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Plant protein prenylation in environmental responses and protein production.

Parul Singh
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PLANT PROTEIN PRENYLATION IN ENVIRONMENTAL RESPONSES
AND PROTEIN PRODUCTION

By

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M.Sc., Biology, University of Louisville, 2016

M.Sc., Biotechnology, Purvanchal University, India, 2003

B.Sc., Biology, M.J.P. Rohilkhand University, India, 2001

A Dissertation

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Department of Biology

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University of Louisville

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A Dissertation Approved on

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DEDICATION

This work is dedicated to my husband and our little girl, to my always encouraging parents, couldn't have done it with you all.

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ABSTRACT

PLANT PROTEIN PRENYLATION IN ENVIRONMENTAL RESPONSES AND PROTEIN PRODUCTION

Parul Singh

July 29, 2019

Lipid post-translational modifications enhance a protein's interaction with membranes and other proteins. In this dissertation, I studied a recently identified *Arabidopsis* gene, *PROTEIN PRENYLTRANSFERASE ALPHA SUBUNIT-LIKE (PPAL)*, by mapping a second-site mutation that rescues the original mutation phenotype and could be an additional factor involved in sugar homeostasis/sensing. I found that mutant phenotype associated with suppressor line was too weak and variable to consistently score, which resulted in not finding any linked markers.

I also generated crosses between *ppal-1* and ABA biosynthesis and signal transduction mutants to find any possible connection between these pathways and *PPAL*. By using several physiological screens, I concluded that *PPAL*'s response is dependent on ABA. A cell wall composition analysis of *ppal-1xaba3-1* showed a severe drop in the lignin content of these plants, which shows promise for biofuels and biomaterial applications and indicates possible crosstalk among ABA biosynthesis, sugar homeostasis, and lignin

biosynthesis. I found that *PPAL* functions in the ABA signal-transduction pathway.

To investigate the possible cause for distorted segregation of *ppal-1* in this study, reciprocal crosses were generated between *ppal-1* and Col-0, and F₁ genotyping again revealed only Col-0 band and no *ppal-1* insertion was found, indicating a possible gametophytic lethality.

I also used a mutant of the moss *Phycomitrella patens* to establish a novel heterologous protein expression system. This mutant, *ggb*, reverts the plants into undifferentiated, unicellular plants resembling green algae. *ggb* was used successfully to express three fungal lignolytic enzymes: aryl alcohol dehydrogenase, glyoxal oxidase and laccase. Western blots of total protein from transgenic lines showed the expected protein bands. I found the laccase activity in extracellular fluid from transgenic lines by development of blue-green color with ABTS; however, similar color was also found in an untransformed *ggb* line. In this dissertation, a bioreactor was developed to test the activity of recombinant proteins. I showed the advantages of *ggb* as a heterologous protein expression system, including its amenability to easy protein isolation compared to other plant-based protein expression systems.

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CHAPTER 1

INTRODUCTION

Posttranslational modifications in plant proteins

Most eukaryotic proteins undergo various posttranslational modifications that aid in performing various protein functions. A protein's function is not only defined by its structure and amino acid sequence but also depends on the groups being attached post-translationally. Such processing can change a protein's activity, structure, function, trafficking, and subcellular localization (Running, 2014; Thompson Jr et al., 2000).

Genetic and molecular evidence has shown that posttranslational lipid modifications play a key role in plant developmental processes such as meristem and flower polar cell elongation, cell differentiation, and hormone responses (Cutler et al., 1996; Running, 2014; Yalovsky et al., 2000). While these lipid modifications are generally conserved among eukaryotes (Crowell et al., 2009; Galichet et al., 2003; Q Zeng et al., 2008) and are carried out by orthologous enzymes, the nature of the targets, the biological effects, and the biological roles, including developmental roles, of these modifications varies among different eukaryotic kingdoms (Running, 2014). Proteolytic cleavage, glycosylation and phosphorylation are already well-known modifications, but, apart from these, lipid modifications also play a key role in protein membrane localization and their interactions with other proteins (Thompson Jr & Okuyama, 2000). Several proteins are known to be modified by these lipid groups, and these modifications are not limited to

signaling components but also involved in modifying structural proteins (Shi, 2015).

There are three main types of lipid posttranslational modifications that are known. S-acylation is the reversible attachment of an acyl group, usually a 16-carbon saturated palmitate group to a cysteine residue of target proteins through a thioester bond (Hallak et al., 1994; Resh, 2006). N-myristoylation is the addition of myristic acid to the N-terminal amino acid of the protein via a thioether bond (Boutin, 1997; Gordon et al., 1991; D. R. Johnson et al., 1994; Thompson Jr & Okuyama, 2000). Prenylation is the attachment of a 15C farnesyl group or 20C geranylgeranyl group to the C-terminal cysteine of the protein (Zhang et al., 1996). The process of lipid modification is conserved among eukaryotes including the use of orthologous enzymes and conserved post-lipidation processing steps (Galichet & Gruissem, 2003; Maurer-Stroh et al., 2003; Yalovsky et al., 2000). However, the process in plants show some distinct features, for instance S-acylation of plant proteins does not seem to depend as much on prior myristoylation or prenylation, and there is variation in the target by these enzymes, however some of the enzymes show broader and overlapping target specificity (Rasteiro et al., 2007; Running, 2014). This chapter will describe in detail the current state of knowledge of plant prenylation enzymes, introduce a previously uncharacterized prenyltransferase-like gene, describe phenotypes of prenylation mutants and their roles in various plant processes, and describe the utility of a specific moss prenylation mutant that shows promise as a novel heterologous protein expression system.

Lipid-modifications and enzymes in *Arabidopsis*

Plants offer a great system to study prenylation processes, especially because, unlike animal and fungal systems studied to date, complete loss of function mutations for many prenylation components are available and are viable and fertile, allowing the study of *in vivo* effects of the loss of prenylation. There are three known prenylation processes in eukaryotes, including plants; though much more is known about these processes in *Arabidopsis* compared to other plants. The three types of prenylation differ in the number and length of lipid groups attached to target proteins, as well as the amino acid sequences that are targeted. During prenylation, the attachment of a 15-carbon (15C) farnesyl group to a cysteine residue near the C-terminus of the target proteins is called farnesylation, while attachment of a single or dual 20-carbon (20C) geranylgeranyl group to the one or two cysteines at or near the C-terminus end of the target proteins is called geranylgeranylation (Zhang & Casey, 1996). Depending on the type of group being attached to the target protein, three different heterodimeric enzymes are involved in the lipid modification. Protein farnesyltransferase (PFT) is involved in protein farnesylation, protein geranylgeranyltransferase-I (PGGT-I) is involved in geranylgeranylation and protein geranylgeranyltransferase-II (PGGT-II) also called Rab-geranylgeranyltransferase (Rab-GGT) is involved in double geranylgeranylation (Maurer-Stroh et al., 2003). Rab-GGT is thought to exclusively prenylate Rab-GTPases, which are involved in the regulation of vesicle transport and organelle biogenesis (Leung et al., 2006).

All these enzymes are heterodimeric and consist of α and β subunits. PFT and PGGT-I share a common α subunit, while the β subunit is the one that provides lipid substrate and target specificity for these enzymes. The β subunits share a weak homology, around 25-

30% (Running, 2014). PFT and PGGT-I target a C-terminal CaaX motif of the protein, (Running, 2014). In the CaaX motif the “C” is the cysteine to which the prenyl group is attached, “a” is typically an aliphatic amino acid, and the X is usually alanine, cysteine, glutamine, methionine, or serine in the case of PFT, and is usually leucine in the case of PGGT-I (and the target sequence is typically referred to as CaaL) (Maurer-Stroh et al., 2003; McTaggart, 2006; Nguyen et al., 2010). *Arabidopsis* prenylation enzymes appear to have a greater tolerance for non-aliphatic amino acids in the “a” position, and biochemical and genetic studies have revealed an unusually large degree of cross-specificity among PFT and PGGT-I in *Arabidopsis*. In the absence of functional PGGT, PFT can prenylate CaaL targets *in vitro*, likely contributing to the lack of visible phenotypes in PGGT mutants (see below).

***Arabidopsis* protein farnesyltransferases and type-I geranylgeranyltransferase**

In *Arabidopsis*, *PLURIPETALA* (*PLP*) encodes the shared α subunit of PFT and PGGT-I (Running et al., 2004). The β subunit of PFT is encoded by *ENHANCED RESPONSE TO ABSCISIC ACID 1* (*ERAI*) (Cutler et al., 1996; Running et al., 1998) and *GERANYLGERANYL TRANSFERASE BETA SUBUNIT* (*GGB*) encodes the β subunit of PGGT-I (C. D. Johnson et al., 2005).

Several genetic studies of PFT and PGGT showed the important role of farnesylation and geranylgeranylation in plant development and growth. *plp* mutants are found to have increased floral organ numbers and hypersensitivity to abscisic acid (ABA) during seed germination assays. *plp* shows phenotypes similar to but more severe than *eral* mutants, as well as additional phenotypes, such as altered cell division planes in the shoot meristem, that clearly indicate its role in both farnesylation and geranylgeranylation

(Running et al., 2004). *eral* mutants show increased sensitivity to abscisic acid (ABA) in seed germination assays, enlarged shoot apical meristem resulting in increased numbers of flowers on the inflorescence stem, enhanced stomatal closure under noninductive concentrations of ABA, and concomitant drought resistance (Cutler et al., 1996; Running et al., 1998; Yalovsky et al., 2000). *ggb* mutants are not distinguishable from wild type plants under normal growing conditions, except they show slightly increased sensitivity to ABA and auxin (C. D. Johnson et al., 2005). While PFT and PGGT knockout mutants are generally lethal in yeast and animals, mutations in *Arabidopsis* PFT and PGGT are mild and provide a tractable model for studying prenylation function *in vivo* (Diaz et al., 1993; Schafer et al., 1990). *eral ggb* double mutants show the same phenotype as *plp*, including equivalent defects in meristem function, floral organs, and in overall growth rate. Together these genetic results indicate that *ERAL* and *GGB* show a partial functional overlap, while PGGT1 compensates the function of PFT in *eral* mutants, and this hypothesis was supported by rescuing the *eral* phenotype by overexpressing the *GGB* (C. D. Johnson et al., 2005) as well as extensive *in vitro* biochemical assays (Andrews et al., 2010; Downes et al., 2006).

While the high degree of functional overlap between PFT and PGGT-I helps explain the mild phenotypes in *Arabidopsis* when *eral* or *ggb* is knocked out (with PFT compensating in the absence of PGGT-I and vice versa), it remains a mystery why a simultaneous knockout of both PFT and PGGT-I (via the *plp* shared alpha subunit mutant or the double *eral ggb* beta subunit mutants) still results in viable and fertile plants, albeit with severe developmental phenotypes (Running et al., 2004). Some possibilities include: redundancy among prenylation target proteins and non-target proteins, compensation for

the loss of prenylation by other lipid modifications such as palmitoylation, hitherto unknown functional overlap with Rab-GGT, and/or the presence of one or more uncharacterized prenyltransferases that can also prenylate PFT and PGGT targets. Indeed, evidence exists for all four of these possibilities. Evidence for the presence of non-prenylated proteins compensating for prenylated proteins is seen in the case of *Arabidopsis* Rop GTPases (Lavy et al., 2002) and the compensation for the loss of prenylation by palmitoylation has been observed in *Arabidopsis* heterotrimeric G proteins (Running, 2014; Qin Zeng et al., 2007). In addition, we have recently demonstrated that *Arabidopsis* Rab-GGT can prenylate non Rab-GTPase target proteins (Shi et al., 2013). This study includes the investigation of a novel putative *Arabidopsis* prenyltransferase-like subunit, which we call *PROTEIN PRENYLTRANSFERASE ALPHA SUBUNIT LIKE (PPAL)*, which may also compensate for the loss of *plp*.

***Arabidopsis* Rab geranylgeranyltransferase**

Rab-GGT is also found throughout eukaryotes but is not as well characterized in plants as PFT and PGGT. Rab-GGT is also a heterodimeric enzyme that consists of distinct α and β subunits. These subunits are distantly related in sequence to the α and β subunits of PFT and PGGT-1 (showing 20-30% amino acid similarity). Unlike PFT and PGGT-1, Rab-GGT requires an additional subunit, called Rab escort protein (REP), for function, and some researchers refer to the enzyme as heterotrimeric (Leung et al., 2006; Running, 2014). Studies of mammalian and yeast RAB-GGT suggest that they exclusively prenylate Rab-GTPases, a family of small Ras-related GTPases involved in eukaryotic organelle biogenesis and vesicle transport (Leung et al., 2006; Running, 2014). Yeast and animals carry single copies of α and β subunits; interestingly, two copies of the gene

encoding α and β subunits are present in the *Arabidopsis* genome, along with a single REP gene (Hala et al., 2005; Lange et al., 2003; Maurer-Stroh et al., 2003). Rab-GGT has a wider range of amino acid target sequences at the C-terminus compared to PFT and PGGT-1; most of these sequences have two cysteine residues which includes XCC, XCXC, XCCX, CCXX, and CCXXX, where C is the potential target for the prenylation and X is a nonspecific amino acid (Maurer-Stroh et al., 2003; Running, 2014). For Rab-GGT target specificity, REP recognizes structural features of the REP-GTPases rather than relying on the C terminal sequence (Pereira-Leal et al., 2003). Recent evidence from our lab shows that of the two alpha subunits, RGTA1 and RGTA2, only RGTA1 is functional, since RGTA2 includes a large amino acid insertion (Shi et al., 2013). The two beta subunits, RGTB1 and RGTB2, are biochemically redundant, though they have different mutant phenotypes when they are knocked out likely due to spatial and temporal differences in expression (Gutkowska et al., 2014; Hala et al., 2005). In addition, *Arabidopsis* Rab-GGTases can efficiently prenylate non Rab-GTPase targets *in vitro*.

The connection between Sugar and Absciscic acid pathway in *Arabidopsis*

Two of the most prominent phenotypes of plant prenylation mutants are hypersensitivity to the plant hormone abscisic acid and to exogenous sugar; two responses that are interrelated in plants. In the past few decades, sugar has emerged not only as one of the most important basic energy sources but also as a signaling molecule that plays critical roles in plant seed development, germination, metabolic and physiological processes, embryogenesis, starch metabolism, flowering, senescence, and nutrient mobilization (Susan I Gibson, 2005; Rolland et al., 2006). Plants produce sugar via photosynthesis, and the internal sugar level coordinates with internal and external environmental cues in

order to control overall developmental and growth (Koch, 1996; Rolland et al., 2006; Sheen, 1999). Sugar plays an important role in overall signaling in the plant and a sugar based genetic screen was utilized to find sugar induced phenotypes in *Arabidopsis*, these phenotypes were later used widely to identify sugar signaling mutants, including both sugar insensitive and sugar hypersensitive mutants (Susan I Gibson, 2005; Rolland et al., 2006).

Sugar molecules are widely distributed at the cellular and subcellular levels in plants and several processes are regulated by these sugar molecules. It is likely that in plants there are several pathways available to sense and respond to these signaling molecules (Susan I. Gibson, 2004). In *Arabidopsis*, HXK1 (HEXOKINASE 1) has been known to play a key role in nutrient, hormone, and light signaling along with its role in glucose metabolism (Moore et al., 2003). There has been evidence that the target of rapamycin (TOR) signaling pathway, which controls meristem activation via several transcriptional activators, is also involved in downstream glucose signaling (Xiong et al., 2013).

In plants a balance needs to be maintained between water loss via transpiration and the intake of carbon dioxide when sugars are being produced via photosynthesis. As a result, all the pathways that involve sugar production and water status inside and outside of the plant body are very closely associated (Rook et al., 2006). Signals involving light, carbohydrates, and water-stress hormone abscisic acid (ABA) regulates the opening and closing of stomata are intimately linked to find the required balance (Rook et al., 2006). It is not clear to what extent these signaling components and regulators are contributing in one particular pathway or sharing the role among various pathways, hence these

interactions are called as ‘crosstalk’ or signaling network (Brocard-Gifford et al., 2003; Susan I Gibson, 2000)

This study includes the role of a newly identified *Arabidopsis* gene, *PROTEIN-PRENYLTRANSFERASE α SUBUNIT-LIKE (PPAL)*, which encodes a similar protein to the known protein prenyltransferase α subunits and has also been found to be involved in ABA and sugar responses.

Prenylation in Moss

Since nearly all the studies on prenylation in plants have been performed in *Arabidopsis*, it remains unclear whether *Arabidopsis* prenylation processes are typical of plants in general. In the past few decades the moss *Physcomitrella patens* has emerged to become an additional prominent plant model organism especially suitable for cellular, developmental, and evolution research (Reski et al., 2018). The unique features of moss as a model organism include a small genome, which facilitates new era omics technology, gene targeting and replacement via homologous recombination with yeast-like efficiency (a feature unique among plants), its use in bioreactors for protein and natural product production (which has been upscaled up to 500L and produces perfect protein homogeneity and stability among different batches), and a stable cryopreservation process for depositing cell lines in master cell banks (Reski et al., 2018). With all these new significant tools being developed for moss, the use of this system for genetic studies is not new to plant biologists. Moss offers several advantages that include a haploid-dominant life cycle, a simple body plan with translucent cells and tissues that are predominantly a single cell layer, and developmental processes leading to rapid genetic studies and easier mutant isolation compared to plant systems with a dominant diploid

phase (David Cove, 2005). Mosses are one of the earliest arising land plants, and can be used to study the developmental and physiological processes that led to the colonization of land (David Cove et al., 2006). To answer these questions, one has to find out how individual cells determine their fate, position and differentiation (Antimisiaris et al., 2014). As in other multicellular organisms, cells constantly signal positional information to each other, which leads to gene expression variation and eventually results in differential cell types (Antimisiaris & Running, 2014). Like *Arabidopsis* and any other system, these signals rely on post-translational modification, which causes the activation or inactivation of the proteins, enhance protein-protein interactions, and affects their localization and interaction with the membrane receptors, among other effects (Antimisiaris & Running, 2014). As mentioned in the above section, the key post-translational lipid modifications are prenylation, acylation and myristoylation; all these processes involve attachment of different lipid groups to the target protein resulting in their enhanced interaction with membrane and other proteins.

Like in other eukaryotes, *Physcomitrella patens* contains three different heterodimeric enzymes that are involved in the prenylation process, as the genes encoding PFT, PGGT-I, and Rab-GGT subunits are present (Maurer-Stroh et al., 2003; McTaggart, 2006). *PLP*, *ERAI* and *GGB* are each present as a single copy in *P. patens*, as in *Arabidopsis*, while two copies of the beta subunit of Rab-GGT (*RGTB1* and *RGTB2*) and single copies of the alpha subunit of Rab-GGT (*RGTAI*) and Rab Escort Protein (*REP*) are present.

Interestingly, two copies of *PPAL* (*PPAL1* and *PPAL2*) are present in *P. patens*, unlike in *Arabidopsis* in which only one copy is found. The functional role of *Arabidopsis* protein prenylation and the effects of mutations in enzyme subunits involved has been explained

in the above section. The Running lab took advantage of the ability to perform gene knockouts via homologous gene replacement to systematically knock out every known prenylation component in *P. patens* (Thole et al., 2014). Of these, knockouts of *RGT1* and *REP* are both lethal, as is the double knockout of *RGTB1* and *RGTB2*. Interestingly, knocking out either of the *PPAL1* and *PPAL2* genes is lethal, suggesting functional divergence of these *PPAL* homologs. Knockouts of *ERA1*, the beta subunit of PFT, result in a dramatic decrease in polar cell elongation and a delay in development of reproductive structures.

Of particular interest is the knockout of *P. patens* *GGB* (*PpGGB*), the beta subunit of PGGT-I. In *Arabidopsis*, *ggb* plants resemble wild type under regular growth conditions (C. D. Johnson et al., 2005), but their phenotype is limited to showing a slightly enhanced response to exogenous ABA in stomatal closure assays and a slightly enhanced response to exogenous auxin treatment in lateral root formation by the exogenous auxin treatment. But in *P. patens*, the phenotype is much more dramatic: *Ppggb* plants form small, round, individual cells that show no signs of differentiation, no cell polarity, and no cell adhesion (Thole et al., 2014). Each cell is converted into an individual, unicellular organism, which gives rise to further unicellular offspring with each cell division. In this way, *Ppggb* mutants resemble the single celled green algae, the progenitors of land plants.

The knockout of the shared PFT and PGGT alpha subunit, *PpPLP*, resembles *Ppggb* knockouts in that cells are round and undifferentiated, but *Ppplp* cells have much slower growth and cell division and a low overall survival rate (*Ppggb* mutants do not show lethality). Thus, as in *Arabidopsis* but unlike in animals and yeast, PFT and PGGT are

not strictly required for the viability of the *P. patens*, but the phenotype of knockouts of PFT and PGGT subunits are overall considerably more severe in *P. patens* compared to *Arabidopsis* (Antimisariis & Running, 2014).

The algae-like *Ppggb* mutant phenotype is of interest for a number of reasons, including its potential use for gaining insights into the establishment of multicellularity in the plant lineage as well as studying the developmental and cell differentiation and specialization processes that facilitated the transition of plants to land. Beyond the insight it may yield for fundamental biological questions, these mutants may be useful for biotechnological applications. Because *Ppggb* mutants have growth rates similar to that of wild type but do not undergo differentiation or senescence as does wild type moss (Thole et al., 2014), they can produce large numbers of cells in both plates and liquid media such as in bioreactors. A major component of this dissertation is development of *Ppggb* as a novel heterologous protein expression system, providing a plant-based alternative for large-scale prokaryotic and eukaryotic protein production systems. Such alternatives are always in demand based on the fact that some proteins are difficult to express in more common systems for a variety of reasons, including problems with protein folding, post-translational modifications, or lethal effects of the expressed protein, among others. In order to demonstrate the potential utility of *Ppggb* as a novel heterologous protein expression system, I chose to express a series of fungal lignolytic enzymes in this line.

Fungal lignolytic system

As we move towards efficient, effective and economical energy options in our everyday life, it is a well-known fact that we cannot rely on fossil fuels indefinitely, especially at the speed we are using these. We have made significant progress in renewable energy

sources that generate electricity (wind, solar, etc.), but these have several limitations, including storage capacity and their unsuitability for certain applications, such as air travel. The development of renewable liquid fuels from plant material also has a number of technical limitations, one of which is that most of the energy stored in agricultural plants is inaccessible due to the presence of lignin. Lignin is found in all flowering plants but is especially prominent in woody plants. Lignocellulose is mainly comprised of three different polymers: cellulose, hemicellulose and lignin (Annele Hatakka et al., 2011). These woody plants utilize photosynthesis to draw carbon dioxide from the air and reduce it to make glucose, which can be polymerized to cellulose outside of the cell membrane. Cellulose provides structural support and protection from pathogens, and represents a major renewable carbon source (Ohm et al., 2014). The estimate for the worldwide production for lignocellulose biomass is 200×10^{12} (Himmel, 2009). Cellulose is also as one of the most abundant biopolymers and makes up to 40% of the dry weight of woody plants, located mostly in the secondary cell wall (Sjostrom, 1993). This plant-based carbon source is found in abundance and is potentially cost effective for mass production with minimal negative effects on the environment. It is a tremendous potential option for biofuels but is difficult to reach the cellulose because of the lignin barrier. Lignin is an aromatic, complex polymer that is recalcitrant to most chemical and biological degradation (Mäkelä et al., 2016). While lignin is recalcitrant to breakdown, a group of fungi are able to break down lignin naturally to free up cellulose as a carbon source for energy. In the carbon cycle of the forest ecosystem, saprophytic wood degrading and decomposing fungi play a key role, since these are the only organisms that can efficiently decompose and mineralize this recalcitrant lignin (Himmel, 2009). As

summarized by (Annele Hatakka & Hammel, 2011), for lignin degradation first the extracellular enzymes and/or mediators like free radicals with small molecular weight are required, secondly the degradation system must be nonspecific, and thirdly the enzymes involved in breakdown have to be oxidative and not hydrolytic. The basidiomycetes white-rot fungi and a few related fungi are the only groups of organisms that are able to depolymerize the complex lignin polymers efficiently by using a set of extracellular lignolytic enzymes (Annele Hatakka, 2005; Kirk et al., 1998). The use of these lignolytic systems and their use in several biological processes including biopulping, biobleaching, pulp mill effluent treatment, and bioremediation of soil was extensively accomplished in the last few decades (Akhtar et al., 1992).

Recently lignolytic enzymes have been used to produce second generation biofuels, and this process needs three further steps to form bioethanol: 1) pretreatment, 2) saccharification, 3) fermentation (Plácido et al., 2015). The lignolytic or white rot fungi group contains three main enzymes for lignin breakdown: laccase (lac), lignin peroxidase (LiP) and manganese peroxide (MnP) (nee'Nigam et al., 2009). Extracellular catalytic activity of the fungal enzymes LiP and MnP requires H_2O_2 , and one of the sources for H_2O_2 are the reactions catalyzed by an additional enzyme, glyoxal oxidase (GLX) (Philip J Kersten, 1990; Philip J Kersten et al., 1987; Whittaker et al., 1999). There are other FAD-dependent extracellular oxidases called aryl-alcohol oxidases (AAO), which are involved in the oxidation of aryl alcohol derivatives into their respective aldehydes by the reduction of oxygen to H_2O_2 (P. Kersten et al., 2007). Secondary metabolites like veratryl (3,4-dimethoxybenzyl) alcohol can either be *de novo* synthesized or can be produced by the reduction of aromatic aldehydes generated during the redox reactions in lignin

breakdown involving another enzyme called aryl-alcohol dehydrogenase (AAD) (Guillén et al., 1994; Gutierrez et al., 1994; Varela et al., 2000).

For biofuel production, these lignolytic enzymes involve two main processes of delignification and detoxification. During delignification there is reduction of lignin content (L. Li et al., 2008; Salvachúa et al., 2011) while detoxification uses these enzymes to decrease the amount of toxic substances in the biomass (Jönsson et al., 1998; Larsson et al., 1999; Palmqvist et al., 2000).

In this dissertation, I have worked mainly on laccase, aryl-alcohol dehydrogenase and glyoxal oxidase; these enzymes are described in more detail below.

Laccase

Laccases belong to the oxidoreductase group of enzymes and are also known known as oxygen oxidoreductase or *p*-diphenol oxidase. These were discovered at the end of the nineteenth century by Yoshida who extracted this enzyme from Chinese or Japanese lacquer trees (*Rhus vernicifera*) and found this is widely distributed in plants, insects and microorganisms with a special function in each (Plácido & Capareda, 2015). Laccases use molecular oxygen as the electron acceptor to catalyze the oxidation of a number of phenolic compounds and aromatic amines (Reinhammar et al., 1981).

In fungi, these laccases have a variety of roles in various processes that include morphogenesis, plant-pathogen interactions, and, above all, lignin degradation to provide carbon as an energy source (Giardina et al., 2010). Generally, laccases are monomeric globular enzymes of 60-70 kDa with the acidic isoelectric point of 4.0, although other variations also exist (Piscitelli et al., 2010). Other organisms, such as certain bacteria,

also express laccases, but laccases from white rot fungi are widely known and most studied. Other advantages of the use of these enzymes include extracellular, inducible, only require oxygen for activity rather than a cofactor like biotin or thiamine, except oxygen (Matera et al., 2008). They also have broad specificity which gives them a wide range of substrates, especially suitable for complex polymers such as lignin with a wide variety of chemical bonds (Plácido & Capareda, 2015). Being an inducible system, laccase production can be increased by the addition of molecules like copper, and other recalcitrant compounds (Minussi et al., 2007). There are four copper molecules that are present in the active site of the laccases; these take part in the reduction of the oxygen and water generation (Dias et al., 2007).

Lignolytic fungi can be divided in two separate groups, one group producing only laccase and MnP while the second group also produces Lip (Munoz et al., 1997). *Pleurotus eryngii* is one of the most studied white rot fungi and belongs to the first group which produces laccase and MnP (Pelaez et al., 1995). This fungus produces laccase in both liquid and solid media and produces AAO additionally when grown in solid media, and, as mentioned in the above section, AAO provides H_2O_2 required for the peroxidase activity (Guillén & Evans, 1994; GUILLÉN et al., 1992).

Aryl alcohol dehydrogenase

While lignin degradation is catalyzed by three main enzymes (laccase, Lip and Mnp), there are additional enzymes that are required to help these enzymes to keep that atmospheric balance inside the cell. These peroxidases are continuously carrying out oxidation and result mainly in aldehydes, quinones, and several acidic groups, which will cause an excessive oxidized state of the polymer under degradation and as a result the activity of

these peroxidases will cease (Reiser et al., 1994). In order to keep the balance of oxidative state, reductive enzymes are also required for lignin degradation (Lewis et al., 1989). In 1988, a model was proposed that extracellular peroxidase works in coordination with intracellular reductive enzymes (Leisola et al., 1988). The activity of these reductive, intracellular enzymes was first described in *Phanerochaete chrysosporium* (Muheim, 1991). The reducing activity of AAD was found using veratryldehyde in agitated and non-agitated cultures of *P. chrysosporium* after 2 days while the maximum activity was found after 6 days and these activities were synchronous with the activity of the lignolytic enzymes while its possible role in lignin breakdown was indicated by its production during secondary metabolism (Reiser et al., 1994).

Glyoxal Oxidase

As mentioned in the above section, *P. chrysosporium* has been found to produce another extracellular enzyme, GLX, which, along with LiP and MnP, is involved in lignin breakdown (Philip J Kersten & Kirk, 1987; Kirk et al., 1987). The role of GLX is to provide H₂O₂, which is required as a oxidant by the extracellular peroxidases (Kirk & Farrell, 1987). GLX is expressed when white rot fungi are growing on media containing glucose or xylose as carbon source. GLX is a non-specific enzyme and its substrates include formaldehyde, acetaldehyde, glycolaldehyde, glyoxal, glyoxylic acid, dihydroxyacetone, glyceraldehyde, and methylglyoxal (Philip J Kersten et al., 1993); however, GLX doesn't use these compounds as it's physiological substrate but rather uses the metabolic intermediates like glyoxal and methylglyoxal as it's substrate (Philip J Kersten & Cullen, 1993).

Expression of recombinant proteins in heterologous systems

Recombinant protein expression is commonly used to generate high amounts of proteins of interest in short durations, vastly reducing the time and production cost (Piscitelli et al., 2010). The range of proteins expressed for commercial purposes include medications, such as insulin, and enzymes used in food production. Smaller scale recombinant protein expression is also an important tool for research applications. The variety of heterologous protein expression options to choose and the upscaling abilities of various expression systems have allowed the new opportunities for industrial applications (Ferrer-Miralles et al., 2009). Recombinant protein expression can be much higher than in native systems due to several factors, including the use of multiple gene copies, use of an efficient organism for the expression of proteins, ability to induce the expression of the desired protein, ability to use strong promoters to express the gene encoding the protein, and ability to design a protein that can be secreted in the extracellular medium to simplify the downstream process. In addition, the activity, stability, and specificity of the recombinant protein can be enhanced and changed as per the user's requirement (Giardina et al., 2010).

In this dissertation I demonstrate the use of moss *Pp ggb* mutant plants as a novel system to express the lignolytic enzymes Laccase, AAD and GLX from two white rot fungi, *Pleurotus eryngii* and *Phanerochaete chrysosporium*.

CHAPTER 2

MAPPING OF A *PPAL* SUPPRESSOR MUTANT, *p*₂₁₂, THAT RESCUES SUGAR SENSITIVITY

Introduction

Post-translational modifications are the attachment of chemical groups to the target protein. In the case of lipid groups addition, known processes include the addition of prenyl, myristoyl, and acyl groups such as palmitoyl (Gordon et al., 1991; Hallak et al., 1994; D. R. Johnson et al., 1994). Prenylation is the attachment of a single 15C or single or dual 20C lipid groups to the target protein (Zhang & Casey, 1996). Attachments of these groups not only enhance protein-protein interaction but also increases their interaction with membranes (Running, 2014; Thompson Jr & Okuyama, 2000). Three different enzymes are known to be involved in the process of protein prenylation. Protein farnesyltransferase (PFT) attaches a 15C farnesyl group to the target protein. Protein geranylgeranyltransferase type I (PGGT-I) attaches a 20C geranylgeranyl group to the target protein. Protein geranylgeranyltransferase-II (PGGT-II) attaches either one or two 20C geranylgeranyl groups to target proteins; it was formerly thought to exclusively prenylate Rab-GTPases and thus is also called Rab-geranylgeranyltransferase (Rab-GGT). These prenylated Rab-GTPases are involved in the regulation of vesicle transport and organelle biogenesis (Leung et al., 2006; Maurer-Stroh et al., 2003). All these enzymes are heterodimeric and made of α and β subunits; the α subunit is the common shared unit between PFT and PGGT-I

while the β subunit provides protein and lipid substrate specificity to these enzymes (Running, 2014). The shared α subunit of PFT and PGGT-1 in *Arabidopsis* is encoded by *PLURIPETALA (PLP)* and, as the name suggests, mutations in *PLP* cause extra floral organs, larger shoot meristems, stem fasciation, and ABA hypersensitivity, and shows much slower growth (Running et al., 2004). *ENHANCED RESPONSE TO ABSCISIC ACID 1 (ERA1)* encodes the *Arabidopsis* β subunit of PFT; *era1* mutants also show enhanced response to ABA, larger meristems, and increased disease sensitivity, though the overall phenotype is much less severe than that of *plp* (Cutler et al., 1996). The *Arabidopsis* β subunit of PGGT-I is encoded by the *GERANYLGERANYL TRANSFERASE BETA SUBUNIT (GGB)* (C. D. Johnson et al., 2005); strikingly, mutations in *GGB* show no detectable phenotype under normal growth conditions. Like PFT and PGGT-I, Rab-GGT is also heterodimeric and consists of α and β subunits, which are distantly related (20-30% similarity) to the α and β subunits of PFT and PGGT-1, but it also requires an additional protein, Rab escort protein (REP), to recognize Rab-GTPase target proteins (Leung et al., 2006).

The isolation and characterization of genetic mutations serves to link molecular genetics with physiological, biochemical, and other plant processes. Full characterization of a mutant, which includes mapping the mutation in the genome and identifying the related protein and its function can give us a more complete understanding of the developmental, biochemical, or environmental response pathways in which the protein is involved. Another potential benefit of mutation studies is the possibility of combining mutations to find out their relative contributions to a phenotype, to get the complete understanding of series of reactions in a pathway, or to find out if there is any critical effect associated when multiple mutations combine in an individual plant line (Wallis et al., 2002).

Because *Arabidopsis plp*, *eral*, and *ggb* mutants are all hypersensitive to abscisic acid (ABA), it is likely that prenylation plays a role in the regulation of ABA biosynthesis or response (Cutler et al., 1996; C. D. Johnson et al., 2005; Running, 2014). There are several additional ABA biosynthesis and signal transduction mutants available, many of which are also altered in sugar response; however, no protein prenylation mutant has been reported to play a role in sugar response or signalling.

Reverse genetics has been used for years to find out the biological role of genes identified in the genome but not associated with a biological function. In this study I characterized a newly identified *Arabidopsis* gene, which we call *PROTEIN-PRENYLTRANSFERASE α SUBUNIT-LIKE (PPAL)*. *PPAL* shares weak homology with the α subunits of PFT and PGGT-1 and Rab-GGT in eukaryotes. Homologs of *PPAL* are found in all plants and many animals, including all mammals, but is absent from all fungi examined to date as well as certain animals, including *C. elegans* (Shi, 2015).

In our reverse genetics approach, Running lab identified in public databases several T-DNA insertion lines that disrupt the *PPAL* gene, ordered the lines from the Arabidopsis Biological Stock Center, and characterized these mutants. Previous studies in the laboratory found that *PPAL* be involved in sugar responses (Shi, 2015), though its biochemical function is yet unknown. Data from our lab also indicated that *ppal* mutants in *Arabidopsis* are viable but grow slower than wild type with shorter root hairs and shorter internodes, and also show male infertility and sensitivity to ABA (Shi, 2015). *ppal* shows an interesting feature of hypersensitivity to small amounts of external sucrose or glucose added to growth media; this appears to be correlated to the hyperaccumulation of sugar in their vegetative tissue. It thus appears that *ppal* mutants fail to maintain sugar homeostasis. Previous lab results also

demonstrated that *PPAL* physically interacts with one of the *Arabidopsis* Rab-GGT β subunits, *RGTB1*, in a direct yeast two-hybrid assay and in co-precipitation with FLAG-tagged RGTB1 in plants, indicating that PPAL may be a previously unidentified component of Rab-GGT, or a direct regulator which plays an important role in protein prenylation (Shi, 2015). The only indication of *PPAL* function from other organisms is evidence from *Drosophila* that the *PPAL* ortholog, called *TEMPURA*, shows weak prenylation activity on a small subset of Rab-GTPases, though we were not able to demonstrate any Rab-GGT activity for *PPAL* despite extensive efforts (Shi, 2015), and its mutant phenotype does not share any similarity to the mutant phenotypes of the β subunits of Rab-GGT, suggesting that *PPAL* function in *Arabidopsis* is largely or completely independent of Rab-GGT activity.

The use of chemical mutagenesis has played a key role for years to examine biological pathways and discover the key players involved in pathways and processes of interest (Dinh et al., 2014). Historically, the use of these screens was limited to wild type backgrounds to find components that play a crucial role, although these screens usually fail to identify regulatory genes with overlapping functions, since generally only one mutant of a pathway is isolated and studied at a time (Dinh et al., 2014). Genetic modifier screens are a more advanced method and are performed on an existing mutant background as opposed to a wild type background. Resulting additional mutants are usually called second site mutations and often result in enhancer or suppressor mutants based on whether they cause a more severe (enhanced) or less severe (suppressed) phenotype compared to the original mutant line (Dinh et al., 2014). Insertional mutations caused by T-DNA often create complete knockouts of the gene, limiting the range of phenotypes that can be acquired and often leading to lethality if the insertion disrupts a gene required for viability. On the other

hand, chemical mutagenesis, such as with ethyl methanesulfonate (EMS) often produces point mutations that can weakly disrupt promotor or protein function (as in the case of missense mutations), sometimes resulting in knockdown rather than knockout, and at the same time, offering more diversity of phenotypes (Jander et al., 2002).

In this chapter I aimed to gain further insight into the role of *PPAL* in *Arabidopsis* by using a suppressor mutant screen. I used the common strategy to find out the role of a functionally- related gene by starting with a line carrying the primary mutation affecting the pathway and then finding secondary mutant lines that modify the phenotype of the primary mutation. Secondary mutants that result in a weaker phenotype compared to the original mutant are called suppressor mutants; some suppressor mutants can completely revert the original phenotype to wildtype. For this purpose, I have obtained two SALK T-DNA insertion mutant lines in *PPAL* (ecotype Colombia [Col-0]), Salk_045793 (*ppal-1*) and Salk_132356 (*ppal-2*) from the *Arabidopsis* Biological Resource Center (ABRC). As per ABRC, *PPAL* has a genomic sequence of 3.12 Kb, with 10 exons and 9 introns (Figure 2-1), and was confirmed previously (Shi, 2015). Previous results showed that *ppal-1* has an insertion in the fourth intron after 1181 nucleotides, while *ppal-2* has an insertion in the third exon after the nucleotide 770 (Figure 2-1). *ppal-1* mutants still express nearly half the transcript and have a very weak developmental phenotype under normal growth conditions, while *ppal-2* appears to be transcript-null and shows an extremely severe developmental phenotype, including infertility. However, *ppal-1* shows extreme sensitivity to added sugar, as noted above (Shi, 2015).

Figure 2-1: Schematic diagram showing T-DNA insertions in *PPAL* used in this study (adapted from Shi, 2015).

Several suppressor lines were generated in the *ppal-1* background using the chemical mutagen EMS and were screened for the rescue of the extreme sugar sensitivity phenotype of *ppal-1*. *ppal-1* single mutants show severely inhibited growth at 50 mM sucrose or glucose, and lethality at 100 mM; wild type plants grow normally on media with these concentrations of sugar. A promising suppressor line, p212, showing no sugar sensitivity on the same sugar concentration, was selected for further use to map the secondary mutation to find some additional players involved in sugar sensing and homeostasis by *PPAL*. p212 also has a late flowering phenotype and slightly reduced fertility, which I thought would simplify the mapping due to these phenotypes being visible and considered to be linked to the suppression phenotype.

Map-based cloning, also called positional cloning or chromosome walking, is a procedure used to identify the genetic basis of a mutant phenotype. It involves determining the genetic distance of the mutation of interest relative to visible or molecular markers and narrowing the position of the mutation on the chromosome until a gene can be identified (Jander et al., 2002). One common set of molecular markers, which I used in this study, is simple sequence length polymorphisms (SSLPs). These molecular markers are short DNA sequences that often vary in length among different individuals of a species, and are

especially useful in *Arabidopsis* due to their variability among different inbred wild type lines, referred to as ecotypes (Bell et al., 1994; Litt et al., 1989; Weber et al., 1989). Specifically, in our study, I crossed the *ppal-1* p212 suppressor line, which is in the Columbia (Col-0) wild type ecotype, with another wild type ecotype, *Landsburg erecta* (Ler). Both ecotypes are sequenced, and abundant and well-characterized sequence length polymorphisms between the two ecotypes have been identified (Bell and Ecker 1994). SSLP makers are codominant in nature, which means both chromosomes of a plant can be genotyped, providing detailed information about mapping populations. Most polymorphisms differ enough in length that these markers can be analyzed using agarose gel electrophoresis, which makes them very easy and inexpensive to analyze. Cross of *ppal-1*-p212 (Col background) to wild type (Ler background) generated a large F₂ population that I used to screen for the p212 visible mutant phenotype, and then isolated DNA from these individuals for linkage analysis with SSLP markers publicly available from various platforms.

Materials and Methods

Plant Material and Growth Conditions

I used F₂ seeds as a segregating population from the cross between the suppressor line p212, which shows rescue of sugar sensitivity, and the wild type Ler ecotype in order to map the secondary suppressor mutant suppressing the phenotype of *ppal-1*. All plants were grown in Sungro Horticulture propagation mix soil (Premium Horticultural Supply, Louisville, KY, cat no.5232601), in a growth chamber, at 22 °C, with 60-70% relative humidity (RH), light intensity 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$ with continuous light. The propagation mix was presoaked overnight in gnatrol (WDG organic BTI fungus gnat control, eBay) and then

seeds were planted next day. All trays were stratified in the cold room at 4°C for 5 days before moving to the growth chamber at 22°C. A large segregating population of around 4000 F2 plants was maintained in the same environmental growth chamber.

Phenotyping

p212 has a late flowering phenotype and slightly reduced fertility, which should simplify scoring and mapping if these visible phenotypes are linked to the suppression phenotype. I started to phenotype the F2 plants as soon as the siliques were mature but not too dry to shed the seeds. Silique data for total 1745 F2 individuals was taken for the fertility and the size of the silique.

DNA extraction

Two week old leaf tissue was collected using the cap of a sterile Eppendorf tube to pinch out a leaf disc in the tube (Edwards et al., 1991). DNA was extracted using the following procedure: an appropriate volume of extraction buffer from the stock solution was prepared in a 50 ml sterile falcon tube {1M Tris-HCl (pH 7.5, 2 ml), 5M NaCl (0.5 ml), 0.5 M EDTA (pH 8.0, 0.5 ml), 10% SDS (0.5 ml), water (6.5 ml)}. 10 ml of the stock solution is suitable for 18 sample preparations. Tissue was ground directly in the microcentrifuge tube with small plastic pestle. 400 µl of extraction buffer was added and tubes were vortexed for 5 seconds, followed by 5 minutes centrifuge at maximum speed (14000 rpm). 300 µl of the supernatant was carefully transferred into the new microcentrifuge tube and 300 µl of isopropanol was added to the supernatant and tubes were inverted gently several times to allow the isopropanol and supernatant to thoroughly mix. Tubes were incubated at room temperature for 2 minutes (at this stage tubes can be left at 4°C for several days to proceed

further). Tubes were centrifuged at maximum speed (14000 rpm) for 10 minutes at room temperature, and supernatant was removed using long-tipped pipet tips without dislodging the pellet. 400 µl of 70% ethanol was added to tubes to remove salts from the pellet with the long-tipped pipet tips. Pellet was allowed to dry and 50µl of sterile distilled water was added directly to dissolve the pellet. Tubes were heated for 3 minutes at 95°C to kill any remaining DNases or microorganisms which can degrade the two strands of DNA. The concentration and quality of the DNA was checked by nanodrop (Nanodrop 2000, Thermo Scientific).

Genotyping of *PPAL-1*

I ordered Salk T-DNA mutant line [Colombia (Col-0)], Salk_045793 (*ppal-1*) from the Arabidopsis Biological Resource Center (ABRC). For genotyping, the *ppal-1* primers were chosen from the SALK website (<http://signal.salk.edu/tdnaprimers.2.html>). To genotype *ppal-1* and wild type lines obtained from ABRC, three primers LBa1, RP and LP were used (Table 2-1). Wild type with no insertion gives a product ~1100 bps (amplifying the fragment from LP to RP), *ppal-1* homozygous lines with insertion in both chromosomes amplified a band 910 bps and for *ppal-1* heterozygous lines with one of the chromosomes with insertion, both bands of 1100 and 910 bps were amplified. The LBa1+RP pair was best amplified using annealing temperature 58°C while LP +RP gave a sharp band at 60°C.

Table 2-1: List of primers for *PPAL* insertion check

Primer	Sequence
LBa1	TGGTTCACGTAGTGGGCCATCG
RP	ACTATCGGCTACATGAAGCCC
LP	TGTATTCCCGAGAGTGACGTC

Genotyping of F2 individuals

For genotyping of the F2 population, SSLP markers from different publicly available databases were used for amplification of the desired product (Bell & Ecker, 1994; Hou et al., 2010; Lukowitz et al., 2000).

PCR condition and reaction mixture

Genomic DNA was extracted using above mentioned protocol and standard PCR reaction with Taq polymerase was set using standard Taq buffer (NEB inc. USA, cat no M0273). Standard PCR program of 95°C for 30 seconds, repeat 30 cycles for 95°C for 30 seconds, 58/60°C for 35 seconds, 68°C for 30 seconds, repeat steps 2 to 4, 68°C for 5 minutes, and held at 4°C.

All reactions were stored at -20°C until visualized by agarose gel electrophoresis.

Gel electrophoresis and visualization of PCR product

All PCR products were run for 30 mins at 157 V using 1.2% agarose gel with 2 µl of loading dye in 0.5X TBE buffer. The gel was stained using ethidium bromide with a working concentration of 0.625 mg/ml and visualized under UV transilluminator.

SSLP primer

The SSLP primer sequences used in genotyping are listed in Table 2-2.

Table 2-2: List of primers used for F2 genotyping (adapted from Lukowitz et al., 2000)

Marker	Forward Primer	Reverse Primer	Chr No.
T23J18	GATATTTGTTTTGCTAACAC	TAATAAAGTTCCAGCTTTGA	1
T22J18	CACTGCAACAAAGTGGAAT	ATCCGTTTCAATATCCACAA	1
F28H19	TGCGGGAGTGTGATAGAATA	TCCTCGAAAGATTCATTGAT	1
F19K23	GAATTCTGTAACATCCCATT	GGTCTAATTGCCGTTGTTGC	1
T17F3	GGACCGACGGTTACGAGAGT	TAACGGGCCGTTGCAAGA	1
T23K3	CGTGTTTACCGGGTCGGA	AAAACCTTGAAGAATACG	2
T12H3	TAGTCTGAGCTTACCAATA	TTACCCTCGACTCGTAAC	2
F3P11	ATGTATTTGTTGCAAAATAA	TGCACAGAAGAAAAAATA	2
T20P8	TCCGATTCGATTAACTC	TTATTCCTATTTCAAGACT	2
T16B24	ATGAACGGAGTAGCTATC	CGCGTAGAACATAATCTGTA	2
F20H23	CAATGGGAAGAAGGTGTGAG	CGCATTTCCATAAGTTTGT	3
MGL6	ACCTGTTCACTCTATGTTAC	GGGAATTATTAACATTATCA	3
K1G2	ATGAGCTTTAGGAGTGTGTA	AATTTTGTCCCAAAAGAATA	3
T32N15	CAAAAGAAATGCAACGAGAC	TTTGATCATGAATGGTAGTG	3
T26I12	GAGCAACATTAAGGATAGAA	ATCTCATACTCATAATATGTA	3
T4I9	TTATAGCAAACGTACAAGTC	CTGCATACACGTCGTCTC	4
F17A8	CTGGACCCTAGTGGATGT	GACGGTCTCCATTAATTAT	4
FCA8	TTCGGAGAAAGAAACGACAT	ATGGAAGTATTCAGGCATTA	4
T15N24	GCAACCGCTGCTGCTTTA	AATATTTGGCTTTGCGTAGA	4
T4L20	ACCCTAAAACAATGTCTCTT	TGCTAACATGGAAATTTGTC	4
MBK20	CTCTGTTGGGGCAAAACC	GATGCTGGAGAGTAGCTTAG	5
MYJ24	TTCATGAGAGCGGCATTC	GCAAAATGTTTGGACAATTA	5
T26D22	CACAGGCCATTGGATGTA	TGTTAGAACCCACCATTTG	5
K6M13	CCTGTTCCAATGAATATG	TGTAGCTGCTGAGTTGTC	5
MQB2	AAAAGGCGACTACTAGCA	GCCATTTATTTGGTCAAC	5
F21M12	GGCTTTCTCGAAATCTGTCC	TTACTTTTTGCCTCTTGTCAT	1
ciw 12	AGGTTTTATTGCTTTTCACA	CTTTCAAAGCACATCACA	1
ciw 1	ACATTTTCTCAATCCTTACTC	GAGAGCTTCTTTATTTGTGAT	1
nga 280	CTGATCTCACGGACAATAGTG	GGCTCCATAAAAAGTGCACC	1
nga 111	CTCCAGTTGGAAGCTAAAGGG	TGTTTTTTAGGACAAATGGC	1
ciw 2	CCCAAAAGTTAATTATACTGT	CCGGGTTAATAATAAATGT	2
ciw 3	GAAACTCAATGAAATCCACTT	TGAACTTGTTGTGAGCTTTGA	2
nga	CGCTACGCTTTTCGGTAAAG	GCACAGTCCAAGTCACAACC	2
nga 168	TCGTCTACTGCACTGCCG	GAGGACATGTATAGGAGCCT	2
nga 162	CATGCAATTTGCATCTGAGG	CTCTGTCACTCTTTTCCTCTG	3
ciw 11	CCCCGAGTTGAGGTATT	GAAGAAATTCCTAAAGCATT	3
ciw 4	G TTCATTAAACTTGCGTGTGT	TACGGTCAGATTGAGTGATT	3
nga 6	TGGATTTCTTCCTCTCTTCAC	ATGGAGAAGCTTACACTGAT	3
ciw 5	GGTTAAAAATTAGGGTTACGA	AGATTTACGTGGAAGCAAT	4
ciw 6	CTCGTAGTGCACTTTCATCA	CACATGGTTAGGGAAACAAT	4
ciw 7	AATTTGGAGATTAGCTGGAAT	CCATGTTGATGATAAGCACA	4
nga	GCGAAAAAACAAAAAATCC	CGACGAATCGACAGAATTAG	4

CTR 1	CCACTTGTTTCTCTCTCTAG	TATCAACAGAAACGCACCGA	5
ciw 8	TAGTGAAACCTTTCTCAGAT	TTATGTTTTCTTCAATCAGTT	5
PHYC	CTCAGAGAATTCCCAGAAAAA TCT	AAACTCGAGAGTTTTGTCTA GATC	5
ciw 9	CAGACGTATCAAATGACAAAT	GACTACTGCTCAAACATATTC	5
ciw 10	CCACATTTTCCTTCTTTCATA	CAACATTTAGCAAATCAACT	5

Results and Discussion

Heterozygous plants of *ppal-1* appear largely similar to wild type but flowers about a week later and have slightly shorter internodes. Map based cloning is based on the linkage analysis between the mutant phenotype and the genetic or molecular marker with known chromosomal location (Jander, 2006; Jander et al., 2002; Peters et al., 2003). Genetic mapping of the secondary mutation was performed using the DNA samples from F₂ plants obtained by the cross between *p212* x Ler and ~ 2000 F₂ seeds were sown in propagation mix. Individual F₂ plants were tagged, phenotyped for the long/medium or short silique as soon as siliques were mature and seeds from individual plants were collected separately. Linkage analysis was performed by counting the recombinants obtained. A total of 1745 F₂ plants were scored for silique length and 200 total individuals were identified with short and medium siliques phenotype and were further used for PCR screening with all 47 SSLP markers. The amplified product was visualized using 4% high-resolution agarose gel electrophoresis and was stained with ethidium bromide.

PCR Results

PCR products amplified using the SSLP makers were used in calculating recombination frequency (1 recombinant in 100 progeny = 1% recombination = 1cM) and linkage distance between the specific maker and the secondary mutation was calculated.

Figure 2-2: First-pass mapping markers from Arabidopsis mapping platform (with permission from Hou et al., 2010).

Twenty-five evenly distributed INDEL markers from the Arabidopsis Mapping Platform and twenty-two markers from The Arabidopsis Information Resources were used for the first round of mapping F_2 individuals. The images from the Arabidopsis Mapping Platform in Figure 2-2 indicate the polymorphisms between Col-0 and *Ler* (Hou et al., 2010).

Mapping requires high density markers saturating the entire *Arabidopsis* genome in order to find the difference between two ecotypes. *Ler* crossed with Col is the most common method for molecular mapping; these ecotypes show molecular diversity from 4 to 11 positions in every 1000 bps (Chang et al., 1988). In this study, p212 was in the Col-0 background and was crossed with *Ler*, and a large F_2 mapping population was created.

In this study the secondary site mutation was recessive, viable and had low fertility, thus based on these traits F₂ plants were phenotyped for short siliques. It was assumed that all the plants at this stage should be recessive homozygous showing the mutant phenotype and will be used for the further analysis for genotyping (Williams et al., 1993).

After screening for all SSLP markers with identified 200 individuals, the closest linked marker was found to be located on chromosome 4 from the Arabidopsis Mapping Platform at the distance of 42.9 cM. In order to fine map any gene/secondary mutation a minimum distance of 20 cM is required, and this distance needs to be further narrowed down with a larger size of mapping population and with higher density of markers in the area. In this study, none of the markers showed genetic linkage with the screened phenotype. In the case of linkage, the population would show a greater than expected number of Col-0 bands with the linked marker; in our case, the population did not deviate significantly than the expected 1:2:1 ratio of Col:Ler alleles amplified by the SSLP primers.

Figure 2-3: Showing the first pass run of genotyping with primer #25.

The SSLP markers are evenly distributed and were successfully used for the first pass mapping run in several studies; however, in our mapping, no marker was found to be closer than 20 cM in the first run of genotyping.

There are a few explanations for our inability to identify linked markers. To resolve and map any mutation or trait, it is necessary to have a strong phenotypic trait that can be associated with the genotype without any ambiguity, and that phenotype can be used reliably to map the mutation in the F₂ population with 100% confidence; in this case the mutation is called 100% penetrant (Griffiths et al., 1999). However, in some cases there is incomplete penetrance, in which the plants may have the desired genotype but don't universally express the corresponding phenotype because of several factors like epistatic genes, modifiers, suppressors in other part of the genome or environmental effects (Griffiths et al., 1999).

In this case the recessive homozygous F₂ plants should have been shown the short silique phenotype from p212 but less than expected 25% of plants were scored as short siliques,

with a variable silique length causing ambiguity in scoring, which clearly shows incomplete penetrance. As mentioned before, positional mapping requires 100% confidence in phenotyping, and in this study the mutant phenotype was too weak and variable to consistently score and the markers were found in the distance of 33 to 50 cM indicating none of the markers that were used could be shown to be linked to the mutation of interest.

Another possible reason of not finding a linked marker could be the segregation distortion (SD) in F₂ plants, which led to deviated genotypic frequencies from the expected ratios (Törjék et al., 2006). There are various mechanisms of SD that have been described for *Drosophila* and mouse. In plants SD has been found in maize (Lu et al., 2002), *Populus* (Yin et al., 2004) and rice (Wang et al., 2005) and can be caused by several genetic, environmental or physiological factors including post-zygotic selection (Lyttle, 1991), or often pistil-pollen incompatibilities (Lord et al., 2002), gametic competition, negative epistatic interaction (Z. Li et al., 1997) or due to gametic abortion (Sano, 1990). In *Arabidopsis*, recombinant inbred lines (RILs) genomic location of SD was found to be associated with reduced fertility and usually caused by the epistatic interaction between the loci on chromosome 4 and 5 (Törjék et al., 2006).

In this study, the F₂ segregation was distorted and showed mixed phenotype which made it impossible to pull out the recessive homozygous candidates for genotyping, and as result I couldn't find a suitable linked marker to map the secondary suppressor mutation. As mentioned before, the possible cause could be any of those factors mentioned above, and it requires further investigation of *ppal-1* pollen by using microscopy to find out any possible distortion leading to gametophytic lethality. Another suppressor line could be

used to generate another mapping population and repeating the mapping again, and to look for any negative epistatic interaction between loci resulting in the SD.

CHAPTER 3

CHARACTERIZATION OF *PPAL-1* SINGLE AND PUTATIVE DOUBLE MUTANTS WITH ABSCISIC ACID BIOSYNTHESIS AND SIGNAL TRANSDUCTION MUTANTS

Introduction

Absciscic acid (ABA) is a weak 15C acid. Often referred to as the plant stress hormone, it is one of the most studied plant hormones. ABA was initially identified as a plant growth inhibitor in abscising leaves of sycamore tree and cotton fruits (Addicott et al., 1968; Nambara et al., 2005). ABA has been found to be involved in essential aspects of plant growth, development, and physiology, including seed dormancy, seed germination, cell division, cell elongation, and induction of flowering and is a key player in plant response to biotic and abiotic stresses (R. Finkelstein, 2013; Zeevaart et al., 1988). ABA has been found in all plants, in algae (Tietz et al., 1986; Tietz et al., 1989), in some phytopathogenic fungi (Dörffling et al., 1984), and in some bacteria and metazoans (Nambara & Marion-Poll, 2005). Actions of ABA mostly involve changes in gene expression at the transcriptional level (Merlot et al., 1997). Scores of genes involved in the ABA response have been identified, and in many cases their functions have been uncovered, including the *trans* and *cis* factors specifically involved directly in the ABA response (Merlot & Giraudat, 1997). ABA has also been found to control the transpiration rate by regulating Ca^{2+} , K^{+} and anion concentrations, which then control

stomatal aperture opening and closing by modulating the turgor pressure of the two surrounding guard cells (Merlot & Giraudat, 1997).

In *Arabidopsis*, two classes of mutants are among the most studied ABA mutants in plant function. One class includes a series of mutants of genes in the ABA biosynthesis pathway; these mutants have lower endogenous ABA levels in the plant. The second class includes mutants that are either ABA insensitive or ABA sensitive mutants; many of these are involved directly in ABA signal transduction or transcriptional regulation.

Mutations in the *PROTEIN PRENYLTRANSFERASE ALPHA SUBUNIT-LIKE (PPAL)* gene of *Arabidopsis* are sugar and ABA hypersensitive, indicating that *PPAL* plays a role in ABA regulation and sugar response/homeostasis as discussed in chapter 2 (Shi, 2015). There may be several mechanisms of crosstalk among genes involved in a single pathway or genes involve in the parallel pathways, regulating the same biological function and response (Boone et al., 2007; Phillips, 2008). In this study I generated putative double mutants of *ppal* with genes involved in ABA biosynthesis or response in order to gain insights into the involvement of ABA in *ppal* mutant phenotypes and to define functional relationships of *PPAL* with other genes mediating ABA responses.

ABA biosynthesis

The level of ABA in any plant tissue is determined by the rate of its biosynthesis and its catabolism; hence to understand how ABA functions it is necessary to know the genes involved and their interactions with each other, and how they affect various developmental/environmental processes (Nambara & Marion-Poll, 2005). Two different biosynthesis pathways for ABA have been suggested. The first direct pathway was found in fungi, in which ABA is synthesized from farnesyl diphosphate (Hirai et al., 2000). In

higher plants, synthesis of ABA is carried out by an indirect pathway (Figure 3-1) that involves the cleavage of a C₄₀ carotenoid precursor (9-cis-epoxycarotenoid) resulting in xanthoxin (C₁₅) and a C₂₅ byproduct. The oxidation of 4'-hydroxy xanthoxin produces a ketone via an NAD- requiring enzyme and as a result there is a nonenzymatic desaturation of the 2'-3' bond resulting in an open epoxide ring to form abscisic aldehyde. The last step of the pathway involves the oxidation of this abscisic aldehyde to ABA (Schwartz et al., 2003). Three commonly studied ABA biosynthesis genes, *ABA1*, *ABA2*, and *ABA3*, encode enzymes in the pathway at points indicated in Figure 3-1.

Figure 3-1: ABA Biosynthesis pathway in higher plants (adapted from Merlot & Giraudat, 1997).

ABA biosynthesis or ABA deficient mutants

Mutants impaired in ABA biosynthesis are referred to as ABA deficient mutants.

Monogenic mutants showing disrupted ABA metabolism have been studied in tomato (M Tal et al., 1973) and isolated (Stubbe, 1957, 1958, 1959). Because guard cells of stomata cannot properly respond to drought conditions without sufficient ABA, these mutants are characterized by extreme wilting (Moshe Tal, 1966). The relationship between the loss of germination inhibition and disturbed ABA metabolism relationship was established by (Smith et al., 1978), and another group showed that the early germination of *viviparous* (*vp*) mutants could be the result of absence of ABA or the inability of these mutants to respond to ABA (Robertson, 1955).

Seed germination is promoted by the synthesis or activation of gibberellins (M Koornneef et al., 1982); however, exogenous spraying of gibberellins also induces the germination of seeds in most plant species (Jones et al., 1977). ABA-deficient mutants were isolated based on their reduced or absent requirement of gibberellic acid (GA) for germination (Koornneef et al., 1982) or by selection of germinating seeds on the GA inhibitor paclobutrazol (Léon-Kloosterziel et al., 1996). The requirement of GA at the time of germination is dependent on the dormancy state, which is determined by the amount of ABA present at the time of germination (Karssen et al., 1986). Reduced dormancy is one of the main characteristics of ABA deficient mutants in *Arabidopsis* and in other plant species (Maarten Koornneef, 1986). ABA deficient mutants have less robust plant growth due to impaired water regulation, precocious germination, a wilted phenotype, and an ability to germinate and grow on media containing a high salt concentration. (Léon-Kloosterziel et al., 1996). Considering the indirect pathway via C₄₀ is the main pathway

of ABA synthesis in *Arabidopsis*, the loci *aba1*, *aba2* and *aba3* are involved in the separate steps of ABA biosynthesis (Rock et al., 1991) (Table 3-1) and these defective mutants showed the same phenotypes as mentioned above (Schwartz et al., 2003).

In this chapter, I also studied the cell wall composition of *aba3-1*, *ppal-1*, the *ppal-1* x *aba3-1* cross (F3 pool) and wild type (X. Li et al., 2010) to find any possible connection and effect of impairment in ABA biosynthesis and sugar sensing.

Table 3-1: List of *Arabidopsis* mutants involved in ABA Biosynthesis (adapted from Schwartz et al., 2003)

Mutation	Dominance	Biochemical Step	Enzyme
<i>aba1</i>	recessive	accumulation of their biosynthetic precursor zeaxanthin	Zeaxanthin epoxidase
<i>aba2</i>	recessive	blocking the conversion of xanthoxin to ABA-aldehyde	Cytosolic short- chain dehydrogenase/reductase
<i>aba3</i>	recessive	blocking the conversion of ABA-aldehyde to ABA	Molybdenum cofactor sulfurase

ABA Signal transduction and response

The first response of any hormone signaling is the binding of the hormone to its receptor (Hubbard et al., 2010). During plant growth, the level of endogenous ABA increases under water stress conditions, reducing the transpiration rate by decreasing the size of the stomatal aperture and by affecting gene expression (Zeevaart & Creelman, 1988). Several transcriptional regulators that are seed specific and targets of ABA have been found to be involved in differential gene regulation, including *ABI4* from *Arabidopsis* and basic

leucine zipper proteins from *Daucus* (R. R. Finkelstein et al., 1998; Kim et al., 1997).

Although several ABA signaling components have been characterized, the longstanding lack of knowledge of the bona fide ABA receptors delayed a full understanding of ABA signaling processes (Hubbard et al., 2010). The identification of PYRABACTIN (4-bromo-N-[pyridin-2-yl methyl]naphthalene-1-sulfonamide) RESISTANCE

(PYR)/REGULATORY COMPONENT OF ABA RECEPTOR (RCAR) proteins as ABA receptors provided a major breakthrough and brought exciting opportunities to test existing hypotheses and to establish new hypotheses for ABA signaling (Hubbard et al., 2010; Ma et al., 2009; Park et al., 2009). ABA signaling occurs through a main signaling system, which consists of three major proteins: PYR/RCARs, which are ABA-binding proteins that interact with protein phosphatase 2cs [PP2Cs]. The phosphatases function as negative regulators of the pathway, while SNF1-related protein kinases 2s [SnRK2s] acts as positive regulators of downstream signaling in this pathway (Ma et al., 2009; Park et al., 2009). In this proposed dual negatively-regulated pathway (Figure 3-2), ABA bound PYR/RCARs hinder the activity of PP2Cs, and as a result PP2Cs activate SnRK2s, which results in the phosphorylation of target proteins (Park et al., 2009; Vlad et al., 2009). In the absence of ABA, the PP2Cs are in the active form and repress SnRK2 activity in downstream signaling.

Figure 3-2: ABA signaling pathway (with permission from Hubbard et al., 2010).

ABA signal transduction mutants or ABA insensitive mutants

The use of single mutants that have different endogenous levels of a specific hormone than wild type offer a great opportunity to elucidate the regulatory functions of plant growth factors (Karssen, 1982; M Koornneef et al., 1982; M Koornneef et al., 1984). The disturbance in hormonal control can be caused by the inability of the target tissue to respond to the specific hormone, making them hormone insensitive mutants; these mutants typically share similar phenotypes for water regulation and seed dormancy as the ABA deficient mutants (M Koornneef et al., 1984). Along with ABA biosynthesis mutants, the Koorneef group isolated a set of *Arabidopsis* insensitive mutants, for which resistance to 10 μ M ABA-mediated seed germination inhibition was used a criterion for the isolation of the mutants (Table 3-2). These insensitive mutants have the higher level of ABA than wild type and show the same phenotypes as ABA deficient mutants. These ABA insensitive mutants have a modified response to ABA and have altered endogenous

ABA levels, and the mutant phenotype can't be reverted to the wild type by the application of exogenous ABA or GA (M Koornneef et al., 1984).

Table 3-2: List of *Arabidopsis* mutants involved in ABA signal transduction and response (adapted from Merlot & Giraudat, 1997).

Mutation	Dominance	Protein	Germination on ABA	Stomatal regulation	Response to stress
<i>abi1</i>	SD	Protein phosphatase	Yes	Yes	Yes
<i>abi2</i>	SD	Protein phosphatase	Yes	Yes	Yes
<i>abi3</i>	R	Transcription factor	Yes	No	No
<i>abi4</i>	R	Unknown	Yes	No	Unknown
<i>abi5</i>	R	Transcription factor	Yes	No	Unknown

In this chapter, I generated crosses between *ppal-1* with ABA deficient and ABA insensitive mutants to find possible roles of *PPAL-1* in ABA biosynthesis and signal transduction pathways. Because *ppal-1* was found to be ABA sensitive (Shi, 2015), F₃ seeds of *ppal-1* with ABA-insensitive mutants should reveal genetic interactions between the two genes. Specifically, I sought to epistatic interactions in order to place the *PPAL* gene in the response pathway. Similar experiments have been conducted with the *Arabidopsis* protein prenylation beta subunit gene *ENHANCED RESPONSE TO ABSCISIC ACID1* (*ERA1*), which was found to act upstream of *ABI3*, *ABI4*, and *ABI5*, and downstream of *ABI1* and *ABI2* (Brady et al., 2003). Generation and study of these crosses will disclose any possible interaction between *PPAL* and other known factors in ABA signal transduction pathway and will place the *PPAL* into a putative location in these pathways. Crosses with ABA biosynthesis pathway will shed information on

whether any aspects of the phenotype of *ppal* mutants is dependent or independent on the presence of ABA.

Materials and Methods

Plant material and Growth conditions of Arabidopsis

I ordered a set of ABA deficient, ABA insensitive, and *ppal* mutants from the Arabidopsis biological resource center (<https://abrc.osu.edu/>) (Table:3-3). All plants were grown in Sungro Horticulture propagation mix soil (Premium Horticultural Supply, Louisville, KY, cat no.5232601), and presoaked overnight with gnatrol (WDG organic BTI fungus gnat control, eBay). Seeds were planted the next day. Seeds were kept at 4°C for stratification for 5 days before transferring to the growth chamber, at 22°C, with 60-70% relative humidity (RH), with continuous light intensity 90 $\mu\text{mol m}^{-2} \text{s}^{-1}$.

Table 3-3: List of *Arabidopsis* lines ordered from ABRC

Stock number	Stock type	Genotype
CS 21 (<i>aba1-1</i>)	Individual line	Homozygous
CS156 (<i>aba2-1</i>)	Individual line	Homozygous
CS157 (<i>aba3-1</i>)	Individual line	Homozygous
CS22 (<i>abi1-1</i>)	Individual line	Unknown
CS23 (<i>abi2-1</i>)	Individual line	Unknown
CS24 (<i>abi3-1</i>)	Individual line	Homozygous
CS8104 (<i>abi4-1</i>)	Individual line	Homozygous
CS8105 (<i>abi5-1</i>)	Individual line	Homozygous
SALK_045793	Individual line	Heterozygous

All seed were grown for a generation in order to increase the seed pool before making any crosses.

Seed Sterilization

50-60 seeds from each line were put in a Eppendorf tube and 200 µl of 70% EtOH was added to the tube, immediately followed by 600 µl of water (treating 2 tubes at once is suggested since the alcohol treatment is harsh and might affect germination if it is left on the seeds too long). 600 µl of the supernatant was immediately pipetted out (water washes were repeated for 3-4 times). At this stage tubes can be left at room temperature until all samples reach this stage. 200 µl of 100% bleach was added to each tube and tubes were allowed to stand for 5 minutes, followed by the addition of 600 µl of water. 600 µl of the supernatant was pipetted out (water washes were repeated 4-5 times) and seeds were used for the plating on appropriate media.

Seed growth media and ABA solution

All single and putative double mutants were tested for their ABA tolerance on ¼ MS Media (Murashige & Skoog, Phytotechnology Laboratories, Cat No: M524) containing 2% sucrose (Sigma), 0.7% agar and containing either 5 µM or 10µM ABA (dissolved in methanol).

Crosses

As soon as the plants started flowering, all crosses were made between *ppal-1* and ABA deficient and ABA insensitive mutants as indicated in Table 3-4.

Table 3-4: List of crosses made between *abi* and *aba* mutants with *ppal-1*

List of crosses
<i>abi 1-1</i> x <i>ppal-1</i>
<i>abi 2-1</i> x <i>ppal-1</i>
<i>abi 3-1</i> x <i>ppal-1</i>
<i>abi 4-1</i> x <i>ppal-1</i>
<i>abi 5-1</i> x <i>ppal-1</i>
<i>aba 1-1</i> x <i>ppal-1</i>
<i>ppal-1</i> x <i>aba 2-1</i>
<i>ppal-1</i> x <i>aba 3-1</i>

Anthers were removed from an unopened flower in the inflorescence used as a female without hurting the stigma and other floral parts. Mature flowers were removed from the same inflorescence to avoid any contamination from the inflorescence. The emasculated flower was marked using a thread for identification. Crosses were performed 24 hours after this step. At this time a mature, healthy flower from the plant used as a male was selected to pollinate the stigma of the plant used as a female and a quick dusting of pollen was added to the stigma.

All crosses were made at room temperature using a Nikon microscope (SMZ1500) and plants were put back into growth chamber after making crosses. Plants were allowed to grow for at least 10 days or until the silique was mature. Collected siliques were kept at least a week at room temperature for further maturation and then were transferred for a week into a Tupperware container holding a thin layer of Drierite at the bottom to remove any leftover moisture before planting.

F₁ seeds were planted in pots containing the same Sungro Horticulture propagation mix soil (Premium Horticultural Supply, Louisville, KY, cat no.5232601), in a growth

chamber, at 22 °C, with 60-70% relative humidity (RH), light intensity 90 $\mu\text{mol m}^{-2}\text{s}^{-1}$ and with continuous light.

Some of the F₁ seeds were planted for genotyping and generating F₂ seeds; the rest of the F₁ seeds were saved for any further use.

F₂ population generation

Around 700 F₂ seeds were planted from all the above crosses and were grown using the same procedure as mentioned above. As soon as the seedling were germinated, individual plants were tagged with a specific number and young leaf tissue was collected for the DNA extraction for genotyping.

DNA extraction

DNA extraction was done following the same procedure as mentioned in chapter 2.

Table 3-5: List of primers for *PPAL* insertion check

Primer	Sequence
LBa1	TGGTTCACGTAGTGGGCCATCG
ppal-1 RP	ACTATCGGCTACATGAAGCCC
ppal-1 LP	TGTATTCCCGAGAGTGACGTC

Gel electrophoresis and visualization of PCR products

Genotyping of *ppal-1* insertions was carried out using the primers listed in Table 3-5. A standard PCR reaction with Taq polymerase was set using standard Taq buffer (NEB inc. USA, cat no M0273). A standard PCR program for NEB-Taq DNA polymerase was used with initial denaturation set at 95°C for 30 seconds and 30 cycles of 95°C for 30 seconds, 58°C (Lba1+RP) or 60°C (LP+RP) for 35 seconds, and 68°C for 1:30 minutes, and a

final extension at 68°C for 5 minutes. The product was held at 4°C at the end of the cycle. All PCR products were run for 30 minutes at 157 V using 1.2% agarose gel with 2 µl of loading dye in 0.5X TBE buffer, and the gel was stained using ethidium bromide with a working concentration of 0.5µg/ml and visualized under UV transilluminator.

Water loss assay for *aba* double mutants

For this study, *aba 3-1*, *ppal-1*, and *ppal-1xaba 3-1* were grown in the growth chamber as mentioned above, and after 30 days plants were transferred to the laboratory and were cut from the roots. 15 plants from each genotype were placed in a beaker on the bench at ambient room temperature, and weight measurements were taken at every 30 minutes to indicate water loss.

Germination test on NaCl for *ppal-1* x *aba 3-1* double mutants

A germination assay was performed using various concentrations of NaCl (50mM, 100mM, 150mM, 200mM, 250mM). Between 50-60 seeds were plated in triplicates on Whatman filter paper (90mm) saturated with the above-mentioned concentrations of the NaCl. Plates were wrapped in aluminum foil and were kept under dark at 4°C for four days and then transferred to the growth room at 22°C under continuous light for the next three days. All germination scores were recorded on the seventh day. The average germination percentage with standard errors of the triplicates were calculated.

Germination sensitivity test of *ppal-1 aba 3-1* double mutants on paclobutrazol

aba deficient mutants were initially isolated on the basis of their resistance to the GA inhibitor paclobutrazol (Léon-Kloosterziel et al., 1996). Here, I tested and compared the resistance of these genotypes on different concentrations of paclobutrazol. In the case of

ABA deficient mutants, a lower GA requirement caused them to germinate in the GA inhibitors like paclobutrazol used in this study (Karssen & Lacka, 1986).

This germination assay as above was performed using various concentrations of paclobutrazol (1 μ M, 10 μ M, 100 μ M), between 50-60 seeds were plated in triplicates on Whatman filter paper (90mm) saturated with respective concentrations of the paclobutrazol, plates were wrapped in aluminum foil and were kept under dark at 4°C for four days and then transferred to growth room at 22°C under continuous light for next three days. All germinations were recorded on the seventh day. The average germination percentage with Standard errors of the triplicates were calculated.

Cell wall composition analysis of *aba 3-1*, *ppal-1* and *ppal-1xaba 3-1*

The whole stems from genotypes and wild type plants were collected from the first internode and above and dried for analysis. The lignin composition analysis of the plant samples was done using standard National Renewable Energy Laboratory procedures (Ruiz et al., 1996; Sluiter et al., 2008). Monomer sugars were analyzed by high-performance liquid chromatography (HPLC) with a Bio-rad Aminex HPX-87P column.

Germination assay on ABA for *abi2-1*, *ppal-1* and *ppal-1x abi2-1*

A germination assay was performed using 5 μ m and 10 μ m of ABA. Approximately 50-60 seeds were plated in triplicates on MS media plate containing the above concentrations of ABA. Plates were wrapped in aluminum foil and were kept under dark at 4°C for four days and then transferred to the growth room at 22°C under continuous light for next three days. All germination was recorded on the seventh day. The average germination percentage with standard errors of the triplicates were calculated.

Cross between Col and *ppal-1*

Several reciprocal crosses between the wild type ecotype Col-0 and two homozygous lines of *ppal-1* were performed in order to investigate the inheritance of the *ppal-1* insertion and in most of the crosses mentioned above (in crosses section). Siliques were collected and maintained as described above, and F₁ seeds were planted and maintained as described above.

Results and Discussion

F₁ genotyping

Four seeds from each cross were planted and maintained as described above. All grown F₁ individuals were wild type looking plants and were genotyped along with *ppal-1* DNA using the *ppal-1* specific markers from SIGnAL (Salk Institute Genomic Analysis Laboratory) parent and out of all of the crosses I made, only *ppal-1xaba3-1* showed the insertion of *ppal-1* in one of the crosses (Figure 3-3a), whereas the rest of the F₁ individuals showed only wild type alleles (Figure 3-3b). All F₂ seeds were collected as soon as the siliques were mature and were kept at room temperature for a week followed by keeping them in Drierite for a week to remove any leftover moisture.

Figure 3-3: Genotyping of F₁ a) showing *ppal-1* insertion in *ppal-1xaba3-1*, b) with genomic primers showing absence of the wild type genomic band.

F₂ Genotyping

Around 108 F₂ seeds for each cross were planted in a tray containing 18 pots (6 seeds/pot) and maintained in the cold room and growth chamber as mentioned above (a total of 631 seeds from all crosses). A young and small leaf tissue from the rosette was collected for DNA isolation to use for genotyping. For genotyping, *ppal-1* specific and genomic primers (as mentioned above) from SIGnAL were used for checking the presence of the T-DNA insertion. A total of 631 plants were genotyped with *ppal-1* specific markers and only one individual from *ppal-1xaba3-1* crosses and 3 individuals from *abi2-1xppal-1* crosses were found to be homozygous for the *ppal-1* insertion (Figures 3-4 and 3-5). Out of the 3 *abi2-1xppal-1* crosses, only one plant produced seeds; the remaining two produced none. However, seed from the rest of the plants were collected and maintained as mentioned above.

Figure 3-4: Genotyping of F₂. a) indicates the presence of the *ppal-1* insertion in the *ppal-1xaba3-1* cross, b) shows the absence of a wild type allele as indicated by the lack of PCR product using genomic primers.

Figure 3-5: Genotyping of F₂. a) indicates the presence of the *ppal-1* insertion in the *abi2-1xppal-1* cross, b) shows the absence of a wild type allele as indicated by the lack of PCR product using genomic primers.

Analysis of putative double mutants *ppal-1xaba 3-1*

F₂ individuals homozygous for the *ppal-1* insertion were considered to include putative double mutants (approximately 1 in 4 F₂ plants that are homozygous for *ppal-1* are predicted to be homozygous *aba3-1* as well), but I were not able to definitively identify plants that were homozygous for *aba3-1* among these homozygous *ppal-1* plants in the F₂ and F₃ populations due to a lack of genetic markers for the *aba-3-1* lesion. *ppal-1*, *aba3-1*, and F₂ and F₃ plants from the *ppal-1xaba3-1* cross were subjected to a number of

different physiological characterizations in order to investigate the effect of ABA deficiency and *ppal-1* hypersensitivity on the phenotypes of these mutants.

Weight loss assay for *ppal-1*, *aba 3-1* and *ppal-1xaba 3-1*

Mutant *ppal-1* and *aba3-1* plants, as well as F₃ plants from *ppal-1xaba3-1*, were detached and measured for water loss over time (Figure 3-6) as characterized by weight loss.

Figure 3-6: Weight loss of *aba 3-1*, *ppal-1* and *ppal-1xaba3-1* F₃ plants.

The weight values were transformed to percent weight loss as shown below.

$$\text{pctwtloss}(0) = 100 \{ \text{weight}(30) - \text{weight}(0) \} / \text{weight}(0)$$

$$\text{pctwtloss}(30) = 100 \{ \text{weight}(60) - \text{weight}(0) \} / \text{weight}(0)$$

$$\text{pctwtloss}(210) = 100 \{ \text{weight}(240) - \text{weight}(0) \} / \text{weight}(0)$$

The trend in “percent weight loss” was analyzed by repeated measures analysis to determine if the three genotypes had different trends over time. The analysis was done with the gls function of R. Both first- and second-degree polynomial models were tested, and for each polynomial degree (1 and 2) nine different covariance structures were examined, giving a total of 18 models. The best fitting of the 18 models was chosen as the one with the lowest AIC value. The best model was a second-degree polynomial with linear spatial correlation (corL in correlation structure in the gls function of R) and is given below.

$$\text{pctwtgain} \sim \text{genotype} + \text{genotype:time} + \text{genotype:time}^2 \quad \text{eq(1)}$$

The global null hypothesis of no genotype effects was tested by comparing the model in eq (1) to a model involving only time given in eq (2) below.

$$\text{pctwtgain} \sim \text{time} + \text{time}^2 \quad \text{eq (2)}$$

A likelihood ratio test was used to determine if model 1 fit the data significantly better than model 2. A significant p-value rejects the null hypothesis of no difference in trend in “percent weight loss” over time among the three genotypes.

Pair-wise comparisons of the three genotype levels were made by again comparing eq (1) to eq (2) by a likelihood ratio test with the genotype effect having each of the three combinations to only two of the three genotypes. Adjustments for multiple testing were made using the Bonferroni method. Residuals of all models were examined to ensure the assumptions of normality and homogeneity of variance were reasonably satisfied. Results are given in Table 3-6.

Table 3-6: Results for the effect of genotype on trends over time for percent weight loss

	Null hypothesis	likelihood ratio p-value	Bonferonni adjusted critical value	reject?
single test	global test of no genotype differences $ppal-1 = aba3-1 = aba3-1 \times ppal-1$	0.0342	0.05	yes
multiple tests	$ppal-1 = aba3-1$	0.0006	0.0167	yes
	$aba3-1 = aba3-1 \times ppal$	0.7386	0.0167	No
	$ppal-1 = aba3-1 \times ppal$	0.0868	0.0167	No

Plot of average percent weight loss +/- s.e. and predicted values 0 denotes predicted values from quadratic model.

In the ABA deficient mutants analyzed here, the wilted (rate of water loss) and plant weight phenotype is correlated with ABA levels, and growth is usually less vigorous in these mutants due to disturbed water regulation (Léon-Kloosterziel et al., 1996). Based on the analyses in this study, I found that *ppal-1* transpires water at a higher rate than *aba3-1* and *ppal-1 x aba3-1* F₃ plants; however, there was not much difference found between the transpiration rate of *aba3-1* and *ppal-1 x aba3-1* F₃ plants. It has been reported previously that the relatively low rate of transpiration in *aba 3-1* plants compared to other ABA biosynthesis mutants was correlated due to presence of relatively higher amount of ABA compared to other ABA deficient mutants; in other words, *aba3-1* is a weak allele (Léon-Kloosterziel et al., 1996). Based on these findings I can conclude that *ppal-1* transpiration under non-water-stress conditions is faster than

aba3-1, and that decreased levels of ABA may cause a increase in transpiration perhaps due to secondary plant growth and metabolic effects.

Germination assay of *ppal-1*, *aba 3-1* and *ppal-1xaba 3-1* with NaCl

The effect of NaCl concentration and genotype on seed germination was determined by linear logistic regression with genotype as a factor (Figure 3-7 and Table 3-7).

Figure 3-7: NaCl sensitivity of *aba 3-1*, *ppal-1* and *ppal-1xaba3-1* F₃ seeds.

The analyses were done using the glm function of R. In all analyses these data were found to be highly over-dispersed with respect to the binomial distribution, so the quasi-binomial distribution was used in all analyses.

Table 3-7: Testing for trend with NaCl dose of percent germination with logistic regression for each genotype

Genotype	p-value for null hypothesis of no trend with dose of NaCl	Conclusion
<i>ppal-1</i>	0.08467	marginally significant
<i>aba3-1</i>	0.6085	not significant
<i>ppal-1xaba3-1</i>	0.6838	not significant

The above result found in accordance with the previous results (Léon-Kloosterziel et al., 1996) that *aba3-1* is insensitive to varying concentrations of NaCl; however, *ppal-1* was found to be slightly sensitive to the NaCl and its germination rate is affected by the presence of NaCl. Seeds from the *ppal-1xaba3-1* cross was found to be insensitive to NaCl as well. Our finding matches previous results (Léon-Kloosterziel et al., 1996) and, as these authors mentioned, it is difficult to describe how a plant with a ABA deficiency can survive on the various concentrations of the salt and stress conditions caused by it. Nevertheless, these results suggest that the sensitivity of *ppal-1* to salt stress may be mediated by ABA, since ABA deficiency (caused by the *aba3-1* mutant) leads *ppal-1* plants to be insensitive to NaCl.

Germination assay of *ppal-1*, *aba 3-1*, *ppal-1xaba 3-1* and wild type with paclobutrazol

Figure 3-8: Paclobutrazol sensitivity of *aba 3-1*, *ppal-1*, *ppal-1 x aba3-1* and wild type.

Statistical analysis

The effect of explanatory variables genotype (*aba3-1*, *ppal-1*, F₃ seeds from the *aba3-1xppal-1* cross, and *Col*) and paclobutrazol concentration (0,1,10,100) on percent germination were modeled with a generalized linear model using the binomial distribution. First, second, third, and fourth degree effects for paclobutrazol concentration were included in the model, which are denoted paclobutrazol concentration, paclobutrazol concentration 2, paclobutrazol concentration 3, and paclobutrazol concentration 4. The initial model included all effects up to three-way interaction among all the explanatory variables. The initial analysis did not indicate the presence of over-

dispersion (Residual deviance:31.308 on 24 degrees of freedom), so the binomial distribution was used for the analysis. In order to simplify the model, backward elimination was used to remove non-significant effects. The highest order effects were removed first until all non-significant effects of that order were removed to obtain the final model.

Result of Statistical analysis

The quartic model is not better than the cubic model as these two models had the same AIC value. The cubic model (AIC= 219.532) is better than the quadratic model (AIC= 367.2047) since it has a smaller AIC value. The cubic model was chosen as the final model for analysis.

Because all interaction terms are significant, it is concluded that genotype and paclobutrazol concentration both have significant effects on percent germination and that these two effects depend on each other, also “percent germination” is non-linearly related to “paclobutrazol concentration” and is approximated very well by a cubic relationship.

Based on previous studies, ABA biosynthesis mutants have no or low need of GA at the time of germination, as shown by their ability to germinate in the presence of the GA inhibitor paclobutrazol (Jacobsen et al., 1993). These mutants were found to be 10 times less sensitive than wild type in paclobutrazol germination assays (Léon-Kloosterziel et al., 1996). However, in this study (Figure 3-8) I have found *aba3-1* seeds to be sensitive to paclobutrazol, and there was no significance difference found among single mutants, F₃ seeds from the *aba3-1xppal-1* cross, and wild type genotypes. However, by using the profile analysis to find out if there is any significant difference exist among all genotypes,

it was found that F₃ seeds from *ppal-1xaba3-1* cross are less sensitive than *ppal-1*. This indicates that depletion of ABA from *ppal-1* mutants allows them to germinate without the addition of GA, and, as in the salt sensitivity assays, suggests that at least part of the *ppal-1* phenotype is mediated by ABA.

Cell wall and carbohydrate composition analysis of *aba 3-1*, *ppal-1*, F₃ plants of *ppal-1xaba3-1* cross, and Col-0 wild type

I found a drastic difference in the lignin content among all genotypes (Table 3-8). F₃ plants from the *ppal-1xaba3-1* cross showed very low lignin content compared to both parental mutants and wild type, which shows it may be significantly easier to degrade the putative double mutant cell walls compared to all other genotypes. Whereas the F₃ plants from the *ppal-1xaba3-1* cross showed similar glucan content as *aba3-1* and wild type, *ppal-1* showed a higher glucan component compared to other genotypes under the study. Xylan levels were found to be similar in all genotypes tested but were found to be slightly higher in wild type. Arabinan abundance was similar for all analyzed samples. Our results indicate that *ppal-1xaba3-1* putative double mutants show promise for use in processes requiring cell-wall degradation and point to strategies for further modification for lower lignin content, although I do not yet have a mechanistic explanation for the dramatic drop in lignin content of these plants. At this stage, it is hard to conclude if this drop in the lignin content is due to possible crosstalk among ABA biosynthesis, sugar homeostasis and lignin synthesis pathways. It will be interesting to investigate the key players and any possible connection of ABA and lignin biosynthesis.

Table 3-8: Cell wall composition analysis for *aba 3-1*, *ppal-1*, F₃ plants from the *ppal-1xaba3-1* cross, and wild type

Composition wt (%)	<i>aba3-1</i>	<i>ppal-1</i>	<i>ppal-1xaba3-1</i>	WT
Glucan	27.24	31.53	21.61	24.04
Xylan	5.20	6.20	4.93	9.97
Arabinan	1.33	1.10	1.35	1.66
Lignin	14.17	11.69	7.65	16.72
Ash	2.14	3.20	2.05	2.42
Others	49.00	46.41	61.99	44.92

Germination assay of *abi2-1*, *ppal-1*, and F₃ plants of *1xaba3-1* and wild type with ABA

Several studies have shown the effects of exogenous application of ABA on germination and growth of young seeds. F₃ seeds from the *abi2-1xppal-1* cross were tested for germination at various concentrations of ABA and compared with wild-type, *ppal-1* and *abi2-1* single mutants (Figure 3-9).

Figure 3-9: Germination assay for *abi2-1*, *ppal-1*, and F3 plants of *ppal-1*×*aba3-* with ABA.

abi2-1 and F₃ seeds from the *abi2-1*×*ppal-1* cross were found to be insensitive at various concentrations of ABA, and *ppal-1* and wild type were found to be sensitive to the same concentrations of ABA. However, no significant difference was found between the sensitivity level of wild type and *ppal-1* to same concentrations of ABA. These results suggest that *ppal-1* functions in ABA signal transduction/response pathway but based on its sensitivity I cannot decipher the position of the *ppal-1* upstream or downstream of *abi2-1* in ABA signal transduction pathway.

Although epistasis is the correct way to find the interaction between genes working in a defined pathway, the architecture of most of the pathways don't allow us to discern a

straight and simple epistasis interaction between several factors. Divergence and convergence of a pathway doesn't allow the simple and scorable double mutant phenotype (McCourt, 1999).

Cross between Col-0 and *ppal-1*

To study the inheritance of the *ppal-1* insertion and to test the possible cause of segregation distortion, specifically an underrepresentation of *ppal-1* alleles in F₁ and F₂ generations of all generated crosses in this study, reciprocal crosses were made between the wild type (Col-0 ecotype) and *ppal-1*. A total of 49 F₁ seeds from all the crosses were planted, and F₁ genotyping was done using *ppal-1* and genomic-specific primers. All the F₁ plants showed the Col type band, while in this experiment no *ppal-1* insertion was found in any of the lines, including lines in which *ppal-1* was used as a female (Figure 3-10). Some obvious explanations for this result include seed contamination, pollen contamination, and mislabeling of seeds; however, extreme care was taken in this experiment to exclude these possibilities. Also, the parental *ppal-1* lines were confirmed as homozygous *ppal-1* both before and after the experiment by genotyping, suggesting that the correct lines were used in the cross. Two other possibilities may be at play. One is that *ppal-1* has a dramatic effect on the haploid phase of the plant, especially in the male gametophyte (pollen). In this scenario, *ppal-1* pollen is defective or delayed in germination and elongation and contaminating wild type pollen can outcompete *ppal-1* pollen in the fertilization process, resulting in reduced or no representation of the *ppal-1* allele in the subsequent generation. However, this does not explain the lack of *ppal-1* alleles in F₁ crosses in which the *ppal-1* plant was used as a female. In this case, it is possible that the *ppal-1* parental plant is a genetic mosaic. Most dicotyledonous plants are

made up of 3 cell layers, referred to as L1, L2, and L3, with L1 referring to the outer layer (epidermis) and L3 representing the deep cell layers. These cell layers are generally clonal, though occasionally a cell can divide to give rise to a daughter cell that will occupy a different layer. Mosaic plants are relatively easily generated and can be seen in many ornamental varieties as white-green mosaics with L1 and L2 layers appearing white due to the lack of chlorophyll in these cells. The L2 layer gives rise to gametes. It is possible that *ppal-1* is a genetic mosaic with L1 and/or L3 containing the insert, and L2 carrying one or two wild type alleles. Further testing is required to rule out this possibility.

Figure 3-10: a) Showing no *ppal-1* insertion, b) showing genomic region amplification in F₁ plants of crosses between Col and *ppal-1*.

Gametophytic lethality

A gene factor responsible for causing the 30% shrunken pollen in a mutant line was found many years ago (Redei, 1964). This gene factor causes the shrinking of pollen when present in mutant heterozygous line. Homozygous lines for this factor were not distinguishable from wild type individuals, and under good growth conditions these lines shed 99% normal healthy pollen (Redei, 1964).

The crosses carrying the *ppal-1* as one of the parents always showed the drastic distortion in the segregation pattern (also discussed in chapter 2). Several species have been shown to carry mutations causing the production of the normal male and female gametophytes leading to distorted populations (Howden et al., 1998). In *Arabidopsis*, several mutants that disturb male and female development during the sporophytic stage have been identified on the basis of reduced fertility. Mutations that are inherited as recessive sporophytically disturb the reproduction and result in self-sterility in homozygous plants; however, in heterozygous states these mutants stay fertile (Howden et al., 1998). In the case of *ppal-1*, it is not yet known if the T-DNA insertion has any effects in the gametophytic stage that would result in an F₁ generation showing no insertion of *ppal-1* present. Among all the crosses generated in this study, including several crosses generated for *ppal-1* x *aba3-1*, where *ppal-1* was used as female and pollen from *aba3-1* were used, only one of the crosses showed the insertion of *ppal-1* in F₁ plants. A possible explanation for this case could be that homozygous lines in *ppal-1* might carry defective pollen which is unable to compete with wild-type pollen and as a result F₁ individuals failed to carry the *ppal-1* allele. Further investigations of the effect of *ppal-1* on gametophytic development and function are ongoing.

CHAPTER 4

EXPRESSION OF FUNCTIONAL LIGNOLYTIC ENZYMES FROM WHITE- ROT FUNGI IN THE MUTANT MOSS *PHYSCOMITRELLA PATENS*

Introduction

The major challenge with processing plant biomass is the presence of lignin in the cell wall. Lignin is a complex aromatic polymer that is recalcitrant to chemical and biological degradation. Lignolytic enzymes, which are able to break down lignin naturally, have been a topic of increased interest from the nineties until now. Interest in lignolytic enzymes has increased because of their use in the pulp industry, in delignification processes in the paper industry, in bioremediation, and in the textile industry (Piscitelli et al., 2010). Delignification of plant cell walls exposes cellulose, which is a potential major renewable carbon source that can be used in bioethanol and biomaterials production. Most of the increasing industrial demand for lignolytic enzymes cannot be fulfilled by the native fungal system due to quality and yield of these enzymes (Bleve et al., 2008). To combat this, several research groups came up with ideas of increasing yield of lignolytic enzymes by finding a suitable heterologous host system that is easy to cultivate and handle, in which expression can be induced, and that offers easy upscale possibilities. While several heterologous organisms, including *Aspergillus sojae* (Hatamoto et al., 1999), *Pichia pastoris* (Soden et al., 2002), *Saccharomyces cerevisiae* (Hoshida et al., 2001) and several other organisms

have been explored as a host system to produce these enzymes as per industry requirement, most of these heterologous organisms have their own challenges including proper protein folding, complications in genetic manipulations, and trouble with scaling up as required by industry (Bleve et al., 2008). As mentioned above, an ideal heterologous system should be easy to handle, affordable, scalable, easily manipulatable genetically, and able to produce active and pure proteins to simplify downstream processing (Piscitelli et al., 2010). The moss *Physcomitrella patens* has been known as a model system to plant biologists for a long time due to its relatively small genome size of 500 Mb with 27 chromosomes, low structural complexity, dominant haploid phase, rapid growth, low production cost, relatively low risk of infection by bacteria and viruses, and, unique among plants, gene targeting by homologous recombination (Cools et al., 2017; Schaefer et al., 1997). In this chapter I describe the use of a mutant version of *Physcomitrella patens* harboring a mutation in the GGB gene, as a heterologous protein expression system. The *ggb* line was obtained by knocking out the β subunit of the PGGT-1 enzyme in *Physcomitrella patens* (as described in Chapter 1). *ggb* mutants grow as individual undifferentiated cells similar to unicellular green algae, and, because *P. patens* does not produce lignin, it is a potentially tremendously useful plant system for expressing these delignification enzymes (Thole et al., 2014).

Figure 4-1: Wild type (A) and *ggb* (B) *Physcomitrella patens* (Images are kindly provided by Dr. Liang, Bao).

In this study, I expressed a series of lignolytic enzymes, including aryl-alcohol dehydrogenase (AAD), glyoxyl oxidase (GLX) from *Phanerochaete chrysosporium* (*P. chrysosporium*) and laccase (lac) from *Pleurotus eryngii* (*P. eryngii*). I took advantage of Gateway cloning technology (Thermo Fisher), a comprehensive cloning strategy that utilizes the site-specific recombination properties of bacteriophage lambda to deliver a fast and highly efficient method to clone inserts into compatible vectors of choice. I attached a 6X-histidine tag to the C-terminus of all recombinant proteins during cloning in order to simplify the purification and downstream processing. I generated several *P. patens* transgenic lines for expression of these proteins and a western blot analysis showed the expected protein band for all recombinant proteins.

To test the activity of the recombinant enzyme and before investing in any production and scaling up process, it is important to test the functionality of the system at all possible levels. I demonstrated enzymatic activity in the extracellular fluid collected from the plates of transgenic lines expressing laccase using a colorimetric assay based on the

oxidative breakdown of the lignin analog 2,2-azinobis-(3-ethylbenzothiazoline-6-sulfonate) (ABTS) by development of an intense blue-green color (A. Srinivasan et al., 2004); however, I found the same activity in wild type and untransformed *ggb* as well which might be caused by the unspecific nature of ABTS. This study shows *ggb* mutants are an excellent heterologous protein expression system but requires some optimization for downstream processing.

These enzymes were expressed with the ultimate goal of binding these active enzymes to nanomembranes for large-scale bioprocessing of lignin-rich biomass for bioenergy and industrial bioproduct applications. Our collaborators Dr. Cindy Harnett and Mohammad Shafquatul Islam (University of Louisville) developed a ravioli style enzyme-based membrane bioreactor to check the activity of the recombinant laccase I produced in the moss *ggb* mutant. For any membrane system the most critical factors are that the enzymes bind densely and securely to the membrane in order to withstand the convective washes, and the enzymes should be able to retain their function and catalytic activity over long periods. There are several advancements in overall attachment of these enzymes including taking advantage of His-tags. The His-Tag was first used in 1991 for the purification of recombinant galactose dehydrogenase using immobilized metal affinity chromatography (IMAC) (LILIUS et al., 1991). As mentioned above, I have attached a 6X His-tag at the 3' end of the primer during the first round of cloning of gene, which resulted in a protein with 6x His-tag at the C-terminus. This His-tag forms coordination bonds with several kinds of immobilized metal ions such as Cu^{2+} , Co^{2+} , Zn^{2+} , Ni^{2+} , Ca^{2+} , and Fe^{3+} . Although any of these ions can all be used for protein purification, Ni^{2+} is most frequently used in practice. His-tagged enzymes immobilized on beads or columns have

been used for enzyme separation and reutilization. A method was reported in which enzymes were immobilized on Ni-NTA-agarose beads (A. Srinivasan et al., 2004), and this method has less denaturation of proteins compared to others because enzyme binding was accomplished through His-tags. Similarly (Dräger et al., 2007) presented a method of immobilizing His-tagged enzymes onto Merrifield resins functionalized with a tyrosine-based Ni-NTA linker on the surface. These His-tagged recombinant proteins are an effective way of protein binding on surfaces to function with any of the above-mentioned metal ions (Gaffney et al., 2010; Ganesana et al., 2011).

Materials & Methods

Fungal strains

The white rot fungus *P. eryngii* (ATCC 90787) was obtained from the American Type Culture Collection (ATCC, Manassas, VA). The fungus was regularly maintained on malt extract agar plates (ATCC Medium: 324) at 28°C for 5-8 days. Inocula were prepared by scraping mycelia from plates and cultivating the fungus in 500 ml flasks with 50 ml of glucose-peptone-yeast extract medium at 28°C, 180 rpm for RNA extraction. Enzymatic activity for laccase reaches its peak at 5-6 days; oxygen was added daily into the culture media until it was ready for RNA extraction. Another white-rot fungus, *P. chrysosporium* strain (RP78), was obtained from the USDA Center for Forest Mycology Research (kindly provided by Dr. Daniel Lindner at Northern Research Station, Madison, WI) and was maintained regularly on potato-dextrose agar plates (ATCC Medium: 336) at 37°C. For RNA extraction the following induction media was used as per (Tien et al., 1988) and cultures were grown at 37°C at 200 rpm for 5-6 days for maximum enzymatic activity.

All samples were collected on the 6th day when the enzymatic activity reaches its peak, which was shown by the developing brown color in the *P. chrysosporium* culture media.

Maintenance and growth of fungal strains

Induction media for *P. eryngii* RNA extraction

4gm glucose, 1 gm tryptone, 0.4 gm yeast, 0.2 gm KH₂PO₄, 0.1gm MnSO₄ was dissolved in 50 ml of distil water and pH 5.5 was maintained.

Induction media reagents for *P. chrysosporium* RNA extraction

For Basal III medium (1L), KH₂PO₄, 20g, MgSO₄, 5g, CaCl₂, 1g, 100 ml of Trace elements solution (for 1 L Trace element solution, MgSO₄, 3g, MnSO₄, 0.5g, NaCl, 1g FeSO₄·7H₂O, 0.1g, COCl₂, 0.1g ZnSO₄·7H₂O, 0.1g, CuSO₄, 0.1g, AlK(SO₄)₂·12H₂O, 10 mg, H₃BO₃, 10mg, Na₂MoO₄·2H₂O, 10mg, Nitrilotriacetate¹³ 1.5g) was added.

Culture Composition for agitated *P. chrysosporium* (50 ml)

Basal III medium (filter sterilized) 5 ml, 10% glucose (autoclaved) 5 ml, 0.1 M 2,2-dimethylsuccinate, pH 4.2 (autoclaved) 5 ml, Thiamin (100 mg/liter stock, filter sterilized) 0.5 ml, Ammonium tartrate (8 g/liter stock, autoclaved) 1.25 ml, Veratryl alcohol (4 m M stock, filter sterilized) 5 ml, Trace elements (filter sterilized) 3 ml, Inoculum from 5-6 days old *P. chrysosporium* 5 ml, 0.05% Tween 20 or Tween 80 and 20.25 ml autoclaved distil water was added.

Figure 4-2: 5-6 days old *P. eryngii* and *P. chrysosporium* (brown color shows peak activity of the lignolytic enzymes).

RNA extraction and cDNA synthesis

5-6 day old fungus in induction media was collected on an autoclaved cheese cloth sitting on the beaker mouth to eliminate the media, and tissue was washed three times with autoclaved distilled water before collection. Tissue weight was measured (up to 100 mg) and flash frozen in liquid nitrogen. RNA extraction was done using the Qiagen RNeasy Mini Kit with RLC buffer to avoid any precipitation, and the RNA extraction protocol for fungi was followed as per manufacturer's instructions. The ImProm-II™ Reverse Transcription System (Promega) was used to convert total RNA into full length cDNA following manufacturer's instructions. All cDNAs were stored at -20°C for further use.

Table 4-1: List of enzymes and their sources

Enzyme	Source	Expected
Aryl- alcohol	<i>Phanerochaete chrysosporium</i>	1176 bp
Glyoxal oxidase	<i>Phanerochaete chrysosporium</i>	1698 bp
Laccase (phenol	<i>Pleurotus eryngii</i>	1614 bp

PCR Primers

Primers for amplification of AAD, GLX, and lac were chosen from available genomic sequences from NCBI. CACC sequence was added to the 5' end of all forward primers as required for the TOPO cloning Gateway technology, and nucleotides encoding the 6X-His tag were added at the 3' end of all reverse primers for future protein purification, detection in western blot and attachment to resins in bioreactors for functionality tests.

Table 4-2: Primers used for cloning

Gene	Forward primer	Reverse primer
lac	CACCatgtttccaggcgcacggattct	ctaGTGGTGGTGGTGGTGGTGagctatgccacctctgtcgg
AAD	CACCatgaacatctgggcacccgc	ctaGTGGTGGTGGTGGTGGTGcttctgggggcggatagcctgtt
GLX	CACCatgttctcgctgctagccgtag	ttaGTGGTGGTGGTGGTGGTGctccagggtcggcggagggg

Ex Taq PCR

A 10 µl reaction using standard Ex Taq polymerase was set (cat no: RR001A, Takara Bio USA, Inc) and a standard PCR program was used with initial denaturation set at 94°C for 3 minutes and 30 cycles of 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. The product was held at 4°C at the end of the cycle.

KOD Polymerase PCR

A standard 10 µl reaction KOD Hot Start DNA Polymerase (cat no: 71086-5, MilliporeSigma) was set. A standard PCR program for KOD polymerase was used with initial denaturation set at 95°C for 3 minutes and 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. The product was held at 4°C at the end of the cycle.

Competent preparation protocol

Competent cells of *E. coli* were prepared as mentioned by open wetware

https://openwetware.org/wiki/Preparing_chemically_competent_cells).

TOPO cloning

For TOPO cloning (the first round of cloning), PCR amplification of the insert was first optimized with Ex-Taq polymerase, and then KOD DNA polymerase was used to avoid any introductions of point mutations in the insert. Ethanol-purified KOD-amplified product was used for TOPO cloning, pENTR/SD/D-TOPO (Invitrogen) was used as the entry vector, and manufacturer's instruction were followed for the cloning procedure.

Competent *E. coli* DH5 alpha cells were generated using above method and used for transformation of the plasmid. Transformed *E. coli* cells were grown overnight at 37°C on LB media containing Kanamycin (50 µg/ml) for selection. All plates were observed next day for putative positive clones carrying the insert. All positive clones with the insert of interest were either confirmed by direct colony PCR from the colony pick or by using gene specific PCR on extracted plasmid DNA from the positive clones (Russell et al., 2001). A second round of confirmation was done with double digestion using the appropriate restriction-digestion enzymes after looking at the insert sequences (all restriction enzymes used in this study were from New England BioLabs, <http://www.neb.com>), and finally all inserts with the attached 6X-His tag were confirmed by DNA sequencing (Eurofins Inc, Louisville, KY, USA).

Figure 4-3: pENTR/SD/D/Topo for TOPO cloning of insert into *E.coli*.

Table 4-3: Primers used for sequencing

Gene	Primer
AAD	ctcagtgcacgattcattgc
GLX seq	tcgccttcgggagatggctg
Glx seq	attcccgaggttcgtctg
lac seq	ttaacgttcccgaccaagct
lac seq	aaatcttcgcgggccaacgc

LR reaction for cloning in destination vector

An LR reaction mediated by LR Clonase II enzyme mix (Invitrogen) was used to replace the insert between the pENTR-clone and the destination vector. In this study the entry vector contains the supercoiled *attL* and destination vector carries supercoiled *attR*, two different types of moss specific destination vectors kindly provided by Dr. Hasebe Mitsuyasu of NIBB, Japan. pT1OG is a constitutive expression vector (C. Li et al., 2017), while pGX8 (Kubo et al., 2013; Zuo et al., 2000) is an inducible vector in which cloned

insert can be induced by exogenous β -estradiol. Induction can be controlled and optimized by β -estradiol concentration and incubation period.

Confirmation of the positive clones

All positive clones were confirmed by restriction-digestion by choosing appropriate restriction enzymes and manufacturer's instructions were followed for appropriate digestion conditions. The next round of confirmation was done by sequencing the insert (Eurofins Inc, Louisville, KY, USA) to confirm the lack of mutations introduced by PCR and the correct orientation and presence of 6X His-Tag in frame.

Preparation of the insert for transformation into the moss

The plasmid DNA from all confirmed positive clones was extracted as per the manufacturer's instruction (QIAGEN Plasmid Midi Kit, cat. no. 12143) or by alkaline lysis and was linearized using the PmeI restriction enzyme. The size of expected bands was confirmed by gel electrophoresis before transforming into moss.

Figure 4-4: pT10G & pGX8 for LR reaction (with permission from above mentioned authors).

Plasmid extraction protocol

4 ml bacterial cells were grown overnight with appropriate antibiotic and modified alkaline lysis protocol was used for extraction of plasmid DNA (Ehrt et al., 2003).

Moss growth conditions

The moss *ggb* was used and cultured on BCDAT agar medium under continuous white light at 25°C as described in (Thole et al., 2014).

Moss transformation and positive clone selection

Polyethylene glycol-mediated (PEG) transformation was performed as described by (Nishiyama et al., 2000). Several lines containing the insert were confirmed by selection on two rounds of media containing appropriate antibiotic (zeocin for pT1OG and hygromycin for pGX8).

Moss Genotyping for positive lines

Any lines that passed the second round of selection were considered as putative positive clones, and genomic insertion was confirmed by using gene specific PCR with extracted moss DNA (IBI plant isolate DNA extraction kit, IBI Scientific) to confirm the presence of correct insert.

β -estradiol treatment

β -estradiol (Sigma, cat no: E8875-1G) was dissolved in 10 mM in dimethyl sulfoxide (DMSO) (Sigma) and stored at -20°C. To use β -estradiol for induction of transformed *ggb* lines, the stock solution was diluted to 1 μ M concentration with sterile deionized water. An appropriate volume of β -estradiol solution was added in 1 L of BCDAT media

and plates were poured and stored at 4°C. For induction, plates with moss lines were incubated at 25°C under continuous light for up to 72 hours and tissue was collected for protein extraction.

Detection of recombinant protein by western blot

This protocol was adapted from the lab of Dr. Menze, University of Louisville. A 10 day old tissue from moss plants with the pT1OG insert (with AAD protein expression) was collected directly; however, moss plants harboring the pGX8 insert were first grown for 5-6 days on BCDAT media and then transferred to plates containing 1 µM β-estradiol and plant tissue was collected after approximately 72 hours incubation. About 20 mg of plant material was collected and flash frozen in liquid nitrogen and transferred to -80°C until protein extraction was performed. Proteins were extracted using SDS sample buffer containing 125 mM Tris-HCl (pH 6.8), 4% SDS, 1M DTT, and 7x proteinase inhibitor (cat no:11836170001, Sigma). The frozen samples were removed from -80°C and transferred to liquid nitrogen until they were ready for grinding. For grinding I used an electronic grinding machine (Cryo homogenizing system, Fisher Scientific). Tissue holding blocks were filled with liquid nitrogen, and the samples were ground to a fine powder (about 2-3 mins/sample) and were quickly transferred to an eppendorf tube. 200 µl of the sample buffer was added, the lid was secured, and the tube was vortexed immediately for 1 minute (at this stage samples were transferred on to ice until all samples reached the same stage) and samples were immediately boiled at 100°C in a heat block for 5 minutes. Samples were vortexed well again and then centrifuged for 10 minutes at 15000 rpm at 4°C. The supernatant was collected as a crude protein extract and stored at -80°C until further use. The protein was quantified using the Bradford assay

(for the Bradford assay a separate set for all samples were processed in the exact same way except 1M DTT was not added to the buffer) (Bradford, 1976). Equal amounts of protein (5 µg) were loaded in each well of SDS-Polyacrylamide gel, and 5% and 10% solutions were used for the stacking and resolving gels respectively. 1x Electrode Running Buffer (30.3 g Tris Base, 144.0 g glycine, 10.0 g SDS, pH8.3, stored at 4°C, but was heated to room temperature before use) was used to run at 120V long enough to ensure adequate protein separation (~1-2h). SDS gels were stained using Coomassie for 2-4 hours using 50% MeOH, 10% HOAC, 40% Purified Water, 0.1% Coomassie stain, Once the gel was completely blue, the Coomassie stain was disposed and the gel was rinsed several times with purified water to remove any remaining stain. The gel was de-stained (5% MeOH, 7% HOAC, 87.5% Purified Water) for 4-24 hours until bands were visible. After Coomassie staining, proteins were transferred to a Nylon membrane using 1x 20% methanol (pH8.3) for 1 hour at 100V. The blot was rinsed in purified water and stained with Ponceau S stain (0.2% (w/v) Ponceau S, 5% glacial acetic acid) and the quality of the transfer was checked. Ponceau S stain was rinsed away with the three washes of TBST (0.1% Tween 20, 20mM Tris-HCL, 500mM NaCl, pH 7.4). Skim milk was used as blocking buffer (5% w/v) and primary antibodies His-Tag XP(R) Rabbit mAb were diluted 1/2500 using this buffer. The blocking buffer was removed, and the antibody/blocking buffer mixture was added to the blot and kept on a rotator for 40 minutes (at 4°C). Next the blot was rinsed 5 times using the TBST buffer. Anti-rabbit IgG HRP-linked antibody was used as secondary antibody using 5% skim milk in TBST and the blot was incubated in the solution for 1 hour at room temperature on a rotator. The blot was rinsed 5 times with TBST.

Detection of biological activity in secreted recombinant laccase protein

Considering laccase is an extracellular enzyme, and to test if it is biologically active and is secreted by the moss into the external environment, I performed a simple plate assay that provides the fast determination of the activity present in the extracellular fluid collected from liquid culture (C. Srinivasan et al., 1995). I have used this plate assay to determine the activity present in the fluid collected from washing the transgenic *ggb* lines on a BCDAT plate. Cells were grown on a cellophane membrane overlaying a BCDAT plate. Autoclaved distilled water was used to wash the cells, and then all cells were collected into 2 ml microcentrifuge tubes. After allowing the cells to settle for few minutes, the supernatant was collected into a new microcentrifuge tube to check the activity of any enzymes present. 1 tablet of ABTS (5mg/tablet) [2,29-azinobis (3ethylbenzathiazoline-6-sulfonic acid); cat no: 11204521001 Roche Diagnostics, Indianapolis, IN, USA] was dissolved in 600 µl of the buffer for ABTS (cat no: 11204530001, Roche Diagnostics, Indianapolis, IN, USA) to use as a substrate. A petri plate containing 15 ml of sterile agarose (0.5%) was poured and five wells of around 5mm diameter were made using the broad end of a sterile Pasteur pipette. 20 µl of the cell wash water from three different transgenic lines were placed in the first, second and third well respectively, whereas 20 µl of the cell wash water from untransformed *ggb* was added in the fourth wells as a negative control. A similar volume (20 µl) of commercially available laccase (*Trametes versicolor*) was added as positive control was added into the last well. 20 µl of dissolved ABTS was added in all the wells and a waiting time of 5 minutes was followed.

Development of a bioreactor to check the laccase activity

A piece of membrane was coated with the Nickel and Cobalt resin solution, and another piece of membrane was attached on the top of it, making it a ravioli-like device, with a pair of membranes carrying resin microbeads trapped between them, a glue based (Figure 4-5) flow through microreactor was developed which was compatible with the ravioli system. This ravioli system was used to capture the His-tagged protein using the flow system (Figure 4-6). Flow throw system was not only efficient in keeping the proteins safely attached to the membrane but also found to be compatible with the ABTS colorimetric assay. After immobilizing the enzymes on the membrane in ravioli system, the activity of the of enzymes was recorded on the microreactor by flowing ABTS solution and as recombinant proteins have lower concentration than commercially available laccase (3mg/ml), residence time for several range was conducted.

Figure 4-5: ABTS based flow-through device showing membrane (yellow disc)

Figure 4-6: Flow-through device with laccase-coated membrane.

Results and Discussion

Sequence confirmation of various inserts in pENTR and point mutations

For cloning in pENTR™/SD-TOPO (pENTR), CACC bases were attached to the 5' end of forward primer in order to directionally clone the insert. Entry vector carries a overhangs GTGG which attacks the 5' end of the PCR product and stabilizes it by annealing to CACC in the correct direction. Expected bands for all genes were amplified from the total cDNA (Figure 4-7). The pENTR vector includes M13 and RVS17 primer sequencing sites, and the PCR product insertion site is flanked by the attL recombination sites; this allows easy verification of the cloned sequences and for further recombination in the destination vector of choice containing attR. For selection and high copy propagation in *E. coli*, a Kanamycin resistance gene and a pUC origin are inserted.

Figure 4-7: Agarose electrophoresis showing KOD polymerase amplification for inserts.

After cloning in pENTR, all insert sequences were matched with the reference sequence from NCBI. AAD and lac inserts showed some sequence discrepancies, whereas GLX insert matched 100% with the reference sequence. The AAD insert matched 96.7% with the reference sequence (GenBank accession no L08964.1) and showed 37 single nucleotide differences; however, when I aligned the protein sequences from insert and the reference, these both expressed the exact same protein despite all point mutations.

GLX matched 100% with the reference sequence (GenBank accession no L18991.1) and expresses the same protein as the reference. lac in this study matches 99.5% of the reference sequence *ERY3* from *P. eryngii* (GenBank accession no AM773999.1) showing a few silent nucleotide changes and a glutamic acid to alanine substitution was found present in the laccase under this study; however, this laccase matches 99.3 % with the *PEL3*, another laccase gene from *P. eryngii* (GenBank accession no AY686700.1), which

also shows the same glutamic acid to alanine substitution as shown by the laccase in this study.

Based on the nucleotide mismatches with the reference sequences, I reason that the silent changes that do not affect predicted amino acid sequence may be the result of evolutionary drift over time in isolates and would likely not affect fitness unless there is a codon bias effect. However, the substitution of an acidic residue for a hydrophobic residue is more likely to affect protein function; it is not yet known, however, if there is an effect in function in our protein.

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AAD primers from mRNAFINAL.apc from 1 to 1318
Alignment to
New CDS for AAD after sequencing (from clone #14).apc from 1 to 1176

Matches ( ) : 1139
Mismatches ( # ) : 37
Gaps ( ) : 142
Unattempted ( . ) : 0

      *      *      *      *      *      *      *      *      *      *
1  atgaacatctggggcaaccgcacactgagcctccgactaagctcgccgctcacaggcagcttgctccggatgtggtcttcattgtatgcccatccagctcg 100
1  ATGAAcATCTTGGGCAccGCACcCTGAGcCTCCGAcTAAgCTCGccCTcACAAGGCAGcCTTgCTCCCGGATGTgGTCTCCATGTATGCCCATCCAGcCTCG 100
      *      *      *      *      *      *      *      *      *      *
101 gtgsaatgagtatcgggacaaagtggcatccctatggcatgggcacacatggacaaggaagcagcttcaagctcctcgagcattctacaacgtggcgg 200
101 GTGSAATGAGTATCGGGCAAAAGTGGcATCCATATGGcATGGGcACCATGGACAAGGAAGCCAGcCTTCAAAGCTCCTCGAGCCATTCTACAACGCTGGTGG 200
      *      *      *      *      *      *      *      *      *      *
201 taaattcattgataccgctaattgtctaccaagacgagacatccgaggaatttattggcagtggtgaggctcggggcaaccgcgacagatggctgtt 300
201 TAACTTCATTGATACCGCTAAATGTCTACCAAGACGAGACATCCGAGGAATTTATTGGcAGTGGATGGAGGCTCGGGGCAACCGGACcAGATGGTGTGTT 300
      *      *      *      *      *      *      *      *      *      *
301 gctaccaagtacagcttggtctcaaaacgggggtgccagcttcgaagagatcccgagaagacgcagtatgtcggaacagcctcaagtgcgatgcacatct 400
301 GCTACCAAGTACAGCTTGGTCTCAAAACGGGGGTGCCAGCTTCGAAGAGATCCCGGAGAAGACGCAGTATGTGGGCAACGCTCAAAGTGCATGCACATCT 400
      *      *      *      *      *      *      *      *      *      *
401 cagtgcacgatttcattgcgaagttgcttacgagacatcagacatattctacgttcacctctctgggactacacctgcaccatcgaggaggtcatgaacgg 500
401 CAGTGCACGATTTCATTGCGAAGTTGCTTACGAGACATCAGACATATATTCTACGTTCACTTCTGGGACTACACTTGCACAAATTGAGGAGGTGATGACGG 500
      *      *      *      *      *      *      *      *      *      *
501 cctgcacaaactctgttgcacaggttaaggtctctttaccttggcgtctcggaacgcccagcctgggtcgtctcgaaaggcgaacactacgcgcgcatggt 600
501 CCTGCACAAACTCTGTTGCACAGGTAAAGTCTCTTACCTGGCCTCGGACACACCCGCTGGGTGTCTCGAAGGCGAACACTATGCCGCATGGCC 600
      *      *      *      *      *      *      *      *      *      *
601 ggcaagacgcctttcgttatctacagggagagtggaacatcactatgcgcgacatggagcgcgacatcattccgatgtgcattcacgaagggatggcca 700
601 GGCAAGACGCCTTTCGTTATCTACAGGGAGAGTGGAAcATCAGATGCGCGACATGGAGCGCGACATCATTCCGATGTGCATTCAcGAAGGGATGGCCA 700
      *      *      *      *      *      *      *      *      *      *
701 tcgcgccttggaattgtcttgcgcgcgaagatacgcgcgagcgcgaggaagagcgcgtctcaagtcocggcaggggtggagctacctcctccagtt 800
701 TCGCGCCTTGGAAATGTGCTTTGCGCGCGCAAGATACGCACGAGCGCGGAGGAGGCGCGCTCTCAAGTCCGGCGAGGTTGGCGCTACGCTCCTACAGTT 800
      *      *      *      *      *      *      *      *      *      *
801 cyacgagtggtccgggaacgagacgcgagcgaaggtcagcaaggtcactcgagaaggtgcgtgaggagatcgggcgaagagatcacgtccgtcgtatt 900
801 CGACGGATGCTCCGGAACGAGACCGAGCGCAAGGTCAGCAAGGCACCTCGAGAAGGTGCTGAAGAGATCGGCGCAAGAGATTCACATCCGTGCGCAATT 900
      *      *      *      *      *      *      *      *      *      *
901 gctacctcatgcagaagttccctacgtcttcccccattgtcgggtgaacgaaggtcgaacacctgtacgccaacctcgaggcgtcgacatctcgtca 1000
901 GCTACCTCATGCAGAAGTTCCCTACGCTCTTCCCCATTGTGCGGTGAGCGCAAGGTGGAACATCTGTACGCCAACTCGAGGCGCTCGACATCTCGCTCA 1000
      *      *      *      *      *      *      *      *      *      *
1001 gccccgcagatgcagttcctgaacgacacagtagccctcaacaagggcttcccatacctcctcttgggtgatgggtcgactacaacatcgtacacaa 1100
1001 GCCCGCAGATGCAGTTCTCTGAACGACACAGTAGCCCTCAACAAGGGCTTCCCATACCTCTCTTCTGCGTGTGGGTCCGACTACAACATCGTACACAA 1100
      *      *      *      *      *      *      *      *      *      *
1101 ggccgcaggtcattatgacaagtgccccgcgcaacaggtatccgccccagaagCACCACCACCACCACtag 1176
1101 GGCTGCAGGTCATTATGACAAAGTGGCCGCGCAACAGGCTATCCGCCCCAGAAAGCACCACCACCACCTAG 1176

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Figure 4-8: AAD insert from pENTR showing mismatching with the reference sequence.


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laccase primers vFINAL.ape from 1 to 1614
Alignment to
Correct ery 2 CD from eurofin with all details.ape from 1 to 1614

Matches():1606
Mismatches():8
Gaps():0
Unattempted():0

1 atgtttccaggcgacgggattctcgctgaacgttaacattagctcttcaccttttacatggcaactcagctgccatcgggcccatgtccgacatgtacatcg 100
1 ATGTTTCCAGGCGCACGGATTCTCGCTACGCTTACATTAGCTCTTCACCTTTACATGSCACTCATGCTGCCATCGGGCCCATGTGCCGACATGTACATCG 100

101 tcaacgaggagctctctcctgacggcttcgctcggtcggtcgctcgctctgtgcccggccacagatccgacgctcgctcggtatcggttctctgg 200
101 TCAACGAGGAGCTCTCTCTCGACGGCTTCGCTCGTTCGGCTGTCTGCTCTGTGCGGGCCACAGATCCGACGCTCGCTCGGTATCGGTTCTCTGG 200

201 cgtctcgtgtaaggaacaggcgataaactttcagctgaacgttcgcaatcaactgtcggaactcgactatgtgaagactactagatccattggcat 300
201 CGTCTCGTGTAAGGAACAGGCGGATAACTTTCAAGCTGAACGTTTCGCAATCAACTGTGCGACTCGACTATGTTGAAGACTACTAGTATCCATTGGCAT 300

301 ggctctcttcaatccggctctacgtggcgagatggacccggttcgtggaatcagtgccccatcgctcggggaatagcttccatgatgactttaacgttc 400
301 GGCTCTCTTCAATCCGGCTCTACGTGGGCGAGATGGACCCGGTTCGTGAATCAGTGCCCCATCGCCTCGGGGAATAGCTTCCTATATGACTTTAACGTTTC 400

401 ccgaccaagctggcagcttctggtaccattcgcatcttccacccagattgtgatggttcttagaggaccattcgtagtatacagatccctccgacccca 500
401 CCGACCAAGCTGGCAGCTTCTGGTACCATTTCGATCTTTCCACCCAGTATTGTGATGCTCTTAGAGGACCATTGTGATATACGATCCCTCCGATCCCA 500

501 cctgtccttatatgacgttgacaacgcccagacactgtcattacacgttgaaagattggtaaccaggttcgggcccctcagggtgcagtgcttctactcctgat 600
501 CCTGTCTTATATGACGTTGACAAACGCCGACACTGTCTTACACTTGAAGATTGGTACCAGTTGCGGGCCCTCAGSGTGCAGTGCTTCTACTCCTGAT 600

601 agcagctcatcaatgtaagggtcgcttcgcccggggccgcaactccctttggctatcatcaactgcaaaagcaacagcgatcggttccgactta 700
601 AGCAGCTCATCAATGTAAGGTGCTTCGCGGGGGCCGCACTTCCCTTTGGCTATCATCAACGTCGAAAGCAACAGCGATCGGTTCCGACTTA 700

701 tctcgatgtcttcgaccccccaatttcacgttctcaatcgacgggtcaactctttgaggtcattgaagcagatgctgcaaatattgtgcccctcgctgga 800
701 TCTCGATGTCTTCGACCCCCAATTTCAGTCTCTCAATCGACGGTCACTCTTTGCAAGTCAATTGAAGCAGATGCTCAATATTGTGCCATCGCTGTGA 800

801 tagtattcaaatcttcgcccgaacagctattcttctgctgaatgcaatcaggctgtcgacaattattggattcgcgcaaatcccaacttgggagcg 900
801 TAGTATCAAACTCTTCGCGGCCCAACGCTATTCTTCTGCTGAATGCCAATCAGGCTGTGCACAATTATTGGATTGCGCAAAATCCCAACTTGGGATCG 900

901 actggttcgaaggtggcatcaattcogctatccttcgctatgctgtgcccactgaaatgacccctgcccagacttcgtcgacgagcaactccctctcgg 1000
901 ACTGCTTCGAAGGTGGCATCAATTCGCTATCCTTCGGTATGCTGCTGCTGCAAAATGACCCCTGCCAGACTTCGTCGACGAGCACTCCCTCTCG 1000

1001 agactaatcttggtgccactcgaaaactcgtggtcctcgtccagctgtccctggaggcgacagacatcaacatcaatcttgctatggggttcgactttac 1100
1001 AGACTAATCTTGTCGCACTCGAAAATCTGCTGCTCTGCTGCGGCTGTCCCTGAGGCGCAGCATCAACATCAATCTTGCTATGGGCTTCGACTTTAC 1100

1101 taactttgaaatgacctcaacggttcccccctcaagcaaccaactgctcgttctgctccagattctgtcaggtgctacgctgcgcttctcgtctct 1200
1101 TAACCTTTGAAATGACCATCAACGTTCCCTTCAAGCACCAACTGCTCTGTTCTGCTCCAGATTCTGTCAGGTGCTACGCTGCGCTTCGCTTCTT 1200

1201 ccttcaggtatgataacgcgtagaagccaacaaagttgtcgaaatcccataccgccttagctgtcggaggaccgcatccttccatcttcaacggac 1300
1201 CCTTCAGGTATGATATACGCGCTAGCAGCCAACAAAGTTGTGAAATCCCATACCCGCTTAGCTGTGAGGAGCCGATCCTTCCATCTTCAGGAC 1300

1301 acacgttcgaagctcatcaggagtgccggctctactacgtataaactcgacaacccctgcgcgacgcatgttgtaacactggaattgacggaacgacaa 1400
1301 ACACGTTGACGCTCATCAGGAGTGCGGGCTCTACTACGTATAACTTCGACACCCCTGCGCGACGCGATGTTGTCAACACTGGAATTGACGGAACGACAA 1400

1401 cgttaccatccgcttctgacggataatccgggcccattgttccctccactgccacattgactggcatctcgaaatcggtctcgcggtcgttttcgcccga 1500
1401 CGTTACCATCCGCTTCTGACGGATAATCCGGGCCATGGTTCTCCACTGCCACATTGACTGGCATCTCGAAATCGGTCTCGCGGTGCTTTTGCCCGAA 1500

1501 gatgtggcgtccatcaaggccccactgcgcgtgggacgcaactgtgtccgatttatgatgctttgagcgattccgacagaggtggcatagctCACCACC 1600
1501 GATGTGGCGTCCATCAAGGCCCACTGCCGCTGGGACGCACTGTGTCCGATTATGATGCTTTGAGCGATTCCGACAGAGGTGGCATAGCTCACCACC 1600

1601 ACCACCACCACTag 1614
1601 ACCACCACCACTAG 1614

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Figure 4-10: lac insert from pENTR showing mismatching with the reference sequence.

Cloning into destination vector pT1OG and pGX8

All three lignolytic enzymes were cloned into either the pT1OG or pGX8 vector using the LR reaction. pT1OG, being a constitutive vector, provides the benefit of continuous expression of the inserted protein, while pGX8 is an inducible vector. In the case of pGX8 an extra effort is needed to optimize the conditions for induction of protein expression, such as β -estradiol concentration and incubation period.

In *P. patens*, the XVE system offers several advantages over other systems, especially its considerable and maximum expression for long period (up to 7 days) without causing any noticeable morphological and growth side effects on *P. patens*. In addition, induction levels can be regulated by adjusting the concentration of β -estradiol from 0.001 μ M to 1 μ M for up to 7 days, though this system needs to be incubated at least 24 hours for induction (Kubo et al., 2013), hence pGX8 made a perfect choice of this study. All the proteins cloned in pGX8 in this study were induced at 1 μ M β -estradiol for up to 72 hours. AAD was effortlessly cloned in pT1OG, and no problem was encountered in expression of the protein, whereas GLX and lac were cloned using the pGX8 vector.

Moss genotyping and maintenance of the lines

Several lines showing the desired insert band for each gene in agarose gels were identified using a gene specific PCR with Ex-Taq polymerase (Figure 4-11). Three lines for each gene was selected and maintained on BCDAT media under continuous light in the growth chamber at 25°C. For further use in Western blotting these lines were maintained in the growth chamber at 25°C as required by the destination vector, whereas

stock tubes of BCDAT media of all selected lines were stored in the 10°C growth chamber.

Figure 4-11: Genotyping of transgenic *ggb* lines for inserts.

Protein extraction and BCA results

Protein was extracted from different lines carrying the insertion from each gene. An equal volume of protein was loaded in SDS-PAGE for optimization of the protocol (Figure 4-12). After looking at the protein yield in SDS-PAGE gels, BCA was done to determine the concentration of the protein, and extraction was repeated to increase the protein yield if necessary. Most of the samples ranged from 250 µg/ml to 1000 µg/ml; protein extraction and BCA was repeated to determine the concentration. Volumes were adjusted for the samples as per required by the western blot.

Western blot results

Western blots were carried out using the transgenic lines carrying the desired insert in moss along with wild type and *ggb* lines as the control. Anti-rabbit IgG HRP-linked Antibody revealed one single band of expected size of all of the proteins. In a few transgenic lines, along with a single band, a smear was found, which likely represents the

degradation of the foreign protein in the host system, but in most cases, a single clean band represents the advantage of using *ggb* as a host system for expressing the foreign proteins. With this assay, I could conclude that the recombinant proteins were present and were expressed by the transgenic plants.

Figure 4-12: Western blot showing expected band size from a) three transgenic lines of GLX, b) two transgenic lines of AAD, c) three transgenic lines of lac.

Recombinant laccase protein activity results

Since laccase is an extracellular enzyme, I reasoned that transgenic lines carrying the laccase-encoding insert may be secreting the enzyme to the surrounding media. I used the water collected from washing the cells grown on induction media to check the enzymatic activity using ABTS for a colorimetric assay. A development of an intense bluish green

color in the wells containing the cell wash water from the recombinant proteins was considered positive for enzymatic activity, a similar color development was found in commercially-available laccase from *Trametes versicolor* (positive control), however, a similar color development was also seen in the negative control (untransformed *ggb*). I used ABTS as a substrate, which reacts with several enzymes, carbohydrates, proteins and other natural substrates and widely used for determining the antioxidant abilities of these products (Sujarwo et al., 2019). Development of the color in the untransformed *ggb* shows that I need to consider other substrates in order to determine the laccase activity, however, the plate assay is easy and quick method for visualization of the presence of the laccase, I can possibly use this assay with other laccase substrates to determine the activity in the extracellular fluid as described by (C. Srinivasan et al., 1995).

Figure 4-13: Plate assay for laccase activity in extracellular culture fluids of *P. eryngii*.

Recombinant laccase activity test on bioreactor

A preliminary model of flow-throw device has been developed and being tested in the lab of Dr. Harnett (University of Louisville), however, in the first pass run there was no activity shown by the recombinant lines developed in this study. As mentioned above, these recombinant proteins have very low concentration compared to commercially

available laccase and a possible reason could be that these proteins are either not being attached to the resins effectively or residence time needs to be optimized to for these recombinant proteins.

***ggb* as a model heterologous system**

The hunt for methods of affordable pharmaceuticals, vaccines, and industrial enzyme production, including lignolytic enzymes, has been one of the major goal for industries (as mentioned in the chapter 1 and in the introduction of this chapter). Several kinds of techniques, including plant- and animal- based protein expression systems, have been explored in order to find out the perfect heterologous system to increase the yield and enhance the functionality of proteins as required by the industry. The moss

Physcomitrella patens has been known for years as a model system in plant molecular biology; it offers the easy disruption of genes via homologous recombination (Decker et al., 2008). *P. patens* can be grown in several different ways, and, for low cost pharmaceutical protein production, it has been easily cultivated in bioreactors with the use of simple inorganic salts and atmospheric CO₂ as a carbon source (Cools et al., 2017). There have been reports of using moss in bioreactors to produce pharmaceuticals, and a successful scaling of this system up to 500L, homogenous and stable protein products from batch to batch, and a stable procedure for cell line cryopreservation in master cell banks (Reski et al., 2018). One of the major advantages of using the plant-based system for producing animal proteins is easy scaleup and low-cost production (Desai et al., 2010); on the other hand, the disadvantage of different modes of glycosylation between flowering plants and humans makes it harder for humans to consume plant based glycosylated proteins, which often results in allergic reactions . Also

many of the proteins are produced at the seed stage, which make downstream processing challenging (Reski et al., 2005). There are also reports that suggest that these plant based sugar-residues can be removed from the glycoproteins (Huether et al., 2005).

The *ggb* mutant line offers all the benefits of using moss as a heterologous system, including an additional feature of staying undifferentiated and unicellular throughout the life cycle. I have clear evidence that transgenic *ggb* lines are expressing and secreting biologically active proteins, although it is important to further optimize the purification and production of the recombinant proteins.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

In this dissertation, I studied protein prenyltransferase mutants in *Arabidopsis* and moss *Physcomitrella patens*. Protein prenylation in plants has been a focus of study for more than two decades, and in *Arabidopsis* extensive studies have been done on the protein prenyltransferase enzymes PFT and PGGT-I. This dissertation was aimed at studying the possible role of a newly identified gene in our lab called *PPAL* that shares weak sequence homology with enzymes involved in protein prenylation. A mutant carrying T-DNA insertion in *PPAL* was ordered from the Arabidopsis Biological Resource Center and called *ppal-1*, these mutants show sugar hypersensitivity, accumulate excess sugar compared to wild type, show slight male infertility and display sensitivity to abscisic acid. A suppressor line (p212), which rescues the *ppal-1* sugar sensitivity phenotype, was identified using chemical mutagenesis and was chosen for mapping to find a possible additional factor involved in prenylation and sugar sensing/homeostasis. An F₂ population was generated by crossing the suppressor line with Ler (wild type), to map the secondary or suppressor mutation. Phenotyping was done on the basis of short siliques (slight male infertility), but it was found that this phenotype is not penetrant enough to consistently score, whereas for positional mapping 100% confidence is required in phenotyping. This weak phenotype with incomplete penetrance resulted in ambiguous phenotyping and no linked marker was found. Further steps can be taken by generating

and identifying another suppressor line, backcrossing it and then creating another F₂ population to repeat the mapping process.

I have also generated crosses between *ppal-1* and different abscisic acid biosynthesis and signal transduction pathway mutants. After the characterization of F₃ seed pools of *ppal-1xaba3-1* and *abi2-1xppal-1*, I have concluded that *PPAL*'s response to stress is dependent on ABA. After analysis of *abi2-1xppal-1* double mutants, I found that *PPAL* functions in the signal transduction pathway but I did not find any difference between the sensitivity level of *ppal-1* mutants and wild type to ABA, so the position of the *PPAL* cannot be determined in the signal-transduction pathway relative to *abi2-1*.

In the future, I can measure the endogenous ABA content of all mutants in this study including the wild type, which will help us disclosing the slower and higher rate of transpiration.

I also studied the cell wall composition of *ppal-1*, *aba3-1*, and *ppal-1xaba3-1* and found out a severe drop in the lignin content of the *ppal-1xaba3-1* pool, and this cross shows the promise for developing plants with easy cell-wall degradation and further modification for lower lignin content. Although I do not currently know the biological basis for the sudden drop in lignin content of these plants. At this stage, it is hard to conclude if this drop in the lignin content is due to a possible crosstalk among ABA biosynthesis, sugar homeostasis and lignin synthesis pathways. It will be interesting to investigate the key players and any possible connection between ABA and lignin biosynthesis. This is the preliminary study of the cell wall composition for all the mutants and this experiment needs to be repeated with larger sample size and replicates in order to make any assumption.

To investigate the possible cause for not finding the *ppal-1* insertion in F1 generation of all the crosses, several reciprocal crosses were made between *ppal-1* and Col-0 using two different homozygous *ppal-1* lines. Considering all individuals are heterozygous at F1 stage, genotyping of 49 F1 individuals was done using *ppal-1* specific primers and with genomic primers, all individuals showed a Col-0 type band; however, none of them showed any *ppal-1* insertion. At this stage, it is hard to explain the problem I encountered with all the crosses generated using *ppal-1*. All *ppal-1* lines used in above-mentioned crosses were homozygous and were repeatedly confirmed by genotyping the parental lines using *ppal-1* and wild type genomic PCR primers.

From the mapping project to generating the double mutants and in crosses with wild-type, I have found the distorted segregation pattern in all the populations carrying *ppal-1* as one of the parents. Genotyping of these populations clearly showed a drastic reduction in the transmission of the T-DNA allele, resulting in a severely distorted population. One leading explanation is that the *ppal-1* allele causes a severe defect in the gametophytic stage, especially in pollen. The next step would be to find out the cause by using microscopy techniques and functional analyses to characterize pollen grains and ovules of *ppal-1* plants.

In the last objective for this dissertation, I used *ggb*, a mutant line of the moss *Physcomitrella patens*, which lacks the β -subunit of geranylgeranyltransferase (a protein prenyltransferase). *P. patens* has several advantages as a model plant system, including low structural complexity, haploid dominant phase, and homologous gene recombination-based gene targeting. *ggb* offers additional benefit over wild type moss by being unicellular and undifferentiated, which makes them an interesting platform to study and

use in the expression of foreign proteins. Lignolytic enzymes have countless uses and advantages for various industrial requirements and I used *ggb* to successfully express three different lignolytic enzymes: AAD and GLX from *Phanerochaete chrysosporium* and laccase from white-rot fungi *Pleurotus eryngii*. Expected protein bands were shown in the western blot and laccase activity was shown in the different transgenic lines carrying the laccase insert; however, a similar activity was shown by the negative control as well, which indicates that I need to further optimize this assay with the laccase specific substrate.

I have used two different kinds of destination vector for cloning these enzymes, a constitutive expression vector pT1OG, and an inducible expression vector pGX8. In the future, q-RT PCR can be used to check and compare the expression of the genes in both kinds of vectors and at which stage the expression of these genes is higher and would be most economical to harvest the moss at that particular stage rather than growing it for a longer and standard period. A further step can be done by adding any fluorescent marker with the 6X His-tag might makes it easier to identify the transgenic line at an early stage. As two of the lignolytic enzymes are extracellular, that means cells secrete these enzymes in extracellular fluid, setting up and optimizing the liquid culture for these enzymes may be an effective way to scale up the process.

By successfully cloning the expressing different recombinant proteins, it is clear the *ggb* shows a promise for an excellent heterologous protein expression system in the future. There are several reports that mention the use of wild type moss for its efficient use in expressing recombinant proteins. Further steps are needed to optimize the *ggb* expression system, but so far it offers cost-effective, consistent protein production and easy

downstream processing (as discussed in chapter 4), all showing it could be the promising heterologous system for expressing proteins.

A ravioli-based bioreactor was developed by the lab of our collaborator Dr. Cindy Harnett (University of Louisville), which supports the binding of His-tagged recombinant on Nickel resin between two membranes. A compatible flow-throw microreactor device was also developed; this supports the colorimetric activity assay of ABTS without washing off the attached enzymes. The development of a blue-green color by ABTS will determine the activity of the attached protein. In the first pass-run, there was no activity was found in the recombinant proteins from this study. This system needs further optimization as there could be several possibilities for not finding the activity in the recombinant proteins during the first trial, including the concentration of these proteins, pH of the resin to support the binding of the recombinant proteins, and optimization of residence time for washing.

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EDUCATION

PhD (Cellular, Molecular & Developmental Biology) **2013-2019**
Dept. of Biology, University of Louisville, Louisville, KY, USA
Title: Plant protein prenylation in environmental responses and protein production

MS (Cellular, Molecular & Developmental Biology) **2013-2016**
Dept. of Biology, University of Louisville, Louisville, KY, USA
GPA: 3.76/4

MS, Biotechnology **2001- 2003**
Dept. of Biotechnology, Purvanchal University, UP, India
Project Title: Cloning and expression of betaine aldehyde dehydrogenase (BADH) from *E. coli*.

BS, Biology **1998-2001**
MJP Rohilkhand University, UP, India

EXPERIENCE

Graduate Research Assistant **2017-2019**
NSF-EPSCOR
Department of Biology, University of Louisville, Louisville, KY, USA

Graduate Teaching assistant **2013-2016**
Department of Biology, University of Louisville, Louisville, KY, USA

Lab Intern **Oct 2010–Dec 2010**
Department of Biology, University of Rochester, Rochester, NY, USA

Volunteer Research Assistant **2009–2010**
Department of Biology, University of Memphis, Memphis, TN, USA
Project Title: Salt, drought and cold tolerance in tomato.

Senior Research Fellow **2006– 2009**
Division of Genetics, Indian Agricultural Research Institute, New Delhi, India
Project Title: Maize Functional Genomics.

Junior Research Fellow **2004–2006**
Division of Biotechnology, U.P. Council of Sugarcane Research, U.P., India
Project Title: Development of mapping population & tagging of high sugar genes using SSR markers in Sugarcane.

Project Assistant **2003–2004**
Division of Tissue Culture, U.P. Council of Sugarcane Research, U.P., India

TEACHING EXPERIENCE (University of Louisville)

- Biol 258 (Introduction to Microbiology lab)- Fall 2014- Fall 2016
- Biol 104 (Introduction to Biological System lab)- Summer 2014
- Biol 331 (Genetic and Molecular Biology Lab)- Spring 2014

ORAL AND POSTER PRESENTATIONS

- Oral presentation, American Society of Plant Biologists Midwestern Section Annual Meeting, West Virginia University, March 16-17, 2019.
- Poster presentation, American Society of Plant Biologists Annual Meeting, Montreal, Canada, July 14-18, 2018.
- Poster presentation, North American Membrane Society Annual Meeting, University of Kentucky, June 11, 2018.
- Oral presentation, Graduate Research Conference, University of Louisville, March 2-3, 2018.
- Oral and Poster presentation, KY-NSF-EPSCoR Supercollider Meeting, University of Kentucky, Feb 22, 2018.

- Poster presentation, KY-NSF-EPSCoR Supercollider Meeting, University of Kentucky, Feb 24, 2017.
- Poster presentation, American Society of Plant Biologists Midwestern Section Annual Meeting, Purdue University, Indiana, February 4-5, 2017.
- Oral presentation, Kentucky Academy of Science, Louisville, KY, Nov 2016.
- Poster presentation, 10th Asian Regional Maize Workshop, Makassar, Indonesia October 20-23, 2008.

TECHNICAL TRAININGS & SCIENTIFIC WORKSHOPS

- STEM- Graduate Teaching Academy, University of Louisville, USA (July 25-29, 2016).
- Generation Challenge Program (GCP) International Training Course on “Molecular characterization of inbred lines and heterogeneous populations in maize” by International Maize and Wheat Improvement Center (CIMMYT), Mexico, and Indian Agricultural Research Institute, Delhi, India, April 1-3, 2007.
- Training on “Biosafety concerns on transgenics and detection of GMOs” at National Bureau for Plant Genetic Resources, India (March 14-21, 2005).
- Molecular techniques in bioprospection and biodiversity analysis, Central Institute of Medicinal and Aromatic Plant, Lucknow, India, June-July 2002.

ACHIEVEMENTS

- Travel award to attend Midwest ASPB 2019.
- Selected speaker at Supercollider 2018, University of Kentucky, Feb 22, 2018.
- Research Grant awarded from School of Arts and Science (University of Louisville, 2017).
- Received First Prize for the poster “Graphical genotyping of genomic resources (QTL-NILs and RILs) and transcriptome profiling of maize genotypes in response to sorghum downy mildew (*Peronosclerospora sorghi*) in India”, in the 10th Asian Regional Maize Workshop (ARMW) at Makassar, Indonesia, October 2008.

OUTREACH

- Career fair judge for Louisville Regional Science and Engineering Fair, March 5th, 2018, Louisville, KY.
- Career fair for Brandeis Elementary School, 2018, Louisville, KY.

RESEARCH PUBLICATIONS:

- Singh R.K., **Singh P.**, Singh S.P., Singh S.B. (2005). STMS marker for tagging high sugar genes in sugarcane. Sugar tech 7 (2&3): 74-76.
- S.B. Singh, R. K. Singh, **Parul Singh**, Kishore Gayakwad & N. K. Singh (2005). Development of cDNA Library for High Sugar Genes in Sugarcane. National Seminar on Plant Physiology at Pune, Dec 27-29 2004, Sugar Tech, 7 (2):18-20