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A Study of Abscisic Acid Regulated Enzymes and Histone Binding Proteins in Plants

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A study of abscisic acid regulated enzymes and histone binding proteins in plants

By

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Plant hormone abscisic acid (ABA) plays a main role in coordinating various stress signals in plants. ABA regulates the expression of genes and activities of enzymes in response to various stress conditions. In the following studies we were able to study the ABA mediated regulation of enzymes in plants. Using in-gel activity analysis we identified that ABA regulates the activity of aspartate aminotransferase (AAT), an enzyme involved in nitrogen assimilation and carbohydrate metabolism. Our results indicate that phosphorylation of AAT by SnRK2.2 and 2.3 kinases results in down regulation of AAT2 and AAT3 isozyme activities in Arabidopsis. AAT was identified as a negative regulator of drought stress and *aat* mutant plants showed improved survival following drought conditions. Using in-gel staining method we were able to visualize sugar phosphatases like fructose 1-6 bisphosphatase family, sedoheptulase-1,7-bisphosphatase, inositol mono phosphatases; protein serine/threonine phosphatases, protein tyrosine phosphatases and studied their response to ABA and drought stress.

Fructose-1-6 bisphosphatase family of phosphatases were identified to be induced by ABA in Arabidopsis and rice.

N-acetylglucosamine (GlcNAc) is present on glycoproteins and as post translational modification (PTM) in cytoplasmic and nuclear proteins. N-acetylglucosamine is removed from target proteins by hexosaminidases. Little is known about the hexosaminidases in plants. Using in-gel activity analysis we were able to identify an ABA induced Beta-hexosaminidase with a neutral pH optimum in soybean.

The nuclear DNA in chromatin is associated with basic proteins called histones. The N-terminal tails of histones contain different PTMs including methylation, phosphorylation, ubiquitination, acetylation, ADP-ribosylation and glycosylation. The histone lysine methylation can serve as a binding site or repel/disrupt the histone binding proteins. The effector/reader proteins specifically recognize the post translational modifications and responsible for the downstream process. Many histone methyl modification effector proteins have been characterized but very few proteins whose binding was disrupted by the presence of a PTM were identified. Using peptide pulldown analysis, far western analyses we identified a WD-40 domain containing histone binding (HB01) protein as direct interactor of unmodified histone. The presence of post translational modifications disrupts HB01 binding to histone H3.

DEDICATION

This dissertation is dedicated to the loving memory of mother, Padma Sasi Rekha Kolli.

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CHAPTER I

INTRODUCTION

Abscisic acid (ABA) is a plant stress hormone required during many developmental stages of plant growth. ABA levels increase in response to stress and regulate the expression of many genes. The expressed genes and their products help plants during various abiotic and biotic stress conditions. Abscisic signaling is a complex regulatory mechanism with cross links to auxin, ethylene and sugar signaling. Abscisic acid is also linked with carbon and nitrogen metabolism. Exogenous application of ABA is known to mimic dehydration/drought stress in plants. Core signaling pathway for ABA signaling has been proposed in the past decade and is well accepted. Large scale RNA expression analysis lead to the identification of transcripts that are regulated by ABA, drought and salt stress. Considerable overlap exists between genes responsive to drought and ABA. Both ABA dependent and ABA-independent pathways exists in response to drought in plants. Promoter analysis of ABA and stress induced genes lead to the identification of cis acting ABA response elements (ABRE), coupling elements (CE), drought response elements (DRE) and ABA responsive transcription factor binding sites. There are many genes that do not contain any of the known ABA response elements, but are regulated by ABA and their mechanism of regulation remains unclear.

Data set of ABA/stress regulated genes is not complete and the ABA mediated regulation of many enzymes and proteins is yet to be identified. Even with significant

amount of research being focused on ABA signaling, many components that function downstream of the SnRk2 kinases of the core signaling pathway, involvement of number of other kinases, phosphatases, and their numerous downstream targets remain unknown.

The nucleosome, a basic repeating unit of the chromatin consists of 147 bp of DNA wrapped (1.65 turns) around the octamer of core histones containing two copies of H2A, H2B, H3, and H4 The N-terminal tails of histones present in the nucleosome contains different post translational modifications like methylation, acetylation, phosphorylation, ubiquitination and glycosylation. Histone methyl post translational marks act as binding sites for histone effector/binding proteins. The effector/reader proteins recognize the post translational modifications and responsible for the downstream biological process. Histone binding proteins have characteristic domains like chromo domain, bromo domain, PHD finger, WD-40 repeats. Since the proposal of "Histone code" number of effector/reader proteins, enzymes regulating the PTMs were characterized and many remain to be identified.

The research reported in this dissertation is focused on improving our understanding of ABA mediated regulation of enzymes in plants. The objectives of the research reported in this dissertation are

1. To study the role of ABA in regulating the nitrogen assimilation. The enzymes aspartate aminotransferase, alanine aminotransferase, glutathione dehydrogenase, and glutamate dehydrogenase were studied using in-gel activity staining.
2. The enzyme aspartate aminotransferase was studied in detail to understand the ABA role in regulating the enzyme during abiotic stress.

3. To identify plant hexosaminidases with neutral pH optimum, the enzymes that catalyze the removal of N-acetylglucosamine (GlcNAc) residues from cytoplasmic and nuclear proteins and to study the role of ABA in regulating their activities.
4. To identify ABA regulated phosphatases in plants using in gel staining.
5. Perform a large scale screen for histone binding proteins in plants by using histone peptide pulldown analysis and confirm the binding of identified proteins using far western analysis.

CHAPTER II

LITERATURE REVIEW

Arabidopsis thaliana

Arabidopsis thaliana is a model plant, belonging to the family of brassicaceae (mustard) and is identified by Johannes Thal (Galun, 2010). Arabidopsis is a well-used model plant considering its ease to grow and small space required for it to grow on agar plates, laboratory settings and green houses. It has a short life cycle about 6 weeks from germination to development to mature seed formation (Meinke et al., 1998). Arabidopsis can be easily transformed using agrobacterium and mutations can be easily introduced into seeds and single mutant plant arising from that seed can be used to produce thousands of seeds. Cost and ease of storing mutants as seeds is another advantage of using Arabidopsis. The availability of natural variants and availability of number of polymorphisms makes it ideal for mutant gene identification (Somerville and Koornneef, 2002).

The Arabidopsis genome initiative (AGI) was established in the year 1996 to initiate genome sequencing of Arabidopsis and it is the first plant to be completely sequenced. Arabidopsis has one of the smallest methylated genome sequence, which is about 125 Megabases and about 115,409,949 bp was sequenced. Arabidopsis genome is distributed in to 5 chromosomes with 25,498 genes and 11,000 protein families (Initiative, 2000). The whole genome sequencing of Arabidopsis helped identify and

characterize the function of many gene families based on the sequence similarities to other genomes. Expression patterns of number of orthologous genes identified in Arabidopsis have similar patterns of expression in rice and barley (Daszkowska-Golec and Szarejko, 2013). Arabidopsis can be used to study epigenetics and gene inheritance. An online resource providing a detailed information about Arabidopsis named "The Arabidopsis Information resource (TAIR)" is available at http://arabiposis .org (Rhee et al., 2003).

Oryza sativa

Rice (oryza sativa) is a major crop plant that has been cultivated for more than 7000 years. It is a staple food for half of the world population with an annual production at 0.5 billion tons. The International Rice Genome Sequencing Project (IRGSP) coordinated by the Japan Rice Genome Program was initiated in the year 1998, and a draft sequence was released in the year 2002. A complete map-based sequence of all the rice chromosomes is now available (Sequencing ProjectInternational Rice, 2005). Rice is considered as a model plant for monocotyledon family of cereals. About 71% of the predicted rice proteins and 89.8 % of the proteins from Arabidopsis have homologues in Arabidopsis and rice respectively (Sequencing ProjectInternational Rice, 2005). Rice has a small, high density genome in the cereal plants with a 4.5Mb (Megabase pairs) genome and 12 chromosomes. The transformation procedures for rice are available for effective transformation. The highly conserved gene order, availability of high number of expressed sequence tags (ESTs), sequence similarities among the cereal plants, efficient transformation makes rice an ideal monocotyledon model plant for crop research and

crop improvement for food security (Goff, 1999; Havukkala, 1996; Izawa and Shimamoto, 1996).

Abscisic acid

Abscisic acid (ABA) is an important plant hormone. It is required during many developmental processes of plant growth like, seed germination, transition from vegetative to reproductive growth, development, maturation and dormancy of seeds. In addition to its role in developmental functions, ABA is also required by the plants to adapt to various abiotic and biotic stress conditions (Finkelstein et al., 2002). An overview of various aspects of ABA pathway including ABA biosynthesis, catabolism, transport, core signaling pathway and signal transduction is detailed below.

ABA biosynthesis

ABA is a carotenoid derived plant hormone. ABA biosynthesis is known to occur in a direct pathway from farnesyl pyrophosphate in fungi or by indirect pathway through a carotenoid precursor in plants (Cutler and Krochko, 1999; Finkelstein, 2013). The early steps of ABA synthesis, the modification of C40 carotenoid precursors to C15 carotenoid precursors of ABA take place in the plastid. Zeaxanthin is one of the first oxygenated carotenoid precursors of ABA in the biosynthesis pathway. Zeaxanthin is epoxidated by zeaxanthin eposidase (ZEP). Zeaxanthin epoxidase is involved in both ABA biosynthesis and in the xanthophyll pathway. ZEP is encoded by genes ABA1 in Arabidopsis and ABA2 in tobacco. ZEP contains an N-terminal chloroplastic transit peptide signaling the translocation of ZEP to the chloroplast. Antheraxanthin, vialoxanthin are the products of the reaction catalyzed by ZEP (Marin et al., 1996; Zhang et al., 2006). The gene

expression analysis revealed that ABA2 is expressed in all tissues, with higher expression in stems, leaves compared to that of roots and seeds. The ZEP transcripts are induced in roots but not in leafs during the drought (Audran et al., 1998). Overexpression of AtZEP increased the tolerance of Arabidopsis plants to salt and drought treatments. The AtZEP overexpression plants had a smaller stomatal aperture compared to the wild type plants (Park et al., 2008).

Conversion of 9-cis–neoxanthin and 9-cis-violaxanthin to xanthasin, a C15 intermediate of ABA is catalyzed by 9-cis epoxy carotenoid dehydrogenase (NCED). NCED is first identified in maize, as a viviparous 14 (vp14) mutant by transposon mutagenesis and mutants are characterized by lower ABA content and subsequent higher transpiration rate (Tan et al., 1997). The recombinant VP14 catalyzes the oxidative cleavage of 9-cis–neoxanthin, 9-cis-violaxanthin to xanthasin and a C25 apo-aldehyde byproduct that is different for both the substrates. The recombinant VP14 is specific to the cis apo-aldehydes and is unable to cleave the trans forms of violaxanthin and neoxanthin (Schwartz et al., 1997b). The notabilis (not) mutant in tomato characterized by Burbige et al., (1999) was similar to the Vp14 mutant in tobacco . The Arabidopsis genome contains 9 NCED genes and they show a localized pattern of gene expression (Tan et al., 2003). NCED is an important rate regulating enzyme in ABA biosynthesis pathway and the NCED genes are upregulated in response to drought (Schwartz et al., 2003; Schwartz et al., 1997b; Tan et al., 1997). Overexpression of AtNCED3 gene in Arabidopsis lead to increased ABA levels and drought resistant phenotype. Whereas the knockout mutants had high transpiration rates and drought sensitive phenotype (Iuchi et al., 2001).

The few final steps in the ABA biosynthesis occur in the cytoplasm. Xanthaxin, is converted to ABA in the cytoplasm via a multi-step reaction with ABA aldehyde as an intermediate. One of the first evidence of conversion of xanthoxin to ABA, was provided by the work of Sindhu and Walton (1987). The cell free extracts from leaves of beans, garden pea, squash, and maize converted exogenous xanthoxin to ABA in a NAD or NADP dependent fashion (Sindhu and Walton, 1987). ABA deficient Arabidopsis mutants, aba2 and aba3 were identified by mutant screening based on their resistance and germination in the presence of paclobutrazol, a gibberellin biosynthesis inhibitor. The aba2 and aba3 mutant plants showed ABA deficient wilty phenotypes and did not accumulate ABA during the drought stress (Léon-Kloosterziel et al., 1996). The aba2 and aba3 mutants were later identified as ABA biosynthesis mutants. Biochemical characterization of the aba2 and aba3 mutants by Schwartz et al. (1997) showed that aba2 mutant plants have a block in the ABA biosynthesis and cannot convert xanthoxin to ABA aldehyde. Screening for mutants that germinate in presence of high salt resulted in the identification of four alleles to ABA2 gene namely: salt resistant (sre1-1, 1-2,) and salabereno (san3-1, 3-2). The biochemical analysis of these four alleles further provided evidence for the conversion of xanthoxin to ABA aldehyde (González-Guzmán et al., 2002). Another allele of ABA2 gene was identified in Arabidopsis as sucrose insensitive (sis1) mutant and the results indicate a crosslink between sugar signaling and ABA signaling pathways (Laby et al., 2000).

Cheng et al. (2002) reported the cloning of GLUCOSE INSENSITIVE 1 (GIN1) and ABSCISIC ACID DEFECIENT 2 (ABA2). These genes are allelic and encode a short-chain dehydrogenase/reductase (SDR) that catalyzes the conversion of xanthoxin to

ABA aldehyde in the cytoplasm. Analysis of gin1/aba2 mutants revealed the crosslink between the signaling of hormones ABA, ethylene and the metabolic glucose signaling in plants. The expression of genes GIN1/ABA2, ABI3 and ABI4 are controlled by collective action of ABA and glucose (Cheng et al., 2002b). The aba3 mutant plants are deficient in the enzyme that catalyzes the last step in the ABA biosynthesis, the oxidation of ABA aldehyde to ABA (Schwartz et al., 1997a). The enzyme aldehyde oxidase (EC 1.2.3.1) catalyzes the oxidation to ABA aldehyde to ABA, the last step in the ABA biosynthesis pathway.

Abscisic acid catabolism

The concentration of ABA in plants is highly regulated and is a net of continuous biosynthesis and catabolism. ABA concentration increases in plants during stress and when the stress reveled, the ABA levels come down helping the plant growth and development. When excised xanthium leaves are subjected to wilting-recovery steps, upon rehydration the accumulation of ABA metabolite phaseic acid (PA) increased and the ABA level reached the pre-stress level (Zeevaart, 1980). This indicates that ABA catabolism is important in maintaining ABA levels in plants. The pathway for ABA catabolism in plants occurs through oxidative degradation of ABA into phaseic acid (PA) and PA is subsequently reduced to dihydrophaseic acid (DPA) or by conjugation of sugars to ABA (Cutler and Krochko, 1999; Finkelstein, 2013; Hauser et al., 2011; Nambara and Marion-Poll, 2005). The evidence showing PA and DPA as the metabolites of ABA catabolism was obtained by using cell free systems and labelled ABA in eastern wild cucumber (Gillard and Walton, 1976), in sugar beets (Daie et al., 1984), in mesophylls of Vicia faba L. and Commelina communis L. (Grantz et al., 1985) and in

chick pea (Babiano, 1995). The first reaction in ABA metabolism process is catalyzed by ABA induced enzyme (+)-ABA 8'-hydroxylase by adding a hydroxyl group at position 8 resulting in the formation of 8'-hydroxy ABA. The intermediate 8'-hydroxy ABA is an inactive intermediate and is rapidly converted to PA by isomerization (Cutler and Krochko, 1999; Nambara and Marion-Poll, 2005). The (+)-ABA 8'-hydroxylase is induced by ABA and exhibited reduced induction during water stress in maize (Cutler et al., 1997). The enzyme (+)-ABA 8'- hydroxylase is later identified as a cytochrome P450 monoxygenase (Krochko et al., 1998). Phylogenetic and microarray analysis of Arabidopsis cytochrome P450 genes resulted in the identification of CYP707A (CYP707A 1 to CYP707A 4) family of genes that encode the enzyme (+)-ABA 8'-hydroxylase. The seeds from CYP707 family mutants have high levels of ABA and exhibit long dormancy period (Kushiro et al., 2004). The expression analysis of CYP707A family genes show their different roles of four members of the family during seed development and maturation (Okamoto et al., 2006).

Abscisic acid is also present in plants in an esterified form mostly as glucosyl ester conjugated ABA (GE-ABA). The enzyme glucosyltransferases adds the glucosyl ester to ABA and this conjugated form of ABA is inactive. The permeability of GE-ABA through the membranes is very low and present mostly in the apoplast, vacuoles are considered as a storage and transport form of ABA (Cutler and Krochko, 1999; Finkelstein, 2013; Ye et al., 2012). The inactivate GE-ABA is hydrolyzed by the drought stress induced enzyme β-glucosidase (AtBG1) in Arabidopsis and the end product of this reaction is ABA in an active form (Lee et al., 2006).

ABA transporters

When plants are subjected to limited water conditions, water stress is first sensed by the roots due to change in the turgidity and ABA is produced in the roots. The ABA produced in roots is transferred to the site of action in leaves and cause stomatal closure even when the turgor of leaves is unaffected. Experiments with air dried roots and roots incubated with ABA from pea and commelina cummis showed that ABA produced or supplied to the roots is transferred to the leaves (Zhang and Davies, 1987). ABA is a weak acid with a pka of 4.8 and in acidic environment the protonated and uncharged form of ABA is predominant compared to the anionic form. The cytoplasm has a neutral pH and the pH difference drives the passive movement of protonic ABA across the lipid bilayer into the cytosol. In the cytosol most of the ABA is present in charged anionic form preventing it from leaving. During water stress the pH of xylem and apoplast increases thus making the pH driven movement difficult during stress (Boursiac et al., 2013; Kang et al., 2010; Seo and Koshiba, 2011; Zhu, 2002) . Considering the fact that ABA is a stress hormone, transport of ABA during stress conditions is important and suggests the presence of ABA transporters. The identification of auxin transporters, presence of ABA in the xylem sap (dead cells) and identification of ABA receptors suggested presence of controlled and efficient transport system for ABA (Davies et al., 2005).

Kuromori et al., (2010) identified a plasma membrane localized ABA transporter AtABCG25 belonging to the family of ATP-binding cassette (ABC) transporters in Arabidopsis. The AtABCG25 gene is induced by ABA and expression analysis indicated that it is expressed mainly in vascular tissues and in roots. The AtABCG25 encodes an

exporter protein, which exports ABA from the site of biosynthesis to guard cells resulting in the stomatal closure. The AtABCG25 overexpressing plants have less transpiration rates and atabcg25 mutants are highly sensitive to ABA treatment (Kuromori et al., 2010). A pleiotropic drug resistance transporter PDR12, also known as AtABCG40, was identified as a plasma membrane located ABA importer in plant cells. The mutant atabcg40 plants have lower ABA transport (30%), guard cells in the mutants showed delayed response to ABA and were sensitive to water stress (wilted), ABA responsive was delayed (Kang et al., 2010). Further studies in the area are needed to identify other transporters in different plant tissues and to understand the fine regulation of ABA transport.

Core ABA signaling pathway

ABA signaling is a very complex pathway, with crosslinks to many other signaling pathways. Some of the components of the ABA signaling pathway have been identified and many components are yet to be identified. The research in the field led to identification of "ABA core signaling pathway" (ABA-PYR/PYL/RCRAR-PP2C-SnRK2s) and is wildly accepted (Cutler et al., 2010). Many components of the ABA signaling pathway directly or indirectly affect each other (Guo et al., 2011; Kuromori et al., 2010; Raghavendra et al., 2010). A brief summary of important discoveries that led to the identification of the well accepted "ABA core signaling pathway" is listed below.

The ABA-insensitive1 (ABI1) gene of Arabidopsis thaliana encodes a serine/threonine protein phosphatase 2C (PP2C) and is one of the first evidence regarding the component of the core signaling pathway (Leung et al., 1994; Meyer et al., 1994). The protein phosphatase activity of PP2C protein is confirmed by using 32P-labeled

casein as a substrate (Bertauche et al., 1996). Later in the year 1997, search for homologues of ABI1 genes resulted in the identification of ABA2 locus, which encodes another protein phosphatase 2C (Leung et al., 1997; Rodriguez et al., 1998).

The ABA-insensitive mutants, abi1-1 and abi2-1 have abnormal reduced seed dormancy and can germinate in the presence of ABA, abnormal stomatal aperture and ABA responses. The ABI1 and ABI2 genes encode for two Mg2+ and Mn2+ dependent PP2C, which are key players in the core signaling ABA pathway. Studies with abi1, abi2 and abi1 and abi2 double mutants show that ABI1 PP2C and ABI2 PP2C play a partially redundant roles in ABA signaling. (Leung et al., 1997). PP2C are evolutionarily highly conserved in both plants and animal kingdoms. The subcellular location of PP2C might help in regulating their activities (Rodriguez et al., 1998). The use of loss of function mutants of ABI1 PP2C1, showed that PP2C acts as a negative regulator of ABA signal transduction (Gosti et al., 1999). ABI2 PP2C is also reported to function as a negative regulator of ABA and the activity of ABI2 PP2C increases in response to ABA (Merlot et al., 2001).

The presence of ABA-activated protein kinase in the ABA signaling pathway is supported by the identification of ABA activated P42 and P44 protein kinases in Arabidopsis suspension cells. The ABA activated P42 and P44 protein kinases were encoded by SNF1- related protein kinase 2 (SnRK2) genes. The snrk2e (p44) mutant showed a wilty phenotype due to the insensitivity of stomata closure to ABA. The SnRK2E/OST1 is induced in response to low humidity/drought (Yoshida, 2002). Later, members of the same group showed that the ABA activated kinase Snrk2E/OST1/Snrk2.6 interacts with ABI1 PP2C but not ABI2 PP2C by using yeast two hybrid binding assay.

The SnRK2E is inactivated by ABI1 but not ABI2 by direct binding at C terminal of SnRk2E (Yoshida et al., 2006). Fujitha et al. (2009) showed that three SnRK2 genes namely SnRk2D/SnRk2.2, SnRK2E/ SnRk2.6/OST1, and SnRk2I/ SnRk2.3 are main positive regulators of ABA signaling. The kinase SnRk2D is shown to interact with ABI1 PP2C and HAI1 PP2C, the upstream components of ABA signaling pathway (Fujita et al., 2009). These results established SnRk2 kinases as targets of PP2Cs and as positive regulators of ABA (Fujita et al., 2009; Yoshida et al., 2006).

Screens for potential binding proteins of ABI2 using yeast two hybrid system lead to the identification of regulatory component of ABA receptor 1 (RCAR1), which belongs to a RCAR family proteins with 14 members grouped into three sub families (RCARI, II, III). The RCAR1 and RCAR1–like proteins (RCAR3, 8, 12) can interact/bind to ABI1 and ABI2 PP2Cs and bimolecular fluorescence complementation results showed the localization of the complexes in both cytosol and nucleus. The phosphatase activity of ABI1 and ABI2 is lost in the presence of ABA and RCAR1, indicating the ABA dependent inactivation/regulation of PP2C by RCAR1 or RCAR1-like protein receptors. The recognition/binding of ABI1, ABI2 PP2C by RCAR proteins is lost in the abi1 and abi2 mutant (both mutants have single amino acid glycine at position 168 changed to aspartine). The phosphatase activity of abi1 and abi2 is unaffected and they effectively regulated SnRk2 kinases in a negative fashion. This indicates that the ABA insensitive phenotype of abi1 and abi2 mutants is due to their inability to bind the ABA receptor. The ABA dependent inactivation of SnRK2s by PP2C is lost in abi1 and abi2 mutant (Ma et al., 2009). During the same time, an independent study using pyrabactin, an ABA antagonist lead to the identification of 12

PYRABACTIN RESISTANCE 1 (Pyr1) mutant alleles. PYR1 has a conserved hydrophobic ligand binding pocket, and is a member of START super family of proteins. Arabidopsis genome contains about 13 genes encoding PYR1 like proteins namely, PYL1-13. The PYR/PYL family of proteins bind class I /clade A PP2C (including ABI1, ABI2, HAB1) and inactivate them in an ABA dependent manner. Similar to RCAR1 proteins, the binding of PYR/PYL proteins is lost in abi1 and abi2 mutants (Park et al., 2009). The crystallographic structure of PYR1 in Arabidopsis is presented by (Santiago et al., 2009) and they described the chemical interactions contributing to the binding of ABA to the ligand. The information from trinary structure of ABA-ligand PYR-PP2C complex explained the increased affinity of receptor (PYR1) to the phosphatases in presence ABA. These results establish PYR, PYL, RCAR family of START domain containing proteins as receptors of ABA, which negatively regulate PP2C activity in an ABA dependent fashion.

In short, the ABA perception and signaling in the "core signaling pathway" is presented (Figure 1). The PYR, PYL, RCAR PYR1 family of protein are ABA receptors recognize, bind ABA and initiate the ABA signaling pathway. The binding of ABA to the receptor PYR1 creates a binding site for the phosphatases PP2C and in the presence of ABA these START domain containing proteins bind to the downstream phosphates with increased affinity. The binding of PP2C to the PYR1- ABA complex results in the inactivation (negative regulation) of PP2C. Inactivated PP2C cannot dephosphorylate the SnRK2s (SNF-related Kinase 2) and the SnRK2 remains in an active phosphorylated state. In their active state the SnRK2 kinases phosphorylate the downstream targets such as transcriptions and ion channels and promote the ABA signal. In the absence of ABA,

the PP2Cs are in the active form and bind to the SnRK2 kinases and deactivate them by dephosphorylating the ser/ther residues present in the activation loop. In the dephosphorylated state the SnRk2 kinases are inactive and the ABA signaling comes to a halt.

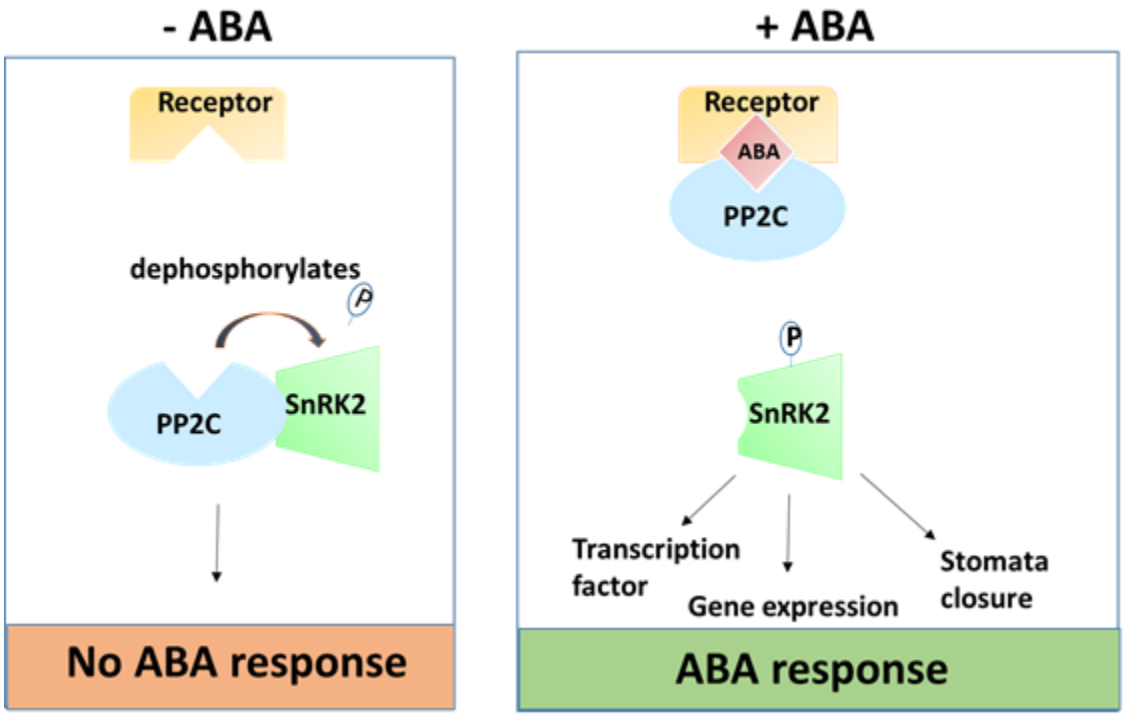

Figure 1 ABA core signaling pathway

ABA core signaling pathway in the absence of ABA (left). In the absence of ABA, PP2C group of phosphatases bind, dephosphorylate SnRK2 kinase rendering the kinase inactive. ABA core signaling pathway in the presence of ABA (Right). In the presence of ABA, The ABA receptors (PYL, PYR and RCAR) and PP2C bind to ABA and form a complex. The SnRK2 kinase is relieved from the negative regulation by PP2C. The active SnnRK2 phosphorylated the downstream target elements like transcription factors, ion channels, target proteins and is responsible for ABA response signal.

ABA signal transduction

The kinases belonging to the SnRK2 family are the positive regulators of ABA signaling mechanism. In addition to the SnRK2 kinases, calcium dependent protein

kinases (CDPKs), mitogen-activated protein kinases (MAPKs, MAPKKs, MAPKKKs), calinerium B-like interacting protein kinases (CIPKs) are involved in ABA signaling (Finkelstein, 2013). The kinases like SnRK2s, CDPKs that are activated by the ABA dependent inactivation of protein phosphatase (PP2c), are known to have many targets including transcription factors, ion channels and many kinases. The CDPK-SnRK2s phosphorylated transcription factors regulate the expression patterns of many ABA-regulated genes. ABA signal is known to play a key role in regulating stomatal closure, and many of the ABA biosynthesis mutants have altered stomatal closure. The role of ABA in regulation of gene expression, stomatal closure, and root growth is detailed below.

ABA regulated gene expression

ABA levels in plants increase in response to stress and regulate the expression of many genes. The expressed genes and their products help plants tolerate and survive the stress. Expression of some ABA responsive genes is slow and they require new protein biosynthesis. Expression of some genes do not require protein biosynthesis and their induction is fast, usually within 20-30 minutes of ABA application or induction of stress. Stress induced the expression of genes involves both ABA-dependent and ABA-independent pathway. DNA sequence analysis of promoter regions of ABA induced genes showed that some of the ABA induced genes contain cis-acting ABA responsive element (ABRE). Some of the main ABA induced genes do not contain ABRE sequence.

One of the first reports of ABRE was by Marcotte et al., (1989). They identified a 9 base pair conserved sequence: ACGTGCCGC as ABRE in a ABA responsive Em gene in wheat (Marcotte et al., 1989). Later, ABRE (TACGTGGR, R can be any purine) was

identified in ABA induced rab-16A gene in rice (Mundy et al., 1990). ABRE (CCACGTGG) involved in drought response were identified in maize rab28 (Pla et al., 1993). There are several reports of cis-acting ABREs in Arabidopsis, rice, maize and tobacco (Fujita et al., 2011). The ABRE is a 9-10 base pairs long consensus sequence with an ACGTG core. The ACGT core containing ABRE is considered as a part of Cis-element called G-Box. Some ABA induced genes have more than one copy of ABRE. For the ABRE to be fully functional it needs other cis acting coupling elements (CE) to be present. A minimal ABRE complex (ABREC), that has a ABRE and a CE are necessary for ABA mediated induction of Late embryogenesis abundant (Lea) gene HVA1 was defined in barley (Shen et al., 1996).

Plant 14-3-3 proteins are acidic in nature and they bind to phosphorylated proteins. They function in regulation of nitrogen and carbon metabolism and ion homeostasis. In barley expression of five 14-3-3 proteins (14-3-3 A, 14-3-3 B, 14-3-3C, 14-3-3D, 14-3-3E) is regulated by ABA and 14-3-3C, 14-3-3D, 14-3-3 E proteins are shown to interact with ABRE/ABF family of bZIP transcription factors (HvABF1,2,3) in a specific manner. The transcription factors (HvABF1, 2, 3) also interact with ABA responsive kinase PKABA1, which mediates the crosslink between the GA and ABA pathways. The OPEN STOMATA (OST)1, a SnRK2 kinase family protein kinase, phosphorylates bZIP transcription factor ABI3 and creates a 14-3-3 binding motif in Arabidopsis (Schoonheim et al., 2009; Schoonheim et al., 2007; Shin et al., 2011; Sirichandra et al., 2010).

Number of transcription factors that recognize and bind to the ABRE sequence have been identified. Many of the identified transcription factors belong to the family of

basic region containing leucine zipper domain (bZIP) transcription factors. The basic domain helps them bind to the DNA and leucine zipper domain is required for dimerization. There are 75 different bZIP transcription factor encoding genes in Arabidopsis. bZIP transcription factors are classified into ten groups and reviewed in detail by Jakoby et al. (2002).

A group of ABA/stress inducible transcription factors (ABF1, ABF2, ABF3) belonging to the family of basic leucine zipper (bZIP) transcription factors with high binding affinity to ABRE sequence have been identified in Arabidopsis seedlings treated with ABA or high amounts of salt by using yeast two hybrid approach. These transcription factors recognize and bind to ABRE and are called ABRE binding factors (ABF). These transcriptions are inducible by ABA and stress with different expression pattern in response to the stress (Choi et al., 2000). The ABI5 gene in Arabidopsis encodes a bZIP transcription factor called ABI5. The abi5 mutant plants have ABA insensitive phenotype and the expression of ABI5 is regulated ABA and other ABI genes. ABI5 regulates the expression of many lea genes (Finkelstein and Lynch, 2000). ABI5 might recruit a transcriptional activator to the promoter regions of lea genes (Jakoby et al., 2002). ABF3, ABF 4 are AREB recognizing bZIP transcription factors and function in ABA signaling. The ABF overexpression mutants are drought resistant, ABA hyper sensitive to germination (Kang et al., 2002)

Many drought inducible genes are regulated by ABA, but ABA independent induction of gens also occurs in response to drought stress and other types of stress. The rd28A and rd28B genes are expressed in response to dehydration in Arabidopsis. The promoter of rd28A contains an ABRE and two dehydration response elements (DRE), a 9

base pairs sequence containing TACCGACAT and its expression is induced in response to both ABA and dehydration. But the rd28B contains only the ABRE sequence and its expression in response to dehydration requires ABA signal (Yamaguchi-Shinozaki and Shinozaki, 1994). Three ABRE-binding proteins (AREB 1, AREB2, AREB3), containing a bZIP domain are induced by ABA, salt stress and drought, and are identified by yeast two hybrid screens. AREB1/ ABF2, AREB2/ABF4 are transcription factors that regulate the expression of rd28B gene in response to ABA by selectively binding to the AREB sequence. The expression of ABF2/AREB1 transcription factor gene is enough to regulate the expression of downstream ABRE containing stress/ABA regulated genes (Fujita et al., 2005; Uno et al., 2000).

Transcription factors AREB1, AREB2 are active in their phosphorylated state and their phosphorylation is regulated by ABA (Uno et al., 2000). The SnRK2 kinase family member and positive regulator of ABA signaling, OPEN STOMATA (OST) 1 phosphorylates bZIP transcription factor ABI3 and creates a 14-3-3 binding motif. ABI3 controls the expression of many ABA induced genes (Sirichandra et al., 2010). These results agree with the fact that transcription factors are the main targets of the SnRk2 family of kinases in the ABA signal pathway. The drought inducible promoter region of gene rd22 does not contain an ABRE but it contains cis acting MYC and MYB elements, and these elements are recognized by transcriptional factors AtMYC2 and AMYB2, respectively. AtMYC2 and AtMYB2 act as transcriptional activators of the expression of rd22 gene in ABA regulated response to drought (Abe et al., 2003). New protein synthesis of transcription factors AtMYC2 and AtMYB2 is required for the action of the genes.

The Arabidopsis drought inducible, ABA regulated rd29A gene also contains an ABRE sequence and DRE element (A/GCCGAC). The DRE element acts as coupling element for ABRE. Both DRE and ABRE are required for the ABA regulated induction of rd29A gene (Narusaka et al., 2003). In addition to the activation of transcriptions factors by phosphorylation ABA also plays a role in mRNA maturation and processing. The RNA polymerase II (RNAP II) is regulated by ABA during stress (Himmelbach et al., 2003).

The AREB1/ ABF2, AREB2/ABF4, ABF3 were previously identified as transcription factors that bind to ABRE sequences. Yoshida et al. (2010) showed that these three transcription factors can form homo or hetero dimers and are activated by ABA. They have over lapping functions and cooperate to regulate the expression of many ABRE containing genes. The triple mutant areb1 areb3 abf3 is very resistant to ABA and the expression of many stress responsive genes are impaired (Yoshida et al., 2010).

In summary, the expression pattern of many genes are altered during osmatic stress. During drought stress regulation of genes can occur in two pathways: ABA independent pathway and ABA dependent pathway. Many ABA responsive genes contain a cis acting elements called ABRE (ACGTGCCGC) that can be present in multiple copies or along with another cis-acting elements called coupling elements (CE). The cis–acting elements are recognized by many transcription factors, like bZIP transcription factors (like AREBs, ABFs, ABI5 etc.). These transcription factors require ABA for activation and the main targets of SnRK2 family of kinases. Some of the drought regulated genes also have a cis-acting element called dehydration response elements (DRE). Some DREs containing genes are ABA responsive while others were

not, suggesting that some of them are in the ABA-independent pathway. In addition to the ABRE and DRE containing genes, there are many other genes that are regulated by ABA but do not contain any known cis acting element and the precise nature of their ABA regulation still remains unknown.

ABA induced proteins

ABA induces the expression of genes and many of these genes encode for proteins that help plants survive during stress. ABA induces the production of enzymes involved in synthesis of solutes like sucrose, proline, tretalose and also reduces the catalytic activity of the enzymes involved in their metabolism. These solutes help in maintaining the osmatic balance and thus maintain the turgor. Aquaporin RD28 is induced in response to ABA/drought stress and it permits increased water flow to the cells. Enzymes involved in protein repair such as heat shock proteins are upregulated during water stress and their expression seems to be independent of ABA. Ubiquitin (UBQ1), which marks the proteins for degradation, is induced during the early stages of drought stress. Proteases are induced by drought and they degrade the misfolded polypeptides and storage proteins during stress conditions. Free radical scavenging enzymes like catalase, superoxide dismutase and ascorbate peroxide increase in response to ABA during stresses conditions. Lea proteins, histone variant (H1), cell wall proteins accumulate in response to ABA and many stress conditions (Campalans et al., 1999).

ABA mediated stomatal closure

The guard cells are a type of epithelial cells that are present in pairs, one on either side of stomatal pore. The opening and closing of the stomatal pore is regulated by

changes in the ionic concentration that is regulated by ion channels present in the membrane and vacuoles. The changes in the ion concentration results on either side of the membrane results in the osmatic diffusion of water. The movement of water causes changes in cell turgor and stomatal movements (Fujii et al., 2009). The Ca2+ is a known second messenger in many signaling pathways and it also participates in ABA signaling. The cytosolic concentration of Ca2+ is known to increase in response to ABA (Hauser et al., 2011; McAinsh, 1990).

The ABA dependent inactivation of PP2C results in the activation of the SnRK2E/OST1, certain members of CDPK-SnRK2 family kinases, these active kinases then phosphorylate and activate the S-type anion channel, SLAC1 and inhibits the inward rectifying potassium channel (KAT1) in the cytoplasm and vacuole. The induction of SLAC1, which permits chloride and nitrate into the cytoplasm, the inhibition of KAT1 channels by SnRK2E results in the outward flow of ions. In addition to induction of SLAC1 channel, ABA also causes the inhibition of Proton-ATPase. These ion channels are also controlled by ABA mediated increase cytoplasmic Ca2+ (cytoplasmic Ca2+ oscillation signature) (McAinsh, 1990). The Ca2+ mediated stomatal closure is through the action of CADPK-SnRK kinases (Cheng et al., 2002a).

Induction of SLAC1 and inhibition of KAT1 and proton coupled ATPase causes initial depolarization of the membrane (Himmelbach et al., 2003; Raghavendra et al., 2010; Sirichandra et al., 2009). The initial depolarization of the membrane caused by action of SLAC1 and KAT1 ion channels causes the activation of the voltage gated K+ outward rectifying channel (GORK), resulting in outward movement of K+ ions and changes in cell turgor and stomata closure in response to ABA (Sirichandra et al., 2009).

Nitrogen assimilation and abscisic acid

Nitrogen element (N) is a very important and often a limiting factor for plant growth. Most plants get their N from soil nitrate (NO3) and ammonium (NH4+) present in the fertilizers that are added to the soil. The process of nitrogen acquisition and assimilation is highly complex and the plants need to sense the availability of nitrogen and regulate their growth, formation of lateral roots and adjusting root to shoot growth based on the nitrogen availability signal (Kiba et al., 2011). The growth and development is regulated by coordination of nitrogen availability signal and signal from hormones ABA, cytokines, auxin. Nitrogen metabolism is linked to carbon metabolism and also the process like respiration and photorespiration. (Kiba et al., 2011; Krouk et al., 2011; Nunes-Nesi et al., 2010) .

High concentrations of nitrate (NO3-) have an inhibitory effect on lateral root growth and low concentrations of NO3- tend to initiate lateral root growth. Using ABA insensitive mutants and ABA synthesis mutants, Signora et al. (2001) showed that ABA regulated transcription factors ABI4 and ABI5 are required for the inhibitory effect of NO3- and elongation of lateral root growth at low concentrations of NO3- (Signora et al., 2001).

In the recent years there have been reports indicating the involvement of ABA in regulation of enzymes in nitrogen metabolism pathway. Expression of certain genes involved in nitrogen transport and assimilation are regulated by ABA (Krouk et al., 2011). The activity of the enzyme nitrate reductase (NR), which catalyzes the conversion of nitrate absorbed by the plants into nitrate (NO2-) in the cytosol is induced by ABA in transgenic tobacco plants. In Arabidopsis the NR is encoded by the genes NIA1 and

NIA2 and the NR produced nitric oxide (NO) is needed for stomata closure in guard cells. The nitric oxide (NO) produced by the enzyme NR is needed for ABA, hydrogen peroxide induced production of antioxidant scavenging enzymes superoxide dismutase (SOD), catalase (CAT), ascorbate peroxide (APX) and stomatal closure (Desikan et al., 2002; Lu et al., 2014). The activity of the enzyme NR was also shown to increase in response to ABA in a dose independent fashion in chicory plant roots. The experiments using chicory plants grown on ABA containing media showed increased expression of mRNA transcripts of nitrogen assimilation pathway enzymes NR and glutamine synthase (GS) (Goupil et al., 1998).

Despite lot of research and understanding in the areas of nitrogen assimilation process, and ABA signaling, our understanding of the ABA mediated regulation of many other enzymes involved in nitrogen assimilation pathway is limited. The enzyme aspartate aminotransferase (AAT) is linked to nitrogen, carbon metabolism pathways, protein biosynthesis and thus plays an important role in regulating the nitrogen and carbon flux in plants. AAT is a part of malate-aspartate shuttle that transports the reducing equivalents between the organelles and cytoplasm (Lam et al., 1996; Schultz et al., 1998; Wilkie and Warren, 1998). The AAT catalyzes the reversible transamination of aspartate, oxoglutarate to form glutamate and oxaloacetate. AAT enzyme is very well studied in animals and is routinely used in diagnostic services as a test for certain liver conditions. AAT in plants exists in multiple isoforms and is characterized by Schultz et al. (1995). The hormonal and stress mediated regulation of AAT isozymes remains to be elucidated.

In-gel activity analysis

In plants many enzymes are encoded by a family of genes and number of isozymes for a particular enzyme are present. For example, glutamine synthase, catalase, nitrate reductase, aspartate aminotransferase, alanine aminotransferase have multiple isozymes and are localized to different cellular compartments. The response of isozymes of a particular enzyme to external stimuli like drought, cold, salt stress or nutrient availability differs from each other. The traditional or commonly used in-solution analysis method measures the total activity of all the isozymes. The increase or decrease in activity of a particular isozyme present in a crude extract might be underestimated when using the in-solution analysis or it needs an extensively purified isozyme. In-gel activity analysis is a very sensitive method for in situ visualization of different isozymes present in a crude extract. In gel activity analysis has been successfully used for the visualization, identification and determination of changes in activities of the enzymes in response to external stimuli.

In–gel activity stain was used for the identification of guanyl-specific ribonuclease in fungus (Blank and Dekker, 1975), cellulases from thermophilic bacteria (Schwarz et al., 1987), glutamine synthase in rice (Zhang et al., 1997). The in-gel activity analysis is quantitative and can be used to study the changes in the activities of isozymes in response to an external stimuli. It is used to study the changes in activities of super oxide dismutase, ascorbate peroxide, glutathione reductase in response to cold in cumber leaves (Lee and Lee, 2000). The expression pattern of catalase isozymes in response to ABA in Arabidopsis is studied using in-gel activity stain (Du et al., 2008).

For the in-gel activity stain, the crude protein extract was separated by poly acrylamide gel electrophoresis (PAGE), after the separation the gel is then incubated in a reaction mixture containing appropriate buffer and substrates specific for the enzyme along with a dye. The dye is changed by the enzymes of interest during the course of reaction and is converted in to a colored or fluorescent insoluble product at the site of the reaction. The colored or fluorescent product stains the site of reaction, enzyme location and is proportional to the activity of the enzyme. Using the in-gel activity analysis the isozymes of a particular enzyme, or different enzymes catalyzing the same reaction can be specifically stained/visualized and quantitatively measured.

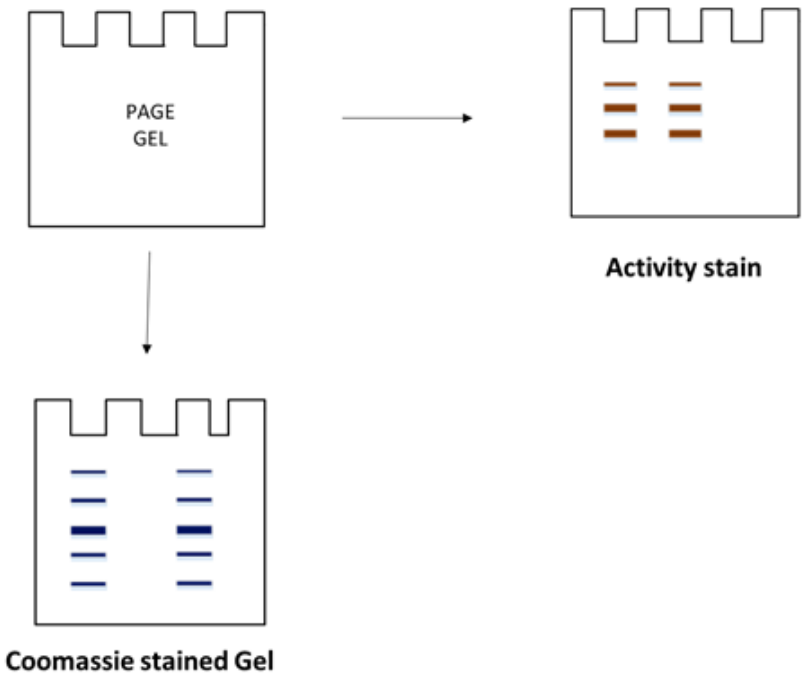

Figure 2 Representation of activity analysis

Crude protein extract was separated by PAGE under native conditions and stained by using in-gel activity stain. In-gel staining is usually performed by incubating the PAGE gel with dye along with a reaction mixture. The dye is changed by the enzymes and is converted in to a colored or fluorescent insoluble product that stains the position of the enzymes. Thus In-gel activity stain leads to the visualization of specific enzymes or isozymes catalyzing the same reaction, whereas coomassie staining of the PAGE gel leads to the visualization of many proteins present in extract.

Histone post translational modifications

The nuclear DNA in chromatin is associated with basic proteins called histones. Nucleosome, the basic repeating unit of the chromatin, consists of 147 base pairs of DNA wrapped (1.65 turns) around the octamer of core histones containing two copies of H2A, H2B, H3, and H4. The mononucleosomal units are connected by a linker DNA 10-50 base pairs along with a linker histone H2A. The nucleosome spacing and positing is generally considered to be a major determinant of chromatin structure. The nucleosome structures are highly dynamic and can be released, swapped and slided to change positions on the DNA. The N-terminal tails of the core histones, that protrude out from the core structure are highly accessible and are modified post-translationally. Methylation, phosphorylation, ubiquitination, acetylation, ADP-ribosylation, glycosylation and sumoylation are few such modifications that are observed on the histones tails. The post translational modifications generate distinct marks on the histones contributing to the diversity of the histones. These post translational modifications of histones can affect the DNA packaging into the nucleosomes, thus affecting the organization of chromatin and chromatin associated process. The effector/reader proteins recognize the post translational modifications and are responsible for the downstream biological process (Johnson et al., 2004; Lachner and Jenuwein, 2002; Martin and Zhang, 2005; Sims et al., 2003).

Histone lysine methylation

Histones can be methylated on both lysines (K) and arginines (R) (Volkel and Angrand, 2007). Lysine (K) residues of histone H3 at positions 4, 9, 27, 36 and 79 and lysine 20 on histone H4 can be methylated. Lysines at these positions can be modified by

addition of mono-, di-, tri- methyl groups. The position of modification and the methylation state affect the chromatin state and structure, thus altering the transcriptional activity of the genes associated. Addition of methyl groups on to the lysines present in the histones does not change the net charge nor add considerable bulk as acetylation, but increases the hydrophobicity and affects the intra and inter molecular interactions. The histone lysine modification occurs in a combinatorial fashion and can be linked to activation or repression of gene expression based on the site and methylation state. Presence of tri-, di- methyl groups at lysine 36 in histone H3 is linked to active transcription of flowering time controlling genes, whereas the presence of monomethyl group is not (Xu et al., 2008). Methyl groups are added on the histone lysines by a group of enzymes called histone lysine methyl transferases (HMKT). All the known HMKTs, except for DOT1L, contain a SET domain (Su(var), Enhancer of zeste, trithorax) and they can add either mono-, di-, tri- methyl groups to the lysines based on their specificity. Enzymes containing SET domains were characterized as histone methyl transferases. Three dimensional structures of SET domain containing proteins explaining the mechanism for catalysis, and product specificity (mono-, di-methyl group) were reported (Volkel and Angrand, 2007; Yeates, 2002; Zhang and Bruice, 2008).

The histone lysine methylation is dynamic and is removed by a group of enzymes called histone demethylases (Liu et al., 2010; Martin and Zhang, 2005; Nottke et al., 2009; Pontvianne et al., 2010). Although different genes encoding putative histone methyl transferases and demethylases are identified in plants and mammals, many of them are yet to be characterized. Many chromatin associated proteins that recognize the specific histone modifications are yet to be identified. The identification of effector/

reader proteins of lysine modifications can provide an insight to the understanding the biological effects of histone lysine modifications.

Histone acetylation

The conserved N-terminal tails of histone H3 in plants and H4 in animals are predominantly acetylated. The histone acetyl transferases (HAT) add a acetyl group from acetyl co A to the Ɛ-amino group of lysines (Sterner and Berger, 2000). The acetylation is dynamic and is removed by the action of a group of enzymes called histone deacetylases. Histone acetyl transferase, HATA, was first identified in terahymena as a homologue of transcriptional coactivator Gcn5 in yeast (Brownell et al., 1996). Acetylation neutralizes/ masks the positive charge of the histone and there by weakening the interaction between the negatively charged DNA and positively charged histones and opens chromatin for transcription machinery.

Acetylation of histone residues is reversible and is often linked to transcriptional activation (Brownell et al., 1996; Sterner and Berger, 2000). Number of histone acetyl transferases and deacetylases have been characterized (reviewed by (Fukuda et al., 2006; Kimura et al., 2005; Lusser et al., 2001)). Mass spectrometry has enabled the identification of many acetyl modified non histone proteins, transcription factors and many remain to be elucidated.

WD repeat proteins

WD-40 domains are abundant, versatile protein interacting domains. WD repeat containing proteins are part of histone, chromatin modifying complexes and are also implicated in RNA processing, cytoskeleton assembly, cell division control, signal

transduction, and regulation of transcription. WD-40 repeat was first identified in GTP binding protein (G protein), bovine-β-transdusin. The WD-40 domain containing proteins have 4- 10 WD domains and most of them contain about 7 WD-40 domains and form a β-propeller structure with seven blades. WD-40 domains are classified into 30 families/groups based on proteins present on the surface. Till to date no WD 40 domain is reported to have an intrinsic enzyme activity. In addition to the WD-40 domain most of the WD 40 domain containing proteins contain other types of domains with enzymatic activity. Even though WD domains have no enzymatic activity, they have many protein interacting sites and act as protein-protein, protein-DNA interacting platforms for complex assemblies (Stirnimann et al., 2010)

WDR5, a WD domain containing protein, is part of histone methyltransferases (Set1, MLL, MLL2, and MLL3) and histone acetyltransferases (MSL, ATAC, MOF). The WD 40 domain containing embryionic ectoderm development (EED) is a part of polycomb repressor protein (PRC 2) complex (Suganuma and Workman, 2010). Binding of WD repeat containing protein p55 to histone H4 causes a change in H4 structure (Suganuma et al., 2008). The WD 40 domain containing protein WDR5 is a part of trithorax (TRX) histone methyltransferase complex and it has identical binding affinity to unmodified, and all three modified states of K4H3 (Wysocka et al., 2005).

Wang et al., (2013) developed a method for detection and secondary structure prediction of WD 40 domains .WD domain containing proteins are very abundant in eukaryotes and are part of many protein complexes. Not all WD domain containing proteins are characterized and many WD domain interacting proteins are yet to be identified.

Peptide pulldown assay

The N-terminal tails of histones have different post translational modifications (PTM). The effector proteins or reader proteins specifically recognize these modifications and are responsible for the downstream biological effects. In recent years, many proteins (readers) including histone methyltransferases, demethylases, acetylases, deacetylases, transcription factors, chromatin remodeling enzymes and many other nuclear proteins that recognize and bind the histone post translational modifications were identified by analyzing the binding of limited group of candidate proteins with different biochemical approaches (Sims et al., 2006). Yet many of the effector proteins and their functions remain to be identified to get a clear understanding of the role of different histone PTMs.

The readers or histone binding proteins recognize histone sequences that is about 10 amino acids in length, thereby histone peptides with PTMs at specific locations can be used to identify the effector proteins. Joana Wysocka (2006) developed a peptide pulldown assay, an unbiased biochemical approach that uses histone peptide-protein interactions to identify the effector/reader proteins.

The peptide pulldown assay can be used to screen the whole nuclear extract to find the effector proteins, instead of screening predetermined set of limited number of candidate proteins and it does not need the generation of recombinant tagged proteins. The principle/mechanism behind the peptide pulldown analysis is represented in Figure 3. The peptide pulldown assay utilizes the histone peptide-protein interactions to identity the histone PTM binding proteins. Biotin tagged purified histone peptides that were either modified or unmodified were immobilized on to avidin beads.

The nuclear protein extract was incubated with bead slurry and after incubation the beads are washed to remove unspecific binding of proteins. The bound proteins are eluted, separated by SDS-PAGE, and visualized by silver stain.

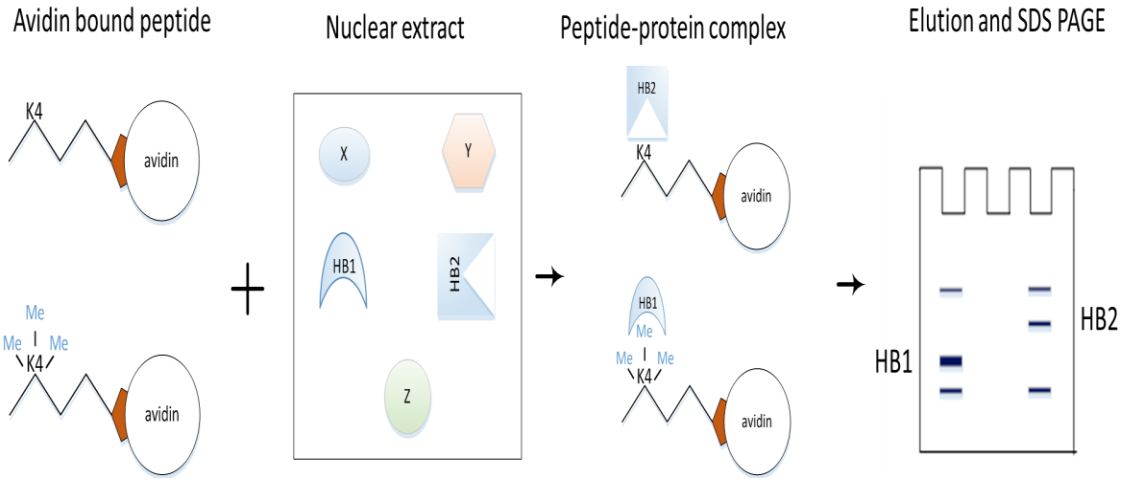

Figure 3 Schematic representation of peptide pulldown assay

Avidin bound modified and unmodified histone peptides are incubated with nuclear extract for 3 hours. Bound proteins are washed, eluted, and separated with SDS-PAGE.

Identification of peptide-protein interactions by far western analysis

Far western analysis is a western blotting like method used to study protein-protein, peptide-protein interactions. It is also known as dot-blot analysis when used to study the peptide-protein interactions. Interactions between DNA repair protein RAD51D and histone (Coleman et al., 2003); yeast nucleosome assembly factor1 and histones (McQuibban et al., 1998); drosophila heterochromatin protein 1 (HP1c) and H3K9me2,3 containing N-terminal histone tails (Font-Burgada et al., 2008) was confirmed using far western analysis. Far western analysis using histone peptides with PTMs dot blotted onto PVDF membrane was used to study the binding affinities of bromo domain containing

proteins (Zhang et al., 2010). Peptides with different post translational modifications immobilized/blotted onto a solid membrane can be used to study the effect of these PTM modifications on protein- protein interactions. Use of peptides in far western analysis also enables the identification of core sequence, and protein domains required for protein interaction. In the far western analysis, peptides are dot blotted on to a solid membrane (PVDF, nitrocellulose), the membrane is blocked and then probed with a recombinant protein (GST-tagged protein probe). The interaction between prey peptide and GST bait can be identified by detecting the bait protein probe with an anti-GST HRP conjugated antibody. Far western analysis can also performed by using peptide as probe instead of blotting them on to a solid membrane. The schematic representation of far western analysis using peptides is presented in Figure 4.

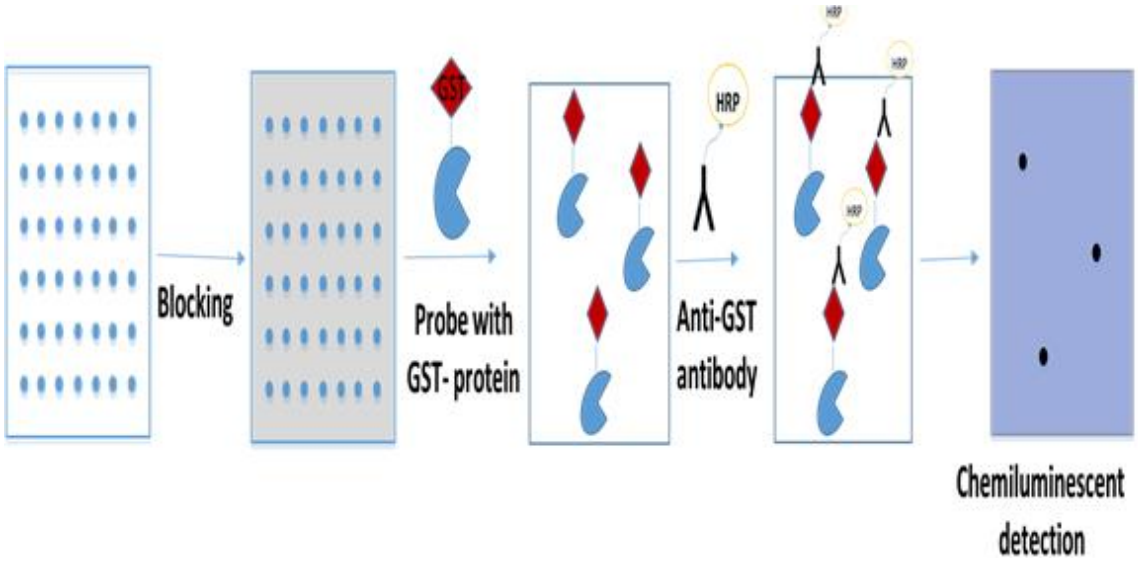

Figure 4 Far western analysis showing the chemilluminiscant based detection of interaction between histone peptides and recombinant GST tagged histone binding proteins

CHAPTER III

IDENTIFICATION OF ABSCISIC ACID REGULATED ENZYMES IN PLANTS

Introduction

In addition to its role in developmental functions, abscisic acid is also required by the plants to adapt to various abiotic and biotic stress conditions (Finkelstein et al., 2002). ABA levels in the dehydrated/wilting plant tissues increase rapidly to reach a new increased state of equilibrium and then ABA levels decrease as the plants are rehydrated and reach pre-stress levels (Zeevaart, 1980). Literature shows that expression of many transcripts, activities of many enzymes are regulated by ABA and many of the drought induced/regulated genes are also regulated by ABA.

Expression analysis of ABA regulated genes in guard cells, mesophylls performed using oligomer-based DNA Affymetrix Genechips in Arabidopsis lead to the identification of 190 transcripts upregulated by ABA in both cell types and 16 of the 190 transcripts were only upregulated in guard cells, 64 transcripts down regulated by ABA. The list of all the genes can be found in Tables 1, 2 and 3 in the paper published by Leonhardt et al., (2004). This result indicates that the expression pattern of ABA regulated genes can be cell type specific and that mRNA levels are modulated by ABA.

Expression and activity analysis of catalase genes in Arabidopsis seedlings showed that the activity of catalase 3 isozyme is upregulated by ABA, drought and oxidative stress (Du et al., 2008; Mhamdi et al., 2010) . Treatment of maize seedling

leaves with low concentrations (10 μM to 100 μM) of ABA resulted in the production of superoxide free radical and hydrogen peroxide, simultaneously launched a defense response against the free radical damage by increasing the activities of radical scavenging enzymes like super oxide dismutase (SOD), catalase (CAT), ascorbate peroxide (APX), glutathione reductase (GR) (Jiang and Zhang, 2001). When rice (Oryza sativa) was subjected to 3 cycles of water stress and rehydration, the activities of the enzymes super oxide dismutase (SOD), guaiacol peroxidase (POX) increased after the first stress cycle. ascorbate peroxide (APX) and glutathione reductase (GR) increased during the during the second stress cycle and catalase (CAT) increased in the final stress cycle and plants survived the stress (Srivalli et al., 2003). Treatment of wheat seedlings with Ca2+ and ABA also resulted in increased activities of the enzymes SOD, APX and CAT (Agarwal et al., 2005). In literature there are many reports indicating the involvement of ABA in stress regulated changes of enzyme activities in plants. The objective was to understand the effect of ABA and drought stress on the enzyme activity profiles in plants. we treated the plants with exogenous ABA, induced abiotic stress and studied the activities of the enzymes like aspartate aminotransferase, phosphatases, hexosaminidase. In-gel activity analysis was used to study the activity profiles of the above mentioned enzymes. Our preliminary analysis data indicate that the activity of free radical detoxifying enzymes catalase, superoxide dismutase and enzyme glutamate dehydrogenase involved in nitrogen metabolism are upregulated by ABA treatment (Figure 23).

Aspartate aminotransferase

Aspartate aminotransferase (AAT; EC 2.6.1.1), is a group I aminotransferase. It exists in the form of a dimer with a molecular mass of 40 kDa. AAT catalyzes the

reversible transamination of aspartate using pyridoxal 5'- phosphate (5' PLP) as co factor in the reaction.

$$\text{Aspartate} + \text{2-oxoglutarate} \leftrightarrow \text{Glutamate} + \text{Oxaloacetate} \quad (1)$$

Molecular analysis by Schultz et al., (1995), indicate that aspartate aminotransferase has multiple isozymes with different cellular localizations and are encoded by a family of four AspAT genes (*AspAT1*, *AspAT2*, *AspAT4 and AspAT5*) in Arabidopsis. The aspartate aminotransferase isozyme AAT1 is localized to the mitochondria and encoded by the gene *AspAT1*. The isozymes AAT2 and AAT4 are present in the cytosol and are encoded by the genes *AspAT2* and *AspAT4,* respectively, but only AAT2 is highly expressed in all the tissues with predominant expression observed in roots. Isozyme AAT3, encoded by the gene *AspAT5,* is localized to the chloroplasts (Schultz and Coruzzi, 1995).

Arabidopsis AAT mutants that are deficient in either cytoplasmic AAT2 (AAT2-1, AAT2-2 and AAT2-3) or chloroplastic AAT3 (aat3-1, aat3-2) were identified by Schultz et al. (1998). The cytoplasmic *AAT2-2* mutants have retarded root growth, altered aspartate and asparagine content. The aspartate levels in the phloem of light grown *AAT2* plants decreased by 80 % and the asparagine levels decreased by 50 % in dark adapted plants. Although, the rest of AAT mutants did not show any signs of delayed root growth or changed amino acid levels. The cytoplasmic AAT2 was hypothesized as major isozyme in aspartate synthesis during light and that aspartate is converted to asparagine during night (Schultz et al., 1998). This hypothesis is further supported by the work of Miesak and Coruzzi et al. (2002) and showed that aspartate and asparagine contents also

decreased in siliques, thus indicating that the cytosolic AAT2 is involved in synthesis of amino acids for storage in seeds (Miesak and Coruzzi, 2002). The subcellular localizations of the AAT isozymes was confirmed in an independent study by Wilkie and Warren (1998). The recombinant AAT isozymes have a molecular weight of 44 -45 kilo Daltons (Wilkie and Warren, 1998). Aspartate along with glutamine, glutamate and asparagine are the main nitrogen carriers/transporting amino acids. Aspartate serves as precursor for the synthesis of the amino acids like asparagine, threonine, isoleucine, leucine, methionine and lysine (Hodges, 2002). The objective was to study the response of AAT isozymes to various external stimuli.

Table 1 Aspartate aminotransferase encoding genes in Arabidopsis and the subcellular localization of their corresponding isozymes

Gene	Isozyme	Localization
AspAT1	AAT1	mitochondria
AspAT2	AAT2	cytosol
AspAT4	AAT4	cytosol
AspAT5	AAT3	chloroplast

Materials and Methods

Plant material and growth conditions

Arabidopsis (Columbia (Col)) ecotype were used in all the experiments and grown as described below. Arabidopsis seeds were surface sterilized by washing them in 70% ethanol followed by 3% bleach. The sterilized seeds were plated onto MS agar

plates and placed at 4º C for 48 hours. Arabidopsis seedlings were grown on MS agar plates for 2 weeks. For the experiments using fully grown/ mature plants, 10 days old seedlings were transferred into soil and grown in growth chambers under 14 hour light and 10 hour dark cycle.

Rice (Oryza sativa) seedlings were grown using hydroponics. Germinated seedlings were floated on half strength Hoagland solution for two weeks by changing the solutions every 4 days. Two weeks old seedlings were used for all the experiments.

Soybean (Glycine max) seeds were grown by using fine sand as a potting mix. One week old seedlings were used for protein extraction from roots. Fully formed first, second or third tripod leaves were used for ABA treatments.

Treatments

ABA treatment

Two weeks old Arabidopsis seedlings grown on MS agar plates were treated with 40 ml of freshly prepared 50µM (±)-ABA for three hours. Mature Arabidopsis plants grown in soil are treated by spraying them with freshly prepared 50µM (±)-ABA for 3 hours.

Soybean seedlings were removed from sand and the roots were washed, blot dried and treated by dipping them in 100 µM (±)-ABA for 4 hours. Soybean plants or seedlings were also treated by spraying them with 100µM (±)-ABA for 4 hours.

Rice seedlings that were two weeks old were treated by dipping the roots in 100 µM (±)-ABA for 3 hours.

Drought treatment

Drought treatment was performed as described by Kim et al. (2011)Two weeks old Arabidopsis, rice and soybean seedlings were removed from their respective growth mediums, washed and blotted with blotting paper to remove excess media and water. They were then placed on parafilm and placed in a growth chamber with 70% humidity for 45 minutes. The control plants were left in the original growing medium.

Salt treatment

Two weeks old Arabidopsis seedlings grown on MS agar plates were treated by adding 40 ml of 150 mM NaCl and incubating for 3 hours. The control plants were treated with double distilled water without NaCl.

After the treatments the plant material was either used immediately for protein extraction or flash frozen in liquid nitrogen and stored at - 80º C.

Protein extraction and quantification

Protein extraction for activity analysis was carried out according to (Zhang et al., 1997). In brief, the plant material is ground into fine powder using motor and pestle in the presence of liquid nitrogen. The ground sample is then quickly transferred in to the extraction buffer (100mM Tris–HCl (pH 7.6), 1mM MgCl2, 1mM EDTA, 1mM DTT, protease inhibitor cocktail (Sigma, catalog number P9599), phosphatase inhibitor) and incubated on ice for 10 minutes. Soluble protein fraction was separated by centrifugation of the homogenate at 12,000 g for 10 minutes. The soluble crude protein extract was quantified using the Bradford assay with purified BSA as a standard.

Native gel electrophoresis

Equal amounts (100 μg) of crude protein extracted from treatment and control samples was separated on either 12% native-PAGE or discontinuous (5-12 %) PAGE. Native-PAGE is performed the same way as the SDS PAGE, except that SDS was omitted from the gel and all the buffers. The electrophoresis was carried out at constant voltage (100V) overnight (14-15 hours) at 4º C. After completion of electrophoresis, the PAGE gel was washed in double distilled water for 10 minutes and then used for the subsequent activity assay.

Activity measurement of aspartate aminotransferase

In-gel activity analysis of aspartate aminotransferase

Equal quantities of crude protein (quantified by Bradford assay) from treated and control Arabidopsis seedlings were separated by 12% native-PAGE as described above. The native-PAGE was then washed with distilled water for 10 minutes and then used for visualization of aspartate aminotransferase isozymes as described by Decker and Rau (1963). The gel was incubated in a reaction mixture containing 50mM Tris-HCl (pH7.5), l-aspartic acid monosodium salt, 2-oxoglutaric acid, and Fast Blue B salt in dark at 37^0 C until dark brown bands appeared on a yellow back ground. After the desired background was achieved the reaction was stopped by removing the reaction mixture and washing the gel with distilled water. The equal loading of the crude protein was ensured/ cross checked by performing a western blot with anti Actin antibody.

In-solution activity analysis of aspartate aminotransferase

Aspartate aminotransferase catalyzes the reversible transamination of aspartate, oxoglutarate to form glutamate and oxaloacetate. The activity of the enzyme aspartate aminotransferase (AspAT) was measured spectroscopically by coupling the formation of oxaloacetate to the oxidation of NADH by malate dehydrogenase (MDH). The assay was performed as follows; AspAT enzyme present in crude extract (100 µg) was added to 2 ml of working reagent mixture (91 mmol/L Tris, 240 mmol/L L-aspartate, 0.16 mmol/L NADH, 0.11 mmol/L pyridoxal phosphate, 0.93kU/l MDH, 0.42kU/l LDH), mixed thoroughly and incubated at 30º C for 10 minutes until a stable absorbance reading was observed. Then the reaction was initiated by adding 200µl of 12 m mol/L 2-oxoglutarate. The decrease in absorbance ($\Delta A/\Delta t$) at 339 nm due to the conversion of NADH to NAD+ is measured and it represents the AspAT activity (Eisenthal and Danson, 1992; Rej et al., 1981; Rej et al., 1973).

Statistical analysis

Two tailed *t*- test was used to analyze the in-solution activity analysis results. The difference/change in activity is considered significant at P value less than or equal to 0.005.

Results

ABA dependent regulation of aspartate aminotransferase

Abscisic acid is known to regulate the expression patterns of many transcripts and activities of many enzymes in plants (Agarwal et al., 2005; Campalans et al., 1999; Chen et al., 2012; Jiang and Zhang, 2001; Schnall and Quatrano, 1992; Srivalli et al., 2003).

Schultz et al. (1998), showed that aspartate aminotransferase (AAT) activity stain is quantitative and very sensitive method. To study the effect of ABA on AAT, we performed in-gel activity analysis with protein extracts from two weeks old Arabidopsis seedlings. The seedlings were treated by exogenous application of 50µM (±)-ABA for 3 hours. Equal amounts of crude extract from Arabidopsis seedlings was separated by using native-PAGE followed by visualization of AAT isozymes as brown bands on a yellow back ground by using in-gel activity stain with Fast Blue B salt as a dye. Equal loading of sample was tested by western blotting (Figure 7). The activity stain with Fast Blue B dye lead to the visualization (Figure 5) of two dominant/major isoforms cytosolic AAT2, and chloroplastic AAT3 and a minor mitochondrial AAT1 isoform, which is only visible when the gel is over loaded. The mitochondrial AAT1 is a minor component in protein extracts from Arabidopsis seedlings and was not always seen in in-gel analysis (Schultz and Coruzzi, 1995).

Different isoforms of aspartate aminotransferase were identified based on their mobilization pattern in comparison with the results published by Schultz and Coruzzi. (1995) . Our results (Figure 5A) show that the treatment with ABA lead to the decrease in both cytosolic (AAT2) and chloroplastic (AAT3) isoforms. The intensity of the signal corresponding to the bands visualized in the zymogram was measured using the "myImage Analysis" software from Thermo scientific and the results indicate that was approximately 65% decrease in activity of the AAT2 isoform and 60% decrease in activity for AAT3 isoform.

To further confirm the decrease in the activity of the aspartate aminotransferase in response to ABA, the total activity/ combined activity of all aspartate aminotransferase

isozymes was measured by using in solution analysis (Figure 5 B) and the results further confirm the decrease in the activity of aspartate aminotransferase enzyme in response to ABA.

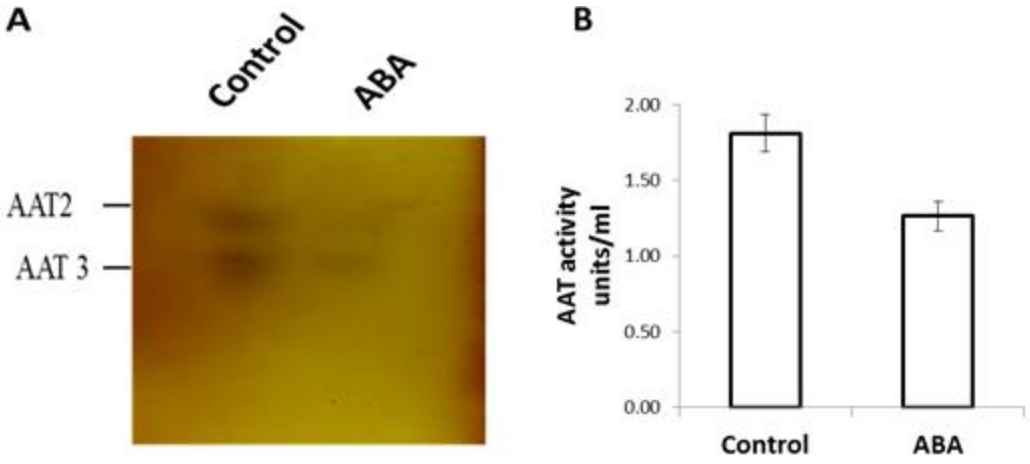

Figure 5 Changes in aspartate aminotransferase activity by ABA treatment

(A) one hundred microgram of crude protein from plants treated with or without ABA was separated by native-PAGE. In-gel activity stain was used to study the changes in activity of aspartate aminotransferase isozymes in response to ABA. Intensity of the band corresponds to the activity of AAT enzyme. (B) Total activity of aspartate aminotransferase was measured spectroscopically at 339 nm due to the conversion of NADH to NAD^+. One hundred µg of crude protein is used for control and ABA treated samples. The activity values are presented as means ± SE in the chart (n=3), statistical significance was calculated with 2 tailed T-test, has a P value of 0.025.

The activity of aspartate aminotransferase isoforms is regulated by drought stress

To assay the activities of the aspartate aminotransferase isozymes under drought conditions, crude protein extracted from water stressed seedlings was separated by native PAGE and AspAT isozymes visualized by in-gel activity analysis. The total activity of AspAT enzymes was measured by in-solution activity analysis. Our results from in gel activity analysis (Figure 6A) showed reduction in the activities of aspartate aminotransferase isozymes cytosolic AAT2 and chloroplastic AAT3 in the seedlings

under limited water conditions. Equal loading of sample was tested by western blotting (Figure 7). The intensity of the signal corresponding to the bands visualized in the zymogram was measured using “myImage Analysis” software from thermo fisher and our results indicate approximately 65% decrease in activity of the AAT2 isoform and 50% decrease in activity for AAT3 isoform. The total activity of the aspartate aminotransferase was measured spectroscopically by coupling the formation of oxaloacetate to the oxidation of NADH with malate dehydrogenase (MDH) and the results (Figure 6) indicate a decrease in activity of the enzyme during water stress.

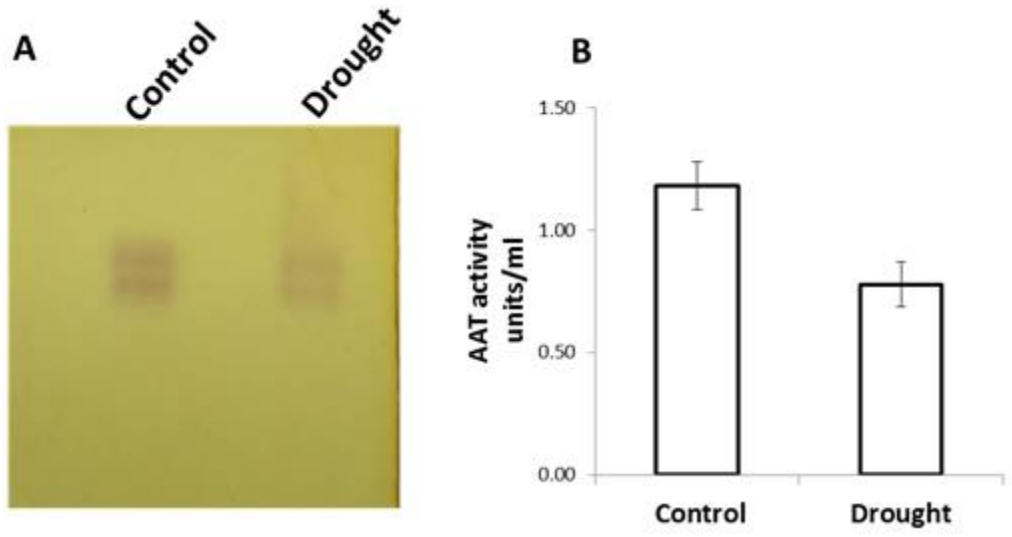

Figure 6 Changes in aspartate aminotransferase activity under drought stress

(A)One hundred microgram of crude protein from plants control and drought stress induced plants was separated by native-PAGE. In-gel activity stain was used to study the changes in activity of aspartate aminotransferase isozymes in response to drought. Intensity of the band corresponds to the activity of AspAT enzyme. (B) Total activity of aspartate aminotransferase was measured at spectroscopically at 339 nm as decrease in absorbance due to the conversion of NADH to NAD^+. One hundred µg of crude protein is used for control and drought treated samples. The activity values are presented as means ± SE in the chart (n=4), statistical significance was calculated with 2 tailed T-test, has a P value of 0.0548.

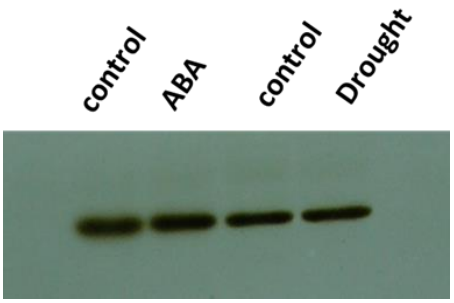

Figure 7 Anti-actin western blot

Equal loading of crude protein in the control and treatments for aspartate aminotransferase activity assay was confirmed by performing a western blot with anti-actin antibody.

The activity of aspartate aminotransferase isoforms in response to salt stress

We used In-gel activity analysis to study the effect of high salt on the activities of the aspartate aminotransferase. The zymogram (Figure 8) obtained by using crude protein extract from 150 mM NaCl treated Arabidopsis seedlings indicate that the change in the activities of the aspartate aminotransferase isozymes during salt stress is not very significant. The intensity of the signal corresponding to the bands visualized in the zymogram was measured using the "myImage Analysis" software from thermos fisher and our results indicate that there was approximately 26 % increase in activity of the AAT2 isoform and 20 % increase in activity for AAT3 isoform in response to salt stress.

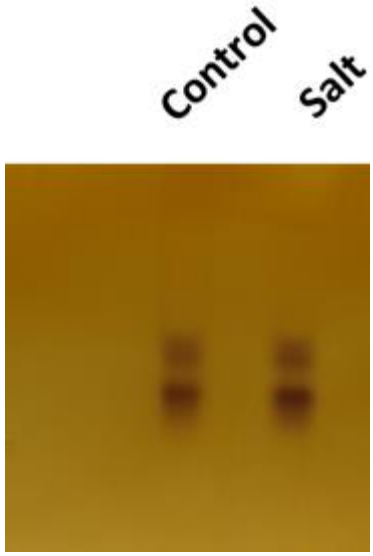

Figure 8 Zymogram of aspartate aminotransferase activity during salt stress

One hundred microgram of crude protein from plants control and plants treated with 150 mM NaCl was separated by native-PAGE. In-gel activity stain was used to study the changes in activity of aspartate aminotransferase isozymes in response to salt stress. Intensity of the band corresponds to the activity of AspAT enzyme.

The kinases SnRK2.2 and SnRk2.3 are needed for ABA dependent regulation of aspartate aminotransferase

The SNF1- related protein kinase 2 (SnRK2s) group of kinases, SnRk2D/ SnRk2.2, SnRK2E/ SnRk2.6/OST1, and SnRK2I/ SnRK 2.3 are major positive regulators of ABA signaling in plants. The SnRK2 family (SnRk2D/ SnRk2.2, SnRK2E/ SnRk2.6/OST1, SnRK2I/ SnRK 2.3) of kinases are activated by ABA and are required for drought, and salt stress in plants. The snrk2 mutants had a wilty phenotype and display a insensitivity of stomata closure to ABA and over expression of kinase SnRK2C was shown to improve drought tolerance (Fujita et al., 2009; Umezawa et al., 2004; Yoshida, 2002; Yoshida et al., 2006). Previously characterized, Arabidopsis snrk2.2 and snrk2.3 mutants were treated with exogenous application of 50μM (±)-ABA for 3 hours and zymogram of in-gel activity assay showed that activities of the isozymes AAT2,

AAT3 slightly increased in the snrk2.2 and snrk2.3 mutants upon treatment with ABA (Figure 9) . This is in contrast to the decrease in activities of AAT2, AAT3 isozymes observed in response to ABA treatments in wild type plants (Figure 9). This results indicates that the kinases SnRK2.2 and SnRK2.3 are needed for ABA mediated decrease in the activities of cytoplastic aspartate aminotransferase (AAT2) and chloroplastic AAT3 isozymes.

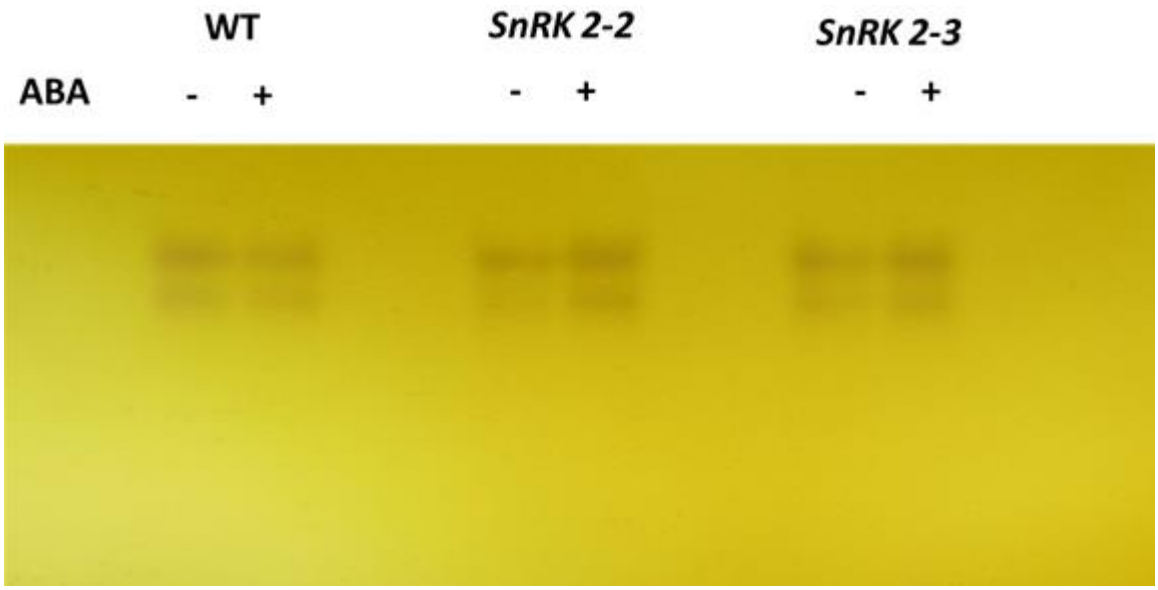

Figure 9 Zymogram of aspartate aminotransferase activity in snrk2.2 and snrk2.3 mutants

Crude protein (100 µg) extracted from wild type, *snrk2.2* and *snrk2.3* mutants treated with or without ABA was separated on native PAGE and AAT enzymes were visualized by activity stain.

Aspartate aminotransferase is a negative regulator of drought stress

The Arabidopsis mutants deficient in the cytosolic AAT2 (aat2) or chloroplastic AAT3 (aat3) were obtained from the "Arabidopsis Biological Resource Center" at Ohio State University. The aat2 and aat3 mutants are characterized by using in-gel activity

analysis. The results form activity analysis (Figure 10) indicate that the plants aat2b-2 and aat3a-1 are truly deficient for AAT2 and AAT3 isozymes, respectively and there is no residual activity present for either of the AAT2 or AAT3 isozymes. Seeds were obtained from tested true mutants and used to study their phenotype and survival under limited water conditions. The aat2, aat3 and wild type Arabidopsis plants grown in soil were used for the study and are watered with equal volume (35ml) of water throughout their growth period. After the plants have a full rosette leaves (3-4 weeks) water is withheld for about 10 days or till the leaves showed signs of wilting. Once the plants showed signs of wilting, plants are then re-watered with equal volume of water. The mutants and the control plants showed signs of wilting, but the wilting in the mutants was slightly more (the phenotype was not very prominent to be visible in photograph) compared to the control plants. Upon re-watering the mutants aat2 and aat3 showed a greater recovery compared to the wild type plants (Figure 11). While all the mutant plants survived the stress, most of wild type plants could not recover the stress treatments. Our results indicate that aat2 and aat3 mutant plants have a greater survival rate.

The role of AAT2 and AAT3 isozymes in germination and sensitivity to ABA during germination was studied using characterized aat2 and aat3 mutants. The wild type, aat2, and aat3 mutant seeds were sown on to MS agar plates and their germination was analyzed two days after placing them in incubator. The aat2, and aat3 mutants have a slow germination or stunted growth during the early stages and were sensitive to ABA during the early stages of germination (Figure 12).

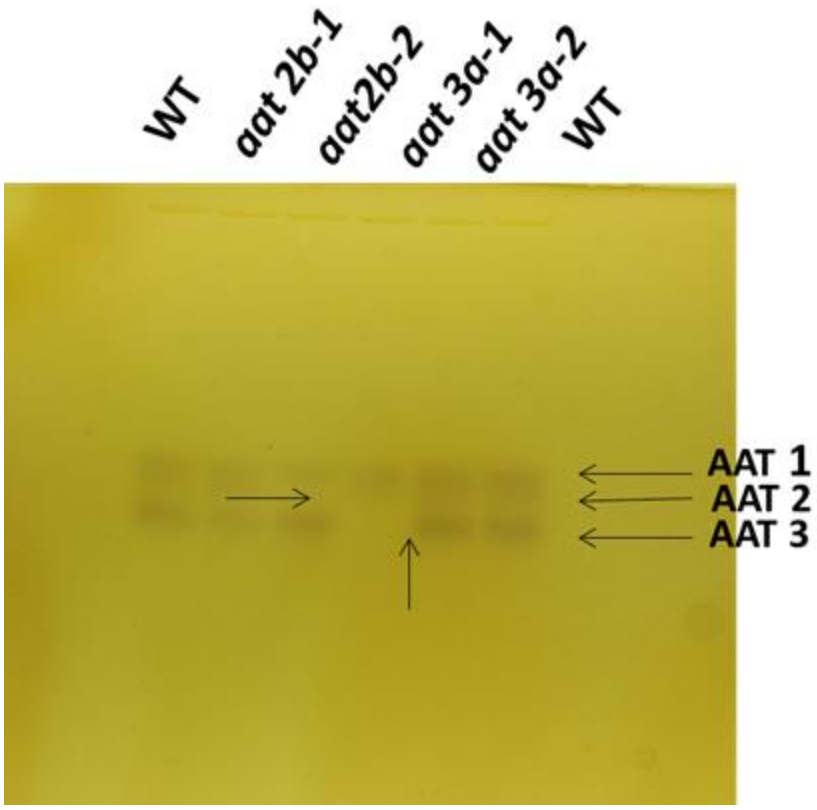

Figure 10 Genotypic characterization of aspartate amino transferase mutants using in-gel activity stain

Wt: wild type Arabidopsis. *aat2b-1, aat2b-2*: names given to two different *aat2* mutant plants, *aat2b-2* is identified as a true *aat2* mutant. Arrows indicate the absence to AAT2 and AAT3 isozyme activity. *aat3a-1*, *aat3a-2*: names given to two different *aat3* mutant plants, *aat3a-1* is identified a true *aat3* mutant.

Figure 11 Enhanced survival of aspartate aminotransferase mutants following drought stress

Drought stress was induced by withholding water for 2 weeks or till the leaves showed signs of wilting and then re-watered. The picture was taken 5 days after re-watering.

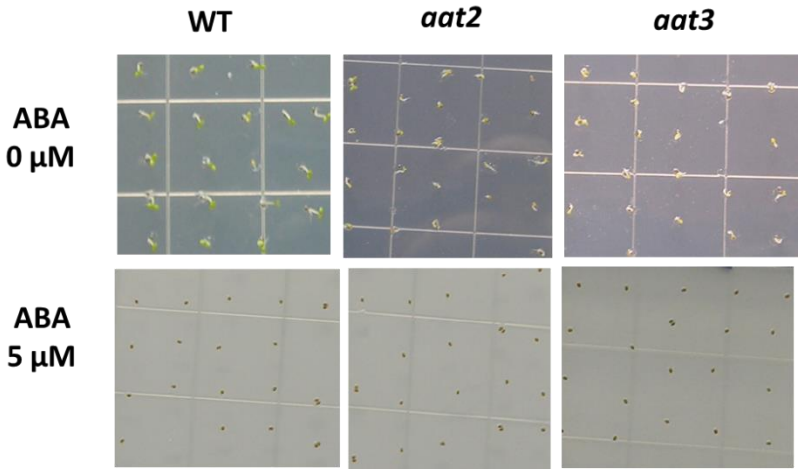

Figure 12 Germination test for aspartate aminotransferase mutants

Arabidopsis wild type (WT) and aspartate mutants (AAT2, aat3) seeds were sown on MS agar media containing 0, 5 µM ABA and the germination was recorded after 2 days.

Discussion

ABA regulates the activities of nitrogen metabolism enzymes like nitrate reductase (NR), glutamine synthetase (GS) (Desikan et al., 2002; Lu et al., 2014; Zhang

et al., 1997). The aim of this work is to study the role of ABA in regulating aspartate aminotransferase, a key enzyme in nitrogen metabolism in young Arabidopsis seedlings. In-gel activity stain with fast blue dye enabled the visualization of cytosolic aspartate aminotransferase isozyme (AAT2) and chloroplastic aspartate aminotransferase (AAT3). The in-gel activity stain is very sensitive and quantitative and was previously used for measuring the activities of superoxide dismutase, ascorbate peroxide, glutathione reductase, catalase in response to stress (Lee and Lee, 2000; Mhamdi et al., 2010). Our in-gel activity analysis of aspartate aminotransferase enzyme in young Arabidopsis seedlings treated with 50µm (±)-ABA, drought stress indicate that activities of cytosolic AAT2, and chloroplastic AAT3 isozymes decreased by 65 %, and 60%, respectively in response to ABA and decreased by 60 %, and 50% during drought stress. Equal loading of crude extract was confirmed by performing a western blot with anti- actin western blot (Figure 7). The total activity or combined activity of all aspartate aminotransferase isozymes present in the crude protein extract from seedlings measured spectroscopically by in-solution analysis also showed that the activity of aspartate aminotransferase decreased in response to ABA. The total activity of aspartate aminotransferase decreased with ABA, and drought treatment. This is supported by Meyer et al., (2014) who showed that aspartate aminotransferase genes along with alanine aminotransferase genes were down regulated in response to drought in switchgrass (Meyer et al., 2014). The cytosolic aspartate aminotransferase AAT2, and chloroplastic aspartate aminotransferase AAT3 showed a small, insignificant increase in activity in wild type plants response to osmatic stress.

The mutants, aat2 deficient in cytosolic aspartate aminotransferase, and aat3 deficient in chroloplastic aspartate aminotransferase showed increased survival rates compared to the wild type plants in response to induced water stress, indicating that AAT2, and AAT3 isozymes are negative regulators of drought stress. Germination assay with aat2 and aat3 mutants showed that even though all the mutants germinate, germination of mutants is slow/delayed or the mutants have stunted growth during the early stages. The germination study shows that isozymes AAT2, AAT3 are required during the early stages of germination. Schultz et al., 1998 studied the root length of aat2 and aat3 mutants and found the mutants exhibited shorter roots compared to wild type. Based on the root length they reported that the aat2 and aat3 have impaired growth. In our experiments, we did observe that the root length of the mutants was short during the early seedling stage (data not shown) and the germination study showed that they have a slow start with germination but the mature plants grown on soil did not exhibit any signs of impaired growth or seed production. Based on these observations we speculate that aat2 and aat3 mutants showed signs of reduced growth phenotype (reduced root length, slow start with germination) only during the early stages of plant growth, which may be due to lower contents of aspartate, asparagine amino acid reserves in aspartate mutant seeds.

The SNF1- related protein kinase 2 (SnRK2s) group of kinases, SnRk2D/SnRk2.2, SnRK2E/SnRk2.6/OST1, and SnRK2I/SnRK 2.3 are major positive regulators of ABA signaling in plants. The SnRK2 (SnRk2D/SnRk2.2, SnRK2E/SnRk2.6/OST1, and SnRK2I/ SnRK 2.3) kinases are activated by ABA and are required for drought and salt stress in plants. The snrk2 mutants have a wilty phenotype

and display the insensitivity of stomata closure to ABA and overexpression of kinase SnRK2C has shown to improve drought tolerance (Fujita et al., 2009; Umezawa et al., 2004; Yoshida, 2002; Yoshida et al., 2006). ABA treatment, drought stress result in decreased activity of aspartate aminotransferase enzymes in wild type plants. In the snrk2 null mutants (snrk2.2, snrk2.3), ABA treatment resulted in slight induction of both cytoplasmic and chloroplastic aspartate aminotransferase and the ABA mediated regulated decrease in aspartate aminotransferase activity was lost in the kinase mutants. In-gel activity analysis with snrk2.2, and snrk2.3 mutants, and wild type seedlings treated with ABA show that the kinases SnRK2.2 and SnRk2.3 are involved and required for the ABA induced down regulation of aspartate aminotransferase isozymes.

ABA treatment causes an increase in ABA levels in plants which induces the expression, and activity of SnRK 2.2, and SnRk2.3 kinases. The induction of SnRK 2.2 and SnRk2.3 kinases results in increased phosphorylation of ABA signaling components, the downstream targets of kinases. The expression analysis of AspAT2 (At5g19550), AspAT3 (AT5G11520) genes using Arabidopsis eFP browser (http://bar.utoronto.ca/efp_arabidopsis/cgi-bin/efpWeb.cgi) indicates that the expression of both genes is not altered by ABA, drought treatments. Based on our results we believe that the aspartate aminotransferase isozymes (AAT2 and AAT3) are regulated by phosphorylation and we believe phosphorylation of AAT2 and AAT3 is responsible for decreased activity of AAT2 and AAT3 isozymes. The aspartate aminotransferase isozymes could be regulated by the same mechanism during drought stress.

In summary, activity analysis results suggests that the ABA mediated down regulation of aspartate aminotransferase isozymes involves and requires SnRk2.2 and

SnRk2.3 kinases. The down regulation of AAT2 and AAT3 isozymes activity is due to the phosphorylation of AAT2 and AAT3 mediated by SnRk2.2 and SnRk2.3 kinases. The cytosolic and chloroplastic aspartate aminotransferase mutants display higher survival rate in response to drought.

CHAPTER IV

ABA REGULATED PHOSPHATASES IN PLANTS

Introduction

Protein phosphorylation is an important reversible post translational modification. The protein phosphorylation status in the cell is maintained by the combined orchestrated actions of protein kinases, the group of enzymes that add phosphate group and the phosphatases that remove the phosphate group from their targets. Extent of phosphorylation at the target site can be altered by changing the activities of either the kinases or the phosphatases or by changing the activities of both. Many signaling pathways and biological process are reversibly controlled by phosphorylation and dephosphorylation. The phosphorylation status of the protein can alter its conformation, stability and there by the activity of the target proteins. In the past, the protein phosphatases are considered to be in constitutively active and remove the phosphate groups added by the kinases. In the recent years it has been proved that the phosphatases act in a much regulated manner (Farkas et al., 2007; Hunter, 1995).

In plants, the protein phosphatases are classified based on their specificity and placed into two major groups; the protein tyrosine phosphates (PTPs) and the protein serine/threonine phosphatases. The members belonging to the protein serine/threonine phosphatases class are further classified to protein phosphatase P (PPP) and

proteinphosphatase M (PPM, metallo-dependent protein phosphatase) (Moorhead et al., 2009).

The PPP family includes the PP1 (protein phosphatase 1), PP2A, and PP2B (calmodulin-dependent protein phosphatase) groups. The PPM family of protein phosphatase is comprised of protein phosphatases PP2C group and pyruvate dehydrogenase phosphatase (Moorhead et al., 2009).

The model plant Arabidopsis contains about 76 protein phosphatases belonging to the family of PP2C and classified further into 10 groups from A to J (PP2C A-J). The Phosphatases ABI1, ABI2 and others require Mg^{2+}, Mn^{2+} for activity belong to the PP2C A group and act as negative regulators of ABA signaling. The members of PP2C group B phosphatases are involved in MAPK signal transduction cascade. All the protein phosphatases have a catalytic subunit (C) present in several copies. The Arabidopsis genome has about 112 copies of catalytic subunit sequences (Schweighofer et al., 2004). The protein phosphatase2C A family of phosphatases (ABI1, ABI2, AHG3, AHG1, HAB1 and HAB2), other protein phosphatases are regulated by ABA (Bhaskara et al., 2012; Zhang et al., 2014).

Kameshita et al., (2010) developed an in-gel protein phosphatase assay for in situ detection of protein phosphatases. The phosphates were separated by native-PAGE or SDS-PAGE and then enzymatic activity was determined by using a florigenic 4-methylumbelliferl phosphate (MUP) as a substrate and MUP is converted to 4-methylumbellifrym by removal of phosphate group. 4-methylumberym is an insoluble florescent product and the protein phosphatase and other kinds of phosphatases can be visualized as florescent bands.

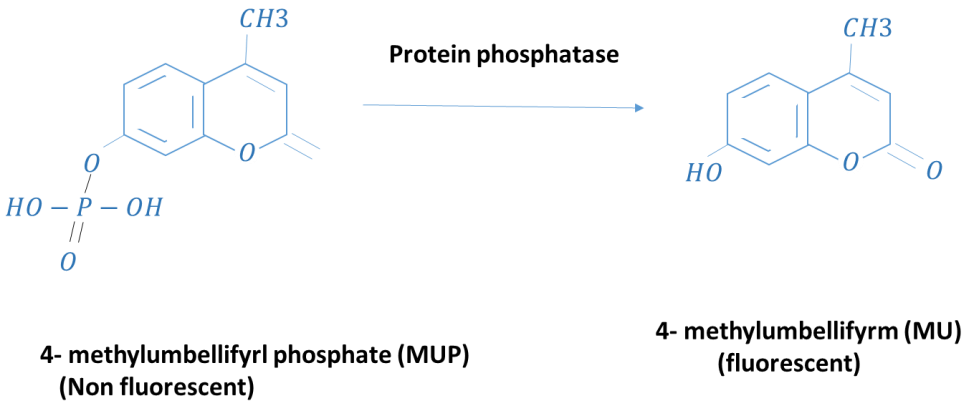

Figure 13 Visualization scheme for phosphatases using MUP

Materials and Methods

Plant material and protein extraction

The Arabidopsis and rice seedlings were grown, ABA, drought treatments are carried out as previously described for aspartate aminotransferase in chapter III. The protein extraction and separation of proteins by native-PAGE was carried out as described in chapter III.

2D- PAGE electrophoresis

A modified version of second dimensional PAGE (proteins are separated by native-PAGE for first dimension and then separated in second dimension using SDS-PAGE) was used for better separation of proteins. In brief, the crude extract was separated in first dimension by native-PAGE as described above. After separation of proteins under native conditions, the gel strip corresponding to the proteins separated under native conditions was cut and incubated in 2 times concentrated SDS sample buffer for 20 minutes at room temperature.

After incubation the gel strip was placed horizontally on top of a 12% separating PAGE gel and is sealed with stacking gel, such that the gel strip is sandwiched between the stacking gel and separating gel. The electrophoresis is carried out under constant current (32 amps) at room temperature.

In-gel protein digestion and mass spectrometric analysis

The protein bands/spots visualized by In-gel activity analysis were cut and placed in a small Eppendorf tube. The proteins/protein present in gel slice were digested with trypsin and the digested polypeptides were identified by Mass spectrometry as described by Li et al. (2002).

In-gel activity analysis of protein phosphatase

The crude protein extract was separated by native-PAGE and the protein phosphatase were visualized as described by Kamehita et al. (2010). The gel was incubated in the reaction mixture containing 50 mM Tris-HCl (pH 8.0), 0.1 mM EGTA, 0.01% Tween 20, 2 mM DTT, 20 mM MnCl2, 0.5 mM 4-Methylumbelliferyl Phosphate (MUP) for 20 minutes and the phosphatases were detected under UV-trans illuminator at 325nm.

For the better separation of phosphatases in the crude extract, the proteins are separated by using a modified version of 2D-PAGE (native PAGE in first dimension followed by SDS PAGE for second dimension) as described above. After the separation under denaturing conditions, In-situ denaturion/ renaturation of proteins was carried out as described by Kameshita et al. (2010). In brief the SDS from the gel was removed by incubating the gel twice in a 50 ml of 50mM Tris-HCl (pH 7.5) buffer containing 20% 2-

isopropanol for one hour. After the removal of SDS from the gel, the proteins are denaturated by incubating the gel twice in a buffer containing 50mM Tris-HCl (pH 7.5), 5mM 2-mercaptoethanol, 8M urea for one hour at room temperature.

After the denaturation step the proteins were allowed to renaturate by placing the PAGE gel in a buffer containing 50mM Tris HCl (pH 7.5), 0.02% tween 20, 20mM 2-mercaptoethanol, 1mM MgCl2, and 1mM MnCl2. The renaturation step was carried out overnight or 14-16 hours with four buffer changes at 4º C to prevent degradation. After renaturation the gel was incubated in reaction mixture and the activity analysis was performed as described earlier.

Results

Fructose-1-6 bisphosphates are regulated by ABA in Arabidopsis and rice

In-gel phosphatase activity analysis of crude protein extracts from Arabidopsis and rice seedlings separated under native conditions lead to the visualization of different phosphatases. The proteins were also electrophoresed using a 2D PAGE (1D by native PAGE followed by 2 dimension SDS PAGE) system for better separation and visualization of proteins.

In Arabidopsis (Figure 14) and rice (Figure 15), phosphatases with different electrophoretic mobility were visualized by activity stain after separation by PAGE. The activity of certain phosphatases increased in response to ABA, drought increased in Arabidopsis (Figure 14A,B) and rice (Figure 15A,B). The crude extract proteins were separated by 2D PAGE (1D by native PAGE followed by 2 dimension SDS PAGE). The 2D separated proteins are stained in-situ after denaturation and renaturation of proteins present in the gel. Six active phosphatases were visualized by 2D PAGE. The visualized

proteins were identified by mass spec analysis in Arabidopsis (Figure 14C and Table 2) and rice (Figure 15C and Table 3).

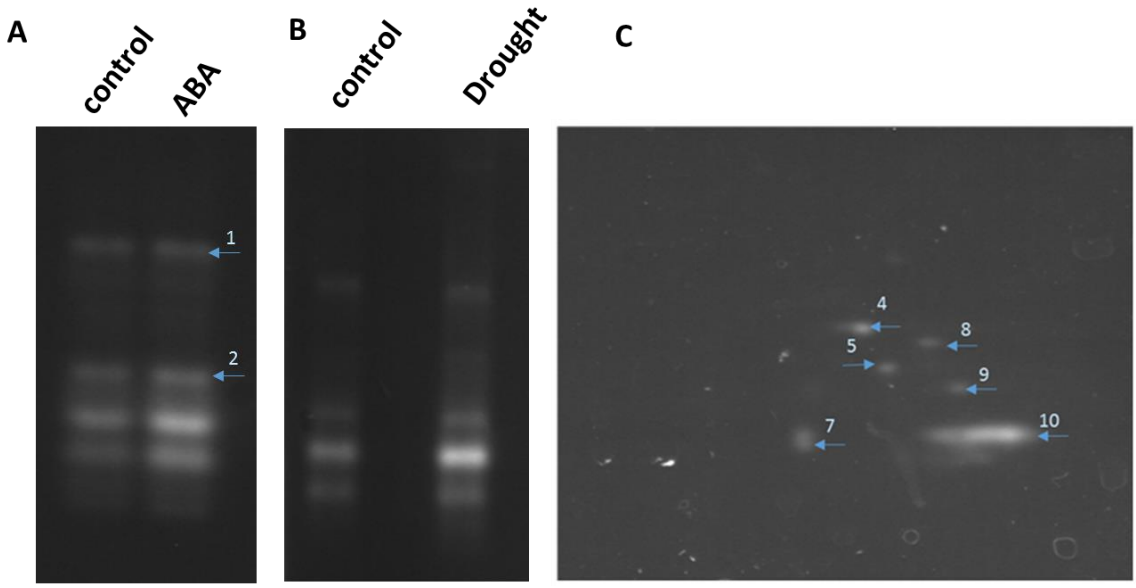

Figure 14 In-gel detection of Arabidopsis phosphatases

(A) One hundred microgram of crude protein from the control and 50 μM (±)-ABA treated Arabidopsis seedlings. (B) one hundred microgram of crude protein from the control and drought treated Arabidopsis seedlings. (C) Two fifty micrograms of crude protein separated by 2D-PAGE. The phosphatase bands that are sequenced by mass spec are marked with arrows.

Table 2 List of proteins Arabidopsis phosphatases identified by activity staining

Band no	Accession	Description	MW
1	IPI00530130.1	Fructose-1-6 bisphosphatase	45.1
2	IPI00542999.1	Protein tyrosine phosphatase 1	37.8
4	IPI00542330.2	Phosphatidylinositol phosphatase	43.5
5	IPI00992657.1	Protein phosphatase 2A-3	19.8
7	IPI00548735.1	Acid phosphatase class B family protein	28.9
8	IPI00544876.1	Sedoheptulose-1-7-bisphosphatase	42.4
9	IPI00523465.2	myo-inositol monophosphatase like 2	41.7

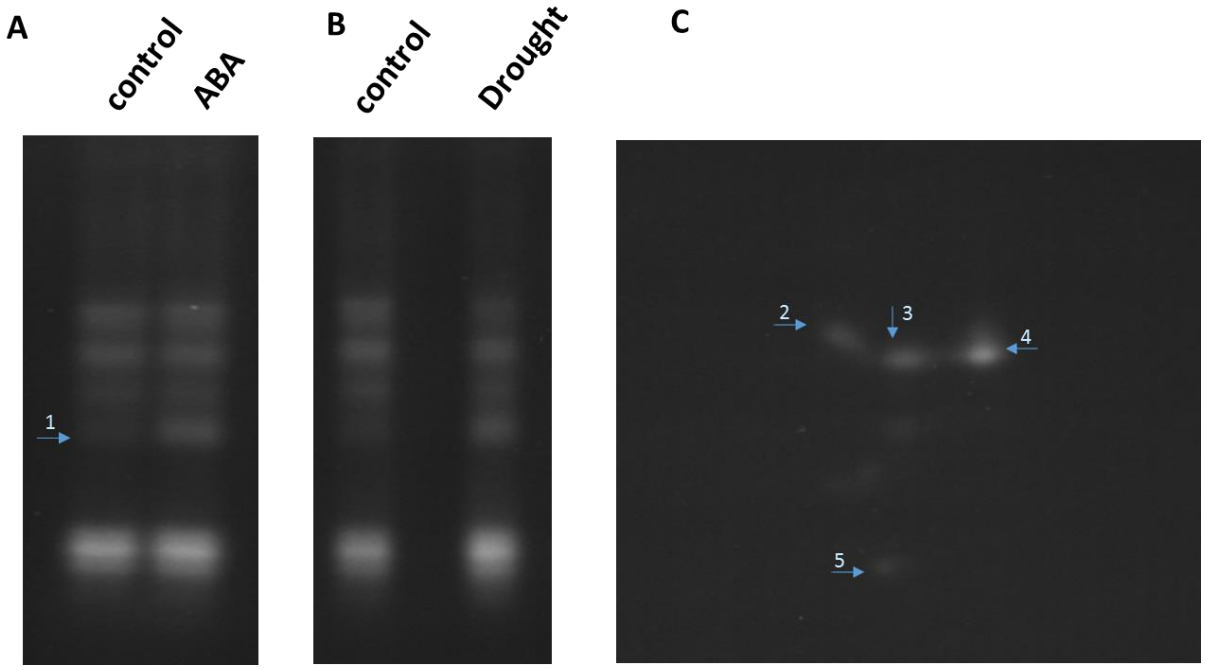

Figure 15 In-gel detection of rice phosphatases

(A) one hundred microgram of crude protein from the control and 100 μM (±)-ABA treated rice seedlings. (B) one hundred microgram of crude protein from the control and drought treated rice seedlings. (C) Two fifty microgram of crude protein extract from rice seedlings separated by 2D-PAGE. The phosphatase bands that are sequenced by mass spec are marked with arrows.

Table 3 List of rice phosphatases identified by activity staining.

Band No	Locus	Description	MW
2	LOC_Os03g16050.1	fructose-1,6-bisphosphatase	43.6
1, 3	LOC_Os04g16680.1	fructose-1,6-bisphosphatase	42.2
4	LOC_Os12g08280.1	inositol-1-monophosphatase	37.5
5	LOC_Os08g44320.1	protein-tyrosine-phosphatase	28.8

Discussion

Both Arabidopsis and rice genomes have a large number of genes encoding for phosphatase enzymes, which are further classified in families and groups. We used in gel activity staining method developed by Kameshita et al. (2010) for visualization and as an

initial/quick screen for phosphatases present in crude protein extracts from Arabidopsis and rice seedlings. Our results indicate that in-gel activity staining of phosphatases can visualize sugar phosphatases like fructose 1-6 bisphosphatase family, sedoheptulase-1,7-bisphosphatase, inositol mono phosphatases; protein serine/threonine phosphatases, protein tyrosine phosphatases, and it will be useful to study these enzymes response to abiotic stress and hormones.

The analysis of sucrose insensitive (sis1) and glucose insensitive (gis1, 2) mutants revealed that they have block in ABA biosynthesis, indicate the crosslink between sugar signaling and ABA signaling pathways and the importance of ABA in sugar signaling (Laby et al., 2000). The role of glucose and sucrose in sugar signaling is well studied. Cho and Yoo (2011) reported that fructose insensitive 1(FINS1), a putative fructose 1-6 bisphosphatase plays an important role in fructose signaling independent of its catalytic activity. FINS1 is reported to act downstream of glucose insensitive 1 (GIS1) in ABA signaling (Cho and Yoo, 2011) . Liu et al (2013) showed that the activity of fructose 1-6 bisphosphatase increased in response to ABA, cold stress in wheat. Our preliminary analysis with in-gel phosphatase staining indicate that activity of fructose-1-6 bisphosphatase family of phosphatases are induced by ABA in Arabidopsis and rice. The activity of fructose-1-6 bisphosphatase family of phosphatases increased in response to drought in both arabidopsis and rice.

Native-PAGE in-gel activity analysis of arabidopsis phosphatases lead to visualization of four phosphasphatses and we were able to identify two of them as fructose 1-6-bisphosphatase (accession: IPI00530130.1) and protein tyrosine phosphatase (accession: IPI00542999.1). Repetition of in gel-activty analysis and mass spec is needed

to identify the other two phosphatases and confirm the ABA mediated induction in activity of protein tyrosine phosphatase.

Phoshstases along with kinases maintain the phosphorylation status of the cell. The activity of many enzymes depends on their phosphorylation status. Our in-gel activity analysis indicate that it can be used as a quick screen to identify the ABA, stress regulated phosphatases in plants. Fructose 1-6 bisphostase family of phosphatses are identified as ABA regulated enzymes in Arabidopsis and rice, implying the cross link between the ABA and sugar signaling pathways.

CHAPTER V

IDENTIFICATION OF PLANT HEXOSAMINIDASES WITH A NEUTRAL pH OPTIMUM

Introduction

N-acetylglucosamine (GlcNAc) is present on the glycoproteins. Many proteins in nucleus and cytoplasm contain an O-linked-N-acetylglucosamine (O-GlcNAc) as a post translational modification on serine/threonine residues (protein O-GlcNAcylation). It is also identified as a novel post translational modification of histones. N-acetylglucosamine is added on the target protein by N acetyl glucosaminyltransferase and removed by hexosaminidases also known as β-N-acetylglucosaminidase (Fischl et al., 2011; Ramot et al., 2004).

Strasser et al. (2007) reported the cloning and characterization of three hexosaminidases (HEXO1, HEXO2, HEXO3) in Arabidopsis, which release the GlcNAc residues from glycoproteins. All three hexosaminidases identified have an acidic pH optimum and subcellular localization studies suggest that HEXO1 is present in the vacuoles and is involved in the degradation of glycoproteins. HEXO2, HEXO3 might act on the glycoproteins present in the plasma membrane or on secreted proteins (Strasser et al., 2007). In humans, the lysosomal hexosaminidases (HEXA) deficiency causes neurogenic disorders like Tay-Sachs disease (Gilbert et al., 1975).

The enzymes responsible for the regulation of glycosylation of nuclear and cytoplasmic proteins have a neutral pH optimum. The nuclear and cytoplasmic proteins have an O-linked-N-acetylglucosamine at serine and threonine residues, and a number of proteins containing these modifications are identified in humans. Protein phosphorylation and GlcNAc are cross linked. The target protein pool for these modifications overlap and occur at the same positions (serine/threonine). The abnormalities in interactions between protein phosphorylation and GlcNAc are linked to diabetes. GlcNAc modification controls transcription by regulating the localization, activity of transcription factors and also by influencing their interactions with binding partners (Butkinaree et al., 2010). A hexosaminidase present in the cytosol that catalyzes the removal of O-Linked- N-GlcNAc with neutral pH was identified in human brain (Gao et al., 2001).

The nuclear proteins (nuclear pore complex) containing GlcNAc modification were identified from tobacco suspension cells and their O-linked-GlcNAc modifications differ from their counterparts observed in animals. Plant nuclear proteins have a oligosaccharide containing more than 5 GlcNAc as modification compared to the single O-linked-GlcNAc observed in animals (Heese-Peck et al., 1995). Compared to their animal/vertebrae counterparts nuclear, cytoplasmic proteins containing O-linked-GlcNAc are less studied in plants and the hexosaminidases responsible for the removal were not identified/characterized yet. Most of the hexosaminidases identified have acidic pH optimum and thus might be involved in glycoprotein catabolism and function in vacuoles.

An increase in activity of β-N-acetyl glucosaminidase with a pH optimum of 5.6 was observed in germinating cotton seeds (Yi, 1981). Gilbert et al. (1975) assigned a locus for hexosaminidase A and B and developed a staining method (visualization

scheme represented in Figure 16) for in-situ visualization of hexosaminidases present in the crude protein extract from human and mouse cell lines. The lysosomal hexosamindases; HEXA, HEXB, HEXC were visualized by using a 4-methylumbelliferyl N-acetyl-β- D-glusaminide as substrate at acidic pH 4.0. (Gilbert et al., 1975).

Our objective was to identify the hexosaminidases that can catalyze the removal of GlcNAc from nuclear and cytoplasmic proteins in plants. We used the same method developed by Gilbert et al., (1975), with small modifications to visualize the hexosaminidases at both acidic and close to neutral pH.

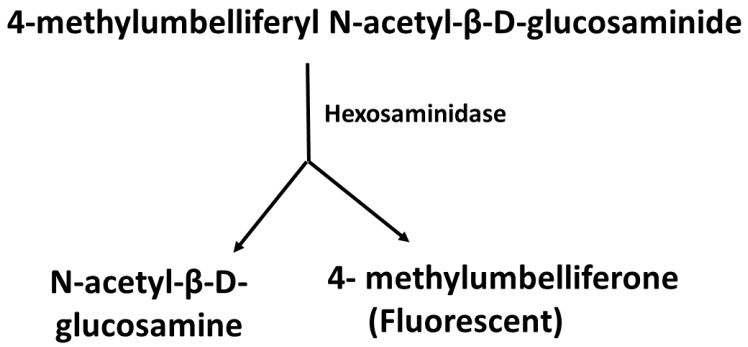

Figure 16 Visualization Scheme for in-situ staining of hexosaminidase

4-methylumbelliferyl N-acetyl-β-D-glusaminide acts as artificial substrate for hexosaminidases and the active hexosaminidase enzymes removes the N-acetyl-β-D glucosamine (GlcNAc) from the substrate resulting in the formation of 4-methylumbelliferone that appears as a fluorescent band under UV light.

Materials and Methods

Plant material and treatments

Soybean and Arabidopsis seedlings were grown and treated with ABA or drought was performed as described in the methods section of chapter III.

Activity analysis of hexosaminidase

The activity stain/ analysis was performed as described by Gilbert et al. (2007), with minor modifications. In brief, crude proteins from plant sample were separated by PAGE under native conditions. The gel is then incubated for 20 minutes at 37º C in sodium citrate buffer at pH 4.5 or sodium phosphate buffer at pH 6.5 containing

3 mM 4-methylumbelliferyl N-acetyl-β- D-glucosaminide with a filter paper overlay. After incubation, the hexosaminidase activity is monitored and captured under long- wave UV-light. Under the UV light the hexosaminidase activity appeared as fluorescent bands.

In-gel protein digestion and mass spectrometric analysis

The protein bands/spots visualized by In-gel activity analysis were cut and placed in a small Eppendorf tube. The proteins/protein present in gel slice were digested with trypsin and the digested polypeptides were identified by Mass spectrometry as described by Li et al. (2002).

Results

Visualization and identification of soybean hexosaminidase

The crude extract from soy bean leaves is separated in PAGE under native conditions and in-gel activity assay using 4-methylumbelliferyl N-acetyl-β- D-glucosaminide as substrate at pH 4.5 and pH 6.5 led to the visualization of one hexosaminidase as a fluorescent band under UV light (Figure 17). The fluorescence is directly proportional to the conversion of 4-methylumbelliferyl N-acetyl-β- D-glucosaminide to 4-methylumbelliferone (fluorescent) by hexosaminidase enzyme and

thus it represents the activity of the enzyme. The visualized hexosaminidase was cut from the gel and identified by using mass spectrometry as β-hexosaminidase. The visualized β-hexosaminidase was more active at pH 6.5 compared to pH 4.5 (Figure 17) indicating that β-hexosaminidase identified has a pH optimum in the neutral range.

β-hexosaminidase is responsive to ABA treatment

The ABA, drought mediated regulation of β-hexosaminidase activity was studied by performing activity analysis with 100 μM ABA treated, drought treated soybean leaves. The results from activity analysis (Figure 17) show that β-hexosaminidase was slightly induced by exogenous ABA treatment. Results, (Figure 17B) show that the activity of β-hexosaminidase was not altered during drought stress under the conditions tested.

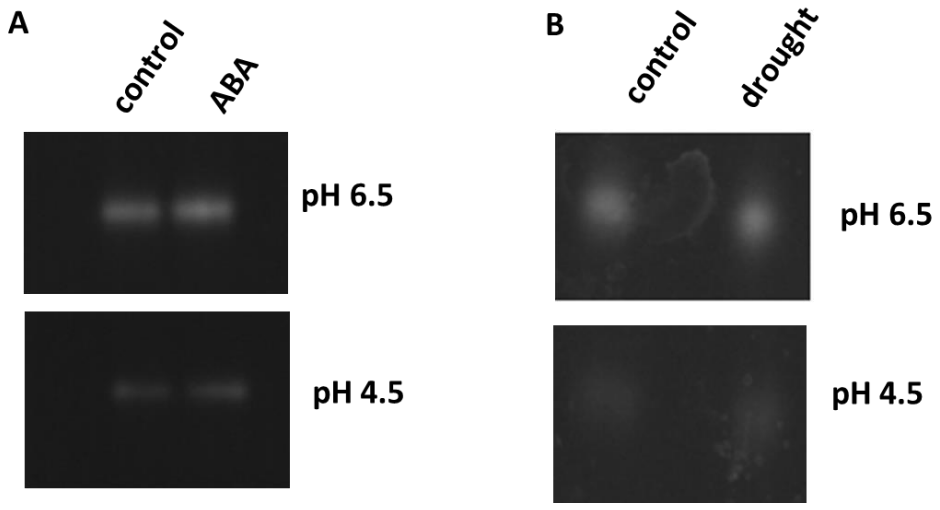

Figure 17 In-gel detection of hexosaminidases in soybean

Equal amounts of crude protein from soybean leaves was separated by native-PAGE and incubated with artificial substrate 4-methylumbelliferyl N-acetyl-β-D-glucosaminide
at pH 4.5 or pH 6.5. The activity of hexosaminidases was monitored under UV light as fluorescent bands due the conversion of substrate to fluorescent product.
(A) Hexosaminidase activity analysis in soybean leaves treated with or without ABA at pH 6.5 or at pH 4.5.
(B) Hexosaminidase activity analysis in soybean leaves treated with or without drought treatment at pH 6.5 or pH 4.5.

Visualization of Arabidopsis hexosaminidase

The crude extract from Arabidopsis seedlings is separated in PAGE under native conditions and activity staining with 4-methylumbelliferyl N-acetyl-β- D-glucosaminide as substrate at pH 4.5 and pH 6.5 lead to the visualization of single hexosaminidase as a fluorescent band under UV light (Figure 18). The visualized hexosaminidase was more active at pH 4.5 compared to that at pH 6.5.

Hexosaminidase visualized in Arabidopsis is not responsive to ABA

The ABA mediated regulation of β-hexosaminidase activity was studied by performing activity analysis with 50 μM (±)-ABA treated Arabidopsis seedlings. The results from activity analysis (Figure 18) show that β-hexosaminidase activity was not significantly altered by exogenous ABA treatment.

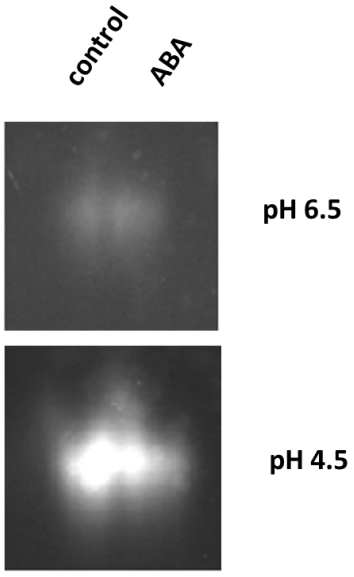

Figure 18 In-gel detection of hexosaminidases in Arabidopsis

Hexosaminidase activity analysis in Arabidopsis seedlings treated with or without ABA at pH 6.5 or at pH 4.5. Equal amounts of crude protein from Arabidopsis was separated by native-PAGE and incubated with artificial substrate 4-methylumbelliferyl N-acetyl-β- D-glucosaminide at pH 4.5 or pH 6.5. The activity of hexosaminidases was monitored under UV light as fluorescent bands due the conversion of substrate to a fluorescent product.

Discussion

Compared to their homologues/counterparts in vertebrates, hexosaminidases in plants are less studied. The hexosaminidase with a neutral pH optimum and thus responsible for removal of GlcNAc residues from nuclear, cytoplasmic proteins were not reported in plants. Using in-gel hexosaminidase stain, mass spec we detected and a β-hexosaminidase was identified in soybean. The β- hexosaminidase in soybean was more active at pH 6.5 compared to that at pH 4.5, indicating that the enzyme has an optimal pH in the neutral range. GlcNAc residues are present on many cytoplasmic and nuclear proteins in animals and plants (Gao et al., 2001; Heese-Peck et al., 1995; Ozcan et al., 2010). The hexosaminidases that catalyze the removal of GlcNAc residues from these proteins have been identified in animals (Dong and Hart, 1994; Gao et al., 2001). Hexosaminidases that catalyze the removal of GlcNAc residues from the cytoplasmic and nuclear proteins should have a neutral pH optimum (Dong and Hart, 1994; Gao et al., 2001). We speculate that soybean β- hexosaminidase, having higher activity at pH 6.5 might be responsible to catalyze the removal to GlcNAc residues from the cytoplasmic and nuclear proteins. Cellular localization study with recombinant β- hexosaminidase such as GFP-fusion protein can help us confirm my speculation that the enzyme is present in the cytoplasm or nucleus. Further experiments with purified β-hexosaminidase to test its capability to remove O-linked-GlcNAc residues from plant proteins are needed to confirm the role of this β- hexosaminidase involved in reversible protein O-GlcNAcylation.

In gel activity stain with soluble crude protein extracted from soybean and Arabidopsis seedlings lead to visualization of single hexosaminidase in both soybean and

Arabidopsis. Our experimental results indicate that extraction procedure used was successful in visualizing multiple isozymes for aspartate aminotransferase, glutamate dehydrogenase, catalase, alanine aminotransferase in Arabidopsis. We speculate that plants might not contain multiple soluble isozymes for hexosaminidases or different extraction procedure is needed to release the hexosaminidases.

Strasser et al. (2007) cloned three hexosaminidase sequences in Arabidopsis and all three hexosaminidases (HEXO1, HEXO2, HEXO3) had acidic pH optimum. HEXO1 is a vacuole protein while HEXO3, HEXO2 are localized to plasma membrane. Our results indicate that the enzyme which catalyzed GlcNAc removal in Arabidopsis was more active at pH 4.5 and we speculate that it might be involved in catalyzing the catabolism of glycoproteins. Sequencing of visualized acidic pH hexosaminidase in Arabidopsis might indicate/confirm if the visualized hexosaminidase is indeed one of the hexosaminidases identified by Strasser et al. (2007).

Protein O-GlyNAc is observed on membrane, cytoplasmic and nuclear proteins and is similar to the cellular distribution of protein phosphorylation. Both these modifications are observed on serine/threonine residues in animals. Protein phosphorylation in plants and animals is regulated by ABA (Kline et al., 2010; Umezawa et al., 2013). The hormonal regulation of enzymes involved in GlyNAc modification is not yet understood.

Our in-gel activity stain results indicated that β- hexosaminidase was induced by hormone ABA and might be involved in ABA signaling in soybean. To understand the role of ABA in regulating the O-GlyNAc modification it is also important to study the effect of ABA on N- acetylglucosaminyltransferase (enzyme that adds the GlcNAc) in

plants. Further studies are needed to further identify/characterize the hexosaminidase in plants and unravel its role of GlcNAc modification.

CHAPTER VI

IDENTIFICATION OF HISTONE BINDING PROTEINS

Introduction

Histone lysine methylation and acetylation are major post translational modifications present at the N-terminal tails of histones. The post translational modifications generate distinct marks on the histones contributing to the diversity of the histones. These post translational modifications of histones can affect the DNA packaging into the nucleosomes, thus affecting the organization of chromatin and chromatin associated process. (Johnson et al., 2004; Lachner and Jenuwein, 2002; Martin and Zhang, 2005; Sims et al., 2003). The acetylation of histone residues neutralizes the positive charge of the histone tails, masks the positive charge of the histone, and thereby weakens the interaction between the negatively charged DNA and positively charged histones and opens chromatin for transcription machinery and is often linked to transcriptional activation.

The H3 lysine methylation modifications act as binding sites for the proteins. The effector/reader proteins that specifically recognize the different H3-lysine modification are responsible for the downstream events. For example, the chromo domain containing heterochromatin protein 1 (HP1) specifically binds to H3K9methyaltion. The recruitment of HP1 protein leads to spread of heterochromatin and recruitment of H3K9

methyltransferase (SUV39H1/2). The polycomb group proteins recognize H3K27 methylation through their chromo domains and lead to gene repression.

The methylation of H3K9, H3K27 are linked to heterochromatin formation and transcriptional silencing resulting in gene repression (Volkel and Angrand, 2007). The X-chromosome inactivation in humans is linked to H3K9 and H3K27 methylation. The H3K4methylation, H3K36 are generally linked to active gene expression (Sims et al., 2003). ChIP-sequencing, histone peptide pulldown assay, histone peptide pulldown analysis with SILAC labeling have been used successfully in animals for large scale screening of histone lysine methylation reader protiens (Vermeulen et al., 2010; Wysocka et al., 2005; Wysocka et al., 2006). Large scale screening for histone effector proteins has not been yet reported in plants. In this report, we used peptide pulldown assay to screen for histone H3 binding proteins present in the rice nuclear protein extract. The binding specificity of the identified proteins was confirmed using far western analysis.

Materials and methods

Isolation of protoplasts

Protoplasts were obtained from rice suspension cells (OC cell line) by enzymatic digestion of the cell wall as described by Tan et al. (2007). In brief, suspension cells are harvested on the 5th day of the subculturing. The cell walls of the suspension cells were digested by adding a filter sterilized enzyme solution (pH 5.7) containing 2.5% cellulose RS, 1% microenzyme R10, supplemented with B5 organic medium, 400mM mannitol, 0.125mM MgCl2, 80mM CaCl2, 0.5mM MES hydrate, 2,4-dichlorophenoxyacetic acid. The suspension cells were incubated in the enzyme solution in dark for about 10 hours at room temperature. The released protoplasts were then

filtered by passing the enzyme solution through a sterilized 25 micron stainless steel sieve. The protoplasts were collected by centrifugation at 120 X g. The pellet containing the protoplasts was then washed 3 times with suspension medium (B5 organic medium supplemented with 400mM Mannitol, 0.125mM MgCl2, 80mM CaCl2, 0.5mM MES hydrate, 2,4-Dichlorophenoxyacetic acid, 0.2 % weight/volume N-Z amine at PH 5.7) to remove cell debris and impurities. The washed protoplast was resuspended in nuclei isolation buffer (10mM Tris pH 8.0, 2mM MgCl2, 1mM CaCl2, 1mM EDTA, 0.25mM sucrose, 0.1mM spermidine, 0.5% ficoll, 1mM PMSF, 1mM DTT, 1% Triton-X-100) and centrifuged at 1800 x g for 10 minutes at 4º C. The nuclei were released from protoplast by gentle shaking for 15 minutes at 4ºC. The nuclei were collected as a pellet by centrifugation at 1800 x g for 10 minutes. The nuclei were then digested by adding micrococcal nuclease to a final concentration of 0.2U/ml and the reaction was carried out for 10 minutes at the room temperature. The digested nuclei were centrifuged at 12,000 x g for 10 minutes and the supernatant contains most of the soluble nuclear proteins.

Peptide pulldown assay

The biotinylated histone H3 peptides that were approximately 22-24 amino acids in length with the following modifications (H3K4me3), trimethylated K9 (H3K9me3), trimethylated K27 (H3K27me3), trimethylated K36 (H3K36me3) and unmodified H3 peptides were obtained from Upsate. The biotinylated peptides were immobilized onto the streptavidin beads. The nuclear proteins obtained by enzymatic digestion of rice suspension cells was added to the peptide bound beads and incubated with continuous gentle rotation for 4 hours at 4°C. The protein bound beads were washed 5 times to remove any unspecific binding proteins. The bound proteins were eluted by SDS sample

buffer. The bound proteins were then separated on a 10% SDS-PAGE gels and visualized by silver staining. By comparing the binding patterns of proteins between the control and the peptides with the modifications, we identified certain proteins whose binding is either enriched or decreased by the presence of modification. The identified proteins were excised from the gel, digested with trypsin enzyme, and analyzed by a mass spectrometer.

RNA isolation and RT-PCR

For RNA extraction two weeks old rice seedlings grown by hydroponics were used. RNA was extracted by using TRIzol® reagent (Invitrogen, Cat. No. 15596-018). In brief, the harvested plant sample was flash frozen in liquid nitrogen and ground to a fine powder in a mortar and pestle. One ml of TRIzol® reagent was added to very 100 mg of round tissue and homogenized by vortexing. Chloroform (0.2ml) was added to every one ml of TRIzol® reagent used, mixed, and incubated for 15min on ice. After incubation the sample was centrifuged at 12,000 g for 10 minutes. The colorless upper aqueous phase containing RNA was transferred to a fresh tube. RNA was then precipitated by adding 0.25 ml of isopropanol and high salt solution (0.8M sodium citrate and 1.2M NaCl) per every 1ml of TRIzol® reagent used. After incubation for 10 minutes at room temperature, mixture was centrifuged at 12,000g. RNA present in the pellet was then washed twice with 75% ethanol and air dried.

RNA was treated with RNase free DNAase I. The RNA was dissolved in RNase-free water and stored in small 10µl aliquots in -80ºC.

Synthesis of cDNA by reverse transcription of total RNA (5µg) from rice was carried out by using "SuperScript ^{TM} First-Strand Synthesis system" from Invitrogen. cDNA synthesis was performed using olio (dT) according to manufacturer's instructions

(Invitrogen,http://tools.lifetechnologies.com/content/sfs/manuals/superscript_firststrand_man.pdf). cDNA was then stored at -80º C.

Cloning of the coding sequence for histone binding 1(HB01) or histone binding 2 (HB02) into a GST vector

PCR reactions were performed using PfuUltra II Fusion HS DNA Polymerase (Agilent technologies, catalog # 600670), the following primers are used HB01: forward primer ATA TGG ATC CAT GGC GTC GTC GGC GTC CA, reverse primer TAT CGC GGC CGC TCA AGG CTT CCA TGT TTT C; HB02: forward primer ATA TGG ATC CAT GCC GAA GGC TAC GGC GG, reverse primer ATA TCT CGA GTC AGG GAG CCT TGG CCG G; The HB01 PCR product was cloned in to BamH 1 and Not 1 restriction sites of pGEX-4T-1 vector (Amersham Biosciences). The HB02 PCR product was cloned in to BamH1 and Xho1 restriction sites of pGEX-4T-1 vector. The resulting constructs were confirmed by DNA sequencing.

Expression of GST tagged recombinant proteins

Respective plasmids containing the HB01, HB02 genes were transformed individually in to E. coli (BL21 strain). The transformed E. coli was grown in ampicillin containing medium. The expression of proteins was induced by addition of 1 mM isopropyl –β-D-thiogalactoside (IPTG) after OD of the culture reached 1.0. The induction of proteins was monitored (1 to 4 hours). The cells were harvested after growing them for 4 hours from the start of protein induction at 28º C.

Preparation of cell extracts

The expression of GST tagged HB01 and HB02 proteins lead to the formation of inclusion bodies. The recombinant proteins were purified from the inclusion bodies according to the protocol previously descried by Steinle et al. (2010). In short, the bacteria was collected by centrifugation at 8,000 x g for 10 minutes. The bacteria was resuspended in lysis buffer ((50 ml per every 1 liter of culture) containing 50mM Tris-HCl, pH 8.0, 100mM NaCl, 5mM EDTA, 0.1% NaN3, 0.5% triton-X-100, 0.1mM PMSF, 1mM DTT and lysed by sonication . To the lysed cells 10mM $MgSO_4$ was added to chelate EDTA and then DNase and lysozyme was added to lysate and incubated at room temperature for 20 minutes to release the inclusion bodies. The inclusion bodies were collected by centrifugation at 5000 g for 10 minutes and the pellet was then washed twice in the lysis buffer. The pellet containing inclusion bodies was dissolved by continuous stirring in solution containing 8M urea, 100mM Tris-HCl, pH 8.0, 50 mM glycine, 5mM glutathione, 0.5mM oxidized glutathione. The denatured proteins were refolded by step by step dialysis in a dialysis buffer (0.1 M Tris-HCl, pH 8.0, 0.4 M L-arginine, 50% glycerol).

Far western analysis

The binding specificity of the recombinant HB01, HB02 proteins was analyzed using far western analysis also known as dot blot analysis as described by Zhang et al. (2010) and Edmondson & Roth, (2001) with minor modifications. Histone peptide array containing 96 spots of purified modified and unmodified histone H3 peptides called "AbSurance TM histone H3 antibody specificity array" was purchased from Millipore (Millipore, catalog no: 16-667). For the far western analysis, the peptide array membrane

was rehydrated by soaking it in methanol for 10 minutes and washed with TBST buffer. The membrane was then blocked with 5% BSA in TBST buffer for 1 hour fallowed by washing with TBST buffer for 5 minutes. The membrane was then incubated with GST tagged recombinant HB01 and HB02 individually for 2 hours at room temperature with gentle shacking. After washing the membrane trice with TBST buffer, the bound GST-tagged proteins were detected by using anti-GST HRP conjugate (GE Healthcare Life Sciences) according to the manufacturer's instruction.

Results

Nuclei enrichment and extraction of nuclear protein from rice suspension cells

Identification of histone interacting proteins requires high quality and large scale purification of nuclear protein. Rice suspension cells was chose as a starting material for isolation of nuclear protein as they do not contain chloroplasts. This avoids contamination of nuclear proteins with abundant chloroplast proteins. My previous experiments with nuclear protein obtained from leaves showed that chloroplast proteins were major contaminating proteins. Tan et al. (2007) and Mujahid et al. (2013) successfully used enzymatic digestion procedure for enrichment of nuclei and extraction of nuclear protein from rice suspension cells. Cell wall digesting enzymes cellulose, and macroenzyme were used to digest the cell wall of rice suspension cells and protoplasts obtained were used for enrichment of nuclei. The purity of the enriched nuclei was observed by staining the nuclei with methylene blue dye. The microscopic observation of methylene blue stained nuclei showed that the nuclei extraction was successful and the nuclei were intact (Figure 19 A). The nuclear proteins (Figure 19 B) were extracted from the enriched nuclei and used for histone peptide pulldown analysis.

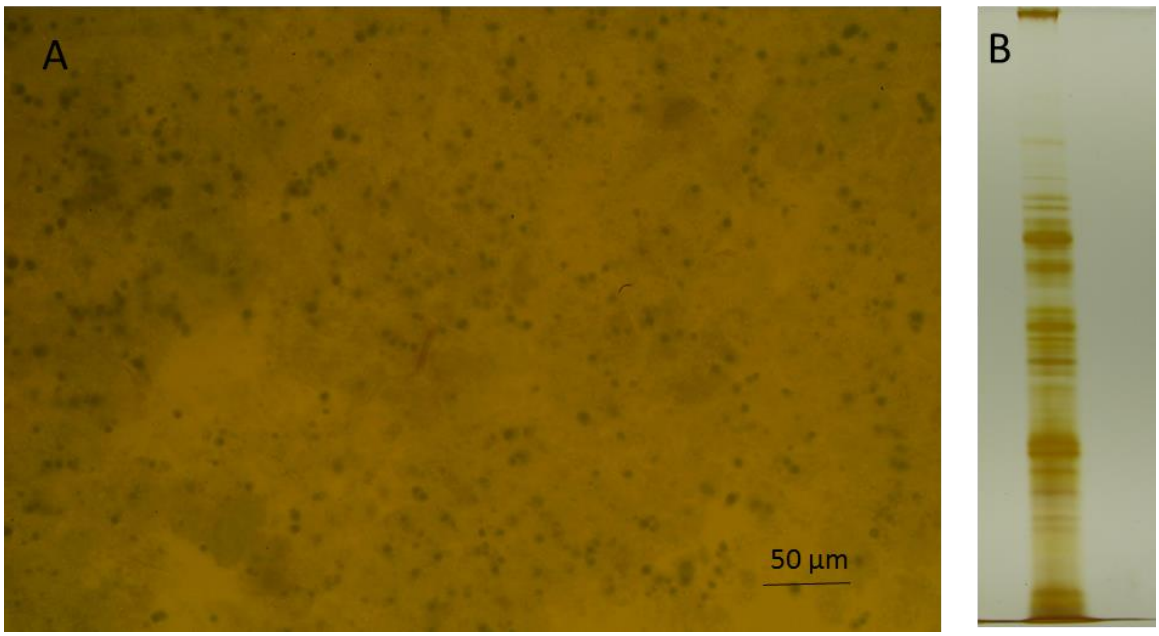

Figure 19 Enrichment of nuclei and extraction of nuclear proteins from rice suspension cells

(A)Image of Methylene blue stained nuclei (B) Silver stained image of nuclear proteins separated by SDS-PAGE.

Peptide pulldown analysis

Peptide pulldown analysis was used to identify the histone binding proteins in rice. For the peptide pulldown analysis, biotinylated histone peptides that were either modified or unmodified control peptides were fused on to avidin beads. Nuclear extracts obtained by enzymatic digestion of rice suspension cells were incubated with unmodified, modified histone H3 peptides. The bound resin was washed and the bound proteins were separated by discontinuous SDS PAGE, visualized by silver staining (Figure 20). The visualized proteins that are enriched by binding to either unmodified or modified histone peptides were identified by mass spec sequencing. Peptide pulldown analyses lead to the identification of many proteins, including some nuclear proteins and ribosomal proteins which are present in the nucleus. All the known histone methyl lysine binding proteins

contain a known histone binding domains, bromo domain, chromodomain, plant homeodomain, WD-40 repeats. Using pulldown analysis and mass spec, we identified two putative proteins (Os01g0322800 and Os03g0640100) that were named HB01, and HB02 respectively. Both HB01, HB02 had WD-40 repeats, and G-box that were characteristic of histone binding domains and they showed stronger binding to the unmodified histone peptides compared to that of the modified lysine methylated histone peptides.

HB01 and HB02 contain a histone binding domain

HB01 and HB02 are encoded by Os01g0322800 and Os03g0640100 genes respectively. Protein domain analysis using InterPro (http://www.ebi.ac.uk/interpro/) indicate that HB01 has six WD-40 repeats, three G-box repeats and HB01 has one LIS 1 (lissencephaly-1 protein) homology motif, seven WD-40 (also known as Trp(w)-Asp(D) motif) repeats. Both HB01 and HB02 showed stronger binding to the unmodified histone peptides compared to that of the modified lysine methylated histone peptides.

The HB01 gene is conserved in Arabidopsis, rice, humans, Caenorhabditis elegans, mosquito, fruit fly, cow, mouse, chimpanzee and rhesus monkey. Search for homologs using "HomoloGene" (http://www.ncbi.nlm.nih.gov/homologene) shows that SMU 1 (suppressor of mec-8 and umc-52) is a human homolog of HB01 and has about 65% homology. The Arabidopsis HB01 homolog SMU 1/ AT1G73720 encodes protein SMU1 involving in RNA splicing and shows 83% homology at protein level.

Gene annotation shows that HB02 is a Histone binding protein MSI (microsatellite instability) protein homolog. Search for homologs using "HomoloGene" (http://www.ncbi.nlm.nih.gov/homologene) shows that HB02 has 17 homologs in

eukaryotes. Arabidopsis MSI is a homolog of HB02 with an 87 % identity at protein level and RBBP (Retinoblastoma-binding protein) protein of humans shows 67.7% identity to HB02 at protein level.

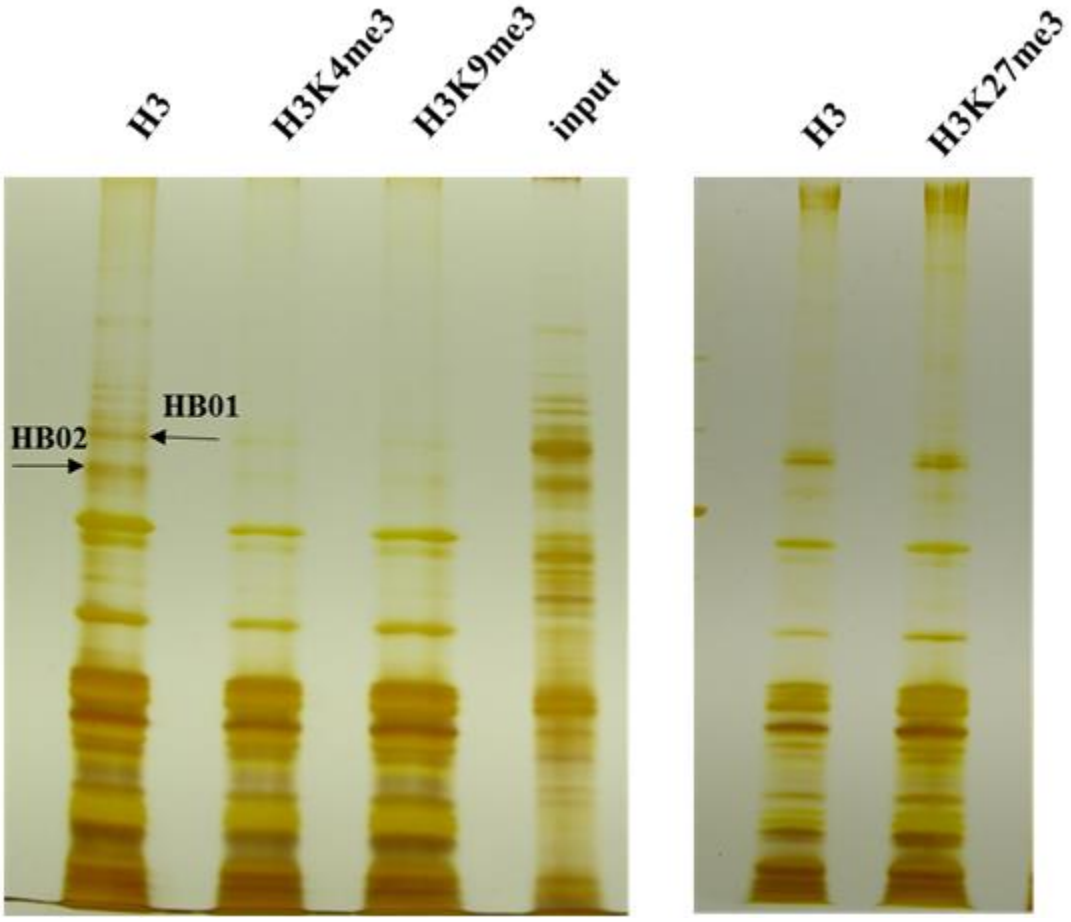

Figure 20 Silver staining of histone pulldown analysis with H3, trimethylated K4, K9 and K27 peptides.

Equal amounts of nuclear protein extract was incubated wither biotinylated modified or unmodified H3 peptides that were linked to avidin beads, washed and the bound proteins were eluted by SDS sample buffer, eluted proteins were separated by discontinuous SDS-PAGE and silver stained.

Generating recombinant proteins of HB01, HB02 resulted in formation of inclusion bodies

HB01 and HB02 proteins were identified by using peptide pulldown assay as histone binding proteins, and the presence of methyl modification at certain H3 lysine residues decreased their binding. Confirming the binding affinity of HB01 and HB02 proteins by far western analysis requires the production of recombinant GST tagged HB01 and HB02 proteins. To produce recombinant proteins, the coding sequences of HB01 and HB02 were cloned into a GST fusion expression vector. The GST-HB01 and GST-HB02 proteins transiently expressed in E.coli cells. Both GST-HB01 and GST-HB02 recombinant proteins were found in inclusion bodies of E.coli cells. The inclusion bodies were purified as described earlier in the Methods and the proteins in the inclusion bodies were isolated. As shown in Figure 21, GST-HB01 and GST-HB02 were the major proteins in the purified inclusion bodies.

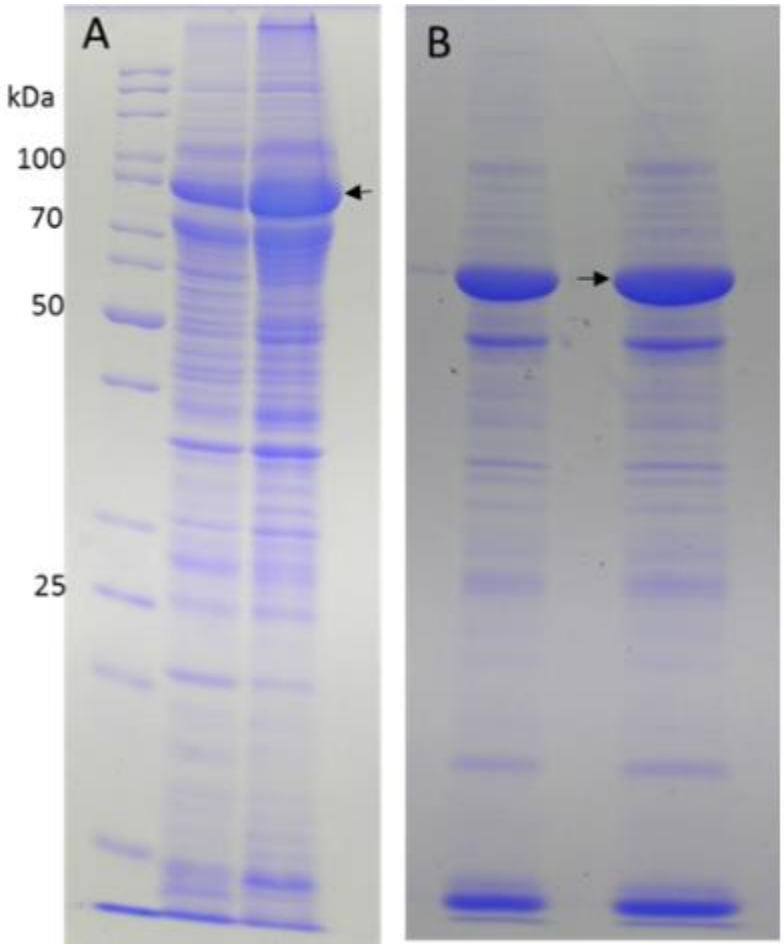

Figure 21 SDS Page analysis of GST-HB01 and GST-HB02 proteins in the purified inclusion bodies

(A) Lane 1 contains molecular weight marker from thermo scientific and the corresponding molecular masses are shown. Lane 2 and 3 contains the proteins present in the inclusion bodies before and after dialysis, respectively. The major band indicated by arrow corresponds to the GST-HB01 protein.
(B) Lane 1 and 2 represents proteins present in the inclusion bodies before and after dialysis respectively. The major band indicated by arrow corresponds to the GST-HB02 protein.

HB01 is a histone binding protein

To test the binding affinities of the hypothetical WD-40 domain containing proteins, the GST tagged recombinant HB01 and HB02 proteins were produced by induction with IPTG and used for far western analysis. In far western analysis, the

binding of individual GST-tagged WD-40 containing proteins to a complete set of histone H3 peptides containing all the known histone H3 modifications was evaluated. "AbSurance TM Histone H3 antibody specificity array" membrane containing all the known histone modifications and the corresponding unmodified peptides was used.

The binding of the GST-tagged recombinant protein to the H3 peptides was detected by western blot like procedure with anti GST-HRP conjugated antibody (Figure 22). The far western analysis performed with purified GST protein serves as a negative control. The far western analysis with GST- HB01 protein shows that it binds to unmodified histone peptide compared to the modified H3 peptide. The far western analysis results agrees with our pulldown analysis results.

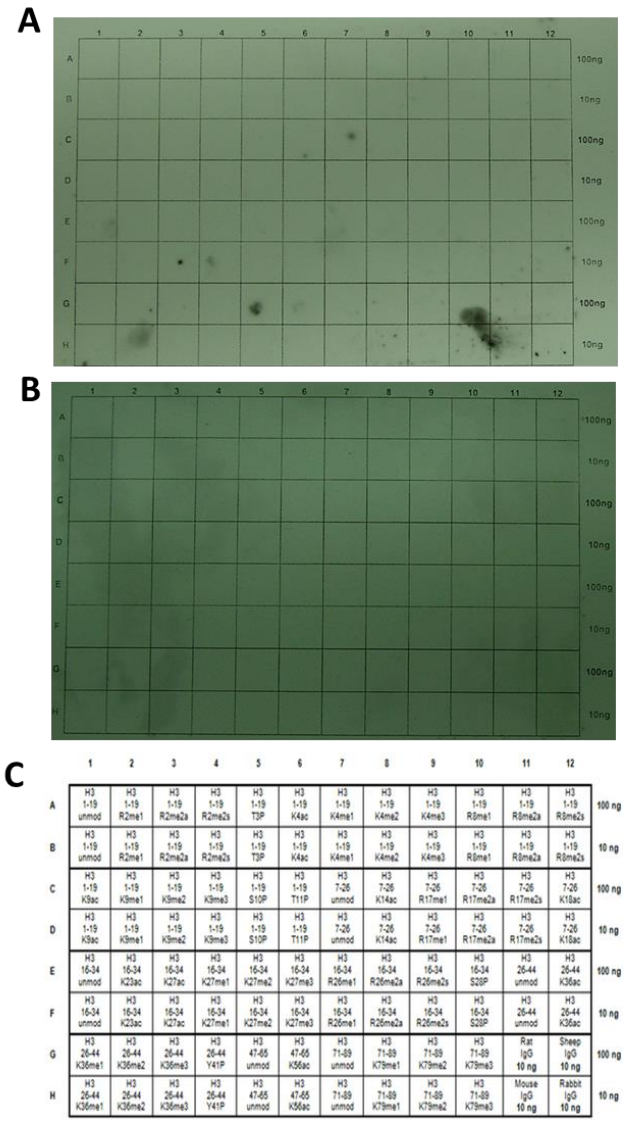

Figure 22 Far western showing the relative binding of GST-HB01, GST protein to histone peptides.

(A) GST-HB01 protein from purified inclusion body is incubated with peptide array containing all the known peptide modifications and corresponding unmodified peptides. The membrane was washed and the binding of GST tagged protein was analyzed with western blot like detection by probing with anti-GST HRP conjugated antibody.
(B) Purified GST protein was tested for binding to the histone peptide array and serves as negative control.
(C) Picture showing positions/placements of different histone peptides in the array.

Discussion

Histone post translational modifications are known to serve as binding sites for effector proteins or their associated protein complexes. Number of histone methylation, acetylation recognizing proteins, enzymes and associated complexes responsible for addition and removal of the post translational modifications have been characterized. Pulldown assays in animals were successful and lead to the identification/ characterization of many effector proteins of histone modifications. Peptide pulldown assay with histone H3 peptides was used for large scale screening of histone binding proteins present in the nuclear protein extract obtained from rice suspension cells. Nuclear proteins were extracted from the enriched nuclei obtained by enzymatic digestion of cell wall of rice suspension cell lines. Nuclear extraction procedure was adopted from Mujahid et al., (2013) protocol for nuclei enrichment. Sequencing of the proteins enriched from the nuclear protein extract by binding to histone peptides matches with the published list of nuclear proteins by Mujahid et al. (2013). The results from pulldown assay with modified and unmodified H3 peptides indicated that binding of WD-40 domain and G-Box containing proteins HB01/Os01g0322800 and HB02/Os03g0640100 was disrupted by the presence of methyl modification. Mass spec sequencing of proteins that bound to the histone modifications also indicated the presence of few ribosomal proteins, 14-3-3 family proteins, a putative uncharacterized WD-40 protein (accession: A2ZMR6). No ribosomal protein has been reported yet for their direct interaction with histone H3 or histone post translational modifications. The enrichment of ribosomal proteins identified in pulldown assay was because ribosomal proteins are abundant in plant nuclei and the abundant ribosomal proteins can compete with other

proteins for binding sites of histone peptides. They might have masked the interactions of other nuclear proteins as the peptide pulldown assay is a competitive binding assay. Histone binding proteins are known to have domains like bromo domain, chromo domain, PHD, WD-40. So we focused on WD 40 domain containing proteins identified in my pulldown assay and further tested their interaction using far western analysis.

The preferential direct binding of HB01 proteins was confirmed by using far western analysis with GST tagged recombinant HB01 protein. Far western analysis indicated that HB01 directly bound to the unmodified histone H3 peptides. This agrees with the pulldown assay results and we speculate that binding of WD-40 domain containing protein HB01 was disrupted by the presence of histone post translational modifications. We were not successful in confirming the direct binding of HB02 protein identified in pulldown analysis by Far western analysis.

It is known for many years that histone post translational modifications act as binding sites to histone binding proteins. In addition to acting as binding sites the presence of post translational modification can also deter the direct binding of some proteins to the histone tails. Pulldown assays in animals were successful in identifying the post translational modification binding proteins, but the disruption of their binding by the presence post translational modifications was ignored or not identified. It was reported that the presence of histone H3K4 methylation disrupts the direct binding of nucleosome remodeling and deacetylase (NuRD) repressor complex to H3 (Zegerman et al., 2002). The direct binding of INHAT (inhibitor of acetyl transferase) to H3 was disrupted by the presence of phosphorylation and acetylation modification on histone H3. Ebrel et al., (2013) reported that the binding of a large group of histone binding proteins (56

members) was disrupted by H3K4me3 modification. The proteins repelled by H3K4 belong to the NuRD complex and other proteins in the group are not yet characterized. One of the members of that group (proteins repelled by H3K4me3) is a retinoblastoma binding protein which is a human homologue of HB02 protein identified in our pulldown assay (Eberl et al., 2013). Till date, to the best of my knowledge these are the only reports showing that the presence of post translational modification on histone tails can disrupt the interaction of histone binding proteins (Eberl et al., 2013; Schneider et al., 2004; Zegerman et al., 2002).

Using pulldown analysis and far western analysis results, we identified that the direct binding of HB01, a homologue of SMU1 protein to Histone H3 is disrupted by the presence of posttranslational modifications. SMU1 protein in Arabidopsis regulates RNA splicing and other developmental process (Chung et al., 2009). WD-40 domain is present in many plant proteins and but none of the identified WD-40 domains have any enzymatic property and because of their structure they have many protein interacting sites and serve as protein interacting platforms. The enzymatic properties of WD-40 proteins come from their adjacent domains and their interacting proteins. Further analysis, tandem affinity purification of HB01 interacting proteins can help us understand the biological role of HB01 binding to histone H3.

CHAPTER VII

SUMMARY

In order to identify and study the ABA regulated enzymes in plants, in-gel activity analysis was performed addressing the enzymes involved in nitrogen assimilation, and enzymes involved in removing the phosphate groups, GlcNAc residues from target protein. The first objective was to identify the ABA regulated enzymes in nitrogen assimilation pathway by measuring the activity of enzymes in response to ABA treatment and study their activity during dehydration/drought stress. In-gel activity analysis, in-solution analysis was used to measure the activity of the enzymes and our results indicate that ABA mediated regulation of aspartate aminotransferase (AAT) results in the reduced activity of the chloroplastic AAT3 and cytosolic isozymes AAT2 isozymes. The activity of AAT2 and AAT3 isozymes reduced during drought/dehydration stress. The phosphorylation by SnRK2.2 and SnRK2.3 kinases is responsible for the reduced activity of AAT 2 and AAT 3 isozymes. It remains to be understood whether the phosphorylation of AAT isozymes by SnRK2.2 and SnRK2.3 kinases occurs either through direct phosphorylation or through indirect phosphorylation of other proteins that might function between the kinases and AAT. The aspartate aminotransferase mutants exhibit higher survival rates in response to drought/dehydration stress. Our results indicate that AAT is a negative regulator of drought stress and further experiments in the area are required to understand the mechanism resulting in the increased drought tolerance. Our preliminary

results also indicate that the activity of the nitrogen assimilation enzyme, glutamate dehydrogenase is upregulated in response to ABA treatment. Our results indicate that ABA regulates the activities of enzymes involved in nitrogen assimilation and adds to the current understating of the role of ABA in nitrogen assimilation.

In-gel activity analysis, mass spec was used to identify and study the activity of the phosphatases in response to ABA and drought stress in plants. We identified that activity of fructose 1-6 bisphosphatase family of phosphates are upregulated in response to ABA and drought stress in both Arabidopsis and rice plants. Fructose 1-6 bisphosphatase plays an important role in fructose signaling (Cho and Yoo. 2011) and our results indicate a crosslink between the fructose signaling and ABA signaling pathways. Our results indicate that in-gel activity staining of phosphatases can visualize sugar phosphatases like fructose 1-6 bisphosphatase family, sedoheptulase-1,7-bisphosphatase, inositol mono phosphatases; protein serine/threonine phosphatases, protein tyrosine phosphatases, and it will be useful to study these enzymes response to abiotic stress and hormones. Further experiments/ repeat experiments were needed to identify few other phosphatases, that we were unable to identify by mass spec and to study the activity of phosphatases in response to ABA and stress.

Soybean β-hexosaminidase with a higher activity in the neutral pH was identified by using in-gel activity analysis and mass spec sequencing. Our results indicate that the activity of the enzyme was induced in response to ABA in soybean. Since the β-hexosaminidase identified exhibited high activity at pH 6.5 compared to that at pH 4.5, we speculate that it might be a cytosolic hexosaminidase. Localization study with GFP tagges β-hexosaminidase can confirm the location. Further studies are needed to further

identify/characterize the hexosaminidase in plants and unravel the role of ABA in controlling GlcNAc modification. The cytosolic β-hexosaminidases were not yet identified in plants and our results indicate that the β-hexosaminidase identified in soybean is a potential candidate.

We identified three enzymes (aspartate aminotransferase, fructose 1-6 bisphosphatase, soybean β-hexosaminidase) whose activities are regulated by ABA. Collectively, our activity analysis results adds to the current understanding of the role of ABA in nitrogen assimilation, sugar signaling and in regulating GlcNAc modification in plants.

The other objective was to identify histone binding proteins in plants using peptide pulldown analysis. Two WD-40 domain containing proteins named Histone binding 01 (HB01) and histone binding 02 (HB02) were identified as histone H3 interacting proteins and the presence of methyl lysine modification had deterred their binding to the histone H3 peptides. Far western analysis with recombinant GST-tagged HB01 protein confirm that HB01 is a histone H3 binding protein and that the presence of post translational modifications deter its binding to histone H3. Further analysis, tandem affinity purification of HB01 interacting proteins can help us understand the biological role of HB01 binding to histone H3.

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APPENDIX A

ACTIVITY ANALYSIS OF CATALASE, SUPEROXIDE DISMUTASE AND GLUTAMATE DEHYDROGENASE

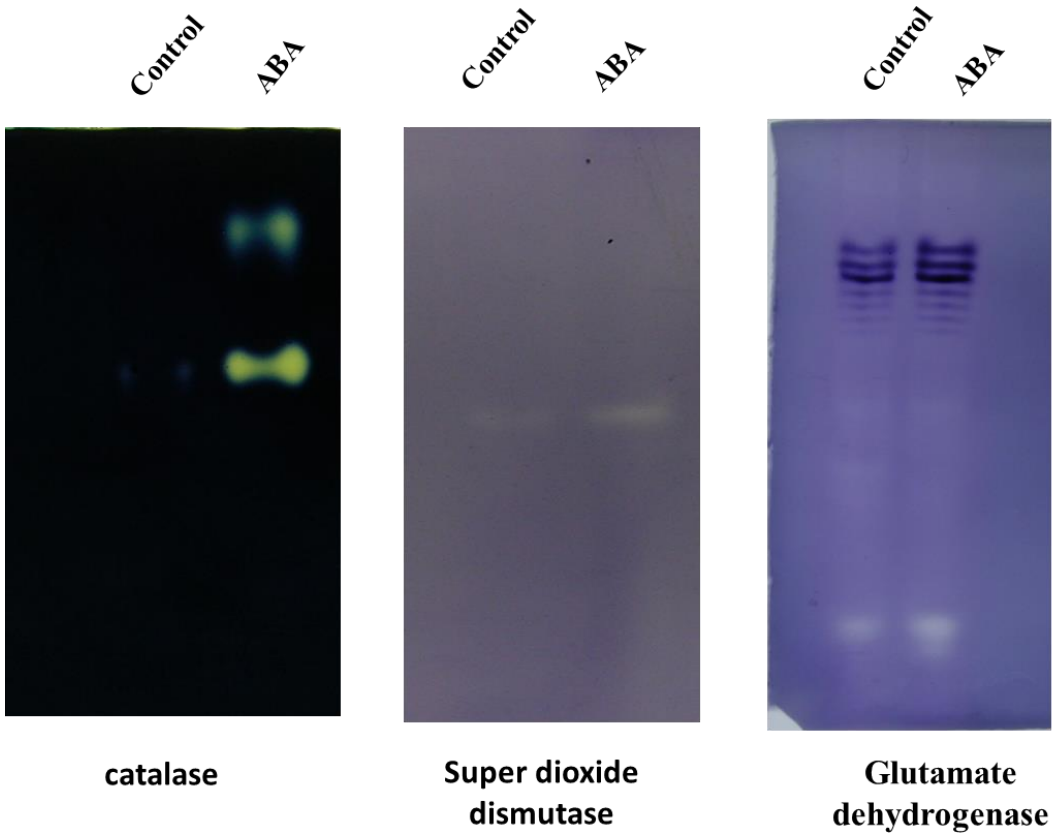

Figure 23 In-gel activity analysis of catalase, super oxide dismutase, glutamate dehydrogenase