a 16/8-hour photoperiod for 20-25 days before experimental treatments.

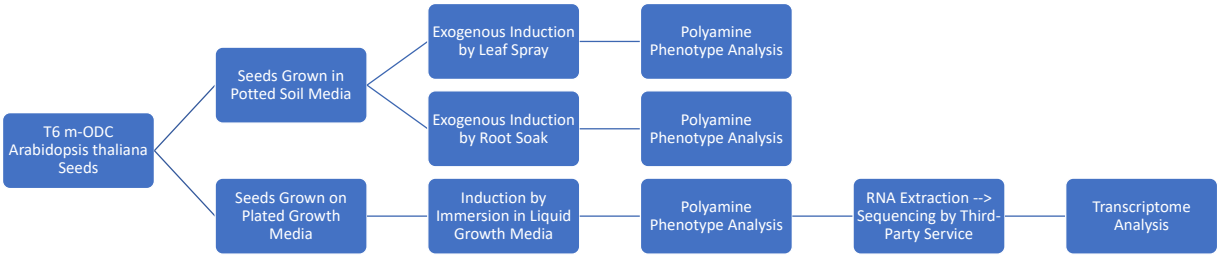


Figure 4. *Overall workflow of complete experimental design. Polyamine quantification was done by HPLC to validate the potential of expression in experimental tissues and to determine best samples that will advance to RNA-seq* analysis.

In-vitro induction of mODC gene in transgenic seedlings by estradiol:

This experiment was designed to investigate in-vitro grown seedlings' response to 5 µM estradiol by floating whole seedlings in liquid test medium. Seedlings grown to 25 days, were removed from their plates (described above) and placed in 5 mL of liquid solutions consisting of the growth medium with or without 5 µM estradiol in 6-well plates (Costar - Corning, Corning NY). Three genetic lines were tested: IND, CON, and WT. Wells containing clusters of ~100 mg of whole seedlings of the three genotypes were distributed among 12 wells of two plates in a random block design (Figure 5). Half of the group received medium with 5µM estradiol and the other half just basal growth medium. The CON seedlings were not exposed to estradiol. The plates were rotated at 100 rpm on a rotary shaker in the same growth chamber used to grow them from seed.

Seedling tissue was collected at time zero, 24 hours, and 48 hours with sample sizes ranging from 30-50 mg for RNA and 40-70 mg for PA analysis. The seedlings were removed from the liquid medium; excess medium was allowed to diffuse onto a sterile paper towel (lightly moistened with sterile double distilled water); seedlings were portioned into sample size

and weighed. For PA analysis, sample biomass was submerged in a 9:1 volume to mass ratio of 5% perchloric acid (PCA) solution. These samples were stored at -20° C. For RNA analysis, samples were folded into a small foil packet and flash-frozen in liquid nitrogen. Packets were kept individually within microfuge tubes and stored at -80°C.

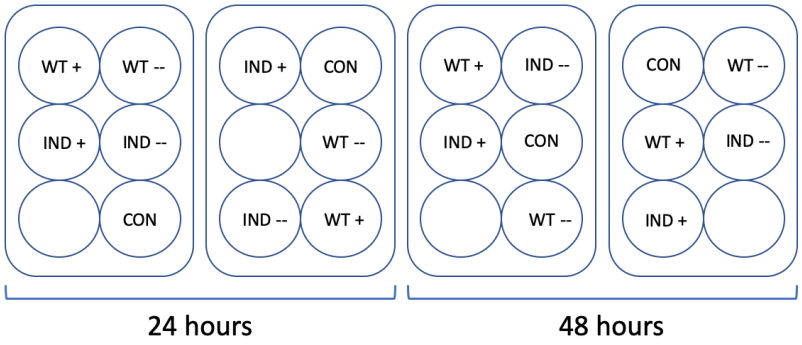


Figure 5. *Random block design distribution of seedling types, treatments, and exposure time. Approximately 100 mg of seedlings were placed in each well for a total of 200 mg of each seedling type and treatment to separate into three replicate samples for RNA extraction and three for PA analysis. Plus (+) symbol indicates presence of 5µM estradiol.*

Induction of *mODC* gene by estradiol leaf spray of mature soil-grown plants:

This experiment investigated the plants' response to 5 µM estradiol sprayed on the leaves. The plants used in this experiment were grown from both CON and IND *mODC* transgenics and WT Arabidopsis seeds in sterilized soil in 9 cm pots as described above. Starting with more than 30 plants per pot, plants were thinned at 19 days to about 10-15 plants per pot. The experimental design included six pots per genotype: CON, IND, and WT. Of those six pots, half were used as control and the other half for the estradiol treatment. This particular experiment was conducted when the plants were 29 days old. By this time, the potted plants were growing robust secondary leaves. Once the plants had been sprayed with test fluids, they were placed back in the growth room under the conditions described above.

The experimental design called for spraying 5 µM estradiol onto the upper leaf surface uniformly one time at the beginning of the study. Time zero collections were made before any

spraying, and 24 and 48-hour collections were completed within one hour of the denoted times. There were six replicates (by plant pots) per genotype – three to receive test treatment and three to receive control treatment. The plants were separated into test and control groups; within these two groups, the pots were randomly arranged in a 3x3 grid; and pots in this grid were set close enough so that the spray will cover evenly across a miniature 'canopy' of leaves. The pots were arranged in plant trays without draining holes as described above. The spray liquid was sterile distilled water with 0.05% Silwet and the test liquid was the same plus 5 μM estradiol. A clean spray bottle was used to spray a measured amount of the solutions to achieve a complete coverage of the leaves. The leaves were to be completely covered with spray solution, but no droplets accumulating or falling to the soil. 20 mL of the control solution was administered to half of the control plants and 18 mL of test liquid was administered to the other half. Samples were collected at time 0, and at 24 and 48 hours after spray. After the 24-hour collection, leaves were sprayed with an additional 10 mL of control or test fluids to maintain leaf moisture for the second 24-hour period.

The strategy for collecting samples for this study was to select leaves at random from several plants in a single pot to comprise each sample. Four to six leaves were used to generate samples for carbon and nitrogen content, PAs, and soluble protein contents. One third of the leaf at its base (not including the petiole) was sequestered in an open microfuge tube to be dried at 40° C for three to five days. The microfuge tubes were then closed for these samples and stored at room temperature. The middle third of leaf tissue was placed into a minimal volume of 5% PCA – enough to immerse the tissue sample - to be later corrected by adding more PCA solution to a final 9:1 ratio of PCA (mL) to plant tissue (mg). The distal third of leaf tissue was immersed in 1 ml of 0.1 M potassium phosphate buffer (pH 6.9) for protein estimation. The PA and protein

samples were all stored at -20° C until analysis. The sample collection for the RNA study was two to three whole leaves per pot that were wrapped in a foil packet, frozen in liquid nitrogen, packed into a microfuge tube, and stored at -80° C.

Induction of *mODC* gene by estradiol via root soak of mature soil-grown transgenic plants:

This experiment was designed to study the plant's response to 5 μM estradiol delivery directly to their root system. The induction of the *mODC* gene is powerful, but short-lived. The same plants in the leaf spray experiment described above were used for this experiment, but approximately ten days after the leaf spray experiment collections had been finished. This amount of time was deemed sufficient to allow the plants to return to their basal physiological state following the leaf spray experiment. Arranged in the same groups, the plants were given test fluids by way of soaking just the roots. The plants were watered and fertilized regularly (as described above), but the night before this experiment they were given only enough deionized water so that the tray would be dry by morning. Zero-hour samples were collected in similar fashion as described in the leaf spray experiment, before the administration of test or control fluids. Collection and storage of leaves were carried out in the same manner as described in the leaf spray experiment as well. Using a pipette, 20 mL of 5 μM estradiol in deionized water and/or pure water were added directly to the soil of each pot, avoiding liquid contact with leaves. Collections were made at 0, 24, and 48 hours.

Confirmation of transgenic activity by quantification of PA production

This analysis used High Performance Liquid Chromatography (HPLC) to quantify PA concentrations from each plant tissue sample. Samples from each of the three experiments, described above, were stored in 5% PCA solution and frozen and thawed three times at -20° C

and room temperature respectively. Following the final thaw and a two-minute vortex, samples were centrifuged at 13,000 rpm for eight minutes. For dansylation of PAs, 100 μL of the supernatant was combined with 20 μL of internal standard of 0.05 mM heptanediamine. Standards were mixed as described in Table 1. Sample solutions were quickly vortexed and spun for 30 seconds at 13,000 rpm, then 100 μL of 2.69 M sodium carbonate solution and 100 μL of dansylchloride solution (20 mg/mL in HPLC-grade acetone) was added to each sample tube. Critical care of volume dispensing is required. Sodium carbonate is used to neutralize the PCA and raise the pH to basic. Samples were vortexed for one minute before a 60-minute incubation at 60° C in a water bath.

Upon removal, samples were rested at room temperature for three minutes before being centrifuged at 13,000 rpm for 30 seconds. Each sample tube then received 50 μL of 20 mg/mL L-asparagine, it was mixed by vortex for one minute, and was centrifuged for 30 seconds. All tubes were incubated in a water bath for 30 min at 60° C and then centrifuged with open lid in a speed-vac for 8 minutes to remove acetone. 400 μL Photrex grade toluene was added to each sample, using a repeater pipettor. Following a one-minute vortex, samples were incubated at room temperature for five minutes. The tubes were centrifuged again for one minute and 200 μL of the top layer of toluene was removed to a fresh microfuge tube. The toluene was evaporated in a 20-minute (until dry) speed-vac cycle with the microfuge tube lids open. One milliliter of filtered methanol (HPLC grade) was added to each tube, vortexed for two minutes followed by a two-minute centrifugation. The samples were transferred to HPLC autosampler vials and used for PA separation by HPLC. Separation and quantification of PAs was done as described in Minocha and Long's 2004 work (Minocha & Long, 2004).

Table 1. *Preparation of PA standard solutions from a pre-mixed working stock solution* (0.04mM Put, 0.02mM Spd and Spm each) *of three PAs in 5% PCA.*

Stock Solution	5% PCA	Total Volume	Final Conc Put	Final Conc Spd and Spm
0 µL	500 µL	500 µL	0	0
25 µL	475 µL	500 µL	0.002 mM	0.001 mM
50 µL	450 µL	500 µL	0.004 mM	0.002 mM
125 µL	375 µL	500 µL	0.01 mM	0.005 mM
250 µL	250 µL	500 µL	0.02 mM	0.01 mM
125 µL	0 µL	125 µL	0.04 mM	0.02 mM

Table 2. *HPLC settings for separation of PAs. Quaternary LC Pump Model 200-Q 410 with Perkins Elmer 900 A/D and Series 200 Autosampler settings.*

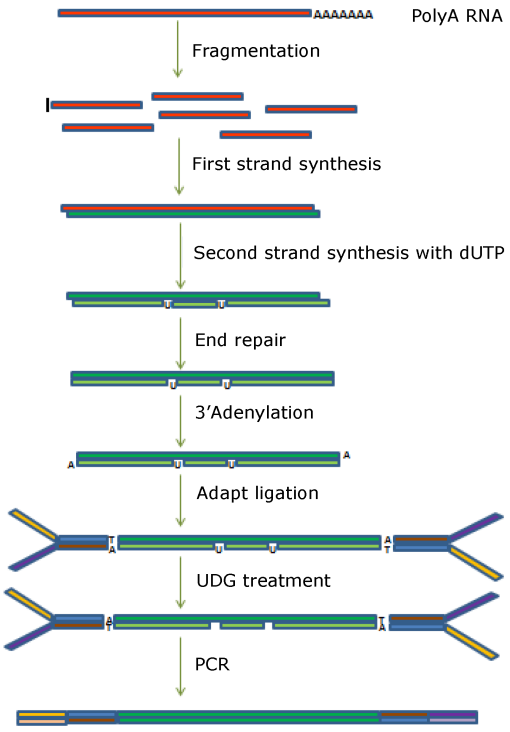
Channel Parameters:	
Delay Time	0 minutes
Run Time	13.5 minutes
Sampling Rate	2.5 parts/second
Autosampler Steps & Parameters	
Injection Volume	20 µL
Loop Size	200 µL
Fixed Mode	Off
Excess Volume	10 µL
Air Cushion	10 µL
Sample Syringe Size	250 µL
Sample Speed	Medium
Flush Volume	700 µL
Flush Speed	Medium
Flush Cycles	2
Pre-injection Flush Cycles	0
Post-injection Flush Cycles	1
Post-method Flush Cycles	0
Needle Level	5%
Inject Delay Time	0 minutes
Pump Parameters:	
Ready Time	15 minutes
Standby Time	30 seconds
Standby Flow	0.5 mL/minute
Minimum Pressure	0 PSI
Maximum Pressure	4000 PSI
Real Time Plot Parameters:	
Offset	0 mV
Scale	400 mV

Isolation of RNA

Based on the results of the PA analysis, a sample set collected from the seedling immersion experiment, explained above, was used for RNA analysis. Total RNA was isolated from tissues, kept frozen at -80° C, using the standard protocol of the Qiagen RNeasy Mini kit (Qiagen, Hilden Germany). The workspace and tools were cleaned with RNaseZap (Millipore Sigma, Burlington MA) according to manufacturer suggested use. Working with no more than three samples for any given protocol run, each sample was ground into frozen powder in ceramic mortar and pestle containing liquid nitrogen. The powder was immediately added to a round-bottom 2 mL microfuge tube containing 450 μL of lysis buffer (RLT Buffer in RNeasy kit) with 1% β-mercaptoethanol (Sigma, St. Louis MO) and vortexed vigorously. The optional three-minute incubation at 56 °C in a Thermomixer (Thermo Fisher, Waltham MA) was employed with all samples. The warmed lysate was transferred to a QIAshredder spin column – designed to both filter out large cellular debris and homogenize the lysate – which was, itself, placed in a 2 mL collection tube and spun for two minutes at 13,000 rpm. Even after filtering through the QIAshredder spin column, some debris passed through to the target lysate and formed a pellet. The supernatant lysate was transferred to a fresh 2 mL microfuge tube and 100% ethanol at a ratio of 2:1 to the total volume of lysate was added and mixed by pipetting. All of this solution was transferred to a RNeasy spin column which was, itself, seated in a fresh 2 mL collection tube and spun for 15 seconds at 10,000 rpm. After discarding the flow through, 700 μL of RW1 buffer was added to the RNeasy spin column and spun for 15 seconds at 10,000 rpm for the first column membrane wash step. The flow through was again discarded and 500 μL of RPE buffer was added to the RNeasy spin column and spun for 15 seconds at 10,000 rpm. This step was repeated with a second portion of RPE buffer but with a longer (two-minute) centrifugation.

Both the RPE buffer spins were a part of the column membrane wash step. Finally, to elute the sample RNA from the membrane, the active spin column was placed in a 1.5 mL microfuge tube, 50 µL of nuclease free water was added, and the unit was spun for one minute at 10,000 rpm. The eluate was initially analyzed for RNA content and purity was checked with a Nanodrop2000 (Thermo Fisher, Waltham MA). Samples reaching this stage were frozen at -20°C while awaiting further processing.

Library preparation and sequencing



RNA samples were shipped (on dry ice) to LC Sciences, Houston TX (http://lcsciences.com), and this organization was tasked with generating cDNA libraries, sequencing the samples, and providing overview analysis of the read data. The integrity of each RNA sample was confirmed using Agilent Technologies 2100 Bioanalyzer and every sample surpassed their quality and quantity threshold. The workflow continued with two rounds of purification using oligo-(dT) magnetic beads for poly-A enrichment.

Figure 6. *Flow chart for post poly-A enrichment, cDNA preparation from RNA, fragmentation, adapter ligation, and cluster formation prior to sequencing. Graphics provided by LC Sciences (https://lcsciences.com).*

Sample fragments were converted to cDNA, fragmented, and adapters were ligated to the fragments. The sequencing preparations concluded with bridge PCR facilitating cluster generation (Figure 6). Illumina NovaSeq 6000 technology was used for performing paired-end sequencing of the sample set.

Data analysis methods

JMP Pro 15 (https://www.jmp.com/en_us/home.html) was used for all variance analysis of HPLC output of the three major PAs. The *mODC* transgene in experimental plants was verified using Burrow-Wheelers Alignment (BWA, version 7.17) by locating only those reads that would map to the *mODC* cDNA as the reference (FASTA index: NC_000078.7:17594809-17601503 *Mus musculus* strain C57BL/6J chromosome 12, GRCm39). Unipro UGENE (version 1.10.3) was used to visualize the alignment (Figures 23-25). LC Sciences made the use of several software packages to produce the analysis of raw sequencing data output. The LC Sciences workflow began with the production of quality statistics using FastQC (version 0.10.1) and Cutadapt (version 10.1) was used to trim primer sequences and low-quality reads. Mapping the reads to a reference genome was done using HISAT (version 2.0) and using the www.arabidopsis.org genome database (https://www.arabidopsis.org/download_files/Genes/Araport11_genome_release/Araport11_blastsets) (subscription required). Transcripts were assembled using StringTie (version 1.3.4); and differential expression analysis was produced using edgeR software package in the R environment. Gene ontology (GO - http://geneontology.org) and Kyoto Encyclopedia of Genes and Genomes (KEGG - https://www.genome.jp/kegg) enrichment analyses were based on fragments per kilobase of transcript per million (FPKM) mapped read values and correlated to annotations in each respective database were done with proprietary scripts within LC Sciences. Samtools (version 0.1.19), and ANNOVAR (version 2017.09) were used for SNP/indel analysis and annotation, respectively. Finally, alternative splice site determination was performed with ASprofile (version 1.0.4).

RESULTS

Effect of estradiol induction by leaf spray on mature plants in soil

There was significant variation in PA contents, specifically Put, as an effect of the *mODC* gene expression. Increase in Put accumulation was seen in all untreated samples with time (0 time vs. 24 and 48 h, Figure 7). Although the WT plants showed some positive response to estradiol in the first 24 hours, the response was smaller than the IND transgenic cell lines. Overall, Put concentrations decreased by 48 hours. The IND cell line responded correctly to estradiol, but showed an insignificant increase in Put accumulation over control IND plants not treated with estradiol over the course of the experiment (Figure 11). The CON transgenic plants showed a higher level of Put accumulation, compared to the WT plants at similar collection times (Figure 10). In the CON set of plants treated with estradiol, Put concentration was lower at both 24 and 48 hours than those not exposed to the inducer. Testing the hypothesis that there will be significant change in CON expression tissue type was supported (p value = 0.0313) but the degree of Put accumulation was minimal. Testing the hypothesis that induction of the IND type will cause a significant change in PA output was not supported (p value = 0.0724). These samples were not considered for RNA-seq analysis.

No clear trends emerged in Spd analysis (Figure 8) in this experiment. Wildtype plants showed slight increase in Spd accumulation when exposed to the inducer. Oppositely, IND cells showed slightly higher Spd production in plants not exposed to the inducer. In CON plants there was a steady drop in Spd production from zero to 48 hours – induced and uninduced mirrored the decline. Spermine variation (Figure 9) was not affected in significant ways in response to treatment with estradiol.

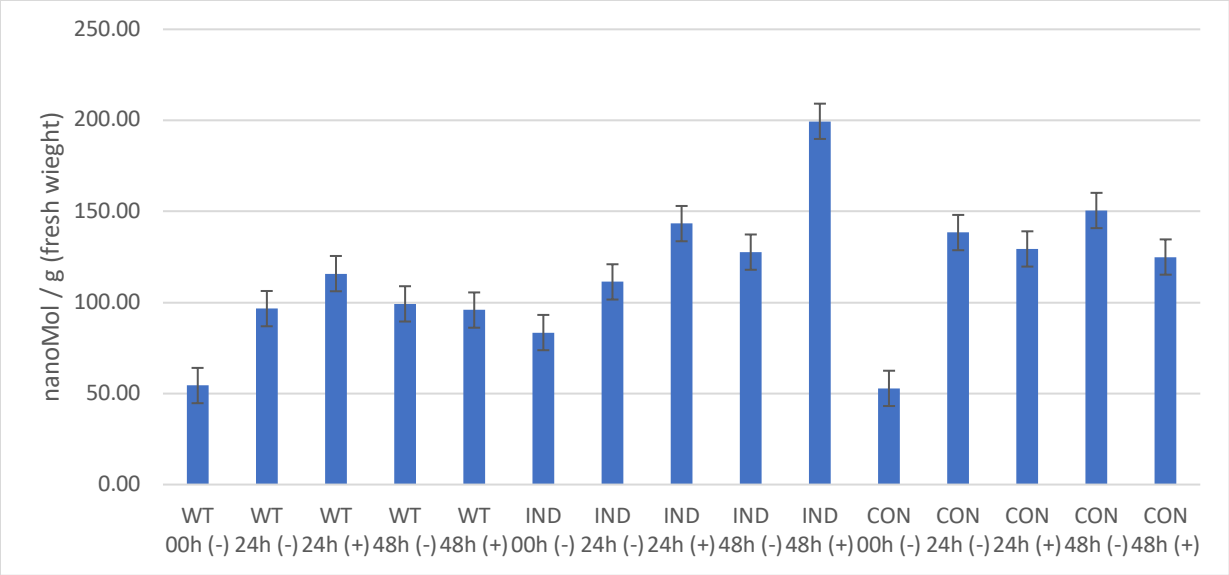


Figure 7. *Accumulation of Put in the leaves of 29-day old Arabidopsis plants sprayed with 5mM estradiol. The pattern unfolding among the IND plants suggests that estradiol properly induced the mODC transgene resulting in an increase in Put accumulation. Plants with constitutive mODC expression showed no significant reaction to estradiol and always had significantly higher accumulation of Put.*

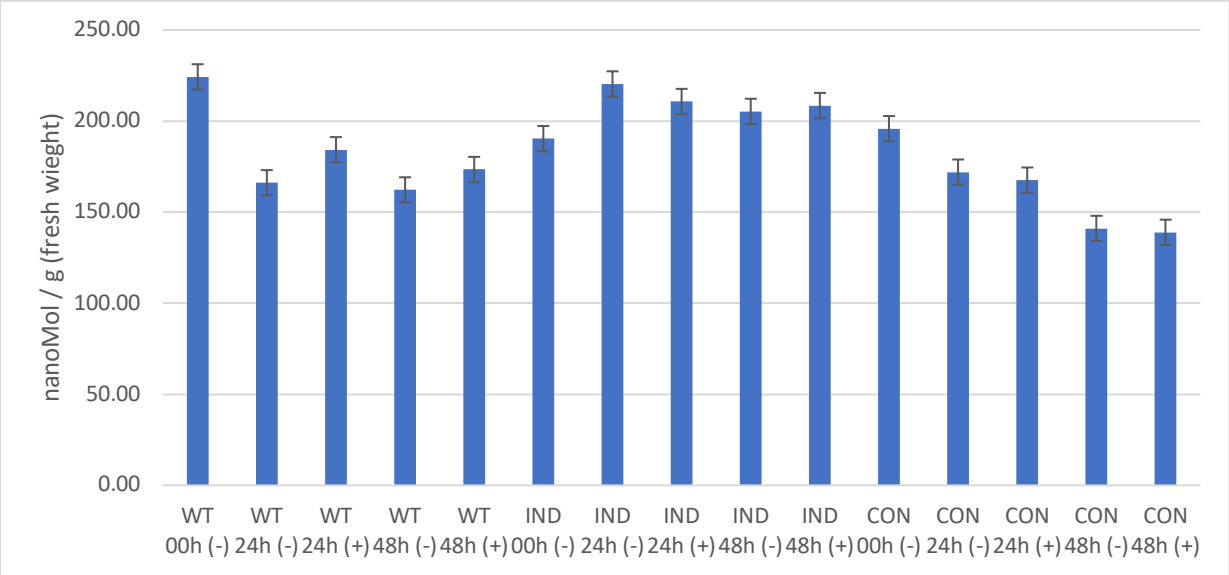


Figure 8. *Average Spd accumulation among samples in the leaf spray induction experiment. There were no significant differences in the Spd results.*

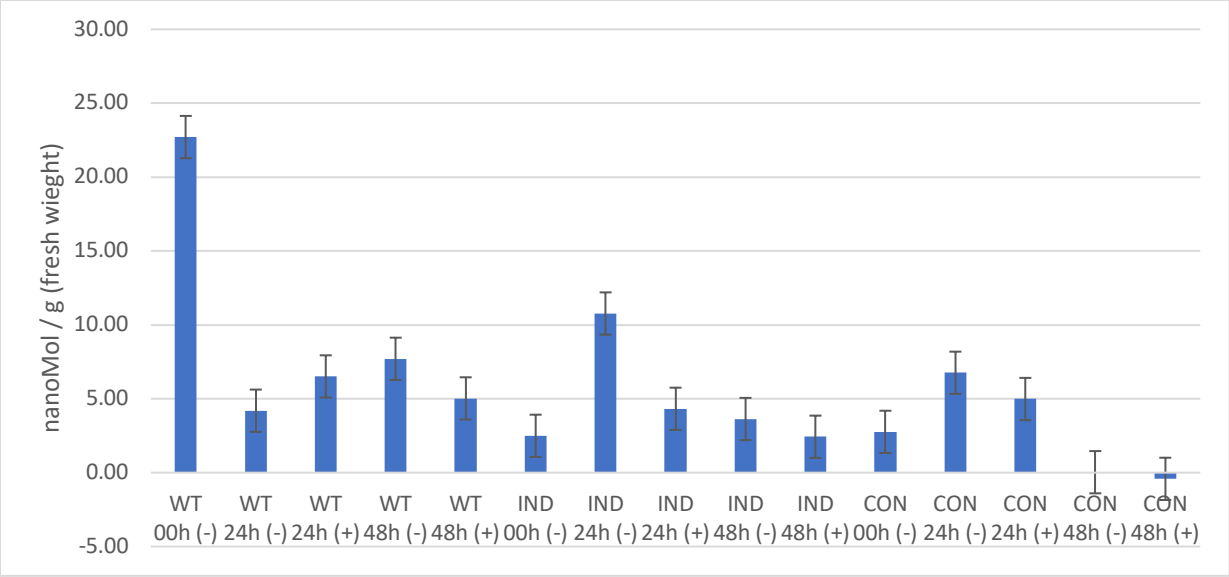
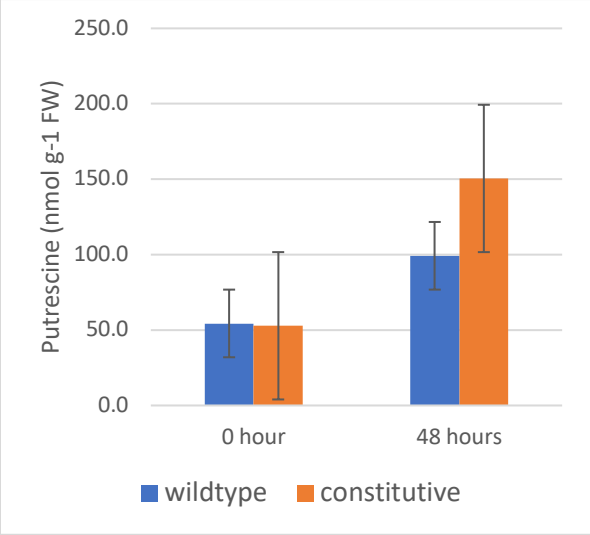
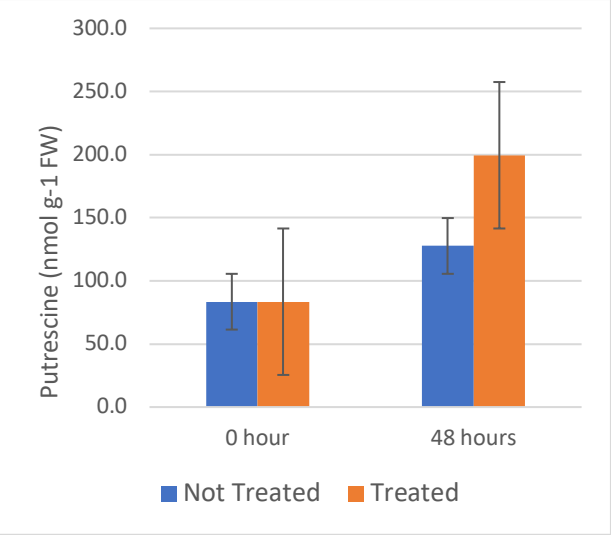


Figure 9. *Average Spm accumulation among samples in the leaf spray induction experiment. Spermine accumulation showed no significant response to estradiol at any time in any line.*





Figures 10 (left). *Putrescine accumulation in WT and CON. The effects of WT and CON show significant difference (p value = 0.0313).*

Figure 11 (right). *Putrescine accumulation in IND plants with and without estradiol inducer. Inducible type, induced vs uninduced, showed differences that were not significant.*

Effect of estradiol on seedlings in liquid medium

Young wildtype (WT) plants (seedlings 25 days old) did not respond to estradiol in terms of change in Put accumulation, even showing a lower concentration at 24 hours, but again rising to net positive Put accumulation by 48 hours. The WT plants were accumulating higher quantities

of Spd at zero-hour, though there was a steady increase to the higher concentrations reached by 48 hours. Spermine production generally fell with time of incubation in the untreated WT seedlings, but a small change was seen on treatment with the inducer (Figure 14). The IND plants showed significant variation in Put production (Figure 12). The slight stepwise decrease in Put production in the uninduced IND plants exemplified the significant increase at 24 hours and 48 hours in induced IND plants. There was a net loss in the accumulation of both Spd and Spm in the IND plants; and little or no variation was observed between induced and uninduced plants. Compared to the WT plants, the CON seedlings accumulated a four-fold greater amount of Put at any time in the experiment (Figure 15). Though CON plants experienced the greatest Put accumulation, there was a net loss, with time, in accumulation at 24 and 48 hours of incubation in the liquid medium. Despite the high Put production, the Spd and Spm accumulation was on par with WT plants and both PAs showed a stepwise decline in concentration over 24 and 48 hours. The most significant variations in Put production were seen in this experiment. There was statistical support for the hypothesis that the CON tissue type would display significant changes in Put compared to wild type (p value = 0.0013). The hypothesis that estradiol-treated IND plants would exhibit significant change was also supported (p value = 0.00021). In each comparison, Put accumulation at 48 h was more than a hundred-fold higher than the untreated plants, approaching levels similar to those in the CON plants at this time. These samples passed the threshold for RNA-seq consideration and were sent for sequencing.

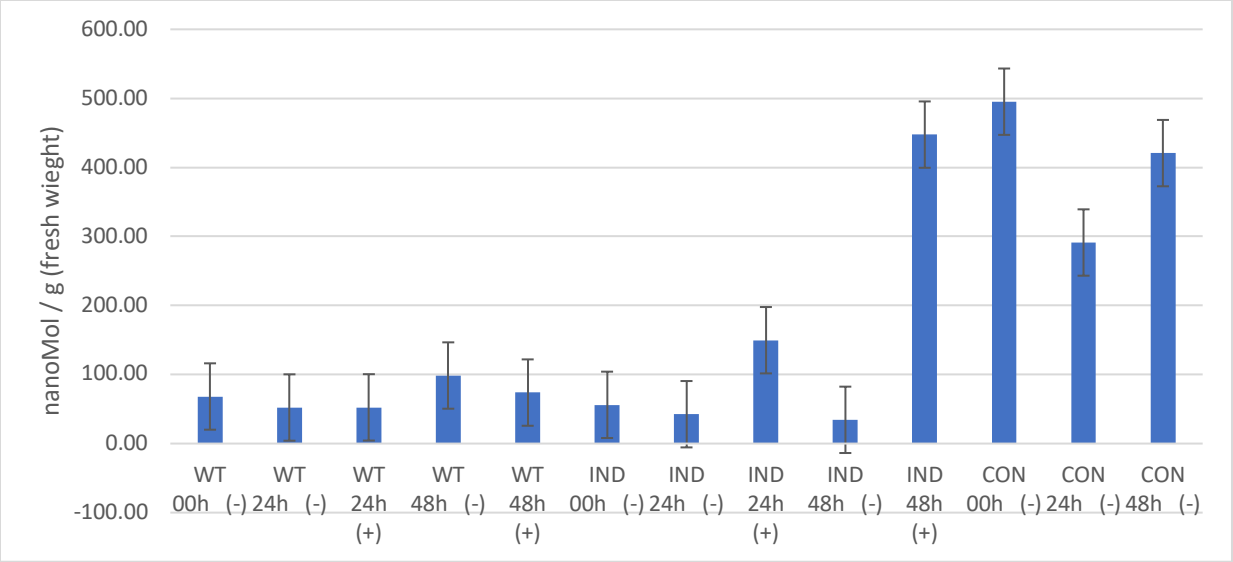


Figure 12. *Putrescene accumulation in the 25-day old seedlings of three genotypes of Arabidopsis at various times in response to estradiol treatment. The CON plants always showed a 5-10-fold higher content of Put vs. the WT plants as well as the uninduced IND plants. Estradiol-treated IND plants also accumulated more than 4-fold amounts of Put over the untreated plants of the same genotype at 48 h. Notable breakout statistics are expressed in Figures 15 and 16.*

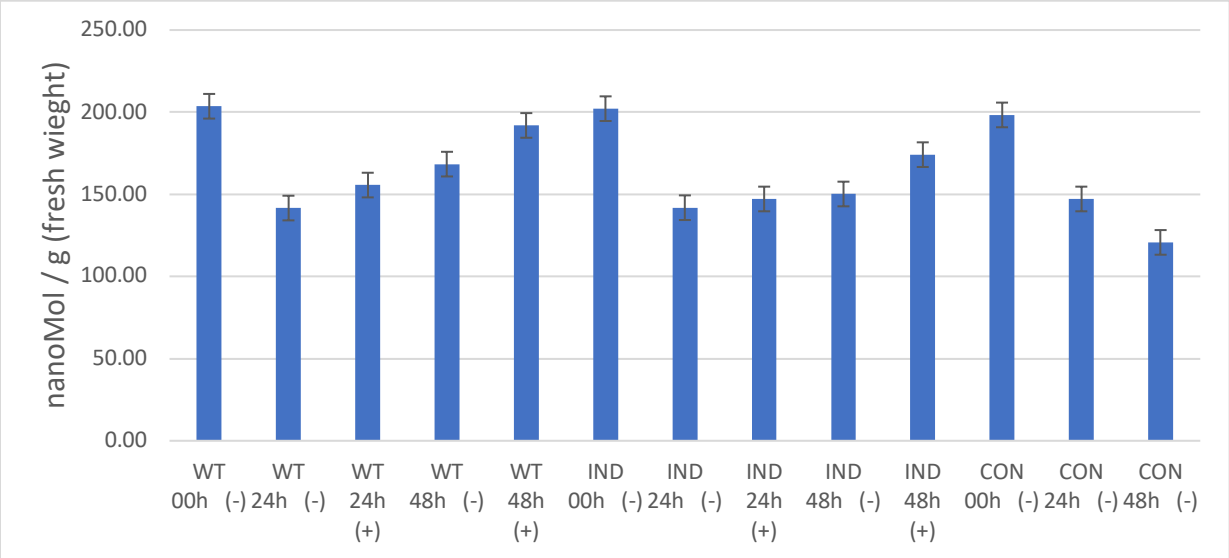


Figure 13. *Spermidine accumulation in the 25-day old seedlings of all three genotypes of Arabidopsis at various times in response to estradiol treatment. Spermidine accumulation showed unremarkable, even antithetical differentiation among groups.*

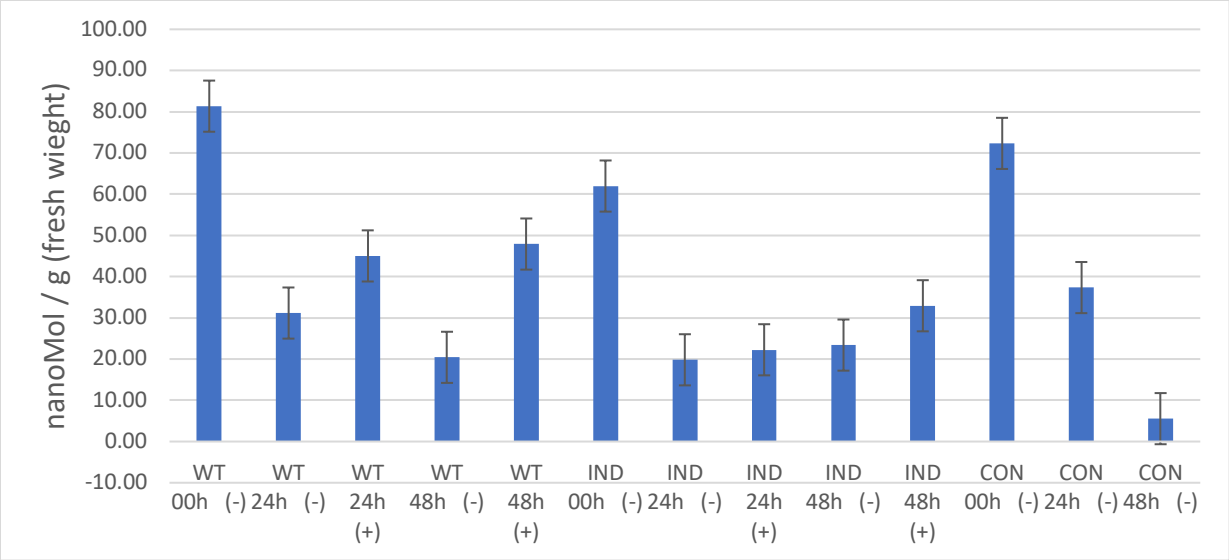
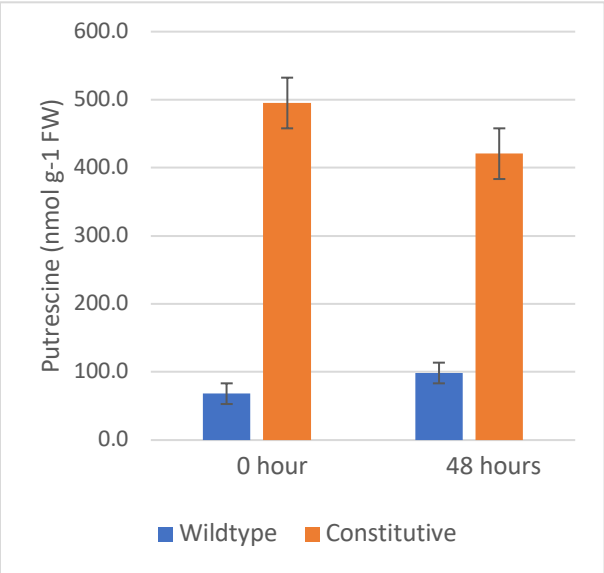
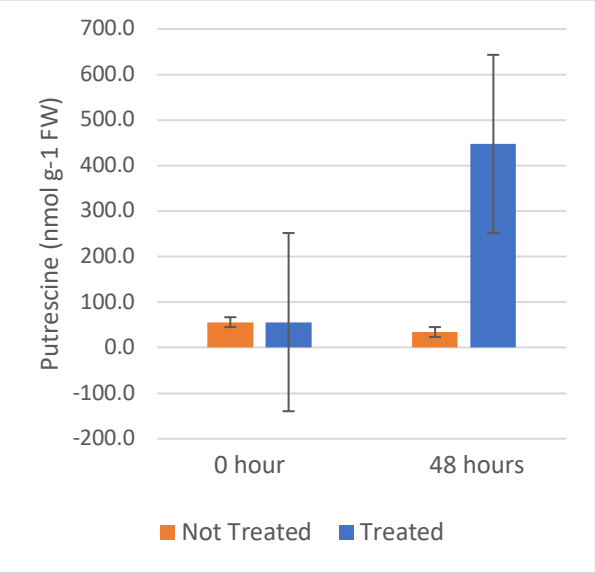


Figure 14. *Spermine accumulation in the 25-day old seedlings of all three genotypes of Arabidopsis at various times in response to estradiol treatment. In each seedling type shows that Spm accumulation was stably high before the experiment.*





Figures 15 (left) *Putrescine accumulation differences in CON and WT plants at zero and 48 h. Comparing WT and CON samples, the accumulation of Put is significant in CON tissue – about 5-fold greater accumulation than WT.*

Fig. 16 (right). *Putrescine accumulation differences in IND plants with and without estradiol treatment. There is a significant difference in the IND sample type – either induced or uninduced. The induced change resulted in more than double the Put accumulation.*

Effect of estradiol induction in root-treated mature plants in soil

As with the leaves of soil-grown older plants, clear trends in Put accumulation were absent in this part of the study. Though CON transgenic plants accumulated more Put, compared to WT, there was wide fluctuation in the maintenance of high Put during the 48 hours of study. In fact, the CON plants exposed to the inducer experienced lower Put, Spd, and Spm accumulation in the roots as compared to uninduced plants at respective times (Figures 17-19). Estradiol-treated WT plants had higher concentrations of Spd at 24 h, but this tapered at 48 h and lost the induced vs uninduced differential effect. Other than a high 48-hour accumulation level, Spd generally dropped in IND plants, regardless of induction status. There was no significant variation in Spm production among all plants (Figure 19). The hypothesis that activating the IND tissue type would cause changes in PAs (specifically Put) was not supported (p value = 0.999). In either comparison, the amount of change in Put output was minimal. These samples were not considered for RNA-seq analysis.

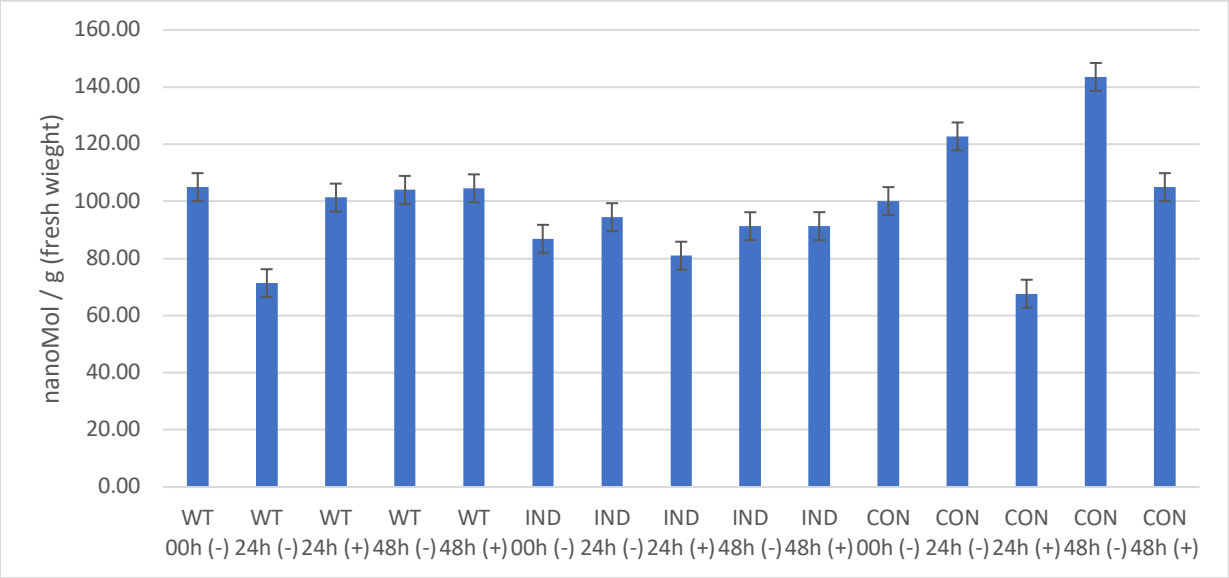


Figure 17. *Putrescine accumulation in the 39-day old seedlings of all three genotypes of Arabidopsis at various times in response to estradiol treatment. Unremarkable differentiation among experimental groups of IND plant type. Similar to the leaf spray experiment, but more dramatically, the CON type treated with estradiol shows lower Put accumulation. Notable breakout statistics are expressed in Figures 20 and 21.*

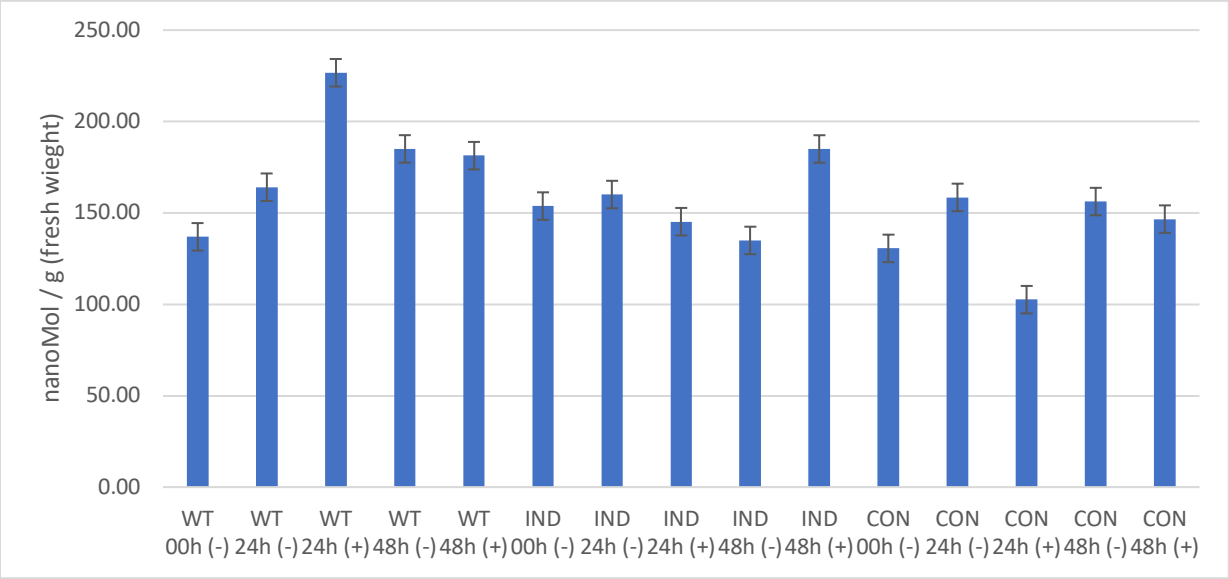


Figure 18. *Spermidine accumulation in the 39-day old seedlings of all three genotypes of Arabidopsis at various times in response to estradiol treatment. Inducible type treated with estradiol shows slight increase in Spd accumulation at 48 hours only. Constitutive type shows lower Spd accumulation when treated with estradiol.*

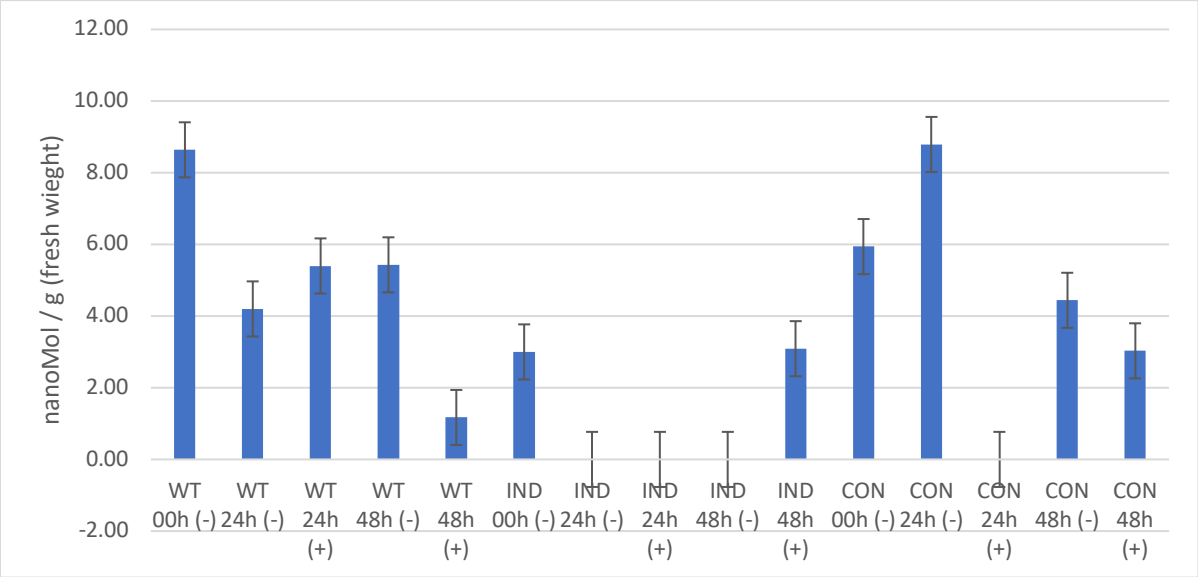
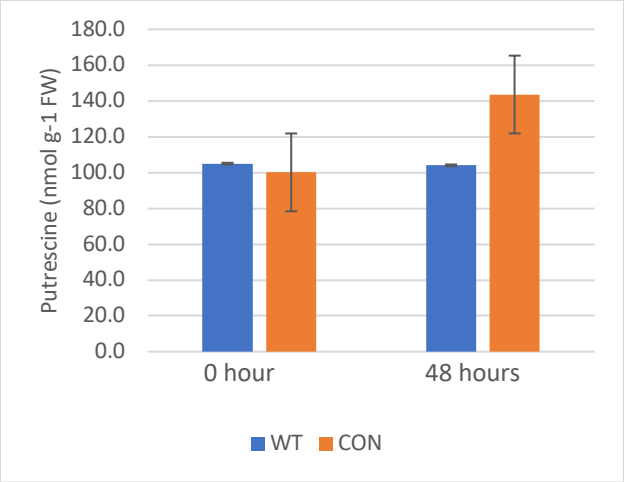
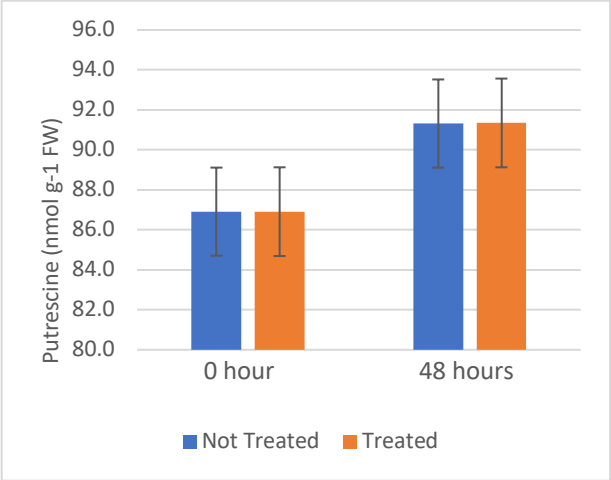


Figure 19. *Spermine accumulation in the 39-day old seedlings of all three genotypes of Arabidopsis at various times in response to estradiol treatment.* Accumulation *of Spm in these leaves was as slight as Spm accumulation in the leaf spray study above.*





Figures 20 (left). *Putrescine accumulation in WT and CON plants over time. There was no significant change between these samples (p value = 0.117).*

Figure 21 (right). *Putrescine accumulation in IND plants with and without estradiol inducer. These IND samples showed no significant difference (p value = 0.999).*

Table 3. *Quality control and RNA yield (tested by Nanodrop spectrometer) of primary samples prepared for RNA-seq showed both acceptable concentration (>100ng/L recommended) and purity within the desirable range (i.e. 260/280 ratio of ~2.0 for RNA) to qualify each sample for the next step in processing* for RNA-seq analyses.

Tissue	Estradiol	Hour	Conc ng/uL	260/280	260/230
WT	no	0	188.0	2.09	0.77
WT	no	0	302.3	2.05	2.19
WT	no	0	185.5	1.87	1.77
IND	no	0	199.6	2.23	2.33
IND	no	0	265.1	2.09	2.33
IND	no	0	287.8	2.15	2.19
CON	no	0	425.5	2.05	2.20
CON	no	0	314.4	2.09	2.36
CON	no	0	340.0	2.12	2.40
WT	yes	48	498.8	2.07	2.35
WT	yes	48	462.3	2.05	2.44
WT	yes	48	272.9	2.14	2.19
WT	no	48	328.2	2.09	2.02
WT	no	48	360.2	2.08	2.25
WT	no	48	134.8	2.09	0.69
IND	yes	48	399.3	2.07	2.25
IND	yes	48	241.8	2.07	2.19
IND	yes	48	423.0	2.09	2.37
IND	no	48	417.3	2.07	2.28
IND	no	48	447.9	2.05	2.02
IND	no	48	395.2	2.08	1.87
CON	no	48	624.1	2.05	2.36
CON	no	48	539.9	2.06	2.28
CON	no	48	684.2	2.09	2.33

RNA extraction and quantification

The extraction of RNA using the Qiagen RNA isolation kit yielded high quality and acceptable concentrations of RNA as characterized by Nanodrop spectrometer (Thermo Fisher, Waltham MA), which also showed a high purity of RNA extract (Table 3). These RNA samples were sent to LC Sciences for library preparation and sequencing.

Raw read processing and mapping

The sequencing process yielded raw paired-end reads with an average of 46.2 million reads per sample or, in terms of base pairs, an average of 6.9 kilobases per sample. Removing primer sequences and reads not meeting a Q30 Phred score (as the sequencer calls a base pair, the quality of that identification is given a Phred score; a score of Q30 is the equivalent to an error rate of 1/1000, or 99.9% accuracy - https://www.illumina.com/documents/products/technotes/technote_Q-Scores.pdf) threshold yielded valid read data averaging 43.5 million reads and 6.5 kilobases per sample. The percent of raw reads surpassing the Q30 quality threshold was 98.6% and the GC content of the valid reads averaged 45% across all samples. Original data files can be retrieved for review through Dr. Subhash Minocha of the MCBS Department at the UNH.

Sample reads were mapped to gene positions along all five of the *A. thaliana* chromosomes (Figure 22), and mapped genes on the chloroplast and mitochondrion genomes were minimal. The sample reads represented 37,686 genes and 59,051 transcripts. Across all samples, 99.08% of mapped transcripts were exons.

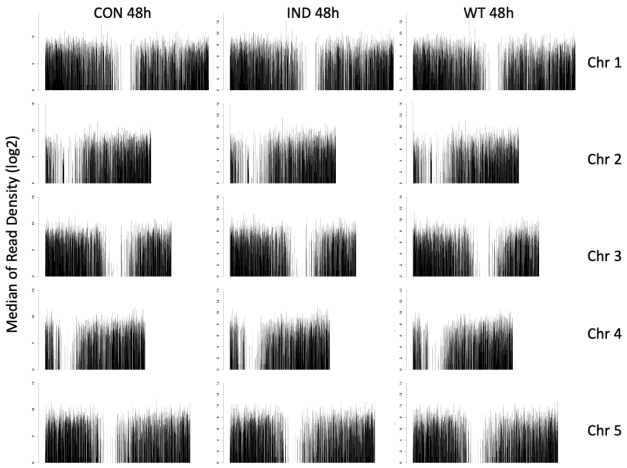


Figure 22. *Read density mapped along the reference genome – https://www.arabidopsis.org version 11- The Arabidopsis Information Resource (TAIR). Each tissue type represented by the reads from a single replicate.*

Verification of *mODC* transgene in experimental plants

A conventional path through RNA-seq analysis uses the verified genome of the species used in the experiment, but in this case, this would not include the transgene transcripts for mouse *ODC* because it is from a heterologous source. In fact, Arabidopsis does not have any sequence matching even plant *ODC* genes because this species does not have this gene (Hanfrey, et al., 2001). Many reads from the transgenic lines mapped specifically to the *mODC* gene, when used as the reference sequence, the *mODC* gene Figures 23, 24). Wild type sample reads showed no alignment with such specificity or robustness with any gene (Figure 25).

The standard method to verify the presence of a gene or group of genes is with qPCR. While this study relies on bioinformatic methods of verification of the *mODC* gene, work with this gene is collaborative. These transgenic plants, of the same generation and seed lot, are the subject of current similar studies in the Minocha Lab. In their work, contributing to the greater *mODC* narrative, colleagues will verify the expression of many of the differentially expressed genes by qPCR. Should this collective work lead to publication, surely multiple transgenic verification pathways will be used to legitimize our claims.

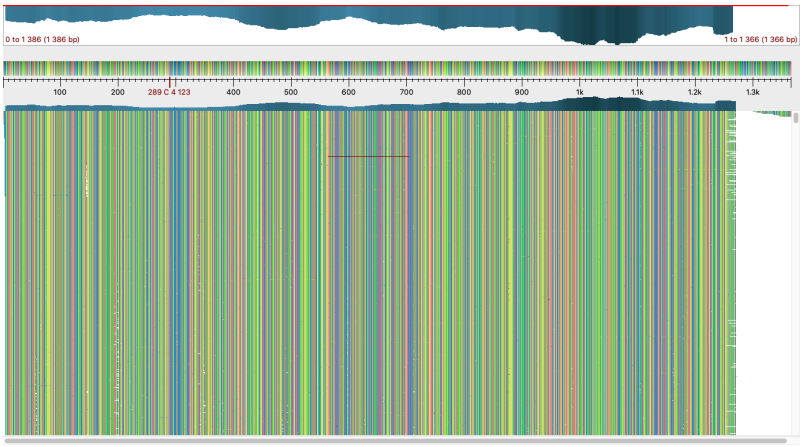


Figure 23. *Constitutive transgenic sample tissue showed >10k read depth to the mouse ornithine decarboxylase 1 (mODC - EU684749.1) as reference sequence.*

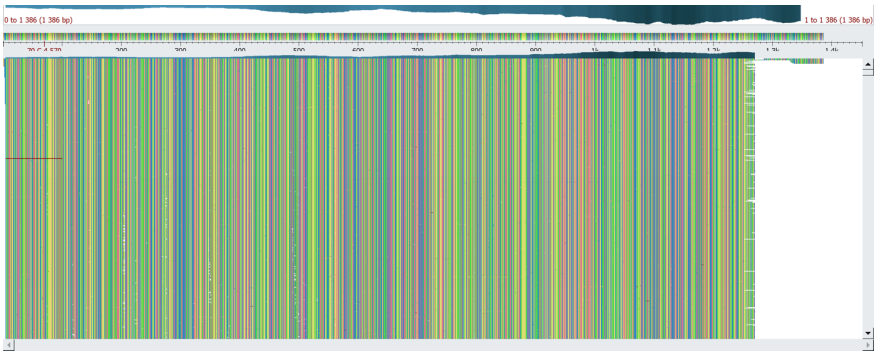


Figure 24. *Substantial read depth in transgenic IND tissue treated with estradiol when mapped to ornithine decarboxylase 1 (mODC - EU684749.1) as a reference.*

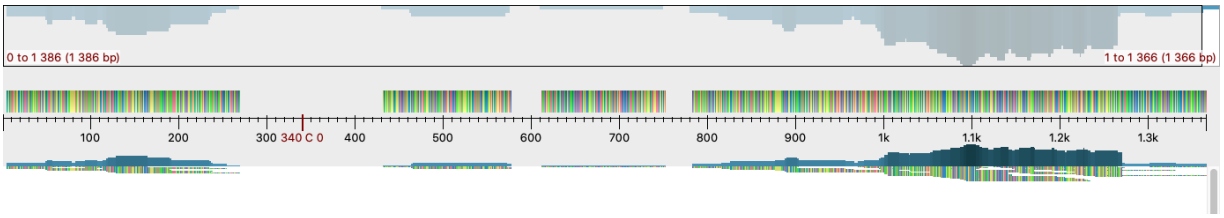


Figure 25. *Wildtype sample tissue showed discontinuous read alignment and low read depth to ornithine decarboxylase 1 (mODC - EU684749.1) as a reference sequence.*

Quantification of differentially expressed genes

In RNA-seq analyses, the kind of data that is highly interesting is what genes were expressed differently given the conditions/treatments of the experiment. The new knowledge about PAs and the pathway which includes *mODC* is derived from the list of differentially expressed genes. Every seedling group had some amount of change in gene expression levels, as shown in Figure 26. Comparing estradiol-treated WT with untreated WT at 48 hours, 68 genes were upregulated and 104 genes down-regulated. By comparison, treated WT at 48 hours compared to untreated WT at zero hours showed 1331 upregulated genes and 510 downregulated genes. This is a 1857% increase in upregulated genes and a 390% increase in downregulated genes with the only difference being the 48-hour time for which the samples were grown in the new liquid medium (untreated estradiol-treated WT samples). Similarly, the difference in untreated WT samples at 48 hours and zero hours showed 1647 upregulated and 658

downregulated genes. This is a 19% and 22% difference in upregulated and downregulated genes, respectively, in treated samples compared to untreated samples given the same time period.

Comparing treated to untreated IND tissue type samples, both at 48 hours, there were 138 upregulated and 168 down regulated genes. Between treated and untreated IND samples collected a 48 and zero hours, respectively, there were 1306 upregulated and 1917 downregulated genes. This is an 846% increase in upregulated genes and 1041% increase in downregulated genes given the difference of 48-hour experimental period. There were 1035 upregulated genes and 1375 downregulated genes in untreated IND tissue samples at 48 hours compared to similar samples at zero hours. The percent differences in gene expression in treated samples to untreated samples over the same experimental time frame was 26% for upregulated and 39% for downregulate genes. Comparing IND samples to WT, both untreated at zero hours, there were 1257 upregulated and 173 downregulated genes.

No treated CON samples were included in this RNA-seq analysis. Constitutive tissue samples at 48 hours compared to similar tissue at zero hours yielded 1943 upregulated and 836 downregulated genes. Constitutive samples compared to untreated WT samples at zero hours showed 750 upregulated and 572 downregulated genes. Constitutive samples compared to untreated WT samples at 48 hours were different by 695 upregulated and 492 downregulated genes. At 48 hours, CON samples compared to treated WT samples showed 740 upregulated and 568 downregulated genes. Constitutive type compared to untreated IND tissue samples at zero hours showed 884 upregulated and 1923 downregulated genes; and CON compared to treated IND samples at 48 hours showed 1020 upregulated and 536 downregulated genes. Comparing

CON samples to IND samples, both at 48 hours and untreated, there were 560 upregulated and 229 downregulated genes. These data are summarized in Figure 26.

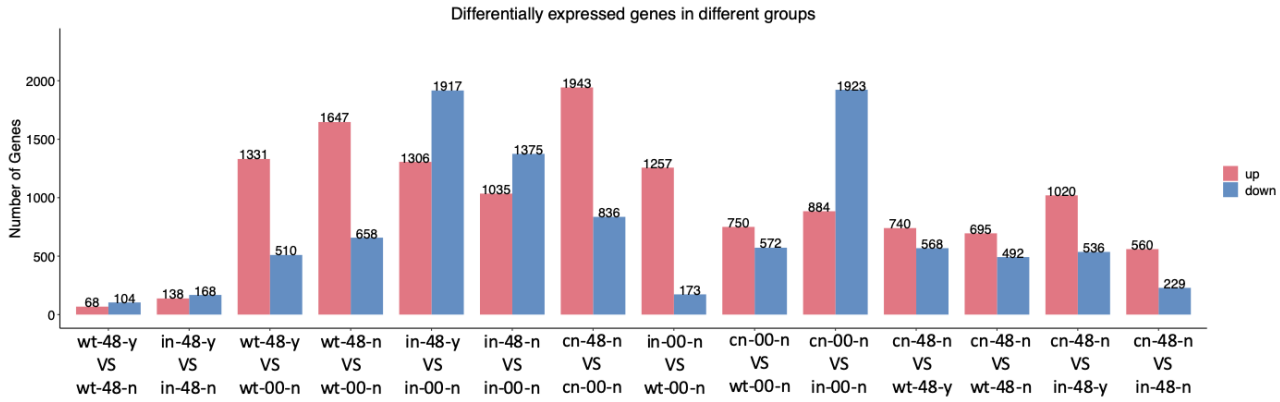


Figure 26. *Differentially expressed genes comparing fourteen combinations. The naming paradigm, slightly different for formatting fit, "wt" is wildtype, "in" is inducible, and "cn" is constitutive. The time of collection is marked as either 00 for zero hours or 48 for the end of the experiment. If the samples received 5μM estradiol treatment, they were marked with "y" and "n" if they were not.*

Gene Ontology (GO) enrichment analysis and functional categorization of DEGs

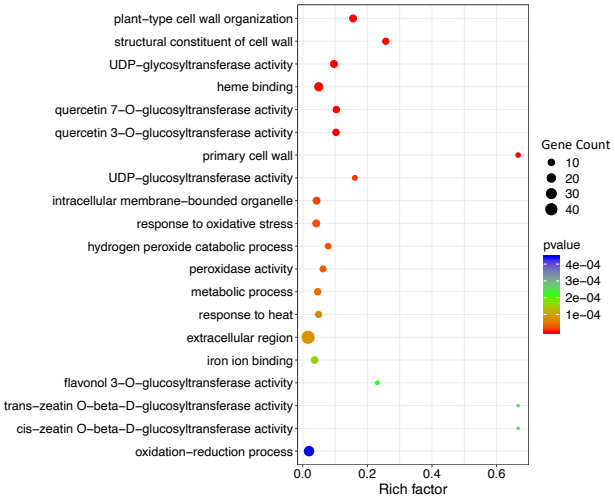
The RNASeq analyses of mRNAs can show large numbers of differences in gene expression among different samples, even if they are experimental replicates. Whereas differences in the total number of genes expressed at a given time between any two sets of samples is indicative of quantitative differences in gene expression, the Gene Ontology (GO – http://geneontology.org/) enrichment analysis is a very useful indicator of the effects of treatments on gene expression. These analyses were conducted in the present study to identify some of the major biological functions of the DEGs in response to stable (CON) and inducible expression of the *mODC* transgene. In other words, the study investigated the gene expression differences between continuous availability of high Put vs. transient change in Put biosynthesis or between homeostatic Put vs. increased production of the diamine due to *mODC* expression.

The number of genes represented in the read data that were mapped to positions with existing GO annotations ranged between 170-3128, depending on the comparison. Using the

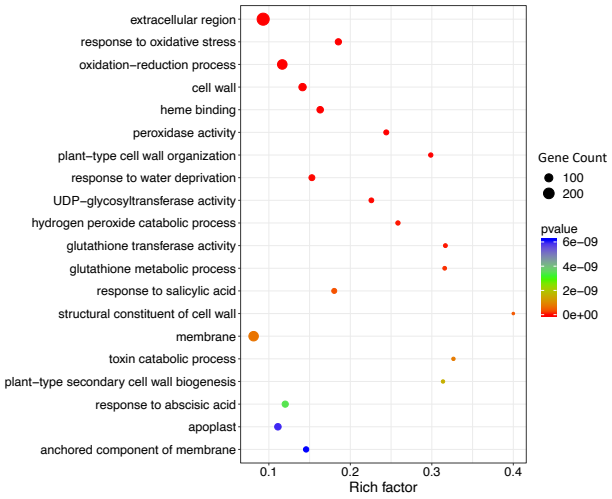
DEGs of fourteen different comparisons looking at nearly every angle of the experiment, GO database enrichment produced somewhat similar representations of various categories across those comparisons (Figure 27). The DEGs were organized into as many as twelve GO annotation classes within three main categories of Biological Processes, Cellular Components, and Molecular Functions (http://geneontology.org/).

The gene annotations with the highest representation were seen across several of the fourteen comparisons and in all the three genotypes. Within the Biologic Process category, regulation of transcription had the greatest representation followed by oxidation-reduction processes. Still with 100 or more DEGs, defense response and protein phosphorylation were represented, but not across all sample comparisons. The terms in the Molecular Function category, DNA-binding annotations, whether generally or specifically for transcription factor, had the greatest representation regardless of sample tissue type. To a lesser extent, metal ion binding was highly represented across sample comparisons. Likewise, gene coding for kinases were highly represented in all three tissue types, but not all comparisons. In the Cellular Component category, the highest representation went to the membrane associated protein genes, whether plasma or organelle membranes, and components of membranes. Though not across many sample comparisons but still with a few hundred DEGs, cytoplasm and extra-cellular regions were commonly represented. All tissue types showed cell wall represented as well. All three tissue types saw representation of similar highly represented GO terms but, comparisons characterizing IND and CON tissue types saw a greater degree of representation over wildtype for any given GO term.

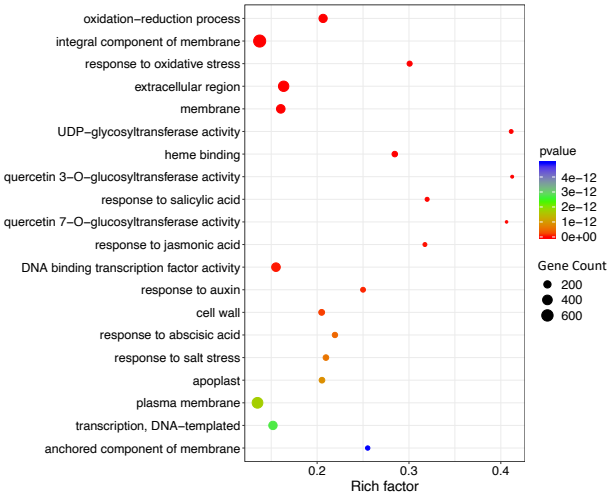
Inducible 48h w/ Estradiol VS Inducible 48h Untreated



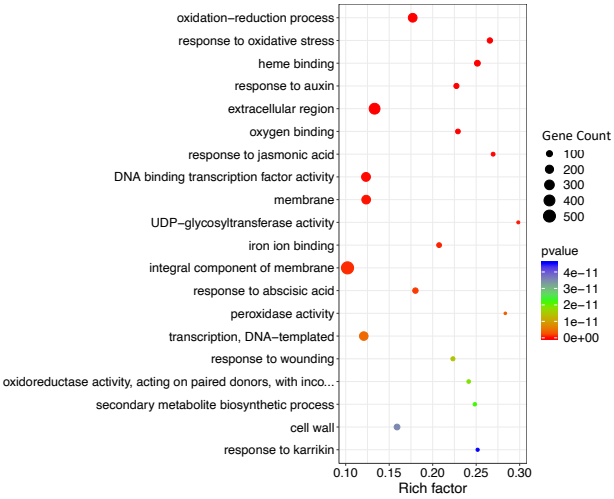
Constitutive 48h VS Inducible 48h w/ Estradiol



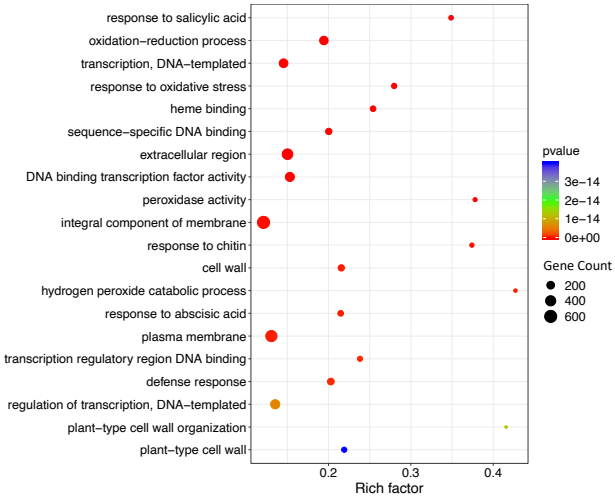
Inducible 48h w/ Estradiol VS Inducible 0h



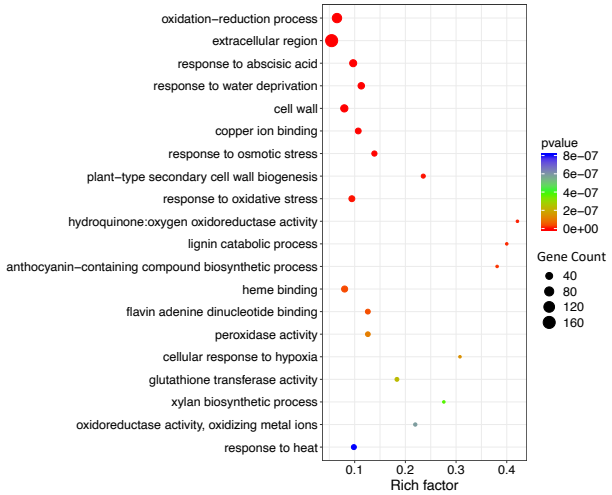
Inducible 48h Untreated VS Inducible 0h



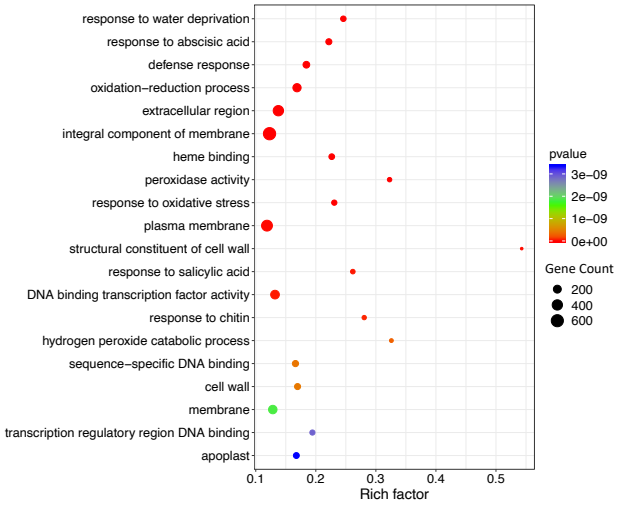
Constitutive 48h VS Constitutive 0h



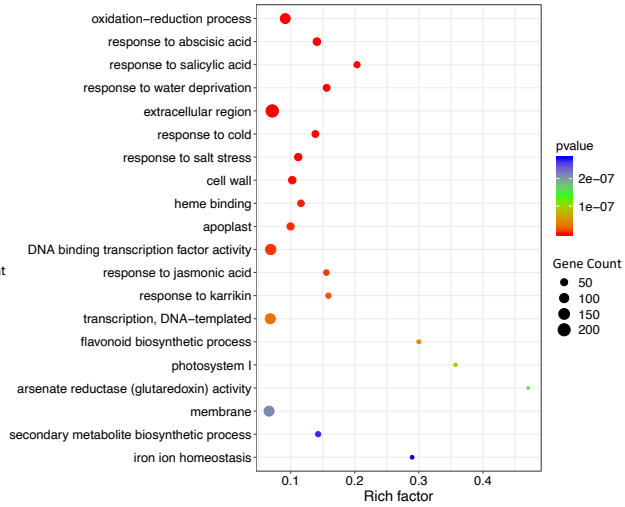
Constitutive 48h VS Inducible 48h Untreated



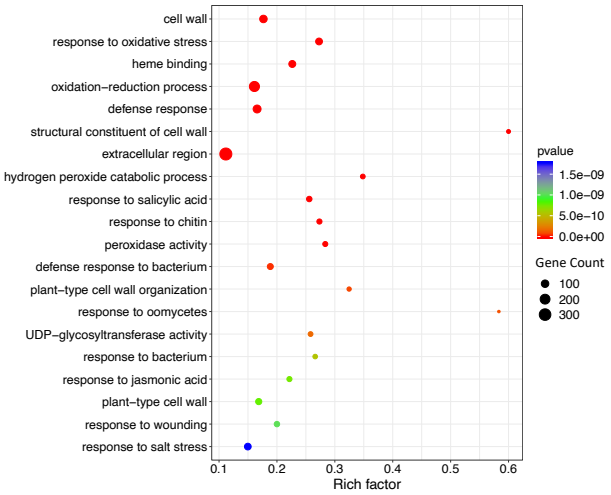
Constitutive 0h VS Inducible 0h



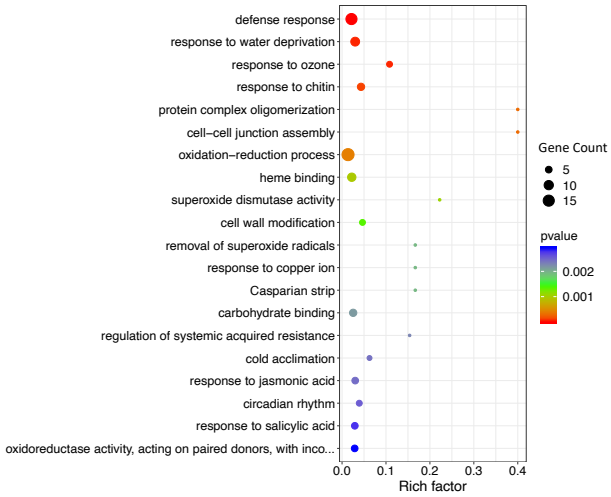
Constitutive 0h VS Wildtype 0h



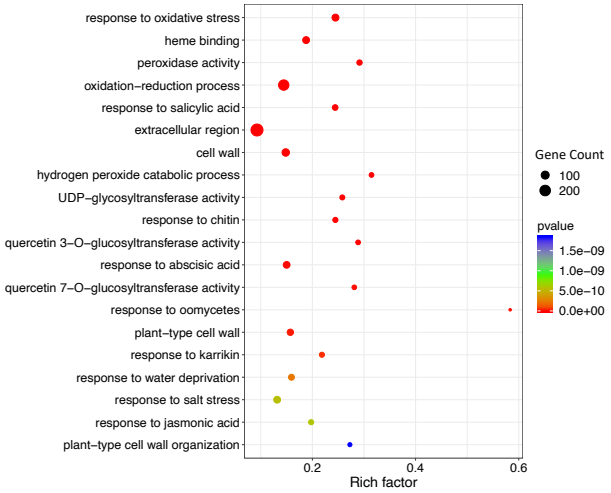
Wildtype 48h Untreated VS Wildtype 0h



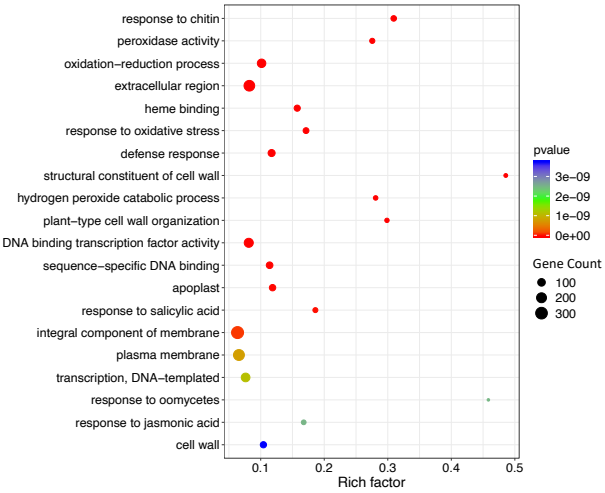
Wildtype 48h w/ Estradiol VS Wildtype 48h Untreated



Wildtype 48h Estradiol VS Wildtype 0h



Inducible 0h VS Wildtype 0h



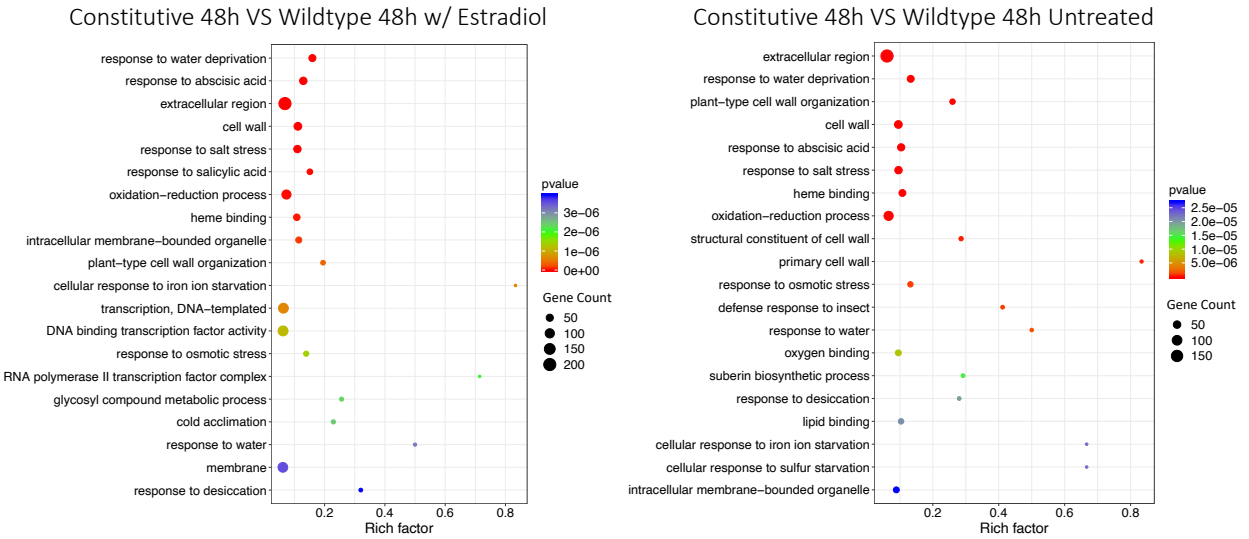


Figure 27 (collection). *Associating DEG with the appropriate GO database annotation, each sample comparison shows unique combination and scale of each term. The ratio of DEGs that have been annotated in a particular pathway to the number of genes in the same pathway is the rich factor.*

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis and functional classification of DEGs

Kyoto Encyclopedia of Genes and Genomes (KEGG - https://www.genome.jp/kegg/) data combines the genome, gene expression, and protein function profiles with the metabolic pathways and their functions to show the connectivity of the various pathways. This database is a commonly used analytical tool to determine the metabolic profiles of cells and tissues at a given time, thus providing a reliable method to compare the impact of transcriptomic changes in each tissue sample.

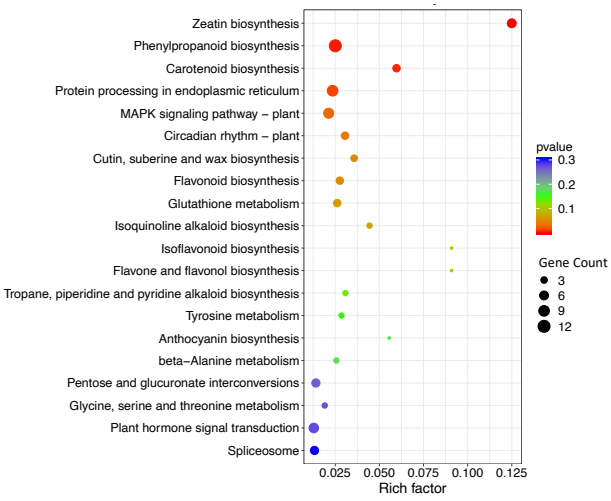
In the present study, the KEGG pathway analysis determined the most highly active biological pathways in the fourteen different comparisons looking at relationships between tissue type, timing, and treatment, as shown in Figure 28. The pathway category that showed the highest enrichment across nine of the fourteen comparisons was plant hormone signal transduction. Estradiol-treated IND tissue compared to untreated IND tissue over time had the

highest degree of enrichment of the entire list with 101 DEGs possessing a p-value less than 0.05. Still, with the number of DEGs close to that maximum, that pathway was enriched in all three genotypes, differing only due to the variety in combinations of testing conditions (their potential Put contents). This result is supported by there being so many individual DEGs with a relationship to ABA signaling (Table 4). The pathway that saw significant enrichment across all fourteen sample comparisons was Ras-Raf-MEK-ERK (MAPK) signaling pathway. This pathway yields a class of kinases integral to plants' response to oxidative, osmotic, cold, and pathogen stress responses. This also aligns with data from those individual high-fold change DEGs involved with the oxidative stress response.

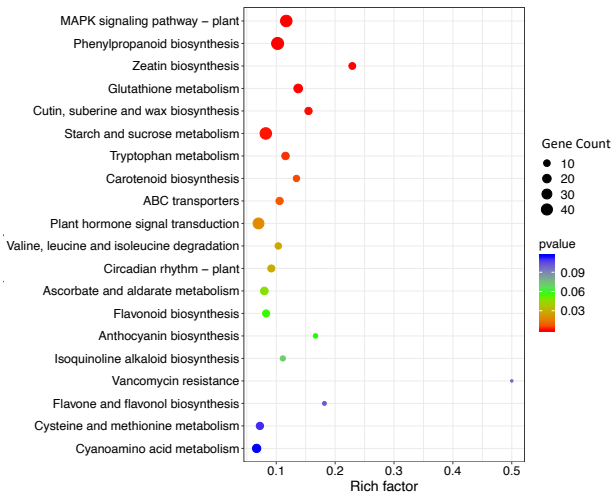
Plant-pathogen interaction is a pathway category that showed DEG enrichment across all sample types, regardless of transgenic or induction status. Wildtype plants over time and untreated IND against untreated wildtype showed this pathway to be highly enriched. Phenylpropanoids are numerous and diverse groups of metabolites in the plants derived from the amino acids phenylalanine and tyrosine. Appearing enriched in ten out of the fourteen sample comparisons, this pathway is an essential contributor to plant responses to all forms of stimuli, both biotic and abiotic stress. Compounds in this phenylpropanoid class have a variety of functions from indicating a response to stressors to supporting reproduction. Though not to the same degree as the three pathways just mentioned, but only occurring within the IND and CON cell lines, starch and sucrose metabolism is a highly enriched pathway. This pathway was primarily enhanced in CON tissue in several comparisons, including to itself over time, to wildtype at any time in the experiment, and to both induced (estradiol-treated) and uninduced IND plants. Table 4 shows the granularity of the most highly differentially expressed genes and hints at the correlation between these genes and the highly enriched KEGG pathways. Not all

comparisons are shown in this format – full data sets can be found in the original data files that can be accessed through the MCBS Department at UNH.

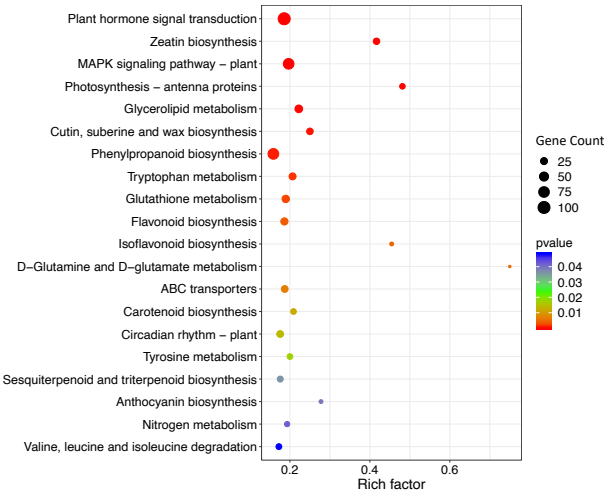
Inducible 48h w/ Estradiol VS Inducible 48h Untreated



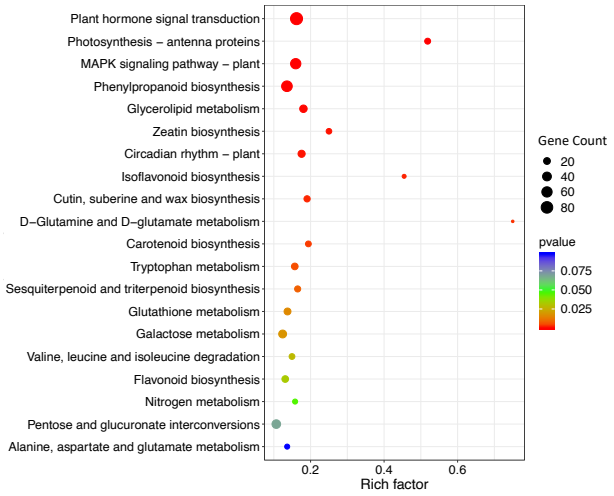
Constitutive 48h VS Inducible 48h w/ Estradiol



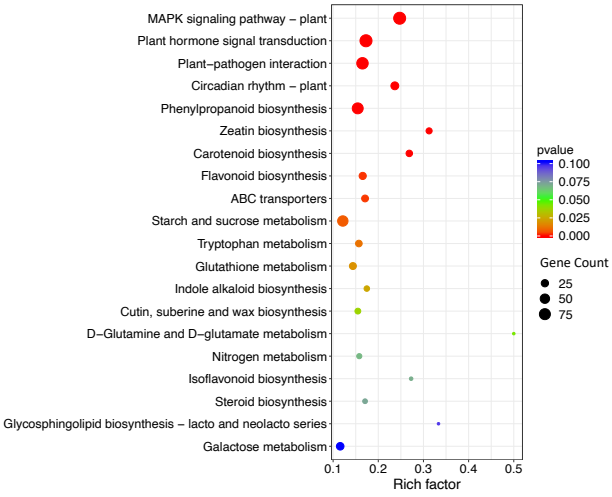
Inducible 48h w/ Estradiol VS Inducible 0h



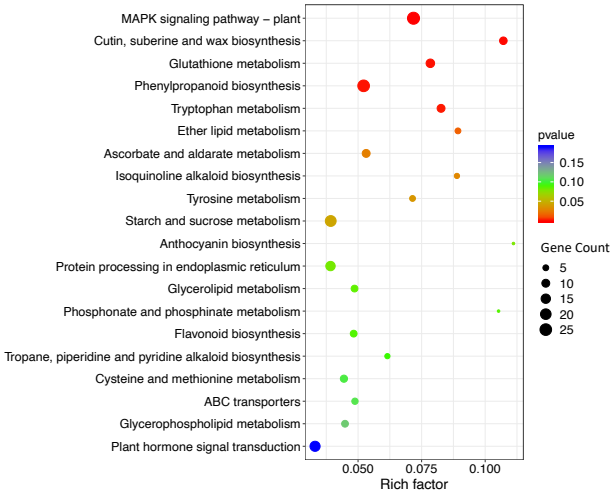
Inducible 48h Untreated VS Inducible 0h



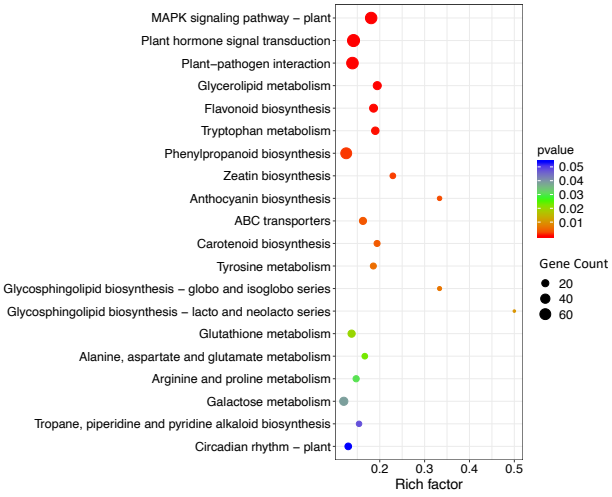
Constitutive 48h VS Constitutive 0h



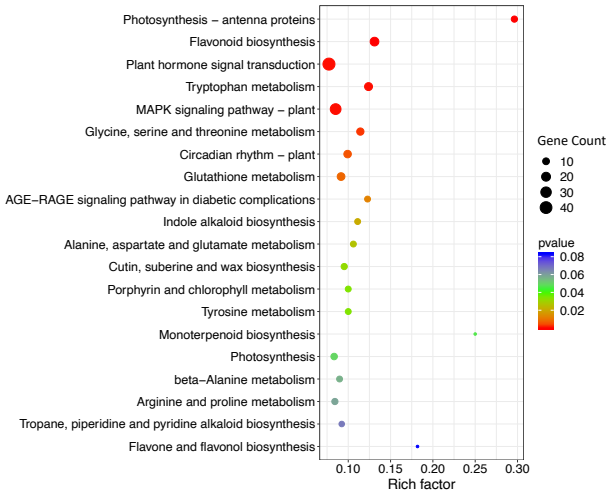
Constitutive 48h VS Inducible 48h Untreated



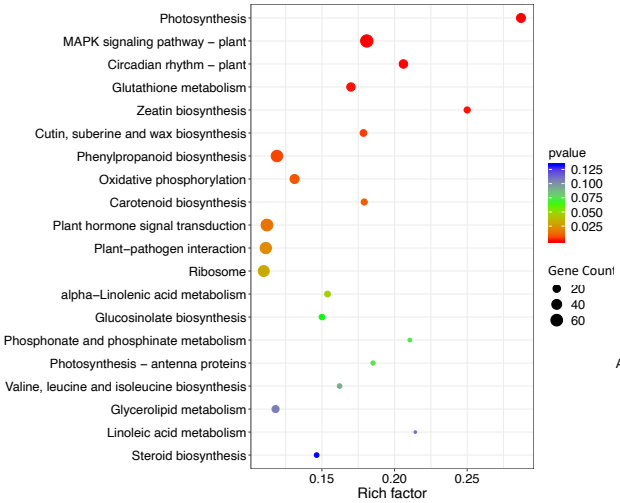
Constitutive 0h VS Inducible 0h



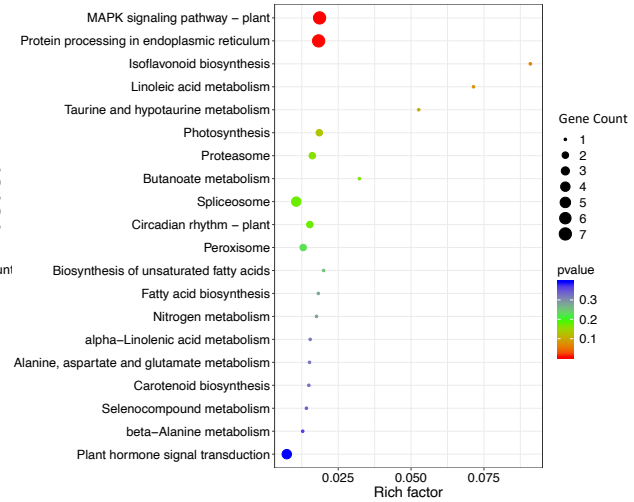
Constitutive 0h VS Wildtype 0h



Wildtype 48h Untreated VS Wildtype 0h



Wildtype 48h w/ Estradiol VS Wildtype 48h Untreated



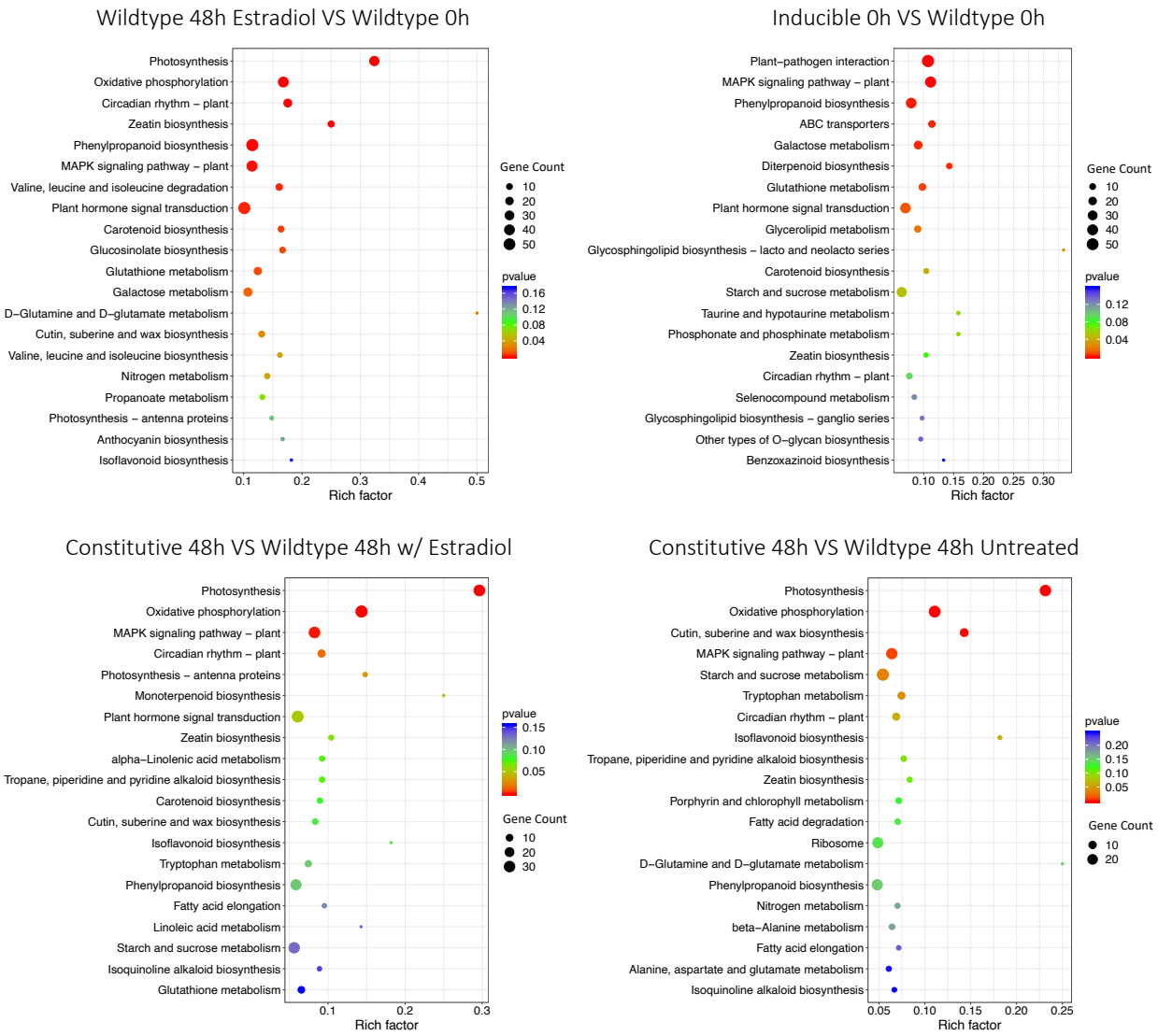


Figure 28 (collection). *Genes are associated with their corresponding metabolic pathways via enrichment with KEGG database of pathway annotation. Each sample comparison shows variation in and unique scale of pathway activation.*

Table 4. *Genes with the ten highest fold change, positive and negative, in each sample comparison. Negative log^2 fold change is down regulated change, positive is upregulated change. These genes are highlights of the KEGG database analysis.*

Constitutive VS Wild Type at 48 Hours

Gene ID	Gene Name	Description	Log^2 of Fold Change
AT5G10530	AT5G10530	Concanavalin A-like lectin protein kinase family protein	-14.87
AT1G74540	CYP98A8	unknown, partial	-14.04
AT2G07723	AT2G07723	cytochrome c biogenesis orf452 (mitochondrion)	-13.64
AT3G57730	AT3G57730	Protein kinase superfamily protein	-13.54
AT4G32208	AT4G32208	heat shock protein 70 (Hsp 70) family protein	-9.73

AT4G13505	AT4G13505	ammonium transporter 1;1	-8.46
AT3G07273	AT3G07273	hypothetical protein AT3G07273	-8.36
AT3G56970	bHLH38	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-7.06
AT2G11810	MGDC	monogalactosyldiacylglycerol synthase type C	-5.38
AT5G01881	AT5G01881	transmembrane protein	-5.28
AT1G12830	AT1G12830	nucleolin	17.78
AT1G21280	AT1G21280	Copia-like polyprotein/retrotransposon	15.53
AT3G21780	UGT71B6	UDP-glucosyl transferase 71B6	14.77
AT1G03790	SOM	Zinc finger C-x8-C-x5-C-x3-H type family protein	14.51
AT3G57510	ADPG1	Pectin lyase-like superfamily protein	14
AT3G49740	AT3G49740	Tetratricopeptide repeat (TPR)-like superfamily protein	13.83
AT3G56890	AT3G56890	F-box associated ubiquitination effector family protein	13.68
AT3G53040	AT3G53040	late embryogenesis abundant protein, putative / LEA protein	13.24
AT5G22470	AT5G22470	poly ADP-ribose polymerase 3	11.76
AT2G25900	ATCTH	Zinc finger C-x8-C-x5-C-x3-H type family protein	7.99

Constitutive VS Treated Inducible at 48 Hours

Gene ID	Gene Name	Description	Log^2 of Fold Change
AT3G57730	AT3G57730	Protein kinase superfamily protein	-14.4
AT3G12030	AT3G12030	transmembrane/coiled-coil protein	-11.84
AT4G28652	AT4G28652	Leucine-rich repeat transmembrane protein kinase family protein	-11.76
AT4G32208	AT4G32208	heat shock protein 70 (Hsp 70) family protein	-9.5
AT3G07273	AT3G07273	hypothetical protein AT3G07273	-7.95
AT4G13505	AT4G13505	ammonium transporter 1;1	-7.88
AT4G06835	AT4G06835	DOGL4 – sequence-specific DNA binding	-6.82
AT3G56970	bHLH38	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-6.44
AT2G34420	LHB1B2	photosystem II light harvesting complex protein B1B2	-6.11
AT4G16983	AT4G16983	hypothetical protein AT4G16983	16.19
AT3G62740	BGLU7	beta glucosidase 7	15.13
AT1G05250	AT1G05250	Peroxidase superfamily protein	14.78
AT3G21780	UGT71B6	UDP-glucosyl transferase 71B6	14.77
AT5G46900	AT5G46900	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein [Arabidopsis thaliana]	14.1
AT2G33790	AGP30	arabinogalactan protein 30	8.21
AT5G60660	PIP2%3B4	plasma membrane intrinsic protein 2;4	7.78
AT2G25900	ATCTH	Zinc finger C-x8-C-x5-C-x3-H type family protein	6.85
AT4G13390	EXT12	Proline-rich extensin-like family protein	6.3
AT1G13635	AT1G13635	DNA glycosylase superfamily protein	6.14
AT1G54970	PRP1	proline-rich protein 1	6.14

Treated Inducible VS Untreated Inducible at 48 Hours

Gene ID	Gene Name	Description	Log^2 of Fold Change
AT4G16983	AT4G16983	hypothetical protein AT4G16983	-16.17
AT5G52190	AT5G52190	Sugar isomerase (SIS) family protein	-15.96
AT2G30360	SIP4	SOS3-interacting protein 4	-15.3

AT3G07615	AT3G07615	DUF740 family protein, putative (DUF740)	-14.44
AT1G74055	AT1G74055	transmembrane protein	-14.27
AT5G60660	PIP2%3B4	plasma membrane intrinsic protein 2;4	-6.5
AT4G13390	EXT12	Proline-rich extensin-like family protein	-4.97
AT5G35190	EXT13	proline-rich extensin-like family protein	-4.2
AT5G27100	GLR2.1	glutamate receptor 2.1	-4
AT3G07273	AT3G07273	hypothetical protein AT3G07273	7.64
AT2G14775	AT2G14775	hypothetical protein AT2G14775	5.05
AT5G46790	PYL1	PYR1-like 1	4.04
AT2G02700	AT2G02700	Cysteine/Histidine-rich C1 domain family protein	3.96
AT3G05080	AT3G05080	hypothetical protein AT3G05080	3.89
AT2G42560	AT2G42560	late embryogenesis abundant domain-containing protein / LEA domain-containing protein	3.37
AT5G01680	CHX26	cation/H+ exchanger 26	3.29
AT5G06665	AT5G06665	unnamed protein product	3.22
AT4G27790	AT4G27790	Calcium-binding EF hand family protein	3.12
AT2G41260	M17	glycine-rich protein / late embryogenesis abundant protein (M17)	3.11

Wild Type at 48 Hours VS Wild Type at 0 Hour

Gene ID	Gene Name	Description	Log^2 of Fold Change
AT1G20070	AT1G20070	hypothetical protein AT1G20070	-15.28
AT4G31540	EXO70G1	exocyst subunit exo70 family protein G1	-15.02
AT3G49740	AT3G49740	Tetratricopeptide repeat (TPR)-like superfamily protein	-14.05
AT3G12030	AT3G12030	transmembrane/coiled-coil protein	-10.82
AT1G22130	AGL104	AGAMOUS-like 104	-5.47
AT2G21200	AT2G21200	SAUR-like auxin-responsive protein family	-4.87
AT3G48060	AT3G48060	BAH and TFIIS domain-containing protein	-4.75
AT1G14960	AT1G14960	Polyketide cyclase/dehydrase and lipid transport superfamily protein	-4.45
AT3G27355	AT3G27355	unknown, partial	-4.24
AT4G12500	AT4G12500	Bifunctional inhibitor/lipid-transfer protein/seed storage 2S albumin superfamily protein	17.44
AT2G26020	PDF1.2b	plant defensin 1.2b	16.61
AT1G55190	PRA7	PRA1 (Prenylated rab acceptor) family protein	16.56
AT2G26010	PDF1.3	plant defensin 1.3	16.20
AT1G69930	GSTU11	glutathione S-transferase TAU 11	16.17
AT1G14540	PER4	Peroxidase superfamily protein	16.16
AT1G26240	AT1G26240		15.85
AT5G19890	AT5G19890	Peroxidase superfamily protein	15.22
AT1G65390	PP2-A5	phloem protein 2 A5	14.82
AT1G49570	AT1G49570	Peroxidase superfamily protein	14.71

Estradiol-treated Wild Type VS Untreated Wild Type at 48 Hours

Gene ID	Gene Name	Description	Log^2 of Fold Change
AT2G07723	AT2G07723	cytochrome c biogenesis orf452 (mitochondrion)	-13.64
AT3G05935	AT3G05935	hypothetical protein AT3G05935	-3.5

AT3G06590	AT3G06590	basic helix-loop-helix (bHLH) DNA-binding superfamily protein	-3.38
AT2G28210	ACA2	alpha carbonic anhydrase 2	-3.34
AT4G14990	AT4G14990	Topoisomerase II-associated protein PAT1	-3.18
AT5G44430	PDF1.2c	plant defensin 1.2C	-2.98
AT1G52690	LEA7		-2.86
AT3G26830	PAD3	Cytochrome P450 superfamily protein	-2.83
AT5G15845	AT5G15845	CONSTANS-like 1	-2.77
AT4G37095	AT4G37095	hypothetical protein AT4G37095	16.92
AT5G52190	AT5G52190	Sugar isomerase (SIS) family protein	14.51
AT3G49740	AT3G49740	Tetratricopeptide repeat (TPR)-like superfamily protein	14.32
AT1G07520	AT1G07520	hypothetical protein, partial [Arabidopsis thaliana];GRAS family transcription factor [Arabidopsis thaliana];F22G5.9	6.2
AT1G15000	scpl50	serine carboxypeptidase-like 50	4.89
AT1G05837	AT1G05837	transmembrane protein	4.25
AT5G28340	AT5G28340	Tetratricopeptide repeat (TPR)-like superfamily protein	4.19
AT3G48060	AT3G48060	BAH and TFIIS domain-containing protein	4.11
AT3G56410	AT3G56410	hypothetical protein (DUF3133)	3.71
AT1G66830	AT1G66830	Leucine-rich repeat protein kinase family protein	3.49

Polyamine biosynthetic pathway

The Put biosynthetic pathway is well characterized in the KEGG database, as are many correlated and connected pathways around Put (Figure 29). Some of these genes were found to have significant differences in expression in the zero-hour CON tissue, when compared to untreated IND also at zero-hour. There was significant upregulation in *AT5G53120*, *AT4G08870*, and *AT4G34710*, the genes producing spermidine synthase III, arginase, and arginine decarboxylase II respectively. The gene for arginine decarboxylase II was also upregulated in treated IND tissue at 48 hours compared to untreated at zero-hour, CON compared to untreated WT at zero hour, and strangely untreated IND at 48 hours compared to the same tissue type at zero hour. Much of these results are aligned with our assumptions, given that CON and treated IND tissues accumulated greatest amounts of Put of all other samples.

Arginine biosynthetic pathway

A precursor to Put, Arg is an important product of the urea cycle (Figure 30). An important enzyme in this biosynthetic pathway, Argininosuccinate synthase (*AT4G24830*), was

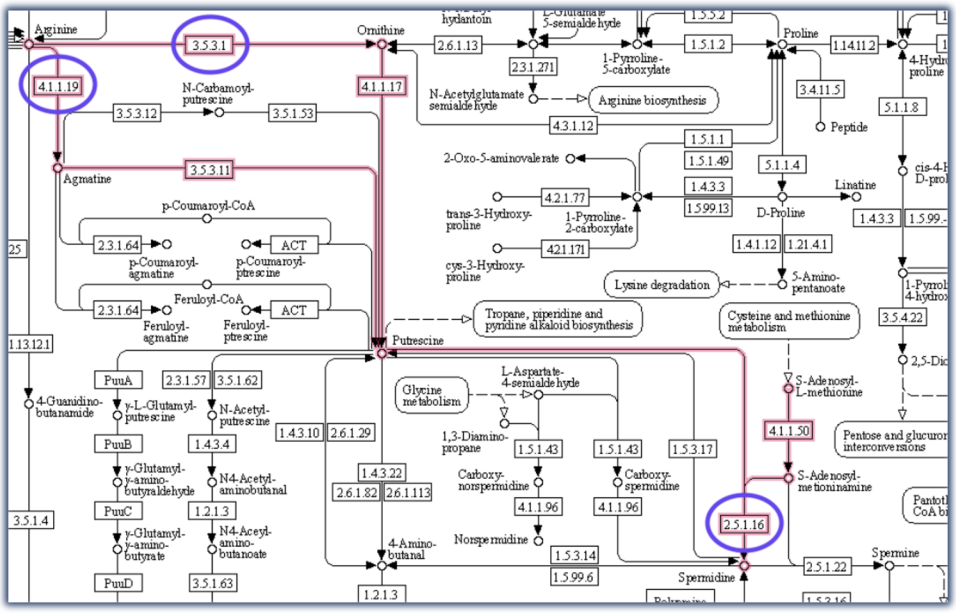


Figure 29. *Polyamine biosynthetic pathway as illustrated in the KEGG database. The highlighted entries showed differential regulation in our study. KEGG index entry 4.1.1.19 includes arginine decarboxylase I and II which transforms arginine to agmatine. Entry 3.5.3.1 categorizes two arginase genes, the enzymes of which (arginase and arginase/deacetylase) convert Arg to Orn. Entry 2.5.1.16 classifies three spermidine synthase enzymes which take part in converting putrescine into spermidine.*

upregulated in CON tissue compared to either untreated WT or treated IND at 48 hours. We expect the pathways that lead to Put to show upregulation in CON tissue (compared to WT). We also expect that this assumption would hold true for

treated IND tissue as well. Though *AT4G24830* was not downregulated in IND tissue compared to CON tissue, the degree of upregulated change was very similar in both comparisons.

Nitrogen metabolic pathway

Though very upstream of the PA biosynthetic pathway, one of the proposed benefits of PA overproduction is that it creates a higher demand for nitrogenous precursors and thus nitrogen itself. As the carbon and nitrogen ratio must be tightly regulated for optimal growth, increasing uptake of environmental nitrogen compounds (nitrate or ammonium) would, on balance, stimulate higher carbon uptake (Oa et al., 2013; Reich et al., 2006; Zheng, 2009). In a

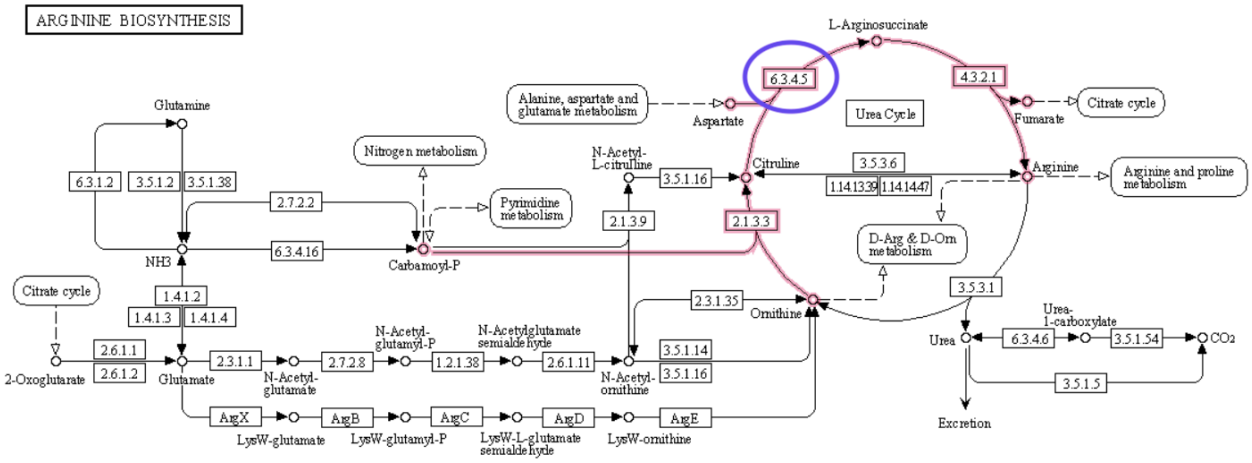


Figure 30. *Arginine biosynthetic pathway as illustrated on the KEGG website. The highlighted entries showed differential regulation in our study. KEGG index entry 6.3.4.5 indicates arginosuccinate synthase converting citrulline and aspartate to form L-arginosuccinate. This is one enzymatic step removed from Arg itself.*

global climate changing rapidly due to increased levels of CO_2 and other greenhouse gases, increased carbon sequestration is a welcomed phenomenon. In zero-time CON seedlings, glutamine synthase (GLN 1;4 – *AT5G16570*), was lower as compared to both untreated IND and WT also at zero time. Both treated and untreated IND tissues at 48 hours showed this gene to be downregulated compared to untreated IND at zero hour. CON tissue at 48 hours, however, showed an upregulation of this gene compared to treated IND at 48 hours. These conflicting and counterintuitive results indicate no correlation to PA overproduction.

Three enzymes related to glutamate production were differentially expressed in our study but showed no clear pattern (Figure 31). Glutamate dehydrogenase [NAD(P)+] I (GDH – *AT5G18170*), II (AT5G07440), and III (AT3G03910) abbreviate to GDH-1, GDH-2, and GDH-3 respectively; this enzyme converts glutamate to α-ketoglutarate and ammonia. Both treated and untreated IND plants at 48 hours showed upregulation of GDH-1 compared to IND plants at zero hour. CON and treated WT plants at 48 hours showed upregulation in GDH-1 compared to CON at zero hour and WT at zero hour, respectively. Upregulating this gene enhances demand for

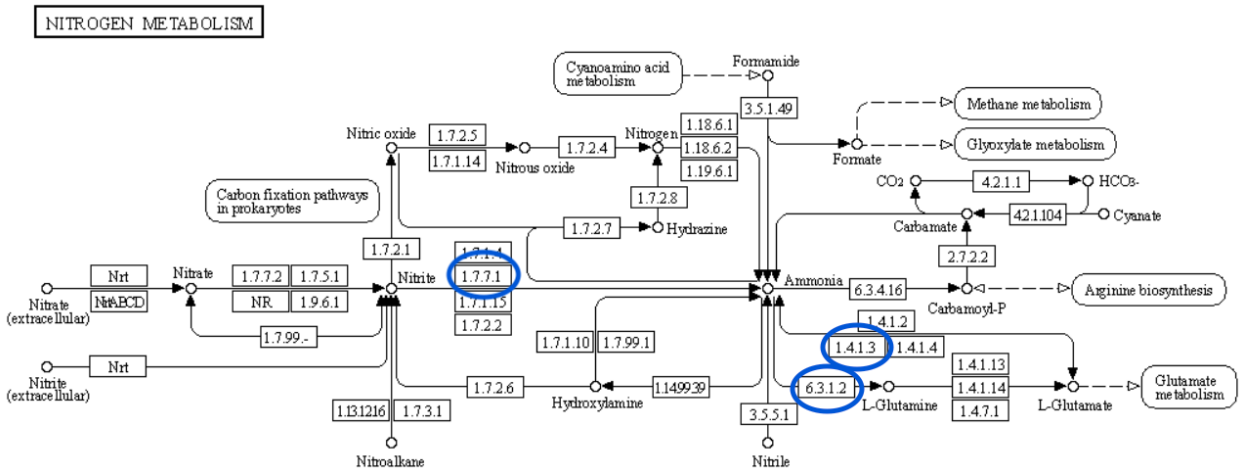


Figure 31. *Nitrogen metabolic pathways as illustrated on the KEGG website. The highlighted entries showed differential regulation in our study. KEGG index entries 6.3.1.2 (glutamine synthase 1;4) and 1.4.1.3 (glutamate dehydrogenase [NAD(P)+] I, II, and III convert ammonia to glutamate either indirectly by way of L-glutamine or directly, respectively. KEGG index entry 1.7.7.1 for ferredoxin-nitrite reductase is one enzyme that converts nitrite into ammonia in Arabidopsis.*

upstream nitrogenous metabolic feedstocks. In both treated and untreated IND plants at 48 hours, there was upregulation of GDH-2 compared to untreated IND at zero hour. Treated WT and untreated CON tissue at 48 hours showed upregulation in GDH-2 compared to WT and CON at zero hour, respectively. Finally, for GDH-2, it was downregulated in CON tissue compared to IND tissue, both at zero hour. Almost opposite to the situation with GDH-1 expression, GDH-3 is downregulated in CON tissue at 48 hours compared to either treated or untreated WT at 48 hours. It is also downregulated in both treated and untreated IND tissue at 48 hours compared to IND at zero hour. Though well represented in significant change in expression among many comparative groups, there may be too much variation in expression for this group to determine if GDHs are correlated to PA overproduction.

Ferredoxin-nitrite reductase (*AT2G15620*) is upregulated in CON tissue at 48 hours compared to CON tissue at zero hour. This enzymatic pathway is the quicker way that Arabidopsis can convert nitrite into ammonia. Though we expect CON tissue to show the most

dramatic differences, given the strong phenotypic performance, the correlation of this enzyme would seem more significant if it was upregulated in CON tissue compared to WT or untreated IND tissues.

DISCUSSION

Having been tested for PA production and several other physiological analyses, the three cell lines used here (WT, CON, and IND) have been well characterized in many studies from the Minocha lab at University of New Hampshire (Majumdar et al., 2016, 2017; Mohapatra et al., 2010; Quan et al., 2002). The methods used to exemplify the efficacy of this transgenic concept varied in effectiveness. Though seedlings grown in vitro and tested in liquid media proved to be the exemplary method, testing seedlings and plants grown in soil should not be overlooked. Only a small effect in Put accumulation was seen in the leaf spray experiment with IND plants indicating that perhaps a higher concentration of estradiol inducer may be what is needed to make the spray more effective. Additionally, waiting 24 or 48 hours to collect and analyze tissue following the experiment may be too long a period; collecting samples more immediately after the spray liquid has been absorbed or dried could be a more telling moment in the metabolic changes brought on by this transgene. The root treatment with estradiol could also be altered with higher concentration, as with the leaf spray method, but a way to potentially improve the root induction method is to use a shallower bed of soil. This way, the aqueous estradiol inducer has more potential to interact with the plants and does not simply get bound by the soil thus reducing its availability to roots. Though the in vitro method was the best, testing this transgene in more natural conditions will be vital to know if it is worth applying this transgenic concept to economically important crop species under natural growth conditions.

There was significant variation in Put accumulation between IND plants induced with estradiol and IND plants that were not induced. The uninduced IND plants also showed similar PA levels to WT indicating that the expression of the *mODC* gene is the specific cause of the increase in Put. The plants of the CON *mODC* genotype demonstrated significantly higher Put

accumulation as compared to WT plants. The efficacy of this transgene to PAs over time was shown in the accumulation of Put. This RNA-seq analysis was conducted to analyze the impact of high Put accumulation on the expression of a wide spectrum of genes affecting various metabolic pathways and other cellular activities. Furthermore, we were interested in the question of long-term impact of overproducing Put temporarily (IND) vs. permanently (CON) during the life cycle of the plants. The assumption is that the CON plants maintain a homeostatic level of higher Put and adjust their overall metabolism throughout their life in all parts of the plant. This is the approach that is most commonly used in genetic engineering of plants to increase the amount of a specific metabolite or altering a pathway, including the approach used in majority of our commercial crops (Andersen et al., 1998; Bastola et al., 1995; Chen et al., 2021; Pandey et al., 2015; Z. Wang et al., 2019; Yu et al., 2021; Zhang et al., 2021). The IND plants, on the other hand, have lower homeostatic levels of PAs, and undergo transient changes in PAs, which is analogous to the situation in plants for making metabolic adjustments in response to short-term changes in their environment, be it a stress factor, change in day length, or the onset of a new developmental stage.

Several promoters are induced with specific chemistries, environmental conditions, or by internal signaling (Gulbitti-onarici et al., 2009; Khurana et al., 2013; Yi et al., 2010). Changes in PAs in response to varieties of signals or stimuli have been studied in many plants in response to stress or during development (Kasukabe et al., 2004; Mehta et al., 2002). During most of its life, a plant must respond to additional PAs through a variety of endogenous induction signals. The three genotypes in this study present a great comparison of the transcriptomic adjustments to ‘normal’ as well as constantly high and temporarily high states of Put accumulation. The results of this study confirm some of our assumptions and provide useful information to understand

interactions in short-term changes in Put production vs long-term changes (continuous production) of low or high PAs with several other genes and their functions.

Prolonged mODC expression is accompanied by Put negative feedback mechanism

High levels of Put accumulation were seen in the CON plants, more so than in the IND plants induced with estradiol for 24–48-hour transient period. Constitutive type plants live in this state of high Put production and accumulation which, in turn, stimulates mechanisms for its metabolic management. The gene that produces nucleolin protein was the most upregulated gene in CON samples compared to any other sample type. This nucleolar protein is responsible for facilitating the synthesis, transport, and assembly of rRNA and ribosomal subunits (Oa et al., 2007). In plants, nucleolin also plays a controlling role in the cellular response to auxin (Medina et al., 2010). There is a nucleolin isoform that binds spermidine/spermine-N^1-acetyltransferase, a PA catabolic enzyme that serves as a homeostatic check on PA over-accumulation (Perez-leal et al., 2012). Though there are many isoforms of nucleolin, the overproduction of this protein could indicate that this negative feedback mechanism to control PA abundance has been activated in the high-Put plants. With Put measuring at 420.7 nmol/g FW in CON tissue at 48 hours, this quantity could be well above the homeostatic threshold of Put accumulation. Thus, it could induce the production of spermidine whose accumulation prevented or slowed down by the action of nucleolin. This, however, was not observed in this situation, which confirms the other studies from our lab by Lin Shao (Ph.D. Thesis – 2013). Were there an outlet or use for the accumulated cellular PA content, perhaps the nucleolin may never reach the point that it would be so powerfully upregulated. Perhaps this gene could serve as a proxy of how much PA accumulation is too much.

High Put correlates with ABA synthesis and variability in cellular sensitivity to ABA

Response to ABA was an enriched GO term in both CON and IND sample comparisons. Abscisic acid is a plant's multi-tool regulator, involved in development and stress response, among other functions, and is the focal molecule for several significant DEGs (Pál et al., 2018; Tuteja, 2007). Surplus Put is metabolized and inherently breaks down (via diamine oxidase) to produce ROS such as H_2O_2. Higher levels of this gas trigger a stress response leading to increased ABA synthesis, but Pyrabactin resistance-like protein (*PYL*) is a regulatory component of ABA receptor (*RCAR*) (Liang et al., 2011); this gene is upregulated in both IND and CON tissues with higher PAs. This is one factor of three ABA core signaling pathway constituents; the other two factors are protein phosphatase 2C (*PP2C*) and sucrose non-fermenting (*SNF1*)-related protein kinase2 (*SnRK2*) (Park et al., 2009; Q. Zhang et al., 2019). The influence of ABA often results in cascading events involved with complex stress responses or highly coordinated developmental stages. An early ABA response event begins with the binding to RCARs (Yin et al., 2009). Left by themselves, *SnRK2s* will phosphorylate downstream transcription factors that manage vital next steps in the ABA stress response cascade. In the absence of ABA, PP2Cs inhibit *SnRK2s* (Liang et al., 2011; Zhang et al., 2019). When ABA is present, it binds PP2C molecules freeing the *SnRK2* molecules to begin a flood of downstream responses. In our study, managing ABA due to high Put metabolism is a likely prelude to the upregulation of AT5G46790 for *PYL1*.

The gene *UGT71B6*, coding for uridine diphosphate glucosyltransferase (*UGT*) is highly upregulated in CON tissue compared to most other comparative samples. This enzyme and its homologues are critical constituents of the ABA metabolic pathway (Dong et al., 2014). Abscisic acid plays an essential role in several stages of development, but its function in stress response, particularly osmotic stress, is vital and may show correlation with PA metabolism and osmotic

stress (Pál et al., 2018). On the anabolic side of ABA homeostasis, several enzymes are involved in its de novo synthesis. When ABA is in overabundance, there are two methods for catalysis of ABA: hydroxylation and conjugation. Hydroxylation involves the degradation of ABA eventually to phaseic acid, which is an irreversible process. Conjugation, on the other hand, is reversible and involves *UGT71B6* binding glucose to ABA, inactivating rather than destroying it (Dong et al., 2014; Rehman et al., 2018). That *UGT71B6* is upregulated so significantly in CON tissue could indicate that ABA levels are also high. Though the experimental design did not include a stress factor, ABA is still a useful indicator of stress response in action. The catabolism of Put produces ROS and perhaps in a great enough quantity to stimulate stress response, despite the lack of a true stressor.

Members of the zinc finger CCCH type protein family (*AtTZF*) have influence throughout the entire development of Arabidopsis. These molecules are known to regulate plant growth as well as stress response, positively regulate ABA production, and have the ability to specifically bind mRNA as post-transcriptional regulator (Bogamuwa & Jang, 2016; Han et al., 2014). *AtTZF* proteins also have the ability to localize processing bodies (P-Bodies) and stress granules which function to preserve pre-translational mRNAs in an inactive, bound form serving to hasten the production of those mRNA products by maintaining post-transcriptional integrity until called upon for translation (Jang et al., 2020; Jang et al., 2019). *AtTZF* is a positive regulator of ABA, which could explain the correlative presence of UGT, produced to manage ABA surplus. This gene is also upregulated in treated IND at 48 hours compared to 0 hour indicating that *AtTZF* is potentially an early-stage reaction to high Put accumulation.

AT3G12030 (transmembrane/coiled-coil protein of unknown function DUF106) codes for tetratricopeptide repeat TPR-like superfamily of protein (*TPR*) and is typically expressed in most

parts of *A. thaliana* (https://www.arabidopsis.org/servlets/TairObject?accession=locus:2088634). Discovered first in yeast, this 34-amino acid canonical sequence is known to require multiple repeats of the sequence to form functional domains for protein-protein interactions throughout all forms of life (Schapire et al., 2006). Further studies into this motif show involvement not only in the cell cycle, but also in protein folding, transcription control, neurogenesis, and hormone signaling and biosynthesis (Greenboim-wainberg et al., 2005). Leveraging mutation studies in Arabidopsis, it is known that *TPR* motifs are found in tetratricopeptide-repeat thioredoxin-like 1 (*TTL1*), which is required for responses regulated by ABA. In fact, *TTL1* positively regulates ABA signaling in stressed early stages of development (Rosado et al., 2006). Another clear connection to the ABA metabolic pathway, albeit indirect is that TPR joins UGT and *AtTZF* as responsive to accumulation of Put (Bogamuwa & Jang, 2016; Dong et al., 2014). Beyond *TTL1*, however, *TPR* motifs are scaffolding complexes essential for the proteins involved in ethylene biosynthesis. Though it was found that overproducing Put did not have an impact on the ethylene biosynthetic pathway, Put and ethylene still both utilize the substrate *dcSAM*, and often have opposing effects on growth and development (Quan et al., 2002). The use of TPR-laden proteins may be supporting ethylene biosynthesis despite the draw on *dcSAM* by the Put pathway to produce Spd and Spm. To say that the Put pathway was inherently winning in the conflict over *dcSAM* is merely conjecture at present, given that Spd and Spm levels were not significantly impacted in our experiment.

Breaking with this theme of increased sensitivity to ABA, heat shock protein 70 (Hsp70) was also downregulated in CON samples. This protein is well characterized as a chaperone protein involved in de novo folding and transport of recently expressed genes, but also as a quality control system that repairs ill-formed proteins and maintains solubility of aggregating,

partially denatured proteins (Lin et al., 2001; Mayer & Bukau, 2005). The name of this protein class denotes a legacy of well-characterized action of thermotolerance, but recent technologies have uncovered roles it has in osmotic, heavy metal, and pathogenic stress response pathways (Leng et al., 2017; Mayer & Bukau, 2005; Yu et al., 2015). It has also been found that this subclass of HSPs play redundant, but critical, roles in developmental signaling (Haq et al., 2019; Kumar et al., 2020). Mutation studies in Arabidopsis reveal that overexpression of *Hsp70* produces an ABA hypersensitivity; conversely, loss-of-function mutants expressing little *Hsp70* show hyposensitivity indicating that *Hsp70* plays an important role in ABA signaling (Leng et al., 2017). In maize, it was found that when facing drought or heat stress *Hsp70* in the cytosol regulates an ABA-induced response that increased production of antioxidant enzymes to manage the ROS being produced from stomatal closures and over-reduction of reactive oxygen (Yu et al., 2015). Future work could focus on Hsp70 to determine which of its many involvements is actually at play in tissues living with high Put.

High Put leads to molecular management of ROS from metabolized Put surplus

The perpetual production of Put in CON tissue for the entirety of its ~30 day life versus the induced production (in IND tissue) over a single 48 hour period may differ in higher accumulation of H_2O_2 biproduct inherent in Put catalysis (Saha et al., 2015; Verma & Mishra, 2005). Constitutive and IND tissues showed enrichment of the GO term, *response to oxidative stress* in many sample comparisons of DEGs. Though it takes at least dozens of genes to indicate enrichment of a GO term, there are several individual genes that were significantly up and down regulated that support the idea that Put catabolism produces ROS which the plant must subsequently manage. Some examples of genes upregulated in this group follow below.

Beta-glucosidase (*AT3G21780 – UDP-Glucosyl transferase 71B6*, *UGT71B6*)) is among the highly upregulated genes in CON tissues. These proteins have a diverse functionality: the control of phytohormones, protection against herbivory, structural support in germination, and mechanical support of stress response (Xu et al., 2004). Regarding abiotic stress, flavanol bisglycosides accumulate in growing tissues to protect against the increase of free radicals that accompany osmotic stress. Beta-glucosidases are necessary for the catabolism of these flavonoid molecules when they have reached critical mass; these enzymes are also triggered independently of flavonoid accumulation (Roepke et al., 2017). Phenotypically, CON and treated IND plants were quite similar in the expression of these genes. That abundant Put in CON plants most immediately yields a stress-response-priming dose of ROS when metabolized, this later-stage beta-glucosidase period could indicate that life-long high Put metabolism may be producing an unhealthy amount of ROS. The induction of these genes happens quickly in response to high Put. Investigating tolerances of flavonoid glycosides using an inducible promoter could show what level of ROS is enough to stimulate this response.

Plant peroxidases, upregulated in CON tissue, come in a variety of isoforms and are involved in many aspects of plants' life; thus, they can be used as markers to denote approximate stage of development (Valerio et al., 2004). They are well characterized as catalyzers of lignin polymers (Shigeto et al., 2013), and are also implicated in many forms of stress response (Cosio et al., 2005; Pinedo et al., 2015). In fact, peroxidases facilitate lignin polymerization by reducing ROS and donating electrons to the process. These electron donations, however, are used to produce hormones and other secondary metabolites, making plant peroxidases a very effective aspect of a stress response (Cosio et al., 2005). The presence of various peroxidase isoforms indicates what kind of stress response is occurring. Whether it is light quality, osmotic stress,

environmental contamination, pathogen infection, or temperature stress, plant peroxisomes are involved (Valerio et al., 2004). This group of genes was significantly upregulated in CON tissue at all times during the experiment (0 as well as 48 hours) compared to treated and untreated IND plants at 48 hours, and treated WT tissue at 48 hours. Though a response to increased intracellular ROS has been suggested by other DEGs in this study, there may be too many functions in this protein family to identify a specific type of stressor. That this gene is as significantly upregulated in treated WT tissue as it is in CON tissue is confounding.

Another upregulated gene in CON tissue, ADPG1 (Arabidopsis Dehiscence Zone polygalacturonase1-*AT3g57510*) produces pectin-lyase which has many functions. The function relevant to this study is that these lyases will promote stress response by catabolizing plant cell wall producing oligogalacturonides (Cao, 2012). Though this action seems counterintuitive to degrade its own cell wall production enzymes in a response to stress, oligogalacturonides stimulate defense-related proteins. These responsive proteins manage ROS, pathogenic infections, and are also involved in developmental processes (Ferrari et al., 2013; Gallego-giraldo et al., 2020). Further studies are needed to understand this connection better.

Increased ABA presence brought on by increased Put stimulates root development

Arabinogalactan protein 30 (*AGP30*) is a proteoglycan molecule expressed only in roots that has the capacity to influence early epidermal cell type differentiation during root generation (Hengel & Roberts, 2003; Jing et al., 2019). This gene is upregulated in CON tissue, living with high Put accumulation, compared to treated IND tissue, ramping Put production over 48 hours, indicating there could be a threshold of ABA that engages this gene. As many regulatory systems can involve negative feedback loops, this gene manages cellular ABA, which independently regulates the expression of the gene itself. Interestingly, the *AGP30* doesn't manage ABA

directly, rather it heightens sensitivity to ABA signaling in receptors around it. Further, there is a notable optimum ABA concentration threshold that must be present before a meaningful level of *AGP30* expression is reached indicating that this system relies on constant signaling to work (Hengel et al., 2004). At the tip of a growing root, just behind the meristem region, an epithelial cell can differentiate into one that will develop a root hair (trichoblast) or one that does not (atrichoblast) thereby spacing out each protrusion along the length of the root. Constant, but asynchronously fluctuating, signaling of ABA and ethylene stimulates this differentiation and *AGP30* plays a key role in amplifying ABA signaling (Hengel et al., 2004). Though ABA has been contextualized as a major player in stress response so far in this work, it also serves in growth and development roles. Considering the implication of high Put production in CON tissue where plentiful Put catabolism yields high level of ABA biproduct, several upregulated genes have been shown to engage in cellular or immune responses because of ABA. In the case of *AGP30*, however, high ABA is necessary to fuel this root growth system (Harris, 2015).

Accumulated Put correlates to changes in some active immune responses

The most downregulated gene in CON tissue is that for the L-type lectin receptor kinase (*LecRK*) which is involved in immune response. In a class of receptor proteins known as Pattern Recognition Receptors, *LecRK* is specifically responsive to Phytophthora, a pathogenic fungus. When resistance is overwhelmed and pathogens infect cells, this gene is independently involved in signaling apoptosis (Wang & Bouwmeester, 2017; Wang et al., 2015). The mitochondrial cytochrome-C biogenesis gene was also significantly downregulated to a similar degree, in both CON at 48 hours vs zero and treated WT at 48 hours compared to untreated WT at the same time. Pivotal in oxidative phosphorylation, this protein-heme complex acts as an apoptosis signaling factor in higher plants (Allen, 2011; Garcia et al., 2016).

AT3G57730 is identified as a protein kinase superfamily in Arabidopsis and it is a transmembrane receptor-like protein that triggers an immune response against *Pseudomonas* pathogens (Liu et al., 2019). Normally, microbe-associated molecular patterns of extracellular pathogens elicit effector triggered immunity (ETI) in plants. Certain pathogens evade or suppress this mechanism by injecting type III secreted effector proteins. In Arabidopsis, *AT3G57730* is a part of a nucleotide-binding leucine-rich repeat protein family, not associated with typical ETI genes, that detects intracellular pathogen effectors and triggers a response (Id et al., 2019; Lewis et al., 2013). In our study, this gene was shown to have consistently high degree of differential expression, but there was no discernable pattern to whether it was up or down regulated throughout the samples. This gene was upregulated in CON tissue as well as treated and untreated IND tissue at 48 hours, compared to IND at zero hour. This gene was downregulated in CON tissue at compared to IND and WT samples.

Transient Put overproduction alters concentrations of ion channel proteins

According to GO enrichment analysis of IND samples, there are hundreds of DEGs involved in integral membrane components. The third most down regulated gene in IND samples, *AT2G30360*, produces an SOS3-interacting protein 4 (*SIP4*), another cell membrane protein kinase involved with regulation of Na^+ and K^+ concentrations and deemed necessary when faced with salinity stress (Halfter et al., 2000). In Arabidopsis, there are more than two dozen members of kinases within the protein family calcineurin B-like interacting protein kinases (*CIPKs*), of which SIP4 is one (Ma et al., 2019).

A notable upregulated gene in IND samples, AT5G01680 is a cation/H^+ exchanger (*CHX*) – an ion-coupled membrane transporter. The Arabidopsis genome contains genes coding for various transport proteins and cotransporters, like *CHX*. Each utilize an electrochemical

gradient of protons to facilitate the transport of specific ions, micronutrients, or biologically significant macromolecules (Maser et al., 2001; Sze et al., 2004). An extensive knock down study of this family of cotransporters showed *CHX17* expression to be induced by abiotic stresses such as K^+ deficiency, lower external pH, increased salinity, and high levels of ABA (Cellier et al., 2004). The particular version of *AT5G01680* in our study was *CHX26*, but further investigation can potentially tie greater transport protein production to increased ABA from metabolized Put.

Conclusions

While the gene expression trends in this work supported some of our assumptions, others surprised us. Though phenotypically, CON tissue and IND tissue induced with estradiol are quite similar, the internal mechanisms for high Put production in each are different, perhaps in the period when similar things would happen. The CON tissue lives with high Put and IND tissue merely visits this state temporarily. Perpetual Put accumulation seems to reveal that ABA, which plays many roles, was synthesized and accumulated naturally taking its place in various pathways of stress response and developmental progression. Further biochemical analysis would likely reveal that accumulated Put in CON tissue is potentially being metabolized at a higher rate as compared to WT; this naturally generates ROS, priming the pipeline of events that occur in response to ROS. Though IND tissue produces high Put for short periods, several responses seen in CON tissue may be inevitable as the response matures but has not yet occurred in IND tissue. The induction period generated high levels of Put in IND samples but had not accumulated long enough for Put catabolism to pass a response-inducing threshold of ROS byproduct. The constitutive expression strategy of the *mODC* gene is ideally suited for conditions in which the plant that bears the gene is in constant stress. Without stress, high levels of accumulated Put can

raise a false flag of stress generating a metabolically expensive, and potentially unnecessary, response.

Several significant DEGs involved in stress response were discovered in this study. To say that the seedlings in this experiment were stressed at all, however, is an overstatement and yet several of these top upregulated genes are important in producing a response to stress. In addition to maintaining photosynthetic and developmental processes throughout the stress experience, the plant also modulates many hormones that delicately do the work of stress response while maintaining vital plant functions. Many hormones stimulate a molecular focus on protecting cellular and intracellular membranes. Repairing membrane damage, detoxifying tissues of destructive ROS, and managing osmotic and ionic homeostasis are some of the highest responsibilities stress pathways have (Ding et al., 2013; Mahajan & Tuteja, 2005; Vos et al., 2005). Polyamines are known to have membrane stabilizing functionality. Polyamines can bind phospholipid head groups with integral proteins; this phenomenon has been known to protect intracellular organelles like mitochondria, lysosomes, and microsomes, as well as the entire cellular membrane (Besford et al., 1993). Though PAs are well known to do a lot of the molecular heavy lifting involved with the stress response there are other gene products that are supporting PAs in this work.

It has been well studied that our *mODC* gene constructs of either CON or IND expression are functionally effective; and the IND phenotypic ability of Put overproduction is very responsive in a short 48-hour period. A compelling theme from this study is the progressive wave of events that happen with high Put over time. The IND tissues show early stages of ABA response in powerful upregulation of *PYL1*, a first responder of sorts for stimulating several ABA-related genes. The later stages of ABA accumulation in CON tissue seem to activate genes

that manage its effects: upregulation of *UGT* inactivates abundant ABA; down regulation of *Hsp70* lowers ABA sensitivity. This study might inspire a tighter time course with the IND tissue looking at induction not just of one or two days, but every day for a week or more. Will the metabolic state of IND tissue ever rival that of CON tissue? And if so, how long of an induction would it take?

Polyamines are compounds that are vital to a plant's growth, development, maturity, and vigor against stress. These molecules, being involved in many metabolic pathways, can bring about systemic changes in plants. Crafting metabolic changes, such as the overproduction of PAs, may seem like an obvious place to start turning the dials of biology. If PAs are such powerful molecules, this could easily be seen as a key to unlock a plant's potential. With the success of the *mODC* gene, shown in this study, this can justify targeting other pivotal steps in the PA metabolic pathway and metabolites in other metabolic pathways for alteration. Being systemic, however, is exactly why metabolic manipulations must be done with care. This study shows broader impacts on plant metabolism that go beyond the effects on PAs and related metabolites in the plant. The best future for this work is to fully investigate the side effects of overproducing PAs in plants in a constitutive way. Beneficial as they may be, overproducing PAs may come along with serious costs; especially if cell/tissue specificity remains unregulated. A highly optimized promoter that prescribes where in plant tissues PAs accumulate could reduce some of the metabolic downside of too much Put, for instance. The overproduction of PAs with these transgene constructs is impressive, making this kind of genetic design an alluring goal for economically important crop plants facing the challenges of climate change. For as much that is known about the importance of PA metabolism, there is still so much more to discover. Some of the lowest hanging fruit in improving the catalogue of knowledge about PAs is to better

characterize some of the unknown genes that turned out to be significantly upregulated in these experiments. The relationship between ROS and PA molecules is another place that would benefit from further study to determine how much direct work PAs do during oxidative stress as opposed to how much PAs are used to modulate other response processes.

This study shows the impacts of the *mODC* gene on the whole metabolome, which offers a glimpse into the inner mechanics of the metabolic engine of plants. The differentially expressed genes found in this study are all avenues for future work. Exploring each of those DEGs could unlock even more precise controls in the design of future crops needing to be tolerant of a changing environment.

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