AUXIN-CYTOKININ RESPONSES AND FUNCTIONAL SIGNIFICANCE OF AUXIN DURING RICE CROWN ROOT DEVELOPMENT

Ph.D THESIS

by

ANANYA NEOGY

DEPARTMENT OF BIOTECHNOLOGY
INDIAN INSTITUTE OF TECHNOLOGY ROORKEE
ROORKEE-247667 (INDIA)
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ROORKEE

CANDIDATE'S DECLARATION

I hereby certify that the work which is being presented in the thesis entitled "**AUXIN-CYTOKININ RESPONSES AND FUNCTIONAL SIGNIFICANCE OF AUXIN DURING RICE CROWN ROOT DEVELOPMENT**" in partial fulfilment of the requirements for the award of the degree of Doctor of Philosophy and submitted in the Department of Biotechnology of the Indian Institute of Technology Roorkee, Roorkee is an authentic record of my own work carried out during a period from December, 2014 to July, 2019 under the supervision of Dr. Shri Ram Yadav, Assistant Professor, Department of Biotechnology, Indian Institute of Technology Roorkee, Roorkee.

The matter presented in the thesis has not been submitted by me for the award of any other degree of this or any other institute.

(ANANYA NEOGY)

This is to certify that the above statement made by the candidate is correct to the best of my knowledge.

(SHRI RAM YADAV)
Supervisor

Date: _____

To my lab mate Anil and brother in law Abhijit da whom I lost during this journey

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ABSTRACT

Rice root system is a fibrous root system, composed of primary/seminal, adventitious/crown and lateral roots. Proper establishment of rice root system is a key determinant of productivity. Plant hormones such as auxin and cytokinin have been shown to play critical role in during crown root (CR) development but the dynamic responses and interactions between these hormones have not been extensively studied during crown root differentiation. The broad aim of this thesis was to study responses of auxin and cytokinin signaling and explore deeper insight on the functional significance of auxin during rice crown root development. The study first focused on identifying auxin and cytokinin responsive domains during various stages of CR development. Auxin and cytokinin responsive synthetic promoter::reporter constructs, DR5::erYFP and TCSn::erGFP, were generated for monitoring auxin and cytokinin responses, respectively. Stable transgenic rice lines generated raised with these constructs were used to monitor hormonal responses in rice stem base containing developing CRs. Using RNA-RNA in situ hybridization and fluorescence analysis, we observed show that in the early crown root primordia, auxin response is abundant in the early root cap cells of the root tips whereas TCSn::erGFP signals were relatively broader in the root tips. Importantly, TCSn::erGFP signals were significantly less in the auxin responsive domains in the root tip. Auxin-cytokinin cross talk is well established during root development in plants. During CR development, we also identified some narrow domains with overlapping auxin and cytokinin responses that might be a domain where auxin and cytokinin signaling directly interact with each other. Next, in order to understand the functional importance of auxin in the auxin-cytokinin interaction domain, we genetically altered active pool of endogenous auxin in the cytokinin responsive domain by expressing auxin inactivating gene, *OsMGH3* using TCSn promoter. We observed that reducing active auxin resulted pleiotropic abnormalities such as stunted plant growth, loss-of apical dominance and root architecture in the transgenic plants, indicating key role of auxin and cytokinin interaction during CR development. Further, to dissect out mechanism of auxin-regulated crown root formation, we selected two plant-specific AP2-domain containing specialized transcription factors (*OsAP2/ERF-40* and *OsAP2/PLT3*) for functional studies using reverse genetics-based approaches. RNA in situ hybridization and RT-PCR analyses showed that both genes are specifically expressed in the developing crown root primordia. Interestingly, and the expression of *OsAP2/ERF-40* was induced upon exogenous auxin treatment induced whereas *OsAP2/PLT3* was not affected in the rice stem base upon auxin treatment. We show

that down-regulation and ectopic over-expression of *OsAP2/ERF-40* in the transgenic rice display defects in the crown root development in the transgenic rice. These, together with the regulatory gene expression analysis revealed that *OsAP2/ERF-40* is sufficient to induce adventitious root and to trigger the root developmental program in a dose dependent manner. *OsAP2/PLT3* is a member of *PLETHORA* gene family, key regulators for stem cell specification and maintenance in the root apical meristem. High genetic redundancy in the PLT family compelled us to use mis-expression approach to decipher the function of *OsAP2/PLT3*. We observed that ectopic over-expression of *OsAP2/PLT3* causes defects in root architecture, radial growth, leaf angle and plant fertility. Overall, our studies not only reveal specific and overlapping auxin and cytokinin response domains but also provide evidence for two novel transcription factors regulating adventitious/crown root developmental program, a key agronomically important quantitative trait.

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ABBREVIATIONS

µg	microgram
µl	microlitre
µM	micromolar
2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-Benzylaminopurine
bp	base pairs
dATP	deoxy adenosine triphosphate
dNTP	deoxy nucleotide tri phosphate
EDTA	ethylene diamine tetra acetic acid
g	genomic
GFP	green fluorescence protein
hrs	hours
IAA	indole acetic acid
IBA	indole butyric acid
Kb	kilobase
mg/L	milligram/litre
min	minutes
MQ	milli-q
mRNA	messenger RNA
NAA	1-naphthaleneacetic acid
NPA	*N*-1-naphthylphthalamic acid
nts	nucleotides
ORF	open reading frame

p	plasmid designation
PBS	phosphate buffered saline
PCR	polymerase chain reaction
Rpm	revolutions per minute
RT	reverse transcription
SDS	sodium dodecyl sulphate
Sec	seconds
SSC	sodium chloride sodium citrate buffer
TBS	tris buffered saline
UTR	untranslated region
YFP	yellow florescence protein
NBT/BCIP	nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl- phosphate

CHAPTER 1
REVIEW OF LITERATURE

1.1 FEATURES OF PLANT DEVELOPMENT

The last common ancestor by far of plants and animals been a unicellular eukaryote which had incorporated a mitochondrial endosymbiont. Then the one that led to green plants incorporated another prokaryotic endosymbiont, the cyanobacterial chloroplast (Delwiche and Palmer, 1997). It has been clear that there are many developmental aspects which are common to all living being in this universe. Pattern formation is a common aspect of animal and plant organ development. Starting from a small insect *Drosophila* embryo to most complicated human embryo to plant flower, all have a specific pattern of development. For example, in *Drosophila*, segmental body pattern development is regulated by a family of homeobox genes (Veraksa et al., 2000). It is surprising that a plant, seemed to be of simpler organization than animals, have more genes than an animal. Animal system doesn't require anything like plant root system for anchorage. Some algae have holdfast only to provide anchorage. Higher plants evolved root system for anchorage as well as water and nutrient absorption because they are sessile in nature.

Development in plant is broadly studied in two parts: genetic and hormonal activity. Amongst many plant hormones, auxin and cytokinin are the ones to play vital role in every facet of development. These hormones are locally synthesized and transported throughout the organisms to perform a wide range of actions. Auxin was discovered hundreds of years ago as a growth promoting substance. The gradient of auxin distribution decides the cue to organogenesis. Cytokinin performs the opposite to restrict auxin only to some regions ensuring a synchronous growth. Auxin and cytokinin interplay maintain the shoot and root meristems (Dello Ioio et al., 2008; Zhao et al., 2010) and many other developmental programmes in plant. In this thesis, I investigate how dynamic auxin and cytokinin response shapes adventitious root development in rice and functional significance of auxin during various stages of crown root development.

1.2 AUXIN IN PLANT DEVELOPMENT

Auxin is a simple molecule, the idea of which was first conceived by Charles Darwin as a tropism promoting factor in plants. In the late 19th century, Darwin who became interested in plant phototropism and geotropism, demonstrated that light and gravity are perceived by the tips of shoots and roots respectively. But an asymmetric stimulus which gets accumulated to the region below the tip responds with differential growth causing it to bend towards the source

of light or the gravitational force (Darwin, 1980). But almost half a century after Darwin's idea, Frits Went succeeded in capturing the substance in his classic experiment on*Avena* coleoptile curvature and named it as auxin (Went, F. and Thimann, 1937). A decade later auxin was isolated from corn kernels in the form of indole-3-acetic acid (IAA) (Abel and Theologis, 2010). Since its discovery auxin has been the most explored phytohormone with diversified functions in every aspect of growth and development starting from new organ formation, maintenance or senescence. The biosynthesis, transport and signalling machinery is well-known in plant but how the whole machinery harmonize plant development is not elucidated completely. Auxin is a self-regulating signalling agent acting in negative feedback manner to keep many physiological processes in line.

1.2.1 Auxin metabolism and transport

The types of endogenous auxins reported are IAA itself, indole-3-butyric acid (IBA), phenyl acetic acid (PAA) and 4-chloro-indole-3-acetic acid (4-Cl-IAA). IBA without any evidence of inherent auxinic activity serves as storage form of IAA and present in considerable amount in *Arabidopsis* as readily available auxin (Ludwig-Müller, 2000). Although PAA is found in many species, 4-Cl has been found in few plant species and both having auxin effects. The biosynthesis pathway of auxin involves precursors generated via the shikimate pathway, primarily L-tryptophan. The shortest and developmentally significant IAA synthesis pathway is a two-step biosynthesis pathway in which amino acid Trp is converted into IAA using indole-3-pyruvate (IPyA) as the intermediate (Figure 1:1). The rate-liming step of IAA synthesis in IPyA pathway is catalyzed by a flavin monooxygenase, encoded by *YUCCA* gene (Zhao, 2014). *YUCCA* genes are expressed in all the suggested sites of auxin synthesis such as meristems, young primordia, vascular tissues and reproductive organs. Several combinations of *yuc* mutants display auxin related phenotypes, *yuc1 yuc4* double mutants are defective in floral and vascular development, *yuc1yuc4yuc10yuc11* quadruple mutants fail to develop a hypocotyl and root meristem in *Arabidopsis* (Cheng et al., 2006; Okada et al., 1991). TCP4 protein of *Arabidopsis* expresses at various developmental stages of leaf (Nath et al., 2003) by reprogramming the mitotic cells to exit division and acquire differentiation competence by activation of *YUCCA5* as well as by direct activation of *HAT2* (Challa et al., 2019, 2016). Other Trp-dependent pathways, which are generally named after an intermediate of the pathway, have been postulated such as the indole-3- acetamide (IAM) pathway, the tryptamine pathway, and the indole-3-acetaldoxime (IAOx) pathway (Woodward and Bartel, 2005). In rice, the

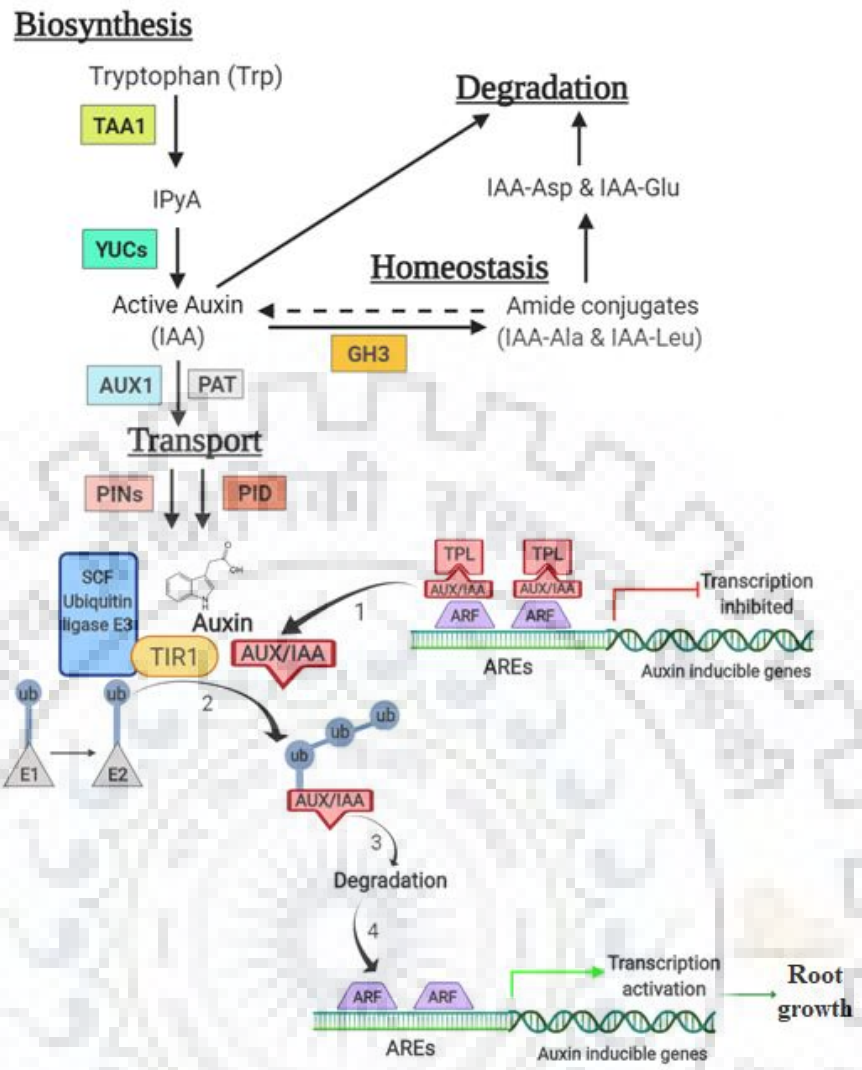

Figure 1:1. Auxin metabolism, homeostasis, transport and signalling pathway in plants. Trp-dependent auxin biosynthesis in two chemical steps, first is catalyzed by the TAA (tryptophan aminotransferase) to generateindole-3-pyruvate (IPyA), 2nd step is catalyzed by the YUC family of flavin monooxygenases to produce indole-3-acetic acid (IAA). Auxin homeostasis is maintained by conjugating auxin to amino acids by GH3 family of amido synthases to form amide conjugates for storage. Polar auxin transport (PAT) involves AUX1 influx, and PIN and PID efflux transporters. Auxin response factors (ARFs) bind to the auxin-response elements (AREs) in promoters of auxin-inducible genes. ARF-mediated gene expression is prohibited by Aux/IAA transcriptional repressors through interaction with their co-repressors TOPLESS (TPL). Auxin is received by auxin receptor, TIR then targeting Aux/IAA for proteolytic degradation. (Figure is adopted from Layser, 2018).

expression of *YUCCA1* is restricted to root tips, stem vascular tissues and leaf tips. Like the eleven *YUCCAs* (*YUCCA1-11*) in *Arabidopsis*, seven *OsYUCCA* genes of rice (*OsYUCCA1-7*) are categorized into three groups based on their sequence homology (Yamamoto et al., 2007). In rice, the expression of *OsYUCCA1* outweigh the expression of other six *YUCCAs*. Over-expression of *OsYUC1* has induced level of auxin biosynthesis which affects overall root growth of rice with increasing crown root number and root hair covering. The antisense RNAi lines of *OsYUC1* is marked with auxin related abnormal phenotypes such as dwarfism. Although the complete pathways of auxin biosynthesis are yet to be elucidated but from the pattern of different *OsYUC* expression, redundancy seems to be less complex in rice (Yamamoto et al., 2007). Also exists a tryptophan-independent auxin biosynthesis pathway forking from the precursor indole-3-glycerol phosphate, while the genes and enzymes involved in this pathway are yet to be discovered.

As auxin is required in all aspects of plant growth and development and it is not synthesized in all the tissues. So, it is necessary to maintain readily available auxin for immediate actions. The auxin pool is perpetuated by biosynthesis, transport, inactivation in the form of conjugates and degradation. The reversible conjugation regulates the auxin homeostasis (Bajguz and Piotrowska, 2009). More than 90% of IAA is found in the form of conjugates in plant (Yadav et al., 2011). IAA is conjugated either at the carboxyl group of sugars or carbohydrate components of glycoproteins via ester bonds or to amino acids, peptides and proteins via amide bonds. In dicots like *Arabidopsis*, amide-linked IAA-conjugates dominate that constitute approximately 90% over the ester-linked IAA-conjugates that constitute 8–10% of the auxin pool (Ljung et al., 2002b). The enzymes that conjugate free auxin to amino acids are encoded by members of the GH3 family of auxin induced genes (Figure 1.1) (Staswick, 2005). In *Arabidopsis*, twenty *GH3* genes represent multiple gene family encrypted in different chromosomes of the genome whereas in rice only 12 GH3 members were identified by sequence homology with the *Arabidopsis* GH3s (Hagen and Guilfoyle, 2002; Jain et al., 2006a). GH3 enzymes are classified into three groups (I, II, and III) based on their sequence homology and substrate specificity. Two from Arabidopsis (*AtGH*-10 and *AtGH3*-11) and four from rice i.e., *OsGH3*-3, -5, -6, and -12, Group I Members of adenylate jasmonic acid (Staswick et al., 2002). Group II has 15 members (eight from Arabidopsis and seven belongs to rice) are involved in IAA adenylation to conjugate amino acids to IAA (Staswick, 2005). Group III probably has undetermined but dicot specific functions as it doesn't include any rice GH3 proteins (Jain et al., 2006a). Two *Arabidopsis* group II *GH3* genes, *dfl1*-D and *ydk1*-D

conjugates auxin to amino acids to regulate hypocotyl elongation and the formation of lateral roots (Nakazawa et al., 2008; Takase et al., 2004). Auxin responsive rice *GH3* genes are expressed in various tissues and organs with most of the expression found in different floral stages (Jain et al., 2006b).

Young *Arabidopsis* seedling usually have very high concentrations of IAA which is known to be synthesized in actively dividing young leaves. But the highest pool of auxin is found to be in the roots and to some extent in the cotyledons which have substantial capacity for IAA synthesis (Ljung et al., 2002a). Auxin synthesized in Cotyledons seemingly useful in the early stages of development while other IAA sources are yet not developed. Developing organisms need to exchange information about relative cellular positions to develop differentiated cell types for proper developmental patterning. The positional cue is mightily depends on concentration gradients of morphogens such as auxin (Friml et al., 2002b). Although IAA synthesis takes place in almost all *Arabidopsis* organs, but the main source of auxin remains young apical tissues which supplies auxin throughout the plant. In plants, there are two independent pathways of auxin transport, most common is transport through phloem in bulk, from the source tissues (young leaves and flowers). The other being the more regulated, slower and carrier-mediated polar auxin transport in the vascular cambium from the shoot towards the base of the plant i.e. root apex, that mediates short-range cell to cell auxin movement in different tissues (Goldsmith, 1977). Polar auxin transport has been explained by classical 'chemiosmotic hypothesis' where cell-to- cell movement happens by the action of auxin specific influx and efflux carriers after identification of putative auxin influx (AUXIN1 [AUX1]/ LIKE-AUX1 [LAX] family) and efflux (PIN-FORMED [PIN] family) carriers (Fig 1.1) (Friml et al., 2003). The efflux carriers are positioned asymmetrically at a particular side of the cell as and when required to ascertain the direction of auxin flow (Raven, 1975; Rubery and Sheldrake, 1974). In *Arabidopsis*, four auxin influx carriers have been characterized functionally and eight members of the PIN protein family (PIN1 to PIN8) have been isolated (Petrásek and Friml, 2009). Five of auxin efflux carriers, PIN1, PIN2, PIN3, PIN4 and PIN7 are localized at the plasma membrane (Mravec et al., 2008; Petrásek et al., 2006). Some of the homologs of both influx and efflux carriers have also been found in several other plants and their concerted action play indispensable roles in plant development such as PIN1, PIN4 and PIN7 efflux carriers are required for the establishment of auxin gradient for proper embryogenesis (Friml et al., 2003b, 2002a). PIN proteins act redundantly as single pin mutants

can form fertile plants, whereas quadruple mutants of *pin1pin3pin4pin7* are strongly defective embryogenesis due to loss in establishment of apical-basal polarity (Benková et al., 2003; Friml et al., 2003). The coordinated shifts and rearrangements in *PIN1* and *PIN7* polarity from apical to basal region of the suspensor cells, also supported by *PIN4* action, lead to proper apical-basal flow of auxin to hypophysis and ensure normal root meristem formation. In the primary root, the asymmetric localization of AUX1 to the upper plasma membrane (PM) of protophloem cell layer helps unloading the auxin into protophloem cells and *AtPIN* localization to the lower PM, facilitate acropetal auxin movement towards the root tip through the phloem (Friml et al., 2002a; Swarup et al., 2001). In the columella, the action of *PIN3* and *PIN7* redirects auxin flow laterally to the lateral root cap and the epidermis (Friml et al., 2003). Till date, rice has 12 PINs, including four PIN1(with one duplicated very recently), one PIN2, three of PIN5, a PIN8, and monocot-specific PIN9, PIN10a, and PIN10b (Wang et al., 2009). Auxin efflux carriers, such as ETHYLENE INSENSITIVE ROOT (EIR) (Luschnig et al., 1998), and AUX1 influx carriers (Swarup et al., 2001) are expressed in specific areas of the root such as epidermal/cortical tissues during lateral root formation, in rice (Wang et al., 2009) and barley (Orman-Ligeza et al., 2013) and are involved in lateral root formation in dicots and monocot cereals. The expression patterns of different *OsPIN* genes in rice are primarily in vascular tissues in the stem-base region, dictates a directional auxin transport towards the root tip (Wang et al., 2009). *OsPIN9* promoter driven GUS expression shows high expression of the gene in pericycle cells, and adventitious root primordia in crown region and at the base of LRP. Also OsPIN10a and OsPIN10b, expression in stem-base pericycle cells suggest role of monocot specific PIN proteins in rice crown root formation (Wang et al., 2009).

1.2.2 Auxin perception and signalling

The diversity of putative auxin receptors is clearly the platform to comprehend the diversity of auxin responses which arises from the multitude of interactors for auxin receptors (TIR1/ AFBs) (Calderón Villalobos et al., 2012). In 2005, Dharmasiri et al. first provided the evidence that TIR1 being the auxin receptor mediates auxin and Aux/IAA interaction which in turn degrades Aux/IAA proteins and auxin-responsive genes express (Figure 1:1). Further studies confirm that TIR1 and Aux/IAA proteins must act as auxin co-receptors for efficient auxin perception by TIR1 (Calderón Villalobos et al., 2012). *Arabidopsis* has 6 members in the TIR1/AFB family and 29 members of Aux/IAA families, most of which undergo rapid turnover in the presence of auxin (Abel and Theologis, 2010). The structure of TIR1-ASK1 was solved

in a complex with auxin and a small Aux/IAA peptide with a C-terminal F-box domain and an N-terminal leucine-rich-repeat region (Dharmasiri et al., 2005). The crystal structure revealed that the LRR domain forms the auxin-binding pocket and also contact with the Aux/IAA peptide, whereas the F-box domain interacts with ASK1 (Tan et al., 2007). Auxin binding stabilizes the interaction of Aux/IAA to TIR1/ASK, as it was shown to create a second binding site for the degron motif of Aux/IAA proteins and enables the SCF^{TIR1} complex (named after three subunits: ARABIDOPSIS SKP1 HOMOLOGUE (ASK1), CULLIN 1 (CUL1) and an F-box protein) (Salehin et al., 2015) to ubiquitinate the transcriptional repressors (Ulmasov et al., 1997), AUX/IAA proteins, targeting them for 26S-proteasome *degradation* (Peer, 2013). Four among the five TIR1/AFB family members promote auxin responses (Dharmasiri et al., 2005). Either single or higher-order mutants of the receptors show either subtle or strong auxin resistance (Parry et al., 2009). In both monocot and eudicot plants the TIR1/AFB2 family is divided into two distinct clades (AFB1 and AFB2) and are maintained since early plant evolution demonstrates distinct function of each clade. It is evident from genetic studies that TIR1 and AFB2 have greater role in auxin response than AFB1 or AFB3 in the root (Parry et al., 2009). The auxin response pathway is of "double negative" nature, in which the presence of auxin directs the degradation of AUX/IAA proteins which in turn inhibit the ARF transcription factors (Weijers and Wagner, 2016). In rice 31 Aux/IAA genes were reported (Jain et al., 2006a). The Aux/IAA proteins are short-lived nuclear targeted proteins, comprised of four highly conserved domains, namely, I, II, III, and IV (Abel et al., 1994; Abel and Theologis, 2010). Domain I contains a conserved EAR domain that can recruit corepressors of the TOPLESS (TPL) family which in turn, can recruit chromatin remodelling proteins that stabilize transcriptional repression (Fig 1.1) (Szemenyei et al., 2008; Tiwari et al., 2004). The degron or Domain II is necessary for the interaction with the SCF^{TIR1} ubiquitin ligase complex (Gray et al., 2001) and subsequent degradation of AUX/IAA. C-terminal Domain III and IV have an acidic and a basic interaction surface that mediate homodimerization of Aux/IAA polypeptides and heterodimerization between Aux/ IAA and ARF proteins (Remington et al., 2004). This dimerization domains (III and IV), also known as PB1 domain (Guilfoyle, 2015), are conserved within the AUX/IAA family and conserved as well in the C-terminal domains of ARF proteins (Tiwari et al., 2003).

The *Arabidopsis* and rice genome contain 23 and 25 ARF (AUXIN RESPONSE FACTOR) loci respectively leading to high levels of redundancy. Presence of multiple AUX/IAA and their ability to modulate transcription of ARFs apparently can contribute to auxin signalling

specificity (Boer et al., 2014; Weijers et al., 2005) in both monocot and dicots. ARFs have been shown to bind in promoters of auxin response elements (AuxRE) with specificity to TGTCTC sequence. ARF activators are hetero-dimerized with Aux/IAA repressors to inhibit auxin response and in turn an auxin-regulated manner. Apart from a C-terminal (carboxy) dimerization domain (CTD), ARFs consist of a N-terminal (amino) plant-specific B3-type, DNA-binding domain (DBD) and an activation domain (AD) or repression domain (RD) in the middle region (Guilfoyle and Hagen, 2007; Tiwari et al., 2003). The modular nature of ARF DBDs has been documented with the Q-rich activation domains, while RDs of ARF are enriched in serine (S), leucine(L) , proline (P), and glycine(G) residues (Tiwari et al., 2003). ARF proteins homodimerize through their B3 domains, and the dimerization assist the cooperative DNA binding (Boer et al., 2014). Protoplast transfection experiments suggest only five major auxin-activated ARFs (ARFs 5, 6, 7, 8 and 19) but the remaining of the *Arabidopsis* ARFs function as transcriptional repressors only weakly interact with the core auxin signalling machinery (Tiwari et al., 2003; Ulmasov et al., 1999; Vernoux et al., 2011). On the other hand, among 25 putative OsARF proteins, nine are predicted to function as activators and the rest fourteen OsARFs as repressors. Rice auxin response factors fall into sister pairs as similar to *Arabidopsis* ARFs. The availability of *Arabidopsis* and rice genomic sequence makes it possible to find a link for comparison of the ARF genes between dicot and monocot plants. *Oryza sativa* contains *OsARF1* to *OsARF25*, dispersed on 10 of the 12 chromosomes (Wang et al., 2007). The AS-*OsARF1* transgenic rice show shorter heights and less vigour including smaller leaves as compared to wild type non-transformed rice plants. AS-*OsARF1* plants were sterile due to failed development heading stage (Attia et al., 2009). It becomes tempting to determine the binding of the twenty-two *Arabidopsis* ARF genes encode proteins with full-length DBDs that are supposed to recognize and compete for the same AuxRE target sites in the promoters and regulate their expression to coordinate growth and development (Abel and Theologis, 1996). The Aux/IAAs do not directly bind to DNA, they dimerize with ARF family transcription factors to support their oligomerization, like a stack of Lego blocks (Dinesh et al., 2015; Korasick et al., 2014). The whole auxin signalling is maintained in equilibrium by a highly dynamic feedback-regulated network which is retuned by one and only auxin. A growing body of information suggest that post-transcriptional regulation apart from transcriptional regulation, control the levels of different ARF proteins in various cells and tissues (Guilfoyle and Hagen, 2007). One such regulation of ARF transcript abundance is controlled by small (i.e. 21–24 nucleotide) endogenous RNAs, knowingly as micro-RNAs

(miRNA) and trans-acting-small interfering RNAs (ta-siRNA). They cleave complementary mRNA of ARF transcripts at widely conserved and target sites both in dicots and monocots. The genes which are induced within 5 to 10 minutes of auxin treatment are known as early, or primary auxin response genes which are categorized into three major classes such as Aux/IAAs, SAURs and GH3s (Hagen and Guilfoyle, 2002).

1.3 CYTOKININ IN PLANT DEVELOPMENT

Century after the discovery of auxin, in 1913, Gottlieb Haberlandt first reported the existence of substances in plant phloem exudates that could stimulate cell division in wounded potato tubers (Kieber and Schaller, 2010). Similar ability of coconut endosperm which stimulated cell division in Datura embryo was eventually discovered (VAN Overbeek et al., 1941). In 1955 for the first time the cell proliferating substance was successfully isolated from herring sperm, which was named kinetin (Miller et al., 1955). Next, in attempt to find out a naturally occurring kinetin in plants, trans-zeatin was identified (Miller, 1961), followed by many more which were reviewed in 2001 by Mok and Mok. Phytohormone cytokinins are adenine derivatives and are classified by configuration of their N6-side chain as unsaturated isoprenoid derived or cytokinins with aromatic side chains. Most prevalent cytokinins are the isoprenoid ones with a trans-hydroxylated N6-side chain such as trans-zeatin and its derivatives (Cebalo and Letham, 1967; Letham, 1963). Although the name cytokinin originated from their cytokinesis promoting nature, they were soon discovered to control multiple aspects of plant growth and development, including cell metabolism, chloroplast development, promotion of shoot and inhibition of root development, and delay of leaf senescence (Kim et al., 2012).

1.3.1 Cytokinin metabolism and transport

In plants, cytokinin synthesis is reported in specific sites like immature kernel, the root tip, and shoot apical meristem (Blackwell and Horgan, 1994; Takei et al., 2001). The first cytokinin biosynthetic gene was identified in the crown gall forming plant pathogenic bacterium *Agrobacterium tumefaciens* which cause abnormal cell proliferation in host plants due to overproduction of cytokinins by transducing IPT (ATP/ADP isopentenyl transferase) genes into plant genome from its Ti-plamid (Akiyoshi et al., 1984). The gene has been found in other bacterial species and proved to biosynthesize active cytokinin in vitro (Lichter et al., 1995). Research with *Arabidopsis thaliana* proposed two biosynthetic pathways of cytokinins, the tRNA pathway and the AMP pathway, with a variety of genes involved for biosynthesis.

The tRNA pathway is considered trivial and only speculated to be a possible source of cytokinins (Chen, 1997; McGaw and Burch, 1995). CK biosynthesis is a multi-step process, but most of the studies focused on the rate limiting step of synthesizing the cytokinin precursor isopentenyladenine (Figure 1:2). To start with *Arabidopsis* which contains utmost ten IPT genes encoded by a small multigene family and all having IPT activity except for *AtIPT2* (Takei et al., 2001). Histochemical analysis and accumulation of AtIPT transcripts in various tissues suggested a dominant role of *AtIPT5* and *AtIPT3* for CK production in roots. Similarly *AtIPT3* is the main CK biosynthesis enzyme in shoots (Takei et al., 2001). *AtIPT1* and *AtIPT3* - *AtIPT8*, only seven genes encode, ATP/ADP isopentenyl transferases in *Arabidopsis*. The cytokinin precursor isopentenyladenine, is then trans-hydroxylated by cytochrome P450 monoxygenases CYP735A1 and CYP735A2. ZR is a riboside of Zeatin, which is one of the most biologically active compounds, along with other ribosides such as iPA (isopentenyladenosine) and DZR (dihydrozeatin riboside). This conversion maintains the homeostasis between iP-CKs and tZ- CKs and is involved also in the regulation of the distant translocation of CKs from roots to shoots via the xylem (Takei et al., 2004). This transport plays a role in CK signalling and metabolism and the formation of hypocotyl adventitious root formation (Takei et al., 2004). Overexpression of *AtIPT4* in Arabidopsis induced shoot regeneration in callus culture even in the absence of exogenous cytokinin supplements (Kakimoto, 2001). Ten candidates of IPT designated as *OsIPT1* to *OsIPT10* is found in rice Phylogenetic analysis. *OsIPT1* to *OsIPT8* were grouped together to mediate the rate limiting step of CK biosynthesis but *OsIPT9* and *OsIPT10* were only involved in tRNA prenylation. Eight of the *OsIPT* genes (*OsIPT1–OsIPT8*) were characterized by Sakamoto et al. in 2006. The long-held idea of multistep reactions ends with the enzymes encoded by the LONELY GUY (LOG) gene family works in the final step of cytokinin metabolism to convert the inactive cytokinins into biologically active forms (Fig 1.2) and is required to maintain shoot apical meristem activity in *Arabidopsis* (Kurakawa et al., 2007). LOG genes were first identified in rice nine and eleven of them were found in the rice and *Arabidopsis* genomes, respectively (Kurakawa et al., 2007; Kuroha et al., 2009). Loss of function mutant of rice LONELY GUY (LOG) gene causes premature termination of the shoot meristem (Kurakawa et al., 2007). High-throughput transcript analysis show five of the *LOG/LOGL* genes (*LOG, LOGL5, LOGL7, LOGL9* and *LOGL10*) were abundant in shoots and the remaining LOGLs (*LOGL1, LOGL6* and *LOGL8*) were highly expressed in roots as compared to the shoot (Tsai et al., 2012). As both synthesis and degradation takes place throughout the plant, the genes

responsible for CK metabolism are widely expressed in the shoots and roots of *Arabidopsis*, but with specific expression patterns of the distinct genes (Miyawaki et al., 2004a; Werner et al., 2003). Oxidative cleavage of the N6 side chain, marks the degradation, breaking the cytokinin into adenine and a aldehyde as the major side-chain derived product (Brownlee et al., 1975). Only CYTOKININ OXIDASE (CKX) family enzymes has been shown explicitly to catalyze specific cytokinins such as isopentenyladenine, zeatin, and their ribosides in several plant species (Houba-Hérin et al., 1999; Schmülling et al., 2003). A family of seven homologous genes coding for CKX is present in the *A. thaliana* genome and clustered only on chromosome 2 and 5 (Bilyeu et al., 2001). Their genomic organization is highly conserved, with five exons and four introns and the amino acid residues are also quite conserved among different CKX enzymes which consists of two domains, one for FAD binding and another for substrate binding (Frebort et al., 2011). The *CKX* gene families of rice comprise of even more members, at least eleven in numbers. Among them only OsCKX4 and OsCKX5 are more closely related to *Arabidopsis* proteins, which is indicative of the separation of CKX family before the divergence of monocot and dicot plants. *OsCKX2* and *4* has been functionally characterized because of their abundance and sustained expression in shoot and root respectively (Ashikari et al., 2005; Gao et al., 2014; Tsai et al., 2012). Similarly, there are a network of cytokinin signalling genes whose expression is differentially regulated at various stages of panicle development (Yamburenko et al., 2017). Loss of function of *OsCKX2* causes excess amount of cytokinin in floral meristems, leading to enhanced numbers of panicles and increased grain yield (Ashikari et al., 2005).

Like any other phytohormones the homeostasis of biologically active cytokinins must be precisely maintained to fine tune growth and development. So, cytokinins can be glucosylated at the N7 and N9 positions by N-Glucosyltransferase with the highest activity on isoprenoid (Entsch et al., 1979). Two such cytokinin glucosyltransferases genes were indeed found in *Arabidopsis* (Ashikari et al., 2005) with no precise report on rice yet. CK derivatives like Zeatin O-glycosyl are resistant to *CKX*, probably to be an emergency storage for CK (Brzobohatý et al., 1993).

Very little is known till date, about how cytokinin transport in plants. A family of *PURINE PERMEASE* (*PUP*) gene encodes broad-affinity transporters and are the most likely candidates for cytokinin transporters as active uptake of various cytokinins by *PUP1* and *PUP2* has been established. Their expression pattern in plants has also led to the suggestion that they may play

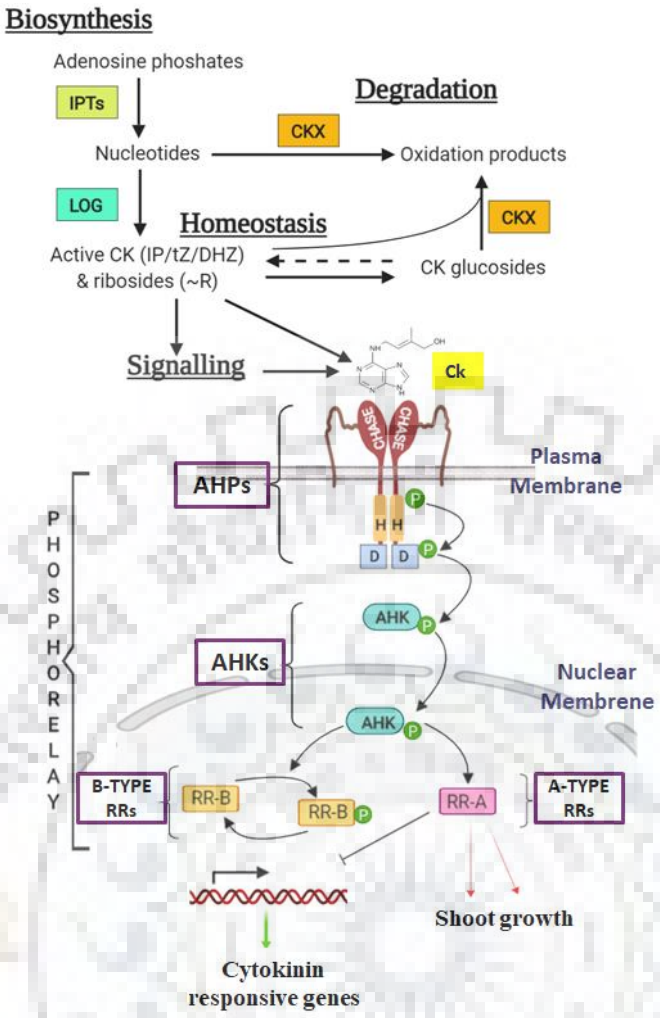

Figure 1:2. Cytokinin metabolism, homeostasis and signalling pathway in plants. Cytokinin biosynthesis is initiated by adenosine phosphate-isopentenyl transferases (IPTs) enzyme to generate iP-nucleotides, converted to the corresponding tZ-nucleotides directly to active free bases by cytokinin nucleoside 5'-monophosphate phosphor ribohydrolases (LOGs). Biologically active cytokinins such as isopentenyladenine (IP), trans-zeatin (tZ), and dihydro zeatin (DHZ) and their respective ribosides (R) are inactivated by cytokinin oxidases/dehydrogenases (CKXs). The core cytokinin signalling circuitry relies on two-component phosphorelay system. Cytokinin receptors or *Arabidopsis* histidine kinases (AHKs), perceive cytokinin at the CHASE domain to trigger a phosphoryl group (P), green circle from a conserved His (H), to an Asp (D), residue within AHK receptor and is then transmitted to *Arabidopsis* histidine phosphotransferase proteins (AHPs). The AHPs in turn phosphorylate the *Arabidopsis* response regulators (ARRs), type-B ARRs regulate the positive the cytokinin response. The AHPs initiate transcription of type A ARRs, which is inhibitory to the cytokinin signalling. (Figure is adopted from Schäfer et al., 2014).

a role in long-distance cytokinin transport (Bürkle et al., 2003). The mechanisms of cytokinin transport in planta are yet to be explored in detail (Kieber and Schaller, 2018; Romanov et al., 2018).

1.3.2 Cytokinin signalling

Genomic sequence analysis confirmed presence of all the and repertoire of genes encoding His kinases, His-containing phospho-transfer (Hpt) proteins, and response regulators that constitute a multistep phosphorelay in cytokinin signalling pathway (Figure 1:2) (Kieber and Schaller, 2018; Verma et al., 2015). The first putative cytokinin receptor was isolated from *Arabidopsis* known as CKI1, (Kakimoto, 1996; Nakamura et al., 1999), which contains three domains, one with two membrane-spanning regions, a receiver domain and a domain with histidine kinase sensor (Mok and Mok, 2001). *Arabidopsis* is the most explored system in which the cytokinin signalling pathway research has been conducted and it has been shown that cytokinin is perceived by three major membrane bound CHK receptors, namely AHKs 2, 3 and 4 (Wulfetange et al., 2011). Thus cytokinin signalling has been argued to be happen in endoplasmic reticulum (ER) membrane and/or at PM (Romanov et al., 2018). CHK receptors has a CHASE (Cyclase/HK-Associated Sensory Extracellular) domain which is a conserved 200–230-amino-acid domain that can (Anantharaman and Aravind, 2001) initiate a two-component signalling (TCS) system (Fig 1.2) upon cytokinin binding that triggers autophosphorylation at a conserved His a phosphoryl group transfer occurs from His to Asp residues of AHKs (Zürcher et al., 2013). *AHK2* and *AHK3* are primarily expressed in aerial tissues, whereas *AHK4* expression is greatest in roots. The three Arabidopsis receptors, *CRE1/WOL/AHK4, AHK2,* and *AHK3* share partial redundancy; single mutants have null phenotypes, whereas the triple mutants produce infertile plant (Higuchi et al., 2004; Inoue et al., 2001). In rice, five genes (CRL-1a, CRL-1b, CRL2, CRL3, CRL4), encoding CHASE domains and other similar domains like CRE1, have been found with functional similarity to AHKs (Han et al., 2004). Once the phosphoryl group is passed on to one of five AHP proteins is transmitted to nuclear HPs, subsequent the activation or repression of transcription occurs through the response regulators (RRs) (Kiba et al., 1999; Mähönen et al., 2006b). Six HPt genes have been identified in *Arabidopsis*, AHP1 to 5, and a pseudo-HPt, *AHP6* lacks the conserved histidine residue necessary for phosphorylation (Mähönen et al., 2006a; Suzuki et al., 2002). When AHP mutants are created, no single or double *ahp* mutants show phenotypic variation, confirms their redundant functions, although higher-order mutants such as quintuple

mutant of *ahp1to* 5 shows abnormal silique development with lesser seeds, diminished vascular development, underdeveloped primary root, and an increase in adventitious roots. Bioinformatic comparison of *O. sativa* and *Arabidopsis* the reveals a multifaceted TCS signalling machinery, comprising of similar number of putative TCS components in the two evolutionarily diverse model. Rice has been identified with eleven histidine kinases (*OsHKs*), five histidine phosphotransferases (*OsHPTs*) and thirty-six response regulators (*OsRRs*) and these components show significant sequence conservation with *Arabidopsis* counterparts (Pareek et al., 2006). The response regulators are divided three groups based on function and domain structure: type-A, type-B, type-C and a fourth group that covers few pseudo-RRs (Schaller et al., 2007). Type-B RRs are essential positive regulators for the cytokinin mediated transcriptional response (Ishida et al., 2008) and the protein possess a receiver domain and a C-terminal Myb-like DNA-binding domain (Ishida et al., 2008). These positive regulators are turned on by AHPs which phosphorylate the Asp residue in their receiver domain (Kieber and Schaller, 2018). The rate of turnover of the type-B RR family is regulated by an E3-ubiquitin ligase complex that comprises the KISS ME DEADLY (KMD) F-box proteins (Kim et al., 2013). The type-A response regulators contain the receiver domain and are the primary transcriptional targets of cytokinin signalling (D'Agostino et al., 2000; Korasick et al., 2014). The type-C response regulators are phylogenetically closer to the type-A RRs and only have the receiver domain sequences (Pils and Heyl, 2009). *ARR7* and *ARR15* are negative regulators of cytokinin signalling are repressed by auxin in the shoot apical meristem to promote shoot growth (Buechel et al., 2010). But the same A-type RRs are induced by auxin to inhibit ck for the maintenance of root apical meristem (Muller and Sheen, 2008). A B-type RR, *ARR10*, functional directly regulate expression of *WUS* that plays key role in the establishment and maintenance of the SAM (Zubo et al., 2017).

1.4 AUXIN-CYTOKININ CROSSTALK

Spatial and temporal homeostasis of auxin and cytokinin is finetuned by their orchestrated crosstalk via signalling. Their interactions are fundamentally antagonistic and can adjust each other's presence or differential distribution in a tissue- or context-specific manner to regulate plant growth and development. The antagonistic roles of auxin and cytokinin in regeneration was demonstrated in 1957 by Skoog and Miller from tissue culture experiments of shoot formation from callus when a high cytokinin: auxin ratio was provided. Recent biochemical

and genetic evidence has revealed that the intricate cross-talk between auxin and cytokinin signalling are required for formation and maintenance of plant meristems which decides the development of the whole plant body (Su et al., 2011; Zhao et al., 2010) The embryonic/primary root meristem forms at the base of the 16-celled globular embryo from a special cell known as the hypophysis which initially divides asymmetrically into two cells, an upper lens-shaped cell (Lsc) and a basal cell (Chandler and Werr, 2015). Upper cells divide to form the quiescent centre (qc) and the lower cells produce the upper layer of the columella (Bishopp et al., 2011). A stem cell niche which divides and differentiate into different tissue types, are organized surrounding the qc in the growing roots. The positional cue of those stem cells give rise to different root specific tissues such as the root cap, ground tissue, vascular tissues etc (Dolan et al., 1993). As the primary root (PR) grows, lateral roots are branched from it throughout the life cycle of the plant. In Arabidopsis, lateral roots develop from xylem pole pericycle (XPP) of PR at a regular interval (Lavenus et al., 2013). These XPP cells undergo a series of asymmetric cell divisions to differentiate into cell layers of lateral root primordia (LRP) (Bishopp et al., 2011). The event of root-pole specification is marked by the asymmetric division of hypophysis in the Lsc which maintains high cytokinin signalling but low auxin response, the lower cell shows vice-versa, upregulation the A-type negative cytokinin response regulator through high auxin response. The event of LR initiation from pairs of XPP cells are primed by oscillating waves of auxin response maxima (Moreno-Risueno et al., 2010). Strong auxin response was detected in the founder cell in stage I LRP (Dubrovsky et al., 2011). Whereas the cytokinin response was located in pericycle cells neighbouring LRPs. Similarly at later developmental stages, mutual auxin-cytokinin response were detected, auxin responsive DR5 reporter expressed at the LRP tips, and the cytokinin reporter TCS expression was restricted to the provasculature (Bielach et al., 2012). The first asymmetric anticlinal division of LR founder cell is antagonised by cytokinin through the repression of cyclins which are mostly involved in the G-M transition independent of auxin, these suggest that both auxin and cytokinin affect various check points of cell cycle (Chandler and Werr, 2015). Auxin along with ethylene regulates adventitious root formation, a key aspect of drought tolerance (Verma et al., 2016). Auxin- gibberellin crosstalk via auxin responsive SAUR36 regulates elongation of hypocotyl, from where adventitious roots emerge in Arabidopsis (Stamm and Kumar, 2013).

1.5 ORIGIN AND EVOLUTION OF ROOT

Root system in plants evolved during the Devonian period (Kenrick and Strullu-Derrien, 2014) after plants made the transition from water to the land . The consolidated evidences from both fossils and living plants suggests that the rooting organs evolved independently in several different clades, with a rapid gain of functionality as well as complexity. The earliest evidence of rooting structures to land plants were found in the form of small rhizome-like traces buried in the paleosols (Retallack 2001) and from lycopods in the sediments (Kotyk et al. 2002). All observations led to a fairly consistent picture of the development of rooting systems which was shallow but extensively specialized to be stabilized in riverbanks. Therefore, in vascular plants, earliest root producing structures were aerial stems or rhizomes that started producing rhizoids. They simultaneously provided plant anchorage and have functioned as perennial growth organ. In lycopod, *Asteroxylon*, rhizoids were truly absent, indicating their non essentiality to all early vascular plants (Edwards, 2003). The rhizome-based root system of horsetails probably emanated from herbaceous plants of Middle to Late Devonian (398–359 Ma) period, in which roots and upright stems were borne along one side of a horizontal rhizome. In progymnosperm, like *Eddya sullivanensis*, a complex taproot system evolved with a strong main root which profuse into small and probably endogenous lateral branches (Beck and B., 1967). Cambial activity was observed in the taproot and rootlets arising from them. Later on gymnosperms possessed much similar type of root system as progymnosperms (Algeo and Scheckler, 1998). The sharedness in the evolutionary history of rhizoids was striking when the development was compared in the moss, *Physcomitrella patens* with the flowering plant, *Arabidopsis thaliana*, root hair formation. Both plants have a highly conserved molecular mechanism controlling their development. (Menand et al., 2007). The evolution of plants with tall heights, particularly trees placed the demand of sophisticated rooting systems. Soils were thin layered because of microbial origin prior to the Devonian Period. The increase in root biomass was witnessed later in Devonian Period, that had an enormous impact on the evolution of soils and the earth atmosphere (Retallack 2001). Specialized rooting systems became necessary for the evolution of plants which would shift from the coastal region to exploit broader range of habitats. In larger trees roots are combined with secondary vascular growth which were essential for the evolution of plants to meet the supply of water and nutrients to aboveground tissues (Boyce 2005). Evidences of fossils indicates that certain aspects of root morphology such as bipolar growth and development (vascular cambium, polar auxin transport) evolved in a piecemeal

fashion in separate groups of vascular plants. The developmental aspects including evolution of endogenous meristems, the root cap, cell layers are still very poorly understood (Gensel et al. 2001). Ultimately, molecular methods has to be deployed to investigate evolution of developmental controls of root system architecture or any other phenotypic development (Menand et al., 2007; Nishiyama, 2007).

1.6 VARIABLE MORPHOLOGIES OF ROOT SYSTEMS

Root system architecture constitutes the primary root, lateral roots and other adventitious root systems and has a prominent role in determining the nutrient and water use efficiency of the plant. Root system organization substantially differs in two angiosperm classes, the dicotyledons and the monocotyledons. Primary roots are the first structure to be derived from the embryo in the form of radicle (Fig1.3). In dicots, the primary root become the main, thick tap root system consists of several lateral roots (Fig1.3). In monocots, embryogenic primary root develops to support the plant at the seedling stage but is very soon replaced by copious numbers of adventitious roots ensuring optimum water and nutrient uptake.

In plants, two major types of root systems have been defined by their origin and patterns of branching. A taproot system is distinctive of dicot plants such as *Arabidopsis thaliana*, pea (*Pisum sativ*um), while monocots such as rice (*Oryza sativa*) and wheat (*Triticum aestivum*), and maize (*Zea mays*) are featured by fibrous root systems (Atkinson et al., 2014). Dicot model plant *Arabidopsis* has a taproot system with an effective role of PR throughout the life cycle along with several lateral roots derived from the PR post-embryonically with occasional secondary orders of branching (Figure 1:3) (Atkinson et al., 2014; Bellini et al., 2014). In monocot emphasizing the cereals, 'seminal' roots, together with shoot-borne roots like 'crown' and 'brace' typify the fibrous root system (Smith and De Smet, 2012). Monocot model rice has a fibrous root system consists predominantly of adventitious root also known as crown root. ARs normally is not developed in *Arabidopsis* but can be induced by conditions like dark-light transitions. Apart from the embryonic roots, rice root system has three more types of the postembryonic roots: crown roots, the large lateral roots (LLR), and the small lateral roots (SLR) (Hochholdinger et al., 2004). Postembryonic crown roots can be called nodal roots as they emerge from the compressed nodes on the stem and tiller base arranged in rows. A phytomer concept in rice is a developmental unit which gives rise to crown roots acropetally. By convention the coleoptilar phytomere (C-th) is the zeroth phytomere

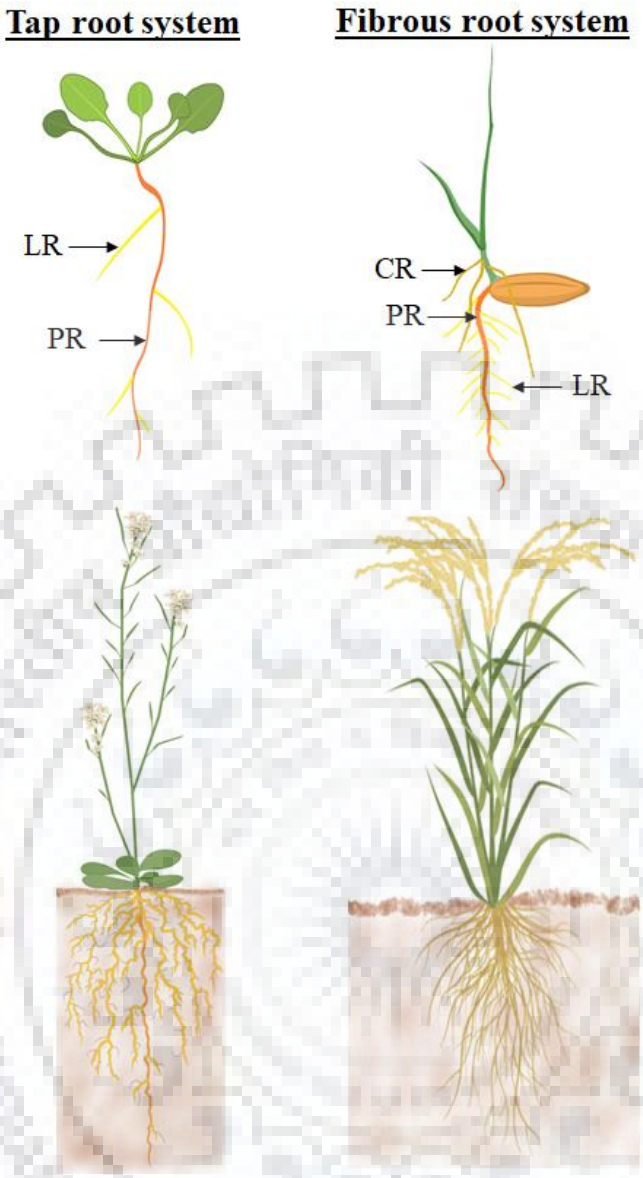

Figure 1:3. Root system architecture in typical dicot and monocot plants. Model dicotyledonous plant *Arabidopsis* in the diagram has taproot system whereas model monocotyledonous plant, *Oryza sativa* with fibrous root system. Upper panel shows roots at early growth phase of plants and lower panel depicts root system in mature Arabidopsis with a perfectly working PR and many orders of LR branching but in mature rice only adventitious roots are evident. PR denotes primary root, LR: lateral root. CR: crown root. (Figure is adopted from Bellini et al, 2014).

where belongs the radicle. A field-grown, healthy rice plant may have thousands of crown roots at mature stage (Itoh et al., 2005). Lateral root initiation in monocots differs somewhat from well-studied dicots. In cereals, both pericycle and endodermal cells has ability to become lateral root cells, but at the phloem poles (Smith and De Smet, 2012). While *Arabidopsis* has only two xylem poles to initiate lateral roots, in rice there can be as many as10 phloem poles are in position to develop lateral roots around any parental root (Coudert et al., 2010). Root development in monocots is recently being explored and a proper staging system of crown root development have been proposed. The crown roots of rice originate from the innermost ground meristem cells, adjacent to the peripheral vascular cylinder (PVC) in the stem, gradually they differentiate various tissues to form the complete organization of the of the crown root and development.

1.7 REGULATION OF ROOT GROWTH

Arabidopsis is the best plant model system till now to address questions related to the processes of root development at the cellular level (Péret et al., 2009). The root branching in dicot Arabidopsis is at the level of lateral roots. However, cereal root system has a different kind of root system than dicots. As rice has been used as monocot model system since 1985, it will address questions that are impossible to answer with Arabidopsis where adventitious roots do not develop naturally. Like, what are the molecular mechanisms happening at differentiation of root primordia at the base of the stem? How the root apical meristems (RAMs) of adventitious roots are generated and maintained? How can we combine functional genomic approaches efficiently to identify genes involved in root development to improve entire root architecture of crops towards limiting soil resources (Coudert et al., 2010)? Many studies have been already done to understand the genetic regulation yet more to be revealed. In any species of plant both ARs and LRs are postembryonic to develop. Origin of LRs is well established in Arabidopsis and rice. The origin of ARs is largely undetermined till now in different species because of the varied organs and tissues they develop from (Bellini et al., 2014). Arabidopsis lateral roots (LRs) develop from the xylem pole pericycle cells of the primary root (PR), LRs or ARs at a regular interval (Lavenus et al., 2013; Péret et al., 2009). The LRs in rice originate from endodermal and pericycle cells located opposite to protophloem (Bellini et al., 2014; Mai et al., 2014). The LR initiation and developmental stages have been thoroughly studied in the model dicot, *Arabidopsis*. Development of LR primordium have been divided in seven stages

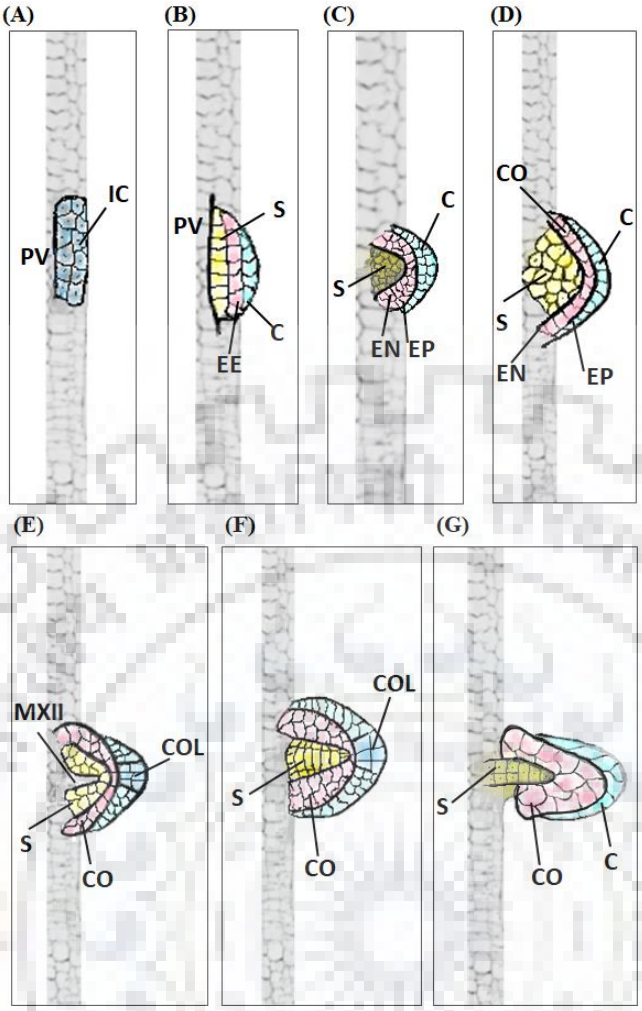

Figure 1:4. Stages of crown root (CR) development in rice. (A) Establishment of IC or the initial cells. **(B)** Establishment of epidermis–endodermis (EE) and root cap initials. **(C)** Differentiation of EE initials into epidermis (EP) and endodermis (EN). **(D)** Differentiation of prominent cortex (CO) layer. **(E)** Formation of central organization of root primordium with meta-xylem. **(F)** Cell vacuolation in cortex and elongation in stele (S). **(G)** Emergence of CR through cell elongation. PV, peripheral cylinder of vascular bundle; C, root cap or its initial; epidermis–endodermis initials; COL, columella; MXII, late meta-xylem vessel. (Figure is adopted from Itoh et al., 2005).

that correspond from acquisition of cell identity till tissue organization (Malamy and Benfey, 1997). Closely spaced cell walls in the pericycle layer in perpendicular orientation to the root axis, increased frequency of anticlinal divisions is clearly seen at XPP marking the stage I of LRP initiation. At stage II periclinal division occurs and cells expand radially, the domed shape of the LRP begins to appear. Next few stages few periclinal and then anticlinal cell divisions occur at next stages that cause LRP to penetrate the endodermal and cortex layer. Stage VI, the LRP has passed through the parent cortex layer and has penetrated the epidermis, and have characteristic vascular elements with much resemblance of a mature root tip with a potential root cap. Stage VII or the last stage, the LRP is ready to emerge through cell expansion (Malamy and Benfey, 1997).

In contrast to lateral roots, adventitious roots can be developed from different tissues but ARs tend to develop from cells close to vascular tissues. The mechanisms of adventitious root development in Arabidopsis is not well studied as compared to LR. Either normal Adventitious roots forming on intact hypocotyls or excision induced ARs, both develop and emerge from hypocotyl pericycle cells (Takahashi et al., 2003). According to Itoh et al, crown root development in rice can be divided into seven stages (Figure 1:4 A- G). Stage 1 is the establishment of initial cells of crown root primordium (CRP), formed in a few layers, by one or two periclinal divisions (Figure 1:4 A). Stage 2 of CRP is formation of epidermis–endodermis, central cylinder and root cap initials by both anticlinal and periclinal divisions to form epidermis–endodermis initial, central cylinder initial and root cap initial (Figure 1:4 B). Stage 3 will be Differentiation of epidermis and endodermis again by periclinal divisions (Figure 1:4 C). At stage 4 endodermal cells begin to form cortical cells by periclinal divisions (Figure 1:4 D). At stage 5, fundamental organization of the root is established, columella divides to form root cap initial cells, a large meta-xylem vessel becomes prominent at the centre of the stele, (Figure 1:4 E). Next stage is commencement of cell elongation and vacuolation, cells in the basal region of the stele show and those of the cortex show vacuolation (Figure 1:4 F). Final or the stage 7 crown roots emerge as all the differentiated cells of the CRP elongate (Figure 1:4 G) (Itoh et al., 2005).

1.7.1 Endogenous control of root development

Mechanisms underlaying root patterning is important to understand as it includes identifying the genes responsible for post-embryonic root growth. The understanding thus could empower

plant breeders to improve crop tolerance to environmental stresses to enable another green revolution (Coudert et al., 2010).

Auxins are the most important group of plant growth regulators so far. Many of them are naturally occurring like IAA and synthetic chemicals like 2,4-D but the most studied are the natural ones which are IAA and IBA. Every aspect of root development from founder cell specification to root elongation involves auxin, be it monocotyledons or dicotyledons. For example, experiments back in 1936 by Thimann, proved the ability of IAA to inhibit *Avena* root elongation (Thimann, 1936). Although auxin biosynthesis is increased specifically in the QC, root stem cell niche and the allied regions (Brady et al., 2007), it is proved that both rootward auxin transport and lateral auxin redistribution helps in auxin accumulation in the primary root tip by polar transport from the shoot, largely via the PINs (Blilou et al., 2005; Grieneisen et al., 2007). To maintain an auxin maximum in the root meristem, auxin reflux occurs at the transition from the root meristematic zone to elongation zone by auxin transport from all the root layers into the root vascular stream by *PIN1*, *3*, and *7*. *GNOM* is one of the most characterized membrane trafficking regulators in plants that activates small GTPases of the ARF (ADP ribosylation factor) class to mediate vesicle budding at endomembrane (Naramoto et al., 2014) and recycles the PIN auxin transporters and ensures their proper distribution to the plasma membrane (Geldner et al., 2003; Steinmann et al., 1999). Genetic perturbation of *gnom* alleles which codes for a a GDP/GTP exchange factor for small G-proteins of the ADP ribosylation factor, in *Arabidopsis* forms small seedlings with very small primary root (Geldner et al., 2004; Kleine-Vehn et al., 2009). Later studies showed that exogenous treatment of auxin has the ability to also induce LRPs from the xylem pole pericycle cells in *Arabidopsis* (Himanen et al., 2002). Auxin is so pivotal that any of its biosynthetic, transport and/or signalling mutants is defective in the density and/or development of LRs (Casimiro et al., 2003). Iterative GUS staining driven by auxin responsive synthetic promoter DR5 study hypothesized that an oscillating auxin response has been regulating the priming of periodic pre-branch site for the LR (De Smet et al., 2007). The best-known example of auxin response in lateral root development is through *SOLITARY-ROOT (SLR)/IAA14*. The dominant mutant of *SLR1/IAA14* lack lateral root initiation as the mutation blocks cell division in the pericycle layer (Fukaki et al., 2002). Further, the hypophysis which gives rise to the radicle, is not specified in mutants lacking *MONOPTEROS (MP)/AUXIN RESPONSE FACTOR5 (ARF5)* (Hardtke and Berleth, 1998), develops no roots. Inhibitory interactions of IAA proteins such as

BODENLOS (BDL)/INDOLE-3-ACETIC ACID INDUCIBLE12 (IAA12) and *ARF* transcription factors such as mp/arf5 prevents embryonic root formation (Hamann et al., 2002; Weijers et al., 2005). *SLR/IAA14* with *ARF7–ARF19* auxin response module directs transcription of the early auxin response *LBD/ASL* transcription factors regulating a set of genes controlling asymmetric pericycle cell divisions during lateral root formation (Okushima et al., 2007). *Arabidopsis* and rice mutants altered in root development facilitated the identification of quite a few components of the auxin transport and auxin signalling machinery being instrumental in LR and CR developmental stages (Lavenus et al., 2013; Orman-Ligeza et al., 2013). In *Arabidopsis*, Exogenous auxin treatment is an efficient promoter of AR initiation through the activation of auxin signalling, which has been shown to occur in many species (Ricci et al., 2008). Additionally, two At mutants *superroot* (*sur*) and *rooty*, have high levels of aux endogenously exhibiting proliferation of adventitious roots (Boerjan et al., 1995; Celenza et al., 1995). The gain-of-function mutant *short hypocotyl2*/*iaa3* was reduced AR formation, while the mutants, *auxin resistant3*/*iaa17* has increased AR formation (Leyser et al., 1996; Tian and Reed, 1999). Certain phytohormone-related genes regulate rice primary root growth, such as auxin importer mutant, *Osaux1* negatively regulates PR length and had a longer primary root than the control wild type, while *OsARF12*, an auxin response factor, positively regulates PR development and knock-out lines had shorter primary roots (Qi et al., 2012; Yu et al., 2015).

While auxin plays as the mastermind, other hormones are also critical for proper establishment and maintenance of root growth. Cytokinin also plays an essential role in root vascular patterning. Mutation of cytokinin receptor gene, *AHK4* also known as *WOODEN LEG* [*WOL/CRE1/ARABIDOPSIS HISTIDINE KINASE4)*], singly or in combination with other receptors *AHK2* and *AHK3*, results in abnormal differentiation of the procambial cells (Scheres et al., 1995). Another proof of essential cytokinin signalling for cell types specification of metaxylem and phloem is *ARABIDOPSIS HISTIDINE PHOSPHOTRANSFER PROTEIN 6* (*AHP6*) which is a known inhibitor of cytokinin and thus promotes protoxylem identity of the root (Mähönen et al., 2006a). Root meristem is enlarged in multiple mutants of cytokinin biosynthetic enzymes (*IPTs*), in the cytokinin receptor *AHK3* and its suggested downstream target *ARR1* (Dello Ioio et al., 2007). *OsCKX4*, a CK oxidase/dehydrogenase (*CKX*) family gene that catabolize CK, plays a positive role in crown root formation by inhibiting Ck signalling. The overall height of the plant was reduced in overexpressing *OsCKX4* but the number of crown roots was more compared to WT, implies its important role in crown root

development (Gao et al., 2014; Kitomi et al., 2011). *OsCKX4* found to be a direct target of both the *OsARF25* and *OsRR2*, *OsRR3*, suggesting that crown root development is co-ordinately regulated by auxin and CK signalling (Gao et al., 2014). Further, reducing endogenous cytokinin by *CKX1* (cytokinin oxidase-dehydrogenase 1) precisely in the zone between dividing and elongating root cells suggest that the hormone inhibit root growth by promoting cellular differentiation (Miyawaki et al., 2004b). Microarray study of roots overexpressing *ARR1* revealed that *SHORT HYPOCOTYL2 (SHY2)/IAA3*, heterodimerizes with *ARF*s, preventing auxin responses, and provides yet another fine example of antagonistic auxin and cytokinin interplay during root growth (Dello Ioio et al., 2008). The same antagonistic auxin-cytokinin network functions at the borderline of meristematic and elongation zone where activation of *SHY2/IAA3*, an *ARR1* direct target, by cytokinin restricts expression domain of a group of auxin transporters (*PIN1*, *PIN3*, and *PIN7*). In summary, mutually exclusive promotion of cell division by auxin and differentiation by cytokinin provides the molecular control for the phenotypic effects observed in root meristem size (Dello Ioio et al., 2008; Miyawaki et al., 2004b).

Endogenous genetic regulation determines the acquisition of cell fate of the very first cell which gets the signal to shift its role from any cell to be the founder cell of either primary root or LRP or CRP. In *Arabidopsis*, *WUSCHEL* related homeobox or the *WOX* genes which encodes transcription factors are the key in the making of embryonic root (Haecker et al., 2004). But soon it was clear that *WOX* is not enough for the embryonic patterning, additional factors are also required. The QC in the RAM region maintains the undifferentiated state of stem cell niche and is specified by two family of TFs: the *PLETHORAs* (PLT) and the *SHORT ROOT (SHR)/SCARECROW (SCR*) (Aida et al., 2004; Galinha et al., 2007; Sabatini et al., 2003). The PLT genes encode AP2-domain containing transcription factors although are found in the niche to promote cell division, their loss-of-function alleles result in loss of QC identity, thus premature termination of root growth. The other group (SHR and SCR) belongs to the GRAS [GIBBERELLIN INSENSITIVE (GAI), REPRESSOR OF GA1–3 (RGA), SCR] family of TFs (Aida et al., 2004; Sabatini et al., 2003). SHR moves from stele to QC to activate SCR transcription which in turn maintains QC as well as the stem cells in a cell-autonomous mechanism (Sabatini et al., 2003). QC cells appear to produce a short-range signal which might be *WOX5* which operates non-cell-autonomously and downstream of *SHR* and *SCR* to prevent root stem cell differentiation (Haecker et al., 2004). Restriction of *WOX5* expression to the QC

maintains the stem cell pool in the root apical meristem by a mechanism similar to that of the transcription factor *WUSCHEL* in the shoot apical meristem (Brand et al., 2000; Mayer et al., 1998). Plant vasculature is vital to transport water and nutrients via xylem and photosynthates via phloem to and from the shoot. In roots, the vasculature is centrally organized into stele, which contains xylem and phloem tissues scattered with procambial cells and surrounded by a pericycle layer (Dolan et al., 1993; Scheres et al., 1994). *LONESOME HIGHWAY* (*LHW*) regulates the bilateral symmetry of two phloem bundles at the poles of the perpendicular xylem axis as *lhw* mutants, form only single xylem and phloem poles (Ohashi-Ito and Bergmann, 2007). Carlsbecker et al. showed that correct patterning of xylem is directed by *SHR* as it moves from the stele into the endodermis, to activate *SCR* and in turn, microRNA165/166 expression. The same miR165/166 returns to the stele to repress *HD-ZIP III* (class III homeodomain-leucine zipper) TF expression differentially. One such HD-ZIP III transcription factors (TFs) *PHABULOSA* (*PHB*) helps in root vascular patterning in *Arabidopsis MONOPTEROS (MP)/ARF5* (Müller et al., 2016).

The rapid development of functional genomics elucidated the genetic control of root development in rice using mutant and quantitative trait loci (QTL) analyses. Here I will summarize the progress till now in identifying genes regulating all the developmental aspects possible regarding rice root system (Figure 1:5). In rice also, the *QHB* and *OsSCR* genes are the first to be expressed in the outer layer cells of stage 2 of crown root (CR) development. Thereafter, *QHB* maintains expression in the QC and *OsSCR* is confined to endodermis (Kamiya et al., 2003a, 2003b). Another QTL related to root trait is *DEEPER ROOTING 1* (*DRO1*), which enhances deep rooting by reducing root growth angle (Uga et al., 1998). *DRO1* expression is regulated by auxin via ARF and modulates root gravitropic response, by epidermal cell elongation orientated towards the pull of gravity. The first crown root specific gene to be identified and functionally characterized was *ARL1/CRL1* (*ADVENTITIOUS ROOTLESS 1/CROWN ROOTLESS 1*) (Liu et al., 2005), as the mutants completely lack all shoot-borne crown roots but an intact primary root system. In these mutants the first periclinal division of the established CRP founder cells did not take place (Inukai et al., 2005, 2001). Without the support from fibrous root system, crops can't withstand, and lodging could be a major issue in harsh environment. Rice *arl1/crl1* encodes a LOB-domain (ASYMMETRIC LEAVES2/ LATERAL ORGAN BOUNDARIES protein family) transcription factor which is homologous to the *Arabidopsis* LBD16 and LBD29 genes (Majer et al., 2012). The shoot

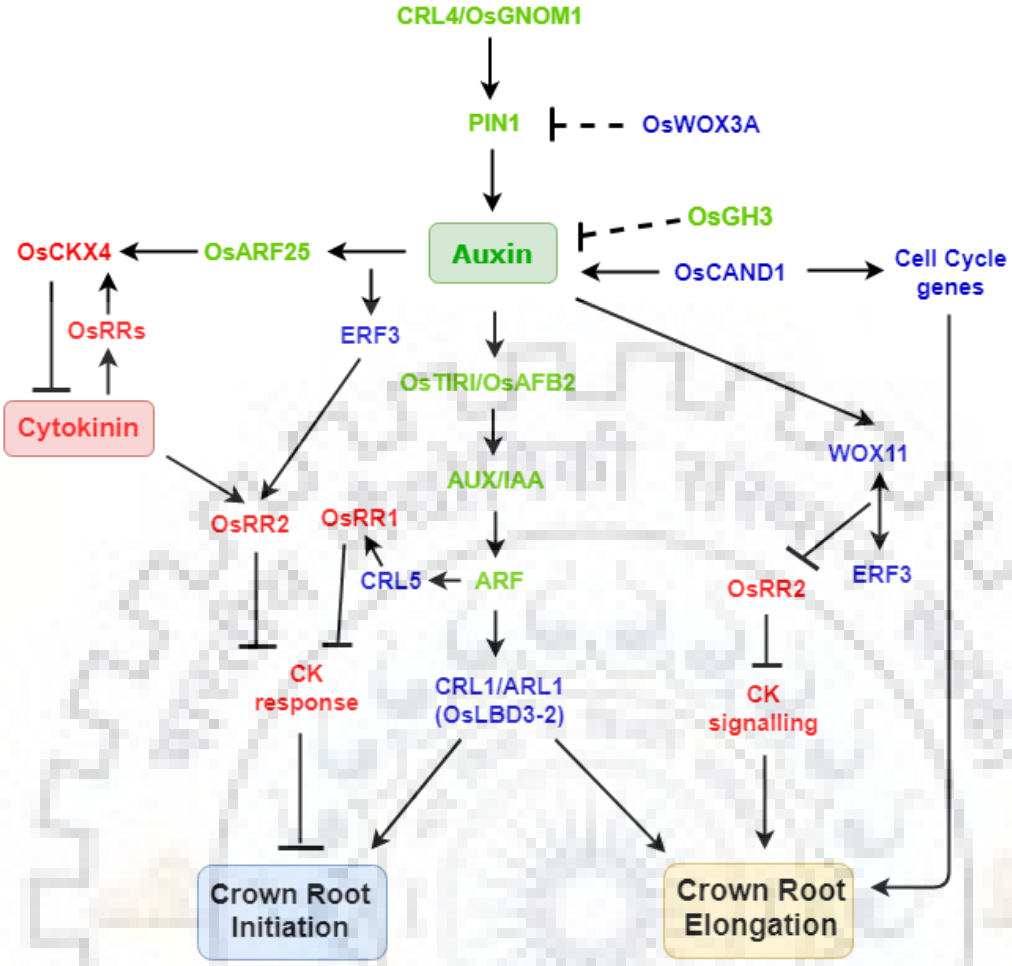

Figure 1:5. Gene regulatory networks controlling crown root initiation and emergence in rice. Auxin and cytokinin signalling components are represented in green and red colour, respectively. Blue colour represents transcription factors and other regulators. Abbreviations: AFB2, auxin signalling F-box2; ARF, Auxin Response Factor; ARL, adventitious rootless; RR2, type-A response regulator2; AUX/IAA, auxin/indole-3-acetic acid; CAND1, Cullin-associated NEDD8-dissociated protein 1; CK, cytokinin; CRL, crown rootless; ERF3, Ethylene-Responsive Factor; GNOM1, membrane-associated guanine nucleotide exchange factor of the ADP-ribosylation factor G protein (ARF-GEF); LBD, lateral organ boundaries domain; PIN, pin-formed auxin efflux carrier proteins; TIR1, transport inhibitor response 1; WOX, WUSCHEL-Related Homeobox. (Figure is adopted from Orman-ligeza et al, 2013).

growth of the mutants was similar to the WT, however, number of LRs emerged from primary root were reduced in the *crl1* mutants (Liu et al., 2005). Furthermore, rice *ARL1/CRL1* acts direct downstream of *OsARF1*, like *LBD16* and *LBD29*, activated by *ARF7/ ARF19* in *Arabidopsis* (Inukai et al., 2005; Okushima et al., 2007). The data available on *ARL1/CRL1* indicates the TF as a specific regulator of crown roots, with a central role in crown root initiation. A mutant defective in CR elongation, *CRL5* (*CROWN ROOTLESS 5*) have also been identified in rice. *CRL5* or *OsPLT8* is an AP2/ERF family transcription factor activate negative type-A response regulator *OsRR1* which represses cytokinin signalling, and promotes crown root initiation in rice (Kitomi et al., 2011). Crown root primordia formation is a developmental process under tight genetic regulation emergence, in contrast, is largely influenced by soil microenvironment. *CROWN-ROOTLESS4*/*OsGNOM1* encodes an ortholog of *Arabidopsis GNOM1* that controls *PINFORMED1* (*PIN1*)-mediated polar auxin transport (PAT). and *crl4/osgnom1* mutants are defective in CR formation, owing to disrupted supply of auxin in region of CRP initiation (Kitomi et al., 2008; Liu et al., 2009). *OsCAND1*, a homolog of *Arabidopsis CAND1* (CULLIN-ASSOCIATED AND NEDDYLATION-DISSOCIATED 1), is involved in auxin signalling to regulate the cell division required for the emergence of CRs (Wang et al., 2011). In addition, *CROWN ROOTLESS 6 (CRL6)*, also known as *CHROMATIN REMODELING 4* (*CHR4*), translates to a member of the CHD (chromodomain helicase DNA-binding) family proteins, and is known to affect crown root development through auxin signalling (Wang et al., 2016; Zhao et al., 2012). Exogenous auxin treatment can partially rescue, the defects of crown root for more or less all the mutants faulty in CR development suggest that all of them plays due role in adventitious root formation through the auxin-signalling pathway (Wang et al., 2016). Cytokinin is also crucial for crown root growth in rice. The loss of function or down-regulation of WUSCHEL-related homeobox gene *OsWOX11*, shows huge reduction in the number and elongation of crown roots. *OsWOX11* is found to be expressed at the root meristem of emerging crown roots and overexpressing rice lines have significantly higher root biomass. Further data disclosed that *OsWOX11* is an important player to block CK signalling in AR development by directly binding to the promoter of *OsRR2*, a type-A RR expressed in CRP (Jiang et al., 2017; Zhao et al., 2009a, 2009b; Zhou et al., 2017). *OsERF3*, an AP2/ERF family TF integrates cytokinin signalling network by interacting with *OsWOX11* protein. The *OsERF3-OsWOX11* interaction limits *OsRR2* expression to aid crown root elongation (Figure 1:5) (Zhao et al., 2015b).

1.7.2 Influence of environmental factors on adventitious and lateral root development

Root systems are continuously reshaped by emergence of new roots throughout plant growth and developmental period to combat the major biotic and abiotic stresses ensuring an optimal response to fluctuating environment. It provides structural support to the plant and enable the uptake of water and nutrients through sensing conditions inside the soil, such as the water level, nutrient content, and trace of toxic elements (Fukai and Cooper, 1995; Gowda et al., 2011). Shoot growth is influenced by the way roots adapt to their surrounding environment. Roots are organs that develop in the rhizosphere which is a heterogeneous milieu where roots must associate with microorganisms.

Most plants have beneficial symbiotic relationship with either mycorrhizal fungi or rhizobial bacteria. These interactions nurture plant by helping water and nutrients uptake [mainly, nitrogen (N) and phosphorus (P)] and the microorganisms get constant supply of carbohydrates from the root interface. Importantly, symbiotic interactions can prompt major changes in the root system architecture (RSA) by affecting length of PR and LR, numbers of LR or AR and their positioning (Contesto et al., 2010; Gutjahr et al., 2009; Mantelin et al., 2006; Oláh et al., 2005). The mutualism between roots and arbuscular mycorrhizal fungi are most beneficial in improving uptake of scarcely accessible soil nutrients such as phosphorus, zinc (Bell et al. 1989; Jakobsen et al. 2005; Bucher 2007), macro elements like calcium (Azcón and Barea, 1997) and iron (Treeby, 1992). The root system architecture is optimized by the accessibility of macronutrients (N, P, K, Ca etc.) and micronutrients (B, Co, Fe, Mo, Zn etc.) as well. High amount of nitrogen strongly inhibits PR and LR elongation, whereas in lower amount it enhances LR elongation. In *Arabidopsis*, nitrate transporters *AtNRT1*.1 and -2.1 (*NITRATE TRANSPORTER 1.1* and *2.1*) and the nitrate-inducible MADS- box transcription factor *ANR1* (*ARABIDOPSIS NITRATE REGULATED 1*) have been identified to aid root's response to the nitrogen available in the environment (Zhang et al., 1999; Zhang and Forde, 1998). Another most influential macronutrient, P and present in the form of inorganic phosphate (Pi), with a very low availability in the soil. Therefore, P starvation may occur which triggers the root system to become shallower with a shorter PR and an increased mass of longer LRs (Rouached et al., 2010) to explore available Pi accumulated at the upper soil layers. In *Arabidopsis*, phosphorus induced root programming seems to use the auxin signalling pathway, through receptor TIR1 and other signalling components such as SLR (SOLITARY ROOT)/IAA14 and *ARF7* and *ARF19*. Targets of AUX-IAA controls multiple developmental pathways even in cotton (Nigam and Sawant, 2013). Together they modulate the expression of genes regulating

Table 1. Rice Mutants Affected in Many Aspects of Root Development

Mutant name	Gene	Phenotype	Refs
adventitiousrootless 1	ARL1	Crown rootless	Liu et al., 2005
altered lateral root formation 1	ALF1/AEM1	Shorter lateral roots and proliferation of lateral roots	Debi et al., 2003
antisense cki1	OsCKI1	Reduced LR number	Liu W et al., 2003
arm1/arm2	OsARM1 OsARM2	Reduced LR number (increased effect in double mutant	Chhun T, et al., 2003
brd1	OsBR6ox	Reduced crown root number	Mori et al., 2002
crl4/osgnom1	CRL4/OsGNOM1	Reduced LR number	Kitomi Y et al., 2008
crown root less 5	OsCRL5	Impaired initiation of crown root primordia	Kitomi Y et al., 2011
crown root less 1	CRL1	Crown rootless	Inukai et al., 2005
lrt1/lrt2	LRT1/RM109 LRT2	Lateral rootless, resistance to 2,4-D, altered root gravity	Chhun et al., 2003; Wang et al., 2006
lower crown root number1 (lcrn1)	OsSPL3	Decreased crown root number	Shao et al., 2019
mir393-ox	OsMir393a, OsMir393b	Reduced crown root number	Bian et al., 2012
osscr	OsSCR	QC, endodermis asymmetric cell division	Kamiya et al., 2003
oscand1	OsCAND1	Normal initiation of crown root primordia but defective emergence	Wang et al., 2011
osiaa11	OsIAA11	No initiation of LR primordia, no effects on crown root	Zhu et al., 2012
osyucca1-ox	OsYUCCA1	Increased crown root formation	Yamamoto et al., 2007
quiescent center specific homeobox	QHB	RAM and QC	Kamiya et al., 2003
radicleless 1 radicleless 2	RAL1, RAL2	Radicle formation, vascular	Scarpella et al.,

		development, procambium	2003, Hong et al., 1995
reduced root length	RRL1, RRL2	Reduced root length	Inukai et al., 2001
deeper rooting 1	DRO1	Increased root angle, deeper rooting	Uga et al., 2013
root growth inhibiting 1	ARM1	Resistance to 2,4-D, fewer lateral roots, shorter roots	Chhun et al., 2003
root hairless 2	RH2	Root hairless	(Suzuki et al., 2003)
short-root 1	SRT1/RM1/RM2	Reduced root length	Ichii and Ishikawa, 1997
oswox11	OsWOX11	Delayed initiation of crown root	Zhao Y et al., 2009, 2015

the cell division in pericycle which gives rise LR development (Narise et al., 2010; Pérez-Torres et al., 2008).

1.8 IMPORTANCE TO STUDY RICE RSA

Rice plant owing a small genome size with known sequence, self-fertilization and has not so lengthy a life span, offers to be an experimental model plant compared with other crops (Sasaki and Burr 2000). It is easy to cultivate and has several standard protocols such as Agrobacterium-mediated transformation to generate mutants. Rice belongs to the Poaceae family together with other grasses of monophyletic origin like wheat, barley and maize (Clark et al., 1995), indicating that research output from rice can be useful to other cereal crops also. Despite roots are the hidden half of the plants considerable effort must be given to characterize, this important biological tissues because of their unique role in modulating soil mass and texture. Plants can many ways alter their root architecture duly but has been largely overlooked by plant breeders in terms crop improvement programmes considering 1960s' green revolution (Waines and Ehdaie, 2007). Ongoing research will expand our understanding of developmental aspects of RSA and ultimately breeders can manipulate root features to increase grain yield and maximize usage agricultural land. A steep–deep–cheap ideotype has been anticipated by Jonathan P. Lynch, to integrate root angles befitting nitrate recovery, deep rooting suitable for water and mineral uptake and a development of additional aerenchyma to lessen the carbon cost of root maintenance (Lynch, 2013). Regardless of the gaps in our understanding of the genetics controlling RSA in crop plants, RSA traits have been modified successfully using overexpression approaches of transcription factors such as *OsNAC5*/9 and *OsMYB2*, the G-protein coding Root Architecture Associated (*OsRAA1*), a cell wall expansion gene *OsEXPA8* gene etc. Auxin negatively regulates the expression of rice *DEEPER ROOTING 1* (*OsDRO1*), a regulator of rice root growth angle resulting increased drought avoidance and crop yield (Uga et al., 2013). Another example of manipulation of RSA is the expression of cytokinin dehydrogenase *AtCKX3* under a root specific promoter to irreversibly degrade cytokinin in root. When *AtCKX3* was, this resulted in increased root meristem. Modification of aquaporins present in root, is another way for increasing water uptake. Increased concentrations of phosphorus by *OsPT1* expression under a constitutive promoter is useful to improve phosphate uptake (Jia et al., 2011). When auxin biosynthetic genes of YUC family are put under root-targeted drought-inducible promoter, the gene is induced upon drought, auxin is synthesised

and subsequently more roots are produced for better adaptation plants in harsh climate (Table 1). *CAMTA1*, a calmodulin binding transcription factor family gene plays a role in the natural plant development under stress condition with altered root development (Pandey et al., 2013). and increased Although several root ideotypes are identified to be suitable for crop improvement, the task is to find the best blend of shoot and root traits that will benefit whole plant growth and productivity, however, a major challenge lies in the ability to improve root phenotypes, under natural field conditions.

CHAPTER 2

STUDYING AUXIN AND CYTOKININ RESPONSES AND FUNCTION DURING CROWN ROOT DEVELOPMENT

2.1 INTRODUCTION

Plant hormones such as auxin and cytokinin are important endogenous regulatory molecules which have diversified roles during every stages of plant growth and development. They function independently as well as in combination of the other plant hormones to regulate key biological processes in plants. The crosstalk between auxin and cytokinin signalling is well known during plant development. During root branching, auxin and cytokinin work in an antagonistic manner; auxin promotes the various events related to lateral root organogenesis whereas cytokinin inhibits both lateral root initiation (LRI) and development (Laplaze et al., 2007; Li et al., 2006).

Auxin is biosynthesized in various plant tissues and is transported to the site of action via PIN-mediated polar auxin transport to function as a morphogen in a dose-dependent manner. Auxin plays a major role during *Arabidopsis* lateral root development. A dynamic local gradient of the auxin distribution generates an auxin maxima at the tip of the lateral root primordia (Benkova et al., 2003). In fact, entire developmental stages of *Arabidopsis* LR differentiation and emergence is regulated by multiple auxin signalling modules through regulating expression of several key transcription factors (De Rybel et al. 2010, Yadav et al. 2010, Lavenus et al. 2013).On the other hand, cytokinin directly acts on the founder cells of the lateral roots and inhibits initiation of the lateral roots (Laplaze et al., 2007).The inhibitory spatiotemporal regulation of cytokinin on LRI, might be crucial for maintaining required distant between recurrent LRI (Bielach et al., 2012). In *Arabidopsis*, cytokinin exerts opposite effect on shoot and root apical meristem (SAM and RAM), as over-expression of cytokinin degradation enzyme CK oxidase (CKX) display opposite effects between shoot and root tissues (Werner et al., 2003).

Auxin signalling pathways are kept repressed in absence of biologically active auxin by inactivating auxin response factors (ARFs) by Aux/IAA proteins and only get activated when concentration of auxin increases. The auxin is received by auxin receptors that eventually targets Aux/IAA protein for proteolytic degradation resulting de-repression of ARFs, a class of transcription factor. ARFs binds to the auxin response elements (AuxREs) present the cis-regulatory elements of down-stream genes. Various DR5-based synthetic promoters have been developed by cloning different combinations of AuxREs upstream of reporter genes and have been extensively used to study dynamic auxin responses in various tissues. Among many early or primary auxin response genes that are identified, GH3 from soybean, has promoter with no

less than three AuxREs that function autonomously of one another (Liu et al., 1994). AuxREs in the GH3 promoter function as composite elements, comprising of a TGTCTC element and a coupling element which together ensure auxin responses in presence of auxin (Ulmasov et al., 1995). The DR5 element as consists of a single type of TGTCTC AuxRE works better than natural auxin responsive promoters. It has been used in combination with several reporter genes such as GUS (Benková et al., 2003), GFP (Friml et al., 2002b), VENUS (Yang et al., 2017), Luciferase (Moreno-Risueno et al., 2010) and hygromycin selectable marker gene for different purpose. Another version of DR5 promoter is DR5rev which consists of nine inverted repeats of the DR5 element (TGTCTC), placed with the minimal CaMV35S promoter (Friml et al., 2002b). DR5rev have been successfully used to track distribution maps at high spatial and temporal resolution in dicot and monocot model plants, approving the importance of polar auxin transport, to monitor dynamic auxin response even in the earliest stages of embryogenesis (Friml et al., 2003).

Similarly, *in vitro* binding studies with the DNA-binding domains of different type-B cytokinin response regulators (ARRs) have identified the consensus sequence of 5'-(A/G)GAT(C/T)-3' (Hosoda et al., 2002; Imamura et al., 2003). This apparent specificity of type-B ARR family members in the DNA binding was exploited to design a synthetic Two Component signaling Sensor (TCS) which reflects the signaling output facilitated by cytokinin (Muller and Sheen, 2008). In transgenic TCS::GFP *Arabidopsis*, the GFP reporter expression aids understanding cytokinin functions during plant development such as shoot meristem patterning) and lateral root organogenesis (Chickarmane et al., 2012; Gordon et al., 2009; Leibfried et al., 2005; Bielach et al., 2012). Further, an improved version of TCS known as TCS new (TCSn) was constructed to enhance its sensitivity and some other limitations (Zürcher et al., 2013).TCSn::GFP was more sensitive to cytokinin in transient transfection assays with brighter and persistent GFP signal in next generation propagation. Although the cytokinin signaling pathway is honed, only recently TCSn::GUS synthetic reporter has been successfully used to track cytokinin signaling output in rice (Tao et al., 2017).

In rice, functions of auxin and cytokinin signalling in regulating root architecture is largely conserved with some species-specific divergence. Similar to *Arabidopsis*, proper auxin biosynthesis, distribution and signalling is required for rice adventitious root (also called crown root) primordia initiation and emergence. In fact, expression of most of the regulators of rice adventitious root development identified till now is directly or indirectly regulated by auxin

signalling pathway, therefore any modulation/alteration in the auxin signalling displays developmental defects in crown root development (Yamamoto et al.2007, Ding et al. 2008, Kitomi et al. 2008, Liu et al.2009, Jun et al., 2011, Yadav et al. 2011; Wang et al. 2011, Wang et al. 2016; Zhang et al., 2018). Similarly, alteration in the endogenous levels of cytokinin by over expression of rice cytokinin degradation enzyme *OsCKX4* produces more number of lateral and adventitious roots whereas reduced expression of *OsCKX2* helps in maintenance of SAM and increased number of inflorescence branching (Ashikari et al., 2005; Gao et al., 2014).

Despite of the key functions of auxin and cytokinin during crown root development, their dynamic spatio-temporal responses and functional role of auxin-cytokinin interaction have not been studied in rice. We, therefore, first aim to study auxin and cytokinin responses durian various stages of crown root development. We have used DR5rev-Reporter constructs to visualize local auxin responses during rice crown root primordia differentiation in transgenic rice and have shown that auxin response is mostly restricted to tip of the crown root primordia and in the vascular tissues Similarly, we have used TCSn-based synthetic cytokinin responsive promoter for monitoring cytokinin responses during rice crown root development and have shown that cytokinin response is relatively broader than the auxin response. We also observed that auxin and cytokinin responses co-exist in endodermal region and the region surrounding the QC. Next, to reveal function of auxin in the auxin-cytokinin interaction domains, we reduced active pools of auxin in cytokinin responsive domains and observed a pleiotropic effect including root architecture.

Some auxin modules also function in root specific manner. The crucial roles of auxin in crown roots are evident from previous studies and once we find the interaction domains of auxin and cytokinin in crown region, it will be exciting to study specific function of auxin in the domain of their co-expression. To study the functional role of auxin in the region where it interacts with cytokinin, we reduce the endogenous free pool of auxin in specified, domain where they co-express. The interactions between different hormonal signaling pathways and their crosstalk often control developmental processes (Bishopp et al., 2011; Chandler and Werr, 2015; El-Showk et al., 2013). Such hormones auxin and cytokinin acts antagonistically to trigger cascades of events leading to AR initiation and development (Pacurar et al., 2014). Biologically active pool of auxin regulates final molecular and cellular outcome of auxin signaling in a specific tissue or spatially localized domain. So, to know the functional significance of this active pool of auxin in the domains where both auxin and cytokinin interacts the free pool of

auxin must be decreased or eliminated. In plant cells excess auxin is stored in inactivated form as conjugates with sugars, amino acids, peptides or proteins (Woodward and Bartel, 2005). Through functional analyses of *OsGH3-8/OsMGH3*, a group II GH3 member, the importance of auxin homeostasis during rice plant development and floral organ differentiation was established (Ding et al., 2008; Yadav et al., 2011). In rice, *OsMGH3* down-regulation phenotypes are elongated internodes and ectopic roots from aerial upper nodes (Yadav et al., 2011). Rice *OsMGH3*, driven by cytokinin responsive TCSn promoter will was be used in our study to reduce the free pools of the auxin in the regions where both auxin and cytokinin co-express. We show that removal of auxin in cytokinin responsive domains and complementation upon exogenous auxin treatment has significant effects on the crown root development.

2.2 MATERIALS AND METHODS

2.2.1 Plasmid Construction

To generate auxin responsive synthetic promoter DR5rev based reporter construct, DR5rev promoter along with erYFP reporter and Nos terminator was digested using *Bam*HI and *Sac*I sites from pHm DR5rev::erYFP-nosT2 (kindly provided by Dr. Ari PekkaMähönen, University of Helsinki, Finland) and the 1.4 kb fragment is sub-cloned into plant expression vector pCAMBIA1300 to generate pCAMBIA1300-DR5rev::erYFP-NosT#1 construct and validated by restriction enzyme digestion (Figure 2:1 B). In order to analyse dynamics of cytokinin response, we have obtained TCSn-min35S::TMVΩmGFP-ER from Dr. Bruno Müller's lab at University of Zürich. To generate TCSn construct for rice transformation, TCSn-erGFP-nosT was taken as *Hind*III-*Bam*HI fragment (1564bp) and sub-cloned into pCAMBIA 1300 to generate p1300-TCSn::erGFP-nosT (Figure 2:1 C). pCAMBIA1300-TCSn:erGFP-NosT #9 is then validated by restriction digestion pattern by multiple enzymes (Figure 2:1 D). pCAMBIA1380OsM1::G2b FL kindly provided by Prof. Usha Vjayraghavan, is digested with *Bam*HI and *Eco*RI and used as vector (Figure 2:5 C). TCSn promoter PCR amplified with TCSnFP and TCSnRP and cloned in pBSSK (*Eco*RV). The clone was validated by restriction enzyme digestion and sequencing and named as pBSSKTCSn #2. Clone #2 is further restriction digested with *Bam*H1 and *EcoRI* to get the insert for sub cloning into pCAMBIA1380G2b FL to generate p1380 TCSn::OsMGH3#1 (Figure 2:5 C) and clone validated by restriction enzyme digestion with several enzymes (Figure 2:5 D).

2.2.2 Rice transformation

All constructs were mobilized to *Agrobacterium tumefaciens* LBA4404 and used to raise transgenic rice lines as described by Prasad et. al. (Prasad et al., 2001). Briefly, embryogenic calli of rice japonica var. TP309 was co-cultivated with *Agrobacterium* harbouring the construct. Co-cultivation was done for 2-3 days in dark (at 26°C) on NB6-AS medium supplemented with 100 mM acetosyringone. Transformed calli were selected on NB6-SEL medium containing 50 mg/L hygromycin. Actively proliferating calli were further transferred to NB6-RM media (50 mg/L hygromycin, 3 mg/L BAP and 0.5mg/L NAA) for shoot regeneration. Regenerated shoots were subsequently transferred to rooting media (1/2 MS with 25 mg/L hygromycin, 0.05 mg/L NAA). Hardening of regenerated plantlets was done in soilrite before transferring to clay for completing the life cycle. Since *OsMGH3* lines with strong

transgene expression did not produce fertile seeds, multiple independent stronger lines were phenotyped in T0 generation for their overall growth and development.

2.2.3 Extraction of rice genomic DNA and polymerase chain reaction

Genomic DNA was isolated from young leaves of rice plant by salt extraction method as detailed in the Aljanabi and Martinez (1997) and Prasad K. 2003 PhD thesis. The quality and concentration of genomic DNA was determined on 0.8% agarose gel. Appx 20 ng of gDNA from leaves of wild-type (var TP309) and from eight and ten independent lines transgenic of DR5::erYFP, TCSn::erGFP respectively were used to determine genomic integration of transgene by PCR. The forward primer, erVENUS/YFP FP, was positioned towards the start of YFP whereas reverse primer, erVENUS/YFP RP, was located in the middle of YFP gene. We validated the lines with PCR amplicon of 609bp. Similarly, forward primer er/m GFP FP and reverse primer er/m GFP RP was designed in the middle region of GFP gene and TCSn::erGFP lines were validated with a PCR amplicon of 542bp. PCR reaction was carried out in 20 ul reaction volume for 34 cycles. The PCR product were separated on 1.0 % agarose gel. For *OsUBQ* control PCR cycle was reduced to 28 with rest of the conditions same for the PCR.

2.2.4 RNA extraction and RT-PCR

Total RNAs from seedlings of DR5::erYFP lines and from seedlings and stem base (2-3 mm) of TCSn::erGFP plants or those from control plants were isolated by TRI-reagent (SIGMA,USA) according to manufacturer's instructions (Appendix A) and then were treated with DNase I (NEB) and precipitated after phenol and chloroform treatment (Appendix B). For characterization of TCSn lines, oligo(dT)-primed cDNA was synthesized from 1-1.5 µg of total RNA using M-MuLV reverse transcriptase (NEB) (Appendix C). A total of 10-20 ng cDNA for semi quantitative PCR using 250 nM of YFP-specific primers and Taq DNA polymerase (NEB). For characterization of DR5rev lines, cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-rad) (Appendix D). 10 ng of diluted cDNA was used for semi quantitative real-time PCR (RT-PCR) using 250 nM of either GFP-specific or *hptII* gene specific primers. 1 ul of RT product was used in a 20 ul reaction for 25-28 cycles to detect Actin or *UBQ5* transcript levels which were used as endogenous normalization control. A list of primer sequences is provided as Appendix H.

2.2.5 Plant Growth and Microtome Sectioning for Microscopy

Seeds of (*Oryza sativa* var TP309) wild-type and were de-husked, surface sterilized and germinated on ½ MS media with 1% sucrose and 0.3% phytagel (Sigma) and grown vertically in sterile square petri dishes (Himedia; 12 cm × 12 cm), at 26 °C in 16/8-hour light/dark period for 6 days. For histological and RNA in situ hybridization analysis, approximately 2mm rice stem base region was dissected from each wild-type and transgenic plants and fixed in FAA (10% formaldehyde, 5% glacial acetic acid and 50% ethanol). Ethanol series was performed to dehydrate the tissues and finally embedded in paraffin (SIGMA) blocks. After solidification and reshaping, materials were cut into 6-8 µm thin sections using microtome (Thermo Scientific) and sections were taken on poly-L-lysine coated glass slides. To observe fluorescent signal ZEISS Axio Scope.A1 microscopy was used; primary roots were visualized in whole mount on MQ water. To visualize tissues of rice stem base, appx. 100um thickness hand sections were made using razor blades and taken onto glass slides and incubated for 1 min in 1% direct red dissolved in MQ water. Sections were washed twice with MQ water by pipetting and cover glass was put on the samples and used for imaging under fluorescent microscope.

2.2.6 RNA-RNA *in-situ* hybridization

For preparing DIG-UTP-labelled riboprobes, 609 bp gene-specific region of YFP and 110 bp specific sequences of GFP was cloned into pBluescript SK+ as a blunt in *Eco*RV site in antisense direction to T7 in pBSSK vector backbone namely pBS SK II erYFP-Venus (as) #3 pBS SK II erGFP probe (as)#1 respectively. The anti-sense probe for YFP and GFP was generated using *Eco*RI-lineralized pBluescript SK+ clones transcribed with T7 RNA Polymerase (Sigma) (Appendix G). Hybridization was performed on cross sections as described by Prasad et al. (2005). The signal was developed using alkaline phosphatase conjugated anti-DIG antibodies (Sigma) and NBT/BCIP substrate (Sigma). Sections were mounted in Entellan (Merck- Millipore) (Appendix F).

2.2.7 Plant material and Chemical Treatments

Tissue parts of planton-plant tissue culture container (Tarson, 7.5x7.5x10 cm) for phenotyping in T1 generation. For optimization of IAA treatment, wild-type TP309 were germinated on ½ MS media containing 10 to 75nM of IAA (Sigma) along with mock treatment. 10 nM of IAA was found to be sub optimal for normal wild type root growth and development. For final IAA

treatment experiment, wild-type and TCSn::*OsMGH3* transgenic lines were grown on ½ MS media supplemented with 10 nM of IAA (Sigma). Emerged CR numbers were counted from 5 seedlings each mock and IAA treated on 6th day post-germination. To study effects of IAA and BAP on CR growth, average length of all emerged CRs were measured on 6-day post-germination (Figure 2:7 A- D). Plants were imaged using Nikon D5300 DSLR camera manually.

2.3 RESULTS

2.3.1 Generating constructs and transgenic rice plants for studying auxin and cytokinin responses

The conserved auxin signalling machineries between *Arabidopsis* and rice, made it possible to use the same auxin biosensor constructs, derived from AuxRE elements, termed as DR5rev::GFP in which a synthetic auxin-responsive promoter (DR5rev) (Figure 2:1 A) was used to drive expression of the green fluorescent protein (GFP) (Friml et al., 2002b; Yang et al., 2017). In order to achieve the goal to monitor dynamic activity of auxin and cytokinin, we have generated constructs in suitable plant expression vectors for rice transformation. For auxin responsive construct, DR5rev promoter was used with ER-tagged VENUS to minimize mobility of fluorescent proteins in neighbouring cells. We have excised 1.4 kb DR5rev::erYFP-nosT fragment from existing pHm DR5rev::erYFP-nosT2 (kind gift from Dr. Ari Pekka Mähönen, University of Helsinki, Finland) and was cloned into pCAMBIA 1300 binary vector (Figure 2:1 A). The clone was validated by restriction digestion pattern analysis with multiple enzymes and sequencing (Figure 2:1 B). The final construct was mobilized to suitable *Agrobacterium* strain for transformation of rice embryogenic calli. We have successfully raised more than ten transgenic reporter lines with p1300DR5::erYFP-nosT construct and these transgenic lines were molecularly characterized. The T1 seeds were collected and T1 plants were used for further analysis.

Recently, TCSn-based cytokinin responsive synthetic promoter-GUS reporter construct was used in rice to monitor cytokinin responses (Tao et al., 2017). Thus, we have generated TCSn-erGFP construct in rice expression vector (TCSn construct was kind gift from Dr. Bruno Müller at University of Zürich) (Figure 2:1 C). We have excised TCSn fragment and sub-cloned with ER-tagged GFP (green fluorescent protein) in pCAMBIA 1300 (Figure 2:1 C). Final construct was validated by several restriction enzyme digestion and mobilized to *Agrobacterium* strain for rice transformation (Figure 2:1 D). We have raised multiple transgenic plants for p1300-TCSn::GFP-nosT construct. Seeds collected from all these putative lines were molecularly characterized and analysed for the expression of reporter genes in T1 generation.

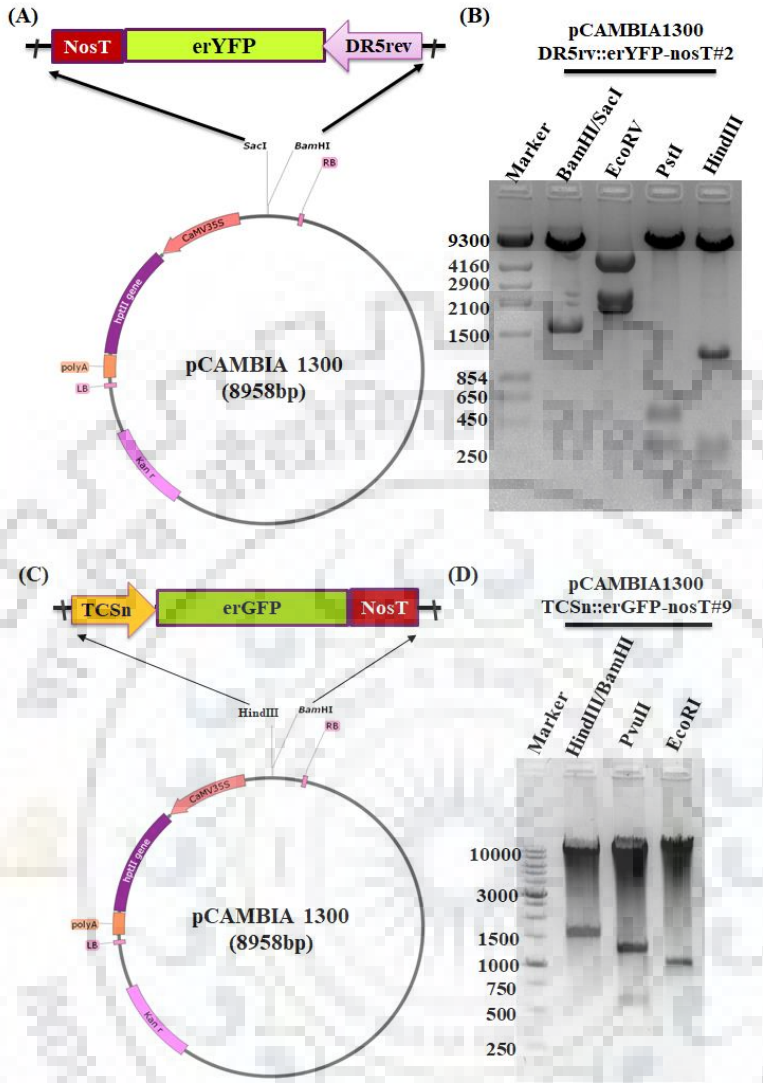

Figure 2:1. Transgene constructs to monitor auxin and cytokinin responses in rice crown root development. (A) Schematic diagram of pCAMBIA 1300 vector containing DR5rev promoter::erYFP reporter-nosT construct. **(B)** Agarose gel image showing validation of final auxin reporter clone pCAMBIA1300-DR5rev::erYFP-nosT #2 with several restriction enzymes. **(C)** Schematic diagram of vector map with T-DNA of the TCSn promoter::erGFP reporter-nosT construct in binary vector pCAMBIA 1300. **(D)** Agarose gel image showing validation of final cytokinin reporter clone pCAMBIA1300 TCSn::erGFP-nosT #9 with several restriction enzymes.

2.3.2 Molecular Characterization of rice reporter lines

After raising transgenic reporter lines, we screened reporter lines with single site insertion and strong expression of the reporter genes. We first germinated these transgenic lines in ½ MS media supplemented with hygromycin to analysed segregation pattern of the transgene. Of ten DR5rev::erYFP lines, we observed 3:1 Mendelian segregation ratio in three lines ((L#3, 4, 6) on the hygromycin plates, suggesting that these lines harbour single transgene. These lines were also PCR genotyped for presence of transgene using YFP specific primers on the genomic DNA extracted from leaf tissues of these lines. We observed strong an expected 600bp band in L#3, 4, 6 which was not seen in wild-type plants (Figure 2:2 A). Seedlings from those three lines were further taken for total RNA extraction and expression analysis of YFP gene through RT-PCR. cDNA synthesized from total RNAs extracted from these lines was used to perform semi-quantitative RT-PCR using YFP-specific primers and our analysis showed that YFP transcripts can be detected in these lines (Figure 2:2 B). Next, to confirm that these lines express YFP proteins in the auxin-responsive domains, we germinated L#3 and 6 on hygromycin containing media and analysed YFP signals in the root tips (Figure 2:2 C, D). As consistent with the earlier report, a prominent YFP signal was visible in the root cap zone of the root tips (Figure 2:2 C) (Yang et al., 2017) and fluorescent signal was not seen in the wild-type control root tips (Figure 2:2 D). Next, we made hand sections at the stem base and analysed on the fluorescent microscope. The cross sections of the transgenic lines show YFP signal (Figure 2:2 E), but signal was absent in the wild-type plants (Figure 2:2 F). These observations suggest that L#3 and #6 were suitable lines for detailed temporal and spatial auxin response analysis.

Similarly, to analyse dynamics of cytokinin responses, we generated multiple transgenic rice plants for p1300-TCSn::erGFP-nosT construct. Seeds were collected from these putative lines and were screened for single insertion lines as described for DR5rev lines. Further, PCR-based genotyping and expression level of GFP transcript was performed using GFP-specific primers with these lines. Semi-quantitative RT-PCR analysis has identified four independent lines (L#4, 13, 15, 18) showing prominent an expected band at of 540bp with GFP-specific primers (Figure 2:2 A). Further, pCAMBIA1300TCSn::erGFP-nosT L#4 and 13 were grown on 1/2 MS media supplemented with hygromycin and stem base of few hygromycin resistant plants were hand sectioned. These sections were analysed in fluorescent microscope. We observed strong

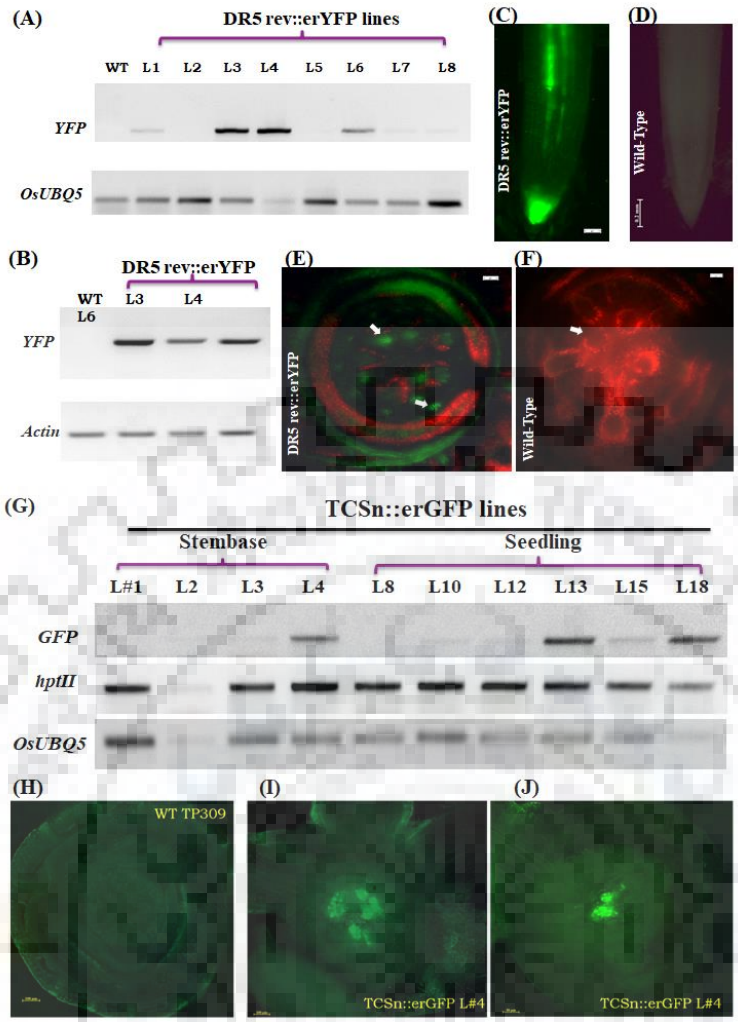

Figure 2:2. Molecular characterization of transgenic rice reporter lines for auxin and cytokinin. (A) PCR on gDNA of wild-type and eight independent transgenic lines for DR5rev::erYFP showing integration of transgene in the genome of transgenic lines (L#1, 3, 4, 6 to 8) which was absent in wild-type. **(B)** RT-PCR analysis in seedlings of control and transgenic lines, showing presence of YFP transcripts in the 6-day old seedling. **(C-F)** YFP signals in 6-days old rice seedlings; in the primary root tip (C, D) and at stem base (E, F). YFP fluorescence is visible in DR5::erYFP lines (C, E) whereas no YFP signal seen in wild-type plant (D, F). **(G)** RT-PCR analysis of GFP expression in seedlings of control and transgenic line shows that GFP transcripts in the 6-day old seedlings. **(H- J)** Fluorescent microscopy images at stem base of 6-days old rice seedlings with visible GFP fluorescence in TCSn::erGFP L#4 (I); TCSn::erGFP L#13 (J); compared to no GFP signal in wild-type (H) negative control. *Actin* and *OsUBQ5* are used as constitutive internal control. Scale bar: 200 µm (C- F) 100 µm in (H- J).

fluorescent signal from GFP in these lines (Figure 2:2 I, J) which was absent in the wild-type plants (Figure 2:2 H). Thus, we have used these lines for detailed expression analysis.

2.3.3 Studying dynamic Auxin responses during crown root development in rice

Stage-specific different auxin modules are known to be involved during *Arabidopsis* lateral root development (Yadav et al., 2010). As several genetic pathways are also regulated by auxin in rice crown root, it is important to understand how auxin response oscillates during crown root development. As described earlier, we used DR5rev-erYFP construct to monitor auxin response during different stages of rice crown root development (Figure 2:3 A- F). Rice crown roots are produced sequentially from successive shoot nodes and the developmental events covering from establishment of initial cells until emergence of crown root are divided into seven stages (Itoh et al., 2005). To study the auxin responses during CR formation, we used 6-day old seedling as this stage contains every stages of crown root primordia (CRP). We have grown reporter lines harbouring p1300DR5rev::erYFP-nosT on ½ MS media, stem bases containing crown tissues (approx.. 2-3 mm) were collected and fixed in FAA solution for paraffin embedding. Similar age wild-type stem base was used as control. 6-8 μm cross sections made from these fixed stem nodes were collected on poly-L-lysine coated glass slides for *in situ* hybridization. These sections were hybridized with DIG-labelled anti-sense RNA probes specific to YFP transcript and detected by alkaline phosphatase-mediated colour reaction. Sense RNA probe against YFP transcript was used as control to rule out non-specific hybridization.

In rice, adventitious roots are initiated from the cells adjacent to the vascular tissues at the stem base (Liu et al., 2005; Bellini et al., 2014). Entire events of rice crown root development can be divided into three broader stages; CRP establishment, differentiation and emergence. Auxin accumulation has been shown to coincides with the very early stage of lateral root priming in *Arabidopsis* thaliana (Benková et al., 2003; De Rybel et al., 2010). We did not observe any signal above than background in the rice stem base prior the CRP establishment, but the auxin responses begin to enhance at some sites near peripheral cylinder of the vascular bundle, marking potential site of CRP (Figure 2:3 A, B). This pattern of auxin response is similar to the pattern seen for lateral roots, suggestion a correlation of auxin maxima with the site of CRP establishment. During later stage of CRP differentiation, strong auxin response is localized to tip of the primordia (Figure 2:3 C, D). The signal is stronger in the initial cells of the root cap

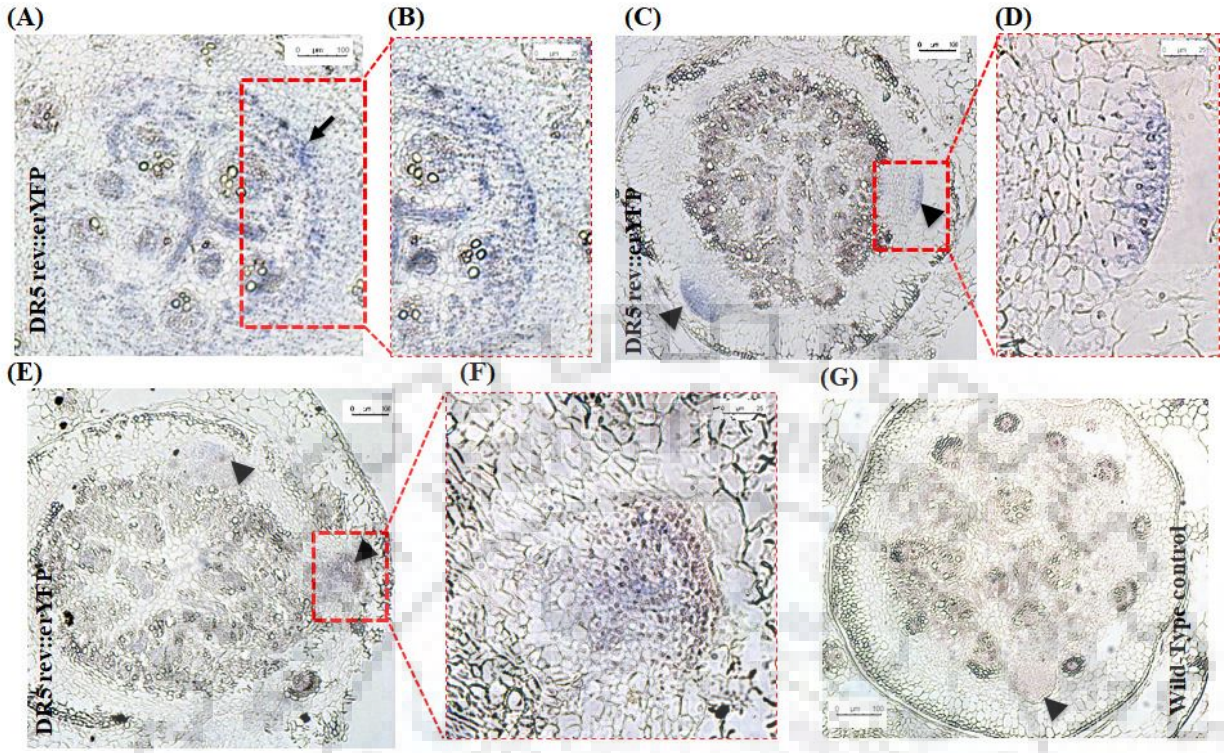

Figure 2:3. Auxin responses during different stages of rice development. (A- B) DR5-YFP expression is visible at the peripheral vascular cylinder (PVC), near the pericycle, the potential layer for CRP initiation. 40X magnification of the PVC and allied region showing colorimetric detection of blue NBT/BCIP substrate (B). **(C- D)** Auxin response in later stages of CRP differentiation; the signal is stronger and localized to tip of the primordia i.e. root cap and root cap initials of the growing roots after emergence. **(E- F)** Signal corresponding to auxin response observed in 40 X magnification in the cells around quiescent centre, stem cell niche and stele tissues. **(G)** No signal detected in the wild-type crown tissue sections probed with the sense probes during crown root development. (A, C, E, G) 10X magnification and scale bar: 100 µm. (B, D, F) 40X magnification and scale bar: 25 µm.

which remains high in the root caps of the growing roots after emergence (Figure 2:3 E, F). Apart from the root cap, we also observed auxin response in the cells around quiescent centre, potential initials of various tissues during differentiation (stem cell niche) and stele tissues (Figure 2:3 E, F). On the other hand, we did not see any signal in the wild-type tissues (Figure 2:3 G) and the sections probed with the sense probes, supporting specificity of the observed auxin responses during crown root development.

2.3.4 Cytokinin response study in TCSn::erGFP lines during rice CRP differentiation

In *Arabidopsis,* synthetic promoters (TCS and TCSn) have been used to visualize the domains of cytokinin action during plant development (Muller and Sheen, 2008; Zürcher et al., 2013). TCSn includes variations in type-B RR regulator binding motif (5′-(A/G)GAT(C/T)T-3′), and 11bp phasing between two such motifs and is a robust and sensitive synthetic promoter for monitoring cytokinin response (Zürcher et al., 2013). These clustered motifs were also abundant in rice cytokinin signalling targets, suggesting that they might be conserved across the species. In fact, TCSn::GUS has been shown to report the cytokinin signalling in meristem of rice seedlings. TCSn::GUS expression was induced by exogenous cytokinin application for 3 hrs in rice seedlings authenticates use of TCSn system in rice (Tao et al., 2017). We, therefore, use TCSn-based reporter to study cytokinin responses during rice crown root development.

For this purpose, we used transgenic rice lines harbouring TCSn::GFP construct. We have germinated the two independent transgenic reporter lines (L#4 and 18) for six days as described previously and used for studying transcript distribution of GFP by *in situ* hybridization. Rice stem base were collected for FAA fixation. Cross sections were made from FAA fixed stem base of these plants along with control (wild-type) and were hybridized with DIG-labelled anti-sense RNA probes for GFP transcript (Figure 2:4 A- G). In contrast to auxin signalling, we did not observe any cytokinin signal in the tissues peripheral to the vascular bundle during the process of CRP establishment, consistent with their antagonistic functions of auxin and cytokinin during CRP establishment (Figure 2:4 A, B). Cytokinin signalling is initiated only during CRP differentiation. In these primordia, the cytokinin response is relatively broader covering most of the differentiating tissues of the primordia (Figure 2:4 C, D). Expectedly, it excludes most of the auxin responsive domains of the root cap, stem cell niche and the xylem tissues (Figure 2:4 E, F) signifying its largely non-overlapping domains with auxin. However, a

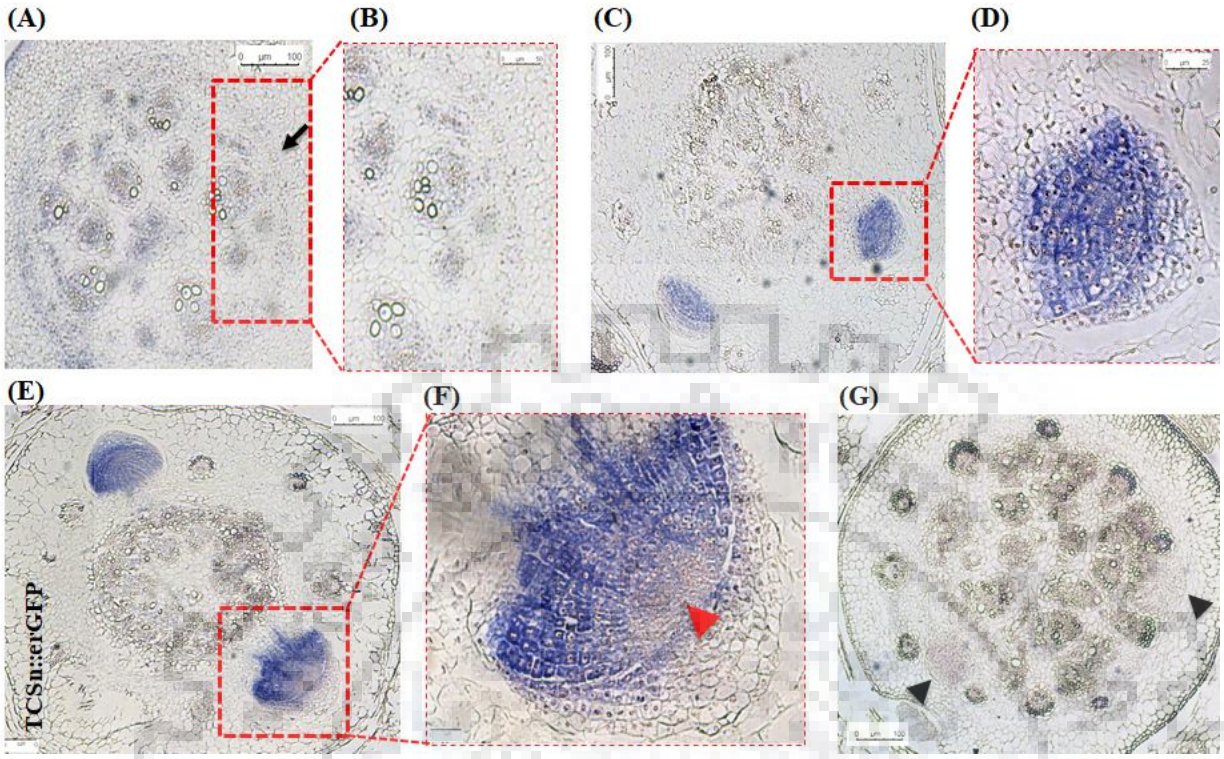

Figure 2:4. Cytokinin responses during different stages of rice development. (A-B) GFP signal not observed at the region around the PVC, before CRP initiation. 40X magnification of the boxed region of the same stem base section (B). **(C- D)** During CRP differentiation, cytokinin response is relatively broader covering most of the differentiating tissues of the primordia, excludingthe root cap, stem cell niche and the xylem tissues. **(E- F)** Little overlap in the auxin and cytokinin response domains, particularly in the QC and the lateral root cap/epidermis tissues. (A, C, E, G) 10X magnification and scale bar: 100 µm. (B, D, F) 40X magnification and scale bar: 25 µm.

careful observation indicates little overlap in the auxin and cytokinin response domains, particularly in the QC and the lateral root cap/epidermis tissues (Figure 2:4 E, F), suggesting a possibility of synergistic functions of these hormones in these tissues. Overall our detailed cytokinin response analysis culminates the role of cytokinin in crown root development and identifies antagonistic and synergistic domains of auxin and cytokinin responses (Figure 2:9).

2.3.5 Phenotypic consequences of endogenous auxin depletion from cytokinin responsive domains

As described in the earlier section that auxin and cytokinin signalling may co-exist in some tissues of the CRP, we next focused our study to understand function of auxin in the cytokinin responsive domains. For this purpose, we aimed to deplete endogenous active pools of the auxin in the cytokinin responsive domains and study the consequences during crown root development (Figure 2:5 A). Members of GH3 gene family are known to inactivate free auxin in form of different conjugates and store them to maintain auxin homeostasis (Figure 2:5 B) (Fu et al., 2011; Jain et al., 2006b; Park et al., 2007; Staswick et al., 2002; Woodward and Bartel, 2005). Previous studies have demonstrated that *OsGH3*-8 (*OsMGH3-8*) significantly alters free auxin levels, when expressed (Ding et al., 2008; Yadav et al., 2011), we therefore used *OsMGH3* for this purpose. We cloned *OsMGH3-8* in plant expression vector under TCSn promoter and used for rice transformation (Figure 2:5 C). We performed several round of rice calli transformation and observe very poor regeneration efficiency with this construct. These calli proliferate well on the selection media with hygromycin but fail to regenerate to plantlets. We could obtain only 4-5 plantlets for this construct, suggesting that presence of auxin in the cytokinin responsive domains has a role in the regeneration. Some of these lines were extremely dwarf and showed bushy shoot appearance (Figure 2:6 A- C), which was similar to the phenotypes observed upon ectopic over-expression of *OsMGH3* (Ding et al., 2008; Yadav et al., 2011). These strong lines could not achieve their reproductive phase and died in the clay. However, two lines displayed moderate phenotype of dwarfism and achieved transition to reproductive stage (Figure 2:6 D- F). These TCSn::OsMGH3-nosT transgenic line produced panicles with reduced branches and very few seeds. These data together suggest that auxin signalling in the cytokinin responsive domain is required for proper growth and development in the plants.

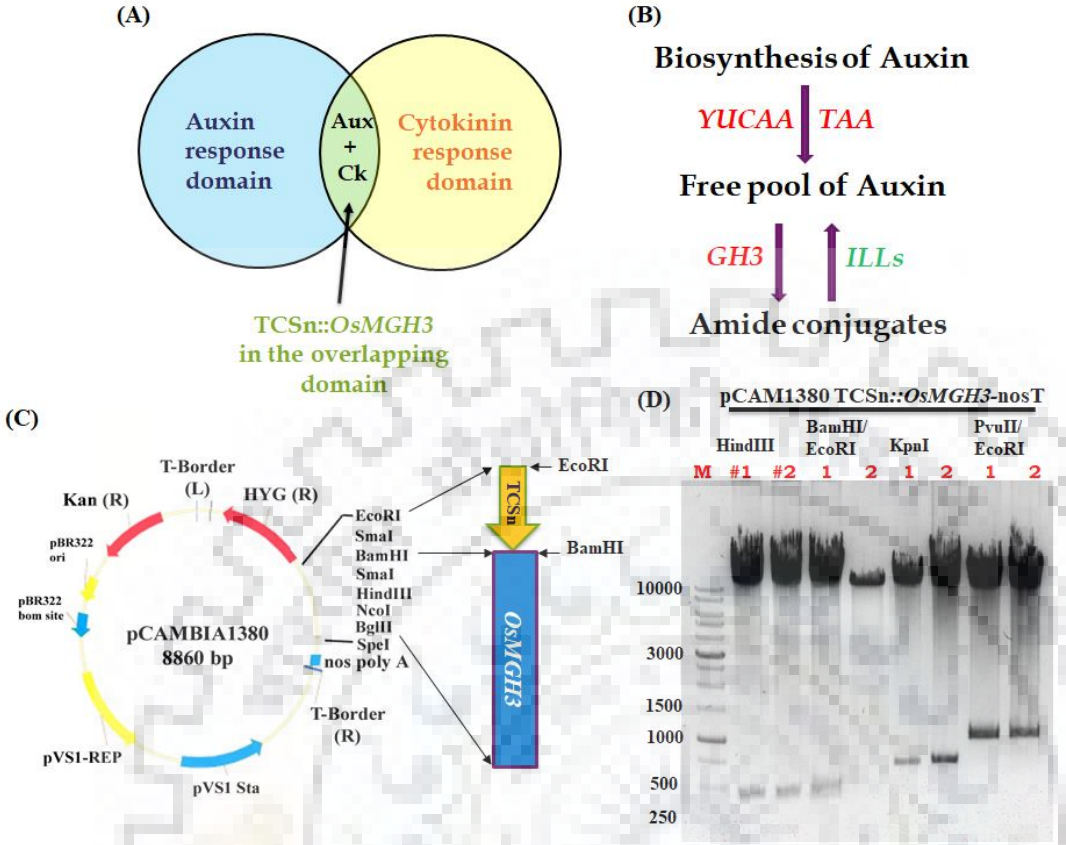

Figure 2:5. Construct to deplete endogenous auxin in the cytokinin responsive domain. **(A)** Schematic showing overlap of auxin (blue circle) and cytokinin (yellow circle) in the green region in between from where auxin will be depleted by TCSn promoter driven *OsMGH3* gene. **(B)** Flow chart of auxin homeostasis by OsGH3 family proteins. **(C)** Transgene construct with *OsMGH3* downstream of TCSn promoter. **(D)** Agarose gel image of verification of two final pCAMBIA1390-TCSn::*OsMGH3*-nosT, plasmid clones (#1 & #2) using restriction enzyme digestion.

2.3.6 Effects of auxin depletion in the cytokinin responsive domains on the root architecture establishment in rice

Next, we analysed two independent lines (L#1 and L#2) in the next generation and compared them with wild-type plants during early stages of the seedling development. Consistent with the T0 phenotypes, these lines were dwarf in the growth and developed fewer and smaller roots as compared with the wild-type plants (Figure 2:8 A, C, E). Line L#1 displays stronger phenotypes where both primary and crown root growth was strongly inhibited (Figure 2:8 C) whereas in line L#2 (Figure 2:8 E) the effect was stronger on the crown root than the primary root, suggesting a dose-dependent and root-specific requirement of the auxin in the auxin-cytokinin interaction domains. Now to gain understanding the effects of endogenous auxin depletion on crown root development T1 seeds were germinated in plant on boxes filled with ½ MS media. The root architecture of both the transgenic lines was significantly compromised as compared to the wild type. Among these lines, L#1 showed stronger root phenotypes that the L#2 in overall root length and crown root numbers. We observed that average length of PR in lines L#1 and L#2 was 1.17 cm and 2.23 cm, respectively which was significantly less than the average PR length of WT (7cm) (Figure 2:8 G, H). Similar effects are also observed on the growth of crown roots in these lines, suggesting that presence of auxin in the cytokinin expression domains has a role in growth of both primary and crown roots (Figure 2:8 G, H). However, the effect of auxin depletion from cytokinin domains does not have strong effects on the CR number, as CR numbers are decreased in these lines with lesser extent (Figure 2:8 G, H). This observation also indicates that the auxin-cytokinin interaction in their overlapping expression domains is more relevant for root growth than the root primordia establishment. In addition to the root, plant height was also reduced in these lines consistent with the phenotypes seen upon *OsMGH3* over-expression (Ding et al., 2008; Yadav, 2011).

The root phenotype indicates that auxin is necessary in the cytokinin responsive domain to ensure proper root growth. If auxin depletion through OsMGH3 expression is causing the altered root structure, we hypothesise that these lines may have altered auxin sensitivity and exogenous application might restore the root architecture. We first identified a sub-optimal concentration of auxin which does not visually affect the root growth of wild type. We germinated wild-type seeds on ½ MS media supplemented with various concentration of IAA, ranging from 10 nM to 75 nM (Figure 2:8 A- D). We observed that IAA higher than 10nM has

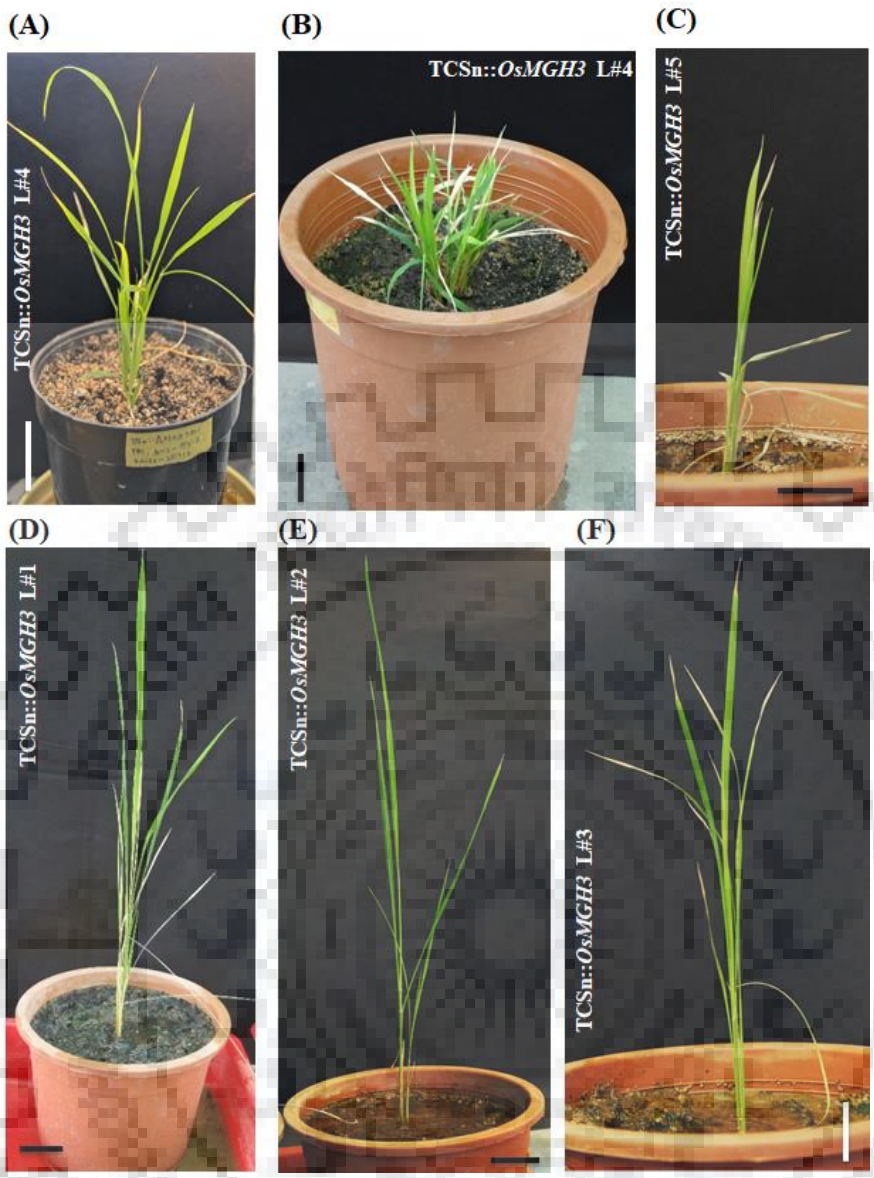

Figure 2:6. Phenotypic consequences of endogenous auxin depletion by targeted expression of *OsMGH3* in the cytokinin responsive domains. (A- F) Phenotypes of TCSn::*OsMGH3* expression in multiple transgenic lines in T0 generation. During hardening (A) and in clay (B- F) in green house conditions. **(B)** A strong transgenic line #4 was severely bushy in appearance when transferred to clay (A) from soilrite. **(C)** Another independent line #5 with TCSn::*OsMGH3* construct showing strong dwarfed phenotype. **(D- F)** Few more independent transgenic lines #1, 2, & 3 showing weak phenotypes and L#1 and 2 among them produced fertile seeds. Scale bar: 4 cm.

some effect on the root architecture (Figure 2:7). For example, at 25nM IAA concentration, the root growth was similar to wild-type but LR number was notably increased (Figure 2:7 C). This suggested that 10 nM IAA could be a sub-optimal concentration of IAA in our laboratory condition (Figure 2:7 B). Next, we germinated five seeds from both the transgenic lines and wild-type on ½ MS media with 10 nM IAA and media without IAA (mock). We compared primary root length and crown root numbers upon IAA treatment with the mock-treated in the 7-d old plants. We observed that IAA treatment could neither restore the PR length nor the crown root number of line L#1 and the effect was similar to the WT level, suggesting that line L#1 has strong depletion of the endogenous auxin and sub-optimal IAA concentration is not sufficient to rescue the effects (Figure 2:8 D). However, we observed near complete rescue in the root phenotypes in the line L#2 upon IAA treatment (Figure 2:8 F). In line L#2, both the PR length and crown root number were fully rescued by IAA similar to the wild-type level (Figure 2:8 G, H). The data suggest that the line with higher level of OsMGH3 transgene expression or lesser available auxin is affected to a greater extent, confirming abundance of the gene in the roots of rice (Figure 2:8 G, H). Cumulatively, all the consequences points towards the necessity of active auxin pool at the domain of synergy and a likelihood of non-canonical auxin and cytokinin partnership.

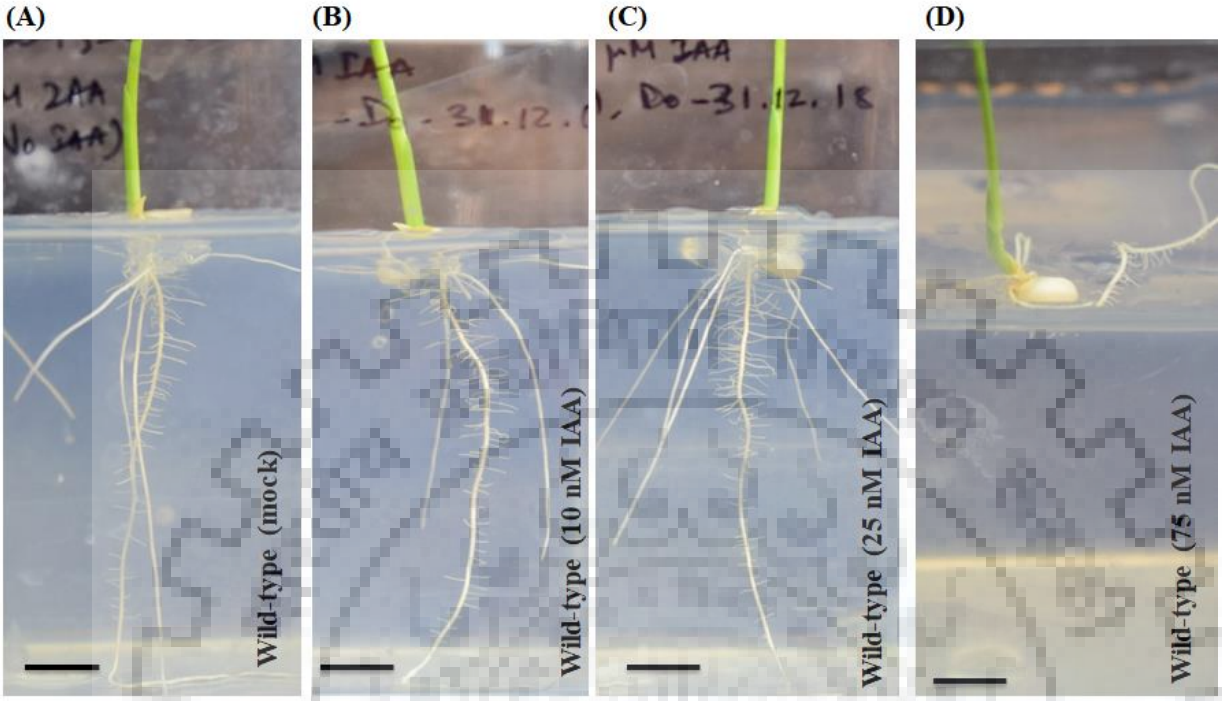

Figure 2:7. Effect of exogenous IAA treatment on wild-type rice seedlings. (A- D)) Rice wild-type plants were grown in ½ MS media supplemented with no IAA (mock treated), with 10nM IAA (B), with 25nM IAA where LR number was visibly increased (C), and with 75nM (D). (A- D) Scale bar: 1 cm.

2.4 DISCUSSION

Phytohormones are pivotal for any aspect of plant development. Auxin and cytokinin being the major ones to focus our study and not very well studied in Indian staple crop rice. Many genetic pathways involved in rice adventitious root development are regulated directly or indirectly by those hormones (Orman-Ligeza et al., 2013) as majority of mutants reported to be affected in root patterning or emergence have been either auxin or cytokinin related (Coudert et al., 2011; Kitomi et al., 2011b; Debi et al., 2005; Rebouillat et al., 2009). Auxin is a mutual regulator for all known monocot root types including rice (Sreevidya et al., 2010). The combined effect and interactions between auxin and cytokinin in development is obvious and established fact in Arabidopsis thaliana (Bishopp et al., 2011; Chandler and Werr, 2015; El-Showk et al., 2013; Pacurar et al., 2014). To add to that knowledge, we trace the role of auxin and cytokinin in rice crown root development individually as well as try to shed some light on their interaction which is necessary for improved root architecture with better water and nutrient use efficiency.

We demonstrated that traditional auxin responsive promoter DR5rev can reveal auxin distribution in rice. Similar hormone distribution maps at high spatial and temporal resolution were developed in Arabidopsis LR development, confirming the importance of auxin in regulating plant morphogenesis both in dicot and monocot species. We found similar DR5::erYFP activity at rice primary and lateral root apex as reported by Yang et al., 2017, validating our sensor lines. Transcriptome analysis of early lateral root development by De Rybel et al. identified few auxin-responsive, differentially expressed genes between xylem and phloem pole pericycle cells of the PR. GATA23 was identified as the key TF for founder cell specification and first asymmetric cell division (De Rybel et al., 2010). Its expression was oscillatory like the auxin during LR organogenesis in Arabidopsis (De Raybel et. al., 2010; Yadav et al., 2010). Rice adventitious roots originate at the stem base within few days of germination from the basal compressed nodes. These nodes do not have pre-existing meristematic cells and requires a signal to dedifferentiate from the current differentiated form to initiate the founder cells to initiate the event of crown root formation. As proceeded to study crown root development at the stem base of those reporter lines, we could locate the presence of auxin even before the initiation of the crown root. Auxin response at the pericycle cell layer and the peripheral vascular cylinder indicates that some auxin module is being activated in these potential tissues giving the cue for first few periclinal cell divisions. Critical for the auxin activity is the dynamic regulation of its distribution mediated through the polar auxin transport

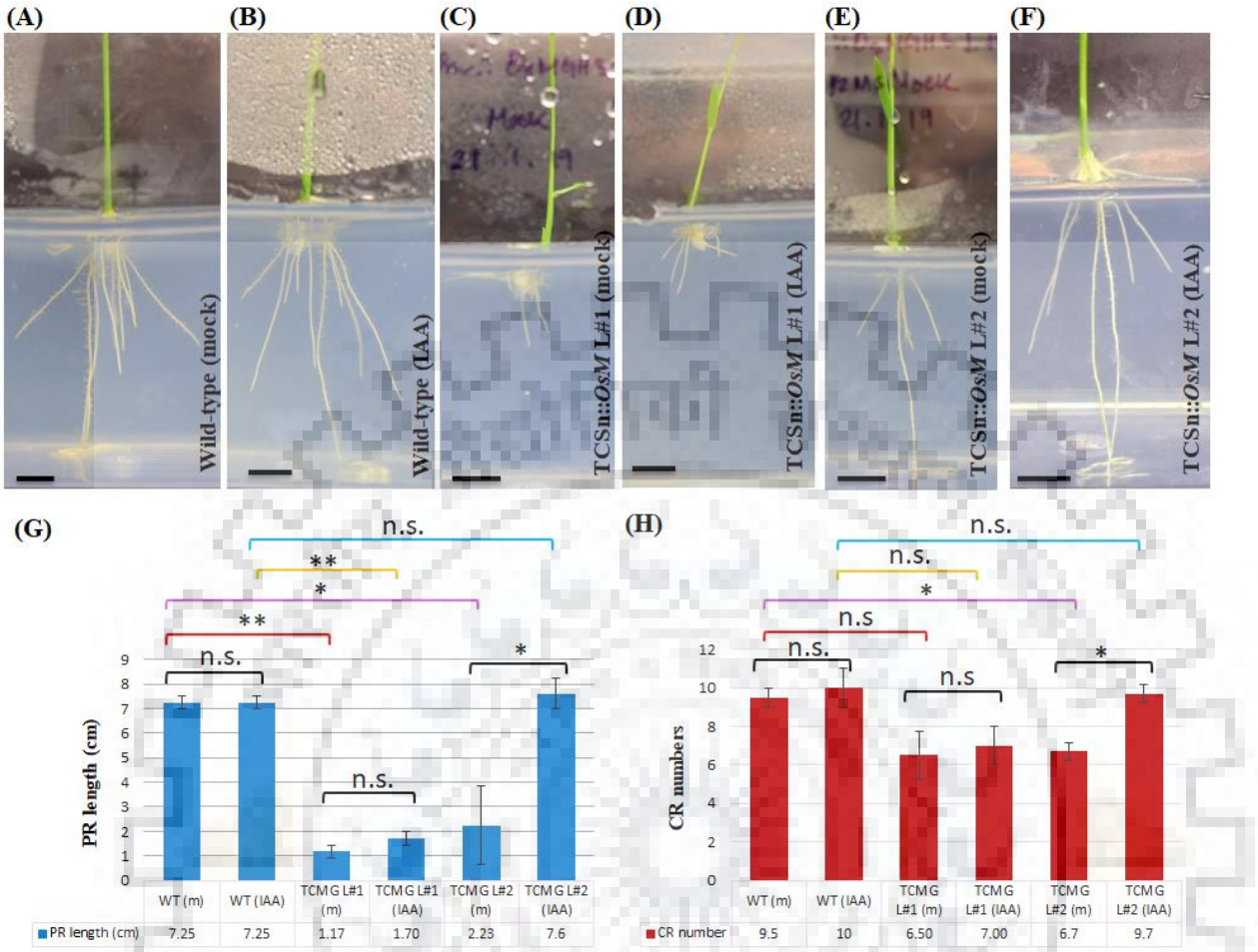

Figure 2:8 Consequence of exogenous IAA treatment on TCSn::*OsMGH3* transgenic rice. **(A- F)** Rescue of root growth phenotype by sub-optimal concentration of IAA treatment (10 nM) on 7 day old TCSn::*OsMGH3* plants in T1 generation (D, F); effect also analysed in wild-type (A, B). TCSn::*OsMGH3*L#1 display stronger phenotypes (C) where both primary and crown root growth was strongly inhibited whereas in L#2 (E) the effect was stronger on the crown root than the primary root. **(G, H)** Graphical comparison of primary root length and crown root number between wild type and transgenic lines. n=2 for WT mock and IAA, and TCMG L#1 mock and IAA; n=3 for TCMG L#2 mock and IAA. Significance is determined by two-tailed Student's t-test, ns, not significant, * p-value <0.05, ** p-value <0.005. (A- F) Scale bar: 1 cm.

machinery (Vanneste and Friml, 2009). At later stages when CRP is visible auxin is restricted to columella region of root cap indicates that the morphogen must be restricted to limit CR initiation at close vicinity of the older ones. Once the QC of the new CRP is made again auxin was required to maintain the stem cell niche and we observe diffused auxin response in the quiescent centre (QC) and surrounding region. In lateral root, auxin response is high in the initial cells of root columella and also present in comparatively lower extent in the QC near root cap (Bishopp et al., 2011). In rice the stele region shows auxin response which seemingly will be essential for the differentiation of the xylem cells. DR5rev helped to map the spatial and temporal auxin distribution during stages of CR development for the first time in greater detail. The auxin distribution pattern suggests root specific modules which is yet to be revealed in rice.

Cytokinins can stimulate cell division in the meristem, so, strong TCS driven GUS expression was detected in the apex of the primary root with root hair and at the top of the coleoptile within few days old germinated rice seeds. When LRP formed at the primary root, GUS expression was found in the primordia of the lateral roots. Our fluorescent microscopy study at the stem base of reporter lines harbouring TCSn::erGFP shows strong GFP signal . Similar results were reported in rice with stronger GUS activity at stem base, when GUS was fused to TCSn promoter (Tao et al., 2017). Strong reporter activity may be the output of high cell division capacity of the axillary buds present at the stem base. To gain deeper insight in rice crown root development stagewise, I have observed cytokinin response using TCSn::erGFP-nosT synthetic reporter lines. We have cut serial cross sections of the stem base and could not trace cytokinin response at the stem base before initiation of crown root. The known inhibitory effects of cytokinin in LR initiation is decisive for the recurrent positioning of LRI. In Arabidopsis, Lateral root organogenesis event takes place in the zone with elevated active cytokinin level but at the same time repressed cytokinin responses by auxin (Bielach et al., 2012; Bishopp et al., 2011). Presence of endogenous cytokinin in the basal meristem counteracts the stimulatory effect of auxin in lateral root initiation (Laplaze et al., 2007; Li et al., 2006). We found similar cytokinin response, which is devoid of the region because probably auxin accumulation inhibits ck response at priming stage of founder cells. Cytokinin response was visibly repressed during the early phases but was sturdily detected in the pro-vasculature in later stages of developing LR primordium (Bielach et al., 2012). To our observation cytokinin was present in the early CRP but expression was mutually separate than that of auxin. Once the differentiation starts, we could locate some zones where auxin and

cytokinin were present together along with zones of antagonism (Figure 2:9). Even if broadly expressed cytokinin response was never found at the root columella zone where auxin was found throughout development. In the case of post embryonic adventitious roots in the differentiating primordia, cytokinin response was found at the cortex region, early stele and endodermal layer indicates specific role of cytokinin in cell division at these regions. But the non-dividing QC shows no cytokinin response suggesting that cytokinin is demanded for cell division in distinct layers of CRP promoting tissue differentiation for CR development. LR morphogenesis in rice is studied using GH3promoter-GUS reporter construct proves that auxin is high during the initiation of the primordium but subsequently the concentrations decreases during differentiation (Sreevidya et al., 2010). In rice auxin in accordance, limits cytokinin response at QC and the stem cell niche but promotes cytokinin response at cortex and central stele region indicates that auxin decides where and when to inhibit or interact with cytokinin to ensure the decorum of adventitious root development.

There's been report that local auxin homeostasis and hormonal crosstalk can play roles in growth and development (Yadav et al., 2011, 2010), abiotic stress response and pathogen interaction (Park et al., 2007; Verma et al., 2016; Zhang et al., 2007). The intracellular pools of inactivated form can provide free aux upon hydrolysis or beta-oxidation (Woodward and Bartel, 2005). In Arabidopsis excess auxin is conjugated with sugars, amino acid, peptides or proteins of which only 1% IAA in the free form. Group II GH3 family members have been shown to play imperative role in amide conjugation of IAA to amino acids (Staswick, 2005; Staswick et al., 2002). The early auxin inducible GH3 family genes is regulated by a negative feedback loop that prevents accumulation of free auxins when present in excess (Delker et al., 2010; Jain et al., 2006b). Mutants of this family manifest various auxin over production phenotypes in *Arabidopsis thaliana* like *dfl1*-D and *ydk1*-D, dominant gain of function mutants of GH3 family genes, produce dwarf plants with reduced apical dominance, reduced lateral rooting and short hypocotyls (Nakazawa et al., 2008; Takase et al., 2004). Functional analysis of *OsMGH3*, provide evidence for the importance of auxin homeostasis during rice plant development (Yadav et al., 2011). In our study we have studied the consequences of the regulated expression of *OsMGH3* in transgenic rice plants with a view to understand the role of auxin in crown root development. Extremely poor regeneration of embryogenic calli when we express *OsMGH3* under TCSn promoter in a regulated manner in aux-ck interaction domain, indicates that perturbation of auxin availability in broad cytokinin response domain of callus

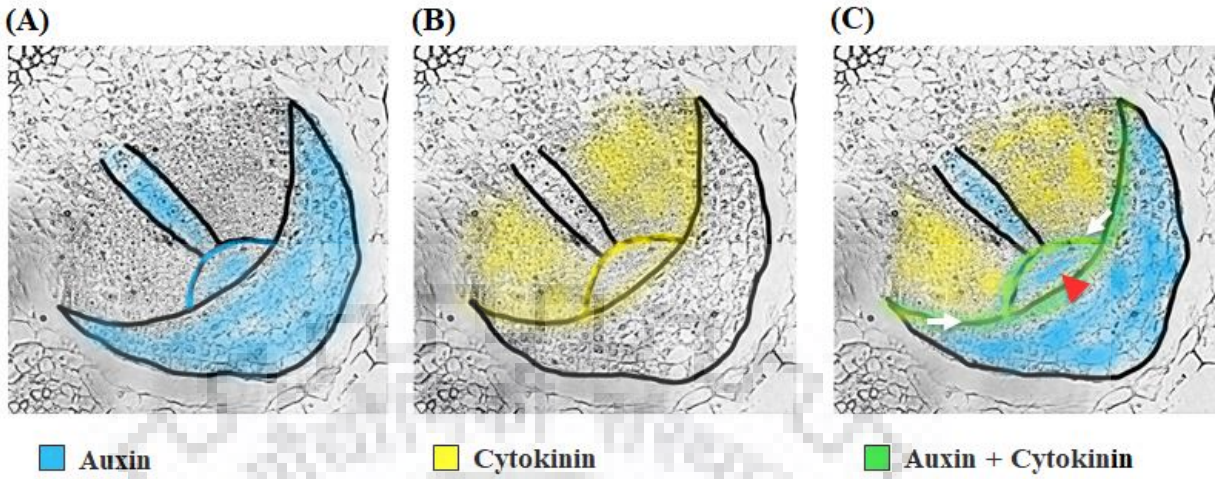

Figure 2:9 Anatagonistic and synergistic domains of auxin and cytokinin responses in developing crown root primordia (CRP). (A-B) Domain of disticnt auxin action (A) and cytokinin action (B). (C) Overlap in auxin-cytokinin domains, suggesting a plausible interaction between auxin and cytokinin (marked in white arrow).

influences the reorganization of shoot meristems from calli. Similar result of very less regeneration frequency of calli was obtained in *OsYUCCA1* down regulation because of decreased auxin production is inhibitory for rice callus regeneration (Yamamoto et al., 2007). In our case, few transgenic plants which regenerated, eliminate apical dominance to such an extent that they never make a transition to flowering. These phenotypes are similar to over-expression phenotypes of *OsGH3* mutants (Yadav et al., 2011). *OsMGH3* when fused to TCSn promoter and expressed in all cytokinin response domain in rice plant, we find that plants are dwarf which explain the importance of absence or maintenance of low level of endogenous *OsMGH3* transcripts in different vegetative organs. Overexpression of auxin biosynthesis gene *OsYUCCA1* root elongation was inhibited, whereas numbers of crown root was promoted (Yamamoto et al., 2007). In T1 generation, I studied the adventitious root development in the transgenic plants at seedling stage to find that auxin depletion in very small auxin-cytokinin interaction domain is so important that the roots turn out to be underdeveloped. In the line with strong expression of transgene, exogenous IAA cannot rectify the growth but the other line with weak transgene expression, pharmacological auxin treatment was enough to recuperate normal root growth. Thus, the expression studies attest the fact that auxin and its crosstalk with cytokinin in specialized domain is an essential factor and mediating crown root development. In inference, comparison of dynamic auxin and cytokinin movement between rice and *Arabidopsis* could help decipher their functions in angiosperms to further improve agronomical traits like root architecture and ultimately crop yield.

CHAPTER 3

AUXIN-REGULATED TRANSCRIPTION FACTOR, *OSAP2/ERF-40* IN CR FORMATION

3.1 INTRODUCTION

Root systems are vital for the survival in the complex environment, for structural support to the plant, and for the acquisition of water and nutrients (Osmont et al., 2007). Plants are continuously reshaping root systems by the generation of new roots throughout the growth period to adapt to biotic and abiotic stresses (Gowda et al., 2011). Understanding the inherent genetic control of root patterning and identifying functions of the genes responsible for post-embryonic root development could therefore expedite crop improvement towards stress tolerance (Meister et al., 2014).

The phytohormones regulate some key transcription factors (TFs) various developmental program including root architecture (Lavenus et al., 2013; Mai et al., 2014). The dicotyledonous plant *Arabidopsis* has a root system comprised of primary root (PR) and lateral roots (LRs), whereas the monocotyledonous rice root system is composed of crown/adventitious roots (CR/ARs) and LRs. Auxin signalling genes like *NONPHOTOTROPIC HYPOCOTYL4 (NPH4)/ARF7*, *ARF5/MP*, *AXR3/IAA17*, *BODENLOS/IAA12* and *ARF19* regulate embryonic PR formation in *Arabidopsis* (Hardtke and Berleth, 1998). Certain phytohormone-related genes control primary root growth of rice. Knock-out lines of such an auxin signaling transcription factor, *OsARF12* had primary root with shorter length (Qi et al., 2012). On the contrary auxin influx carrier mutant *Osaux1* had lengthier primary root than the wild type (Yu et al., 2015). Auxin perception and downstream signaling is regulated by microRNA miR393 to mediate primary root and crown root development through auxin receptor OsTIR1 and auxin response factor OsAFB2 (Bian et al., 2012). Silencing of *TOP1,* an essential DNA topoisomerase, has consequence of reduced root elongation and mis-regulation in gravitropism because of disrupted auxin-related gene loci (Shafiq et al., 2017). Gaseous phytohormone ethylene promotes rice root elongation through transcription factor *OsEIL1*, a positive regulator of ethylene signaling pathway (Mao et al., 2006). An AP2/ERF transcription factor, *SHB*, affects the elongation and proliferation of root meristem cells by gibberellic acid biosynthesis (Li et al., 2015).

Despite of gross similarity between PR, LR and CR, there are some root-specific mechanisms. For example, in rice *Osaux1* mutants, LR development was affected while CR development was normal (Zhao et al., 2015). On the other hand, in *arl1/crl1* mutants only CR development is affected whereas PR is developed normally (Liu et al., 2005) suggesting that some root-type specific auxin signaling modules function during primary, lateral and crown root development. *ADVENTITIOUS ROOTLESS 1/CROWN ROOTLESS 1* was the first gene to be identified as

crown root development gene (Inukai et al., 2005). Other TFs, such CROWN ROOTLESS 5 (*OsCRL5*), AP2/ETHYLENE-RESPONSIVE FACTOR3 (*OsERF3*) and WUSCHEL-Related Homeobox 11 (*OsWOX11*), *CYTOKININ RESPONSE REGULATOR 2* (*OsRR2*) and *QUIESCENT-CENTER-SPECIFIC HOMEOBOX* (*OsQHB*) have been shown to play crucial role during CR development in rice (Kitomi et al., 2011a, 2008; Liu et al., 2005; Wang et al., 2016; Y. Zhao et al., 2015). Entire process of LRs specification and emergence in Arabidopsis is regulated by different auxin modules that control expression of many down-stream transcription factors (De Rybel et al., 2010; Lavenus et al., 2013; Yadav et al., 2010). The expression of TFs known to control CR development in rice, is regulated by auxin and/or cytokinin signalling pathways. *CROWN-ROOTLESS4/OsGNOM1 (OsCRL4/OsGNOM1)* encodes an ortholog of Arabidopsis *GNOM1* that controls *PINFORMED1* (*PIN1*)-mediated polar auxin transport (PAT) and *crl4/osgnom1* mutants are defective in CR formation (Steinmann et al. 1999, Kitomi et al. 2008, Liu et al. 2009). Many genes show effect on more than one type of roots such as the gain-of-function mutant of negative regulator of auxin signalling, *OsIAA23* had defects in the initiation of both crown roots and LRs, and their QC maintenance at the root tip (Jun et al., 2011). Another controller of auxin signalling, *OsCAND1*, *Arabidopsis* homolog of *CULLI-N-ASSOCIATED AND NEDDYLATION-DISSOCIATED 1* (*CAND1*), halts cell division at G2/M transition at crown root primordia to hinder emergence of crown roots (Wang et al., 2011). In addition, CRL6, encodes a member of the large CHD (chromodomain helicase DNA-binding) family proteins, is highly expressed at stem base region and is known to affect crown root development in rice (Wang et al., 2016). The defective crown root formation in *crl6* is due to defect in auxin signalling down regulation of *OsIAA* genes and thus can be rescued by auxin treatment (Wang et al., 2016). *OsIAA11/OsIAA13* auxin module negatively regulates LR formation in rice. A conserved function of IAA–ARF module promoting LR formation is also found in *Arabidopsis* where auxin module of *SLR/IAA14-ARF7/ARF19* stimulates LR formation (Fukaki et al., 2005, 2002) along with a second auxin module involving *IAA12* and *ARF5* (De Smet et al., 2007). Root hair elongation is regulated by *OsWOX3A* through the altered auxin transport (Yoo et al., 2013), further suggesting that transcription factors directly or indirectly regulated by auxin are required all aspects of plant root development.

Cytokinins are also crucial for rice adventitious root development. Cytokinin inducible *OsRR6*, an ARR suppresses root and vegetative development when overexpressed (Hirose et al., 2007). The crosstalk between auxin and cytokinin is known during CR development in rice. Cytokinin Oxidase/Dehydrogenase 4 (*OsCKX4*) encoding a cytokinin degrading enzyme, integrates auxin

and cytokinin signalling pathways in CRs by being a direct target of both the *OsARF25* and type-A cytokinin response regulators *OsRR2* and *OsRR3* (Gao et al., 2014). Auxin and cytokinin directly regulate expression of *OsCKX4* in rice (Gao et al. 2014). Similarly, auxin-inducible genes like *OsCRL5*, *OsWOX11* and *OsERF3* regulate (Kitomi et al., 2011; Zhao et al., 2015, 2009b). While these studies have begun to provide insights into the making of rice root system, an interconnected gene network controlling rice root architecture yet remains obscure.

In this study, we have identified an auxin-responsive transcription factor of plant-specific AP2-domain containing gene family and studied its detailed temporal and spatial expression pattern in developing crown roots. Furthermore, our detail functional studies of *OsAP2/ERF-40* in loss- and gain-of-function mutant reveals that *OsAP2/ERF-40* is necessary and sufficient for development of crown roots in rice. Interestingly, it regulates auxin and OsERF3-OsWOX11-OsRR2 signalling pathways in a dose-dependent manner in the shoot tissues. Taken together, our studies not only identify putative transcriptional regulators down-stream of auxin and cytokinin signalling pathways, but also demonstrate unknown function of an auxin responsive target and gene regulatory network in activating root-specific developmental program in rice.

3.2 MATERIALS AND METHODS

3.2.1 Plasmid Construction for functional characterization of *OsAP2/ERF-40*

For generating ectopic over-expression construct for *OsAP2/ERF-40*, 1.14 kb full-length gene was PCR amplified on rice genomic DNA using primers FPI-AP2 and RP-AP2-*Bam*HI and was cloned in pBluescript SK+ at *Eco*RV site in antisense orientation as blunt to generate pBSSK *OsAP2/ERF-40* #11 and the clone is sequenced subsequently after RE digestion validation. Next, *Bam*HI fragment of *OsAP2/ERF-40* was subcloned into the plant expression vector pUN under maize Ubiquitin promoter to generate pUbi-*OsAP2/ERF-40*-nosT and final clone validated through RE digestion.

A 410bp fragment is PCR amplified using primers FP-II and RP-*Bam*HI from 3'UTR of *OsAP2/ERF-40* using cDNA amplified from IR64 rice variety (Figure 3:7 B). PCR product cloned in pBluescript (*Eco*RV) as blunt fragment. pBSSK ds*OsAP2/ERF-40* is screened for sense and antisense orientation. Then the fragment is taken out from pBSSK ds*OsAP2/ERF-40* antisense clone #2 as *Sma*I-*Bam*HI fragment and cloned in existing pBSSK with a 534bp linker (truncated GUS gene fragment) to generate pBSSK ds*OsAP2/ERF-40*(s) #5 in sense orientation. Then the same 410 bp fragment is taken from pBSSK ds*OsAP2/ERF-40* #2 as *Sma*I & *Hinc*II restriction enzyme digestion and cloned into *Hinc*II site of pBSSK ds*OsAP2/ERF-40* (sense)#5 and validated for antisense orientation to assemble final construct in pBSSK to generate pBSSK ds*OsAP2/ERF-40* (antisense) (sense)#7 (Figure 3:7 A). The insert is taken as *Bam*HI from pBSSK ds*OsAP2/ERF-40* (antisense) (sense)#7 and *OsAP2/ERF-40* hairpin was then sub cloned in pUN plant expression vector in *Bam*HI site under maize ubiquitin promoter to generate pUN::ds*OsAP2/ERF*-40-nosT #1 (Figure 3:7 A).

3.2.2 Rice transformation Generating Transgenic Lines and Phenotyping

OsAP2/ERF-40 over-expression (pUbi-*OsAP2/ERF-40*-nosT) and RNAi (pUN::ds*OsAP2/ERF*-40-nosT) constructs were mobilized to *Agrobacterium tumefaciens* LBA4404 and used to raise transgenic rice lines as described by Prasad et. al. (2001). Briefly, embryogenic calli of rice japonica var. TP309 was co-cultivated with *Agrobacterium* harbouring the construct and selected on NB6-SEL medium containing 50 mg/L hygromycin. Actively proliferating calli were further transferred to NB6-RM media (50 mg/L hygromycin, 3 mg/L BAP and 0.5mg/L NAA) for shoot regeneration. Regenerated shoots were subsequently transferred to rooting media. Hardening of regenerated plantlets was done in soilrite before transferring to clay for completing the life cycle. Since *OsAP2/ERF-40* over-expression lines with strong transgene

expression did not produce fertile seeds, multiple independent stronger lines were phenotyped in T0 generation for their overall growth and development. Rice transformed with ds*OsAP2/ERF-40,* were monitored in green house but as there was no vivid phenotype at T0, we collected seeds from several independent lines and detailed phenotyping for RSA was done in T1 generation.

3.2.3 Extraction of rice genomic DNA and polymerase chain reaction

Genomic DNA was isolated from young leaves of rice plant by salt extraction method as detailed in the Aljanabi and Martinez (1997) and Prasad K. 2003 PhD thesis. The quality and concentration of genomic DNA was determined on 0.8% agarose gel. Appx 20 ng of gDNA from leaves of wild-type (var TP309) and few independent over expression lines of *OsAP2/ERF-40* were used to determine genomic integration of transgene by PCR. The forward primer located in the gene TOC168 FPIII and reverse primer nosT-RP located in the construct specific nos terminator together gave a desired 740bp band. PCR reaction was carried out in 20 ul reaction volume for 35 cycles. The PCR product were separated on 1.2 % agarose gel. For appx. 20 ng of gDNA from leaves of wild-type (var TP309) and few independent RNAi lines (L#5, 6, 8, 9, 10) of *OsAP2/ERF-40* were used to determine genomic integration of transgene by PCR. To validate the presence of RNAi construct pUN::ds*OsAP2/ERF*-40-nosT, RP TOC168 was used as forward and linker GUS fragment specific, RP GUS was used as reverse primer which gave a PCR amplicon of 817bp desired size. For *OsUBQ* control PCR cycle was reduced to 28 with rest of the conditions same for the PCR.

3.2.4 RNA extraction and RT-PCR

RNA was extracted from either shoot tissue or stem base region of rice plants. Total RNAs were treated with DNase I (NEB) and precipitated after phenol and chloroform treatment. For over expression analysis, through semi quantitative RT, cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-rad) and PCR was performed using Taq-Polymerase (NEB) (Appendix D). A total of 10 ng cDNA was used for semi quantitative real-time PCR (qRT-PCR) using 250 nM of gene-specific primers. Actin or *UBQ5* transcript levels were used as endogenous normalization control. For expression of various genes in *OsAP2/ERF-40* over-expression lines, cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-rad) and qPCR was performed using iTAQ Universal SYBR Green Supermix (Bio-rad) as described in Appendix E. A list of primer sequences is provided as Appendix H.

3.2.5 Plant Growth and Microtome Sectioning for Microscopy

Seeds of (Oryza sativa var TP309) wild-type and were de-husked, surface sterilized and germinated on ½ MS media with 1% sucrose and 0.3% phytagel (Sigma) and grown vertically in sterile square petri dishes (Himedia; 12 cm × 12 cm), at 26 °C in 16/8-hour light/dark period for 6 days. For histological and RNA in situ hybridization analysis, approximately 2mm rice stem base region was dissected from each wild-type and transgenic plants and fixed in FAA (10% formaldehyde, 5% glacial acetic acid and 50% ethanol). Ethanol series was performed to dehydrate the tissues and finally embedded in paraffin (SIGMA) blocks. After solidification and reshaping, materials were cut into 6-8 µm thin sections using microtome (Thermo Scientific) and sections were taken on poly-L-lysine coated glass slides (EMS).

3.2.6 RNA-RNA *in-situ* hybridization

DIG-UTP-labelled, 220 bp *OsAP2/ERF-40* anti-sense probe was prepared by in *vitro* transcription using T7 RNA Polymerase (Sigma) on *Oli*I digested pBluescript SK+-ds*OsAP2/ERF-40* clone. Hybridization was performed on cross sections as described by Prasad et al. (2005). The signal was developed using alkaline phosphatase conjugated anti-DIG antibodies (Sigma) and NBT/BCIP substrate (Sigma). Sections were mounted in Entellan (Merck- Millipore).

3.3 RESULTS

3.3.1 Spatio-temporal expression patterns of *OsAP2/ERF-40,* a putative regulator of crown root development in rice

Our aim is to study rice crown root development and functional characterization of key genetic regulators involved in the developmental events covering from establishment of initial cells to emergence of crown root, divided in seven stages from root initiation, then development and growth (Coudert et al., 2010; Itoh et al., 2005). Our RNA sequencing data obtained upon auxin and cytokinin treated rice crown tissues was used to identify a putative transcription factor *OsAP2/ERF-40* with putative functions during rice crown root development (Figure 3:1 A). Since the expression of the selected TF, *OsAP2/ERF-40* was lower in the stem base as compared to emerged CRs and PRs (Neogy et al., 2019), we surmise that its expression of may be restricted to only CR primordia in the stem base. To explore that, We, first studied detailed temporal and spatial expression pattern of *OsAP2/ERF-40* across various developmental stages of CR primordia and in emerged CRs. DIG-labelled anti-sense RNA probes were hybridized on 8 µm cross sections of wild-type rice stem base containing CR primordia at different stages. The expression is initiated in the early CR primordia and is continued during later stages of CR differentiation and emergence. *OsAP2/ERF-40* is expressed both early as well as in late stage CR primordia (Figure 3:1 D- G). Prior to CR primordia establishment, its expression is low in the tissues peripheral to vascular cylinder (Figure 3:1 D). During later stages of development, its expression is specifically enhanced in the developing primordia (Figure 3:1 E, F). In emerged CRs, the expression of *OsAP2/ERF-40* is restricted to the root meristem (Figure 3:1 G). As control, cross sections were hybridized with sense probe and no signals found above the background. All the observations together prompted us to study function of *OsAP2/ERF-40* during adventitious root development.

3.3.2 Functional study of an auxin-responsive gene, *OsAP2/ERF-40* in transgenic rice

An auxin-inducible gene with single AP2-domain of AP2/ERF gene family, *OsAP2-ERF-4* (also called *OsERF3*), functions during early and late stages of rice CR development (Rashid et al., 2012; Zhao et al., 2015). To further investigate the role of another auxin regulated TF of AP2/ERF gene family, *OsAP2/ERF-40* (also called OsTOC168, LOC_Os04g46400) we validated the auxin responsive expression of *OsAP2/ERF-40* through q-RT. We observe that its expression is indeed induced by auxin in the rice stem base (Figure 3:1 B). Rice *DRO1* was used as control to ensure that auxin treatment was robust, and we observed expression of *DRO1* was reduced upon auxin treatment (Figure 3:1 B),

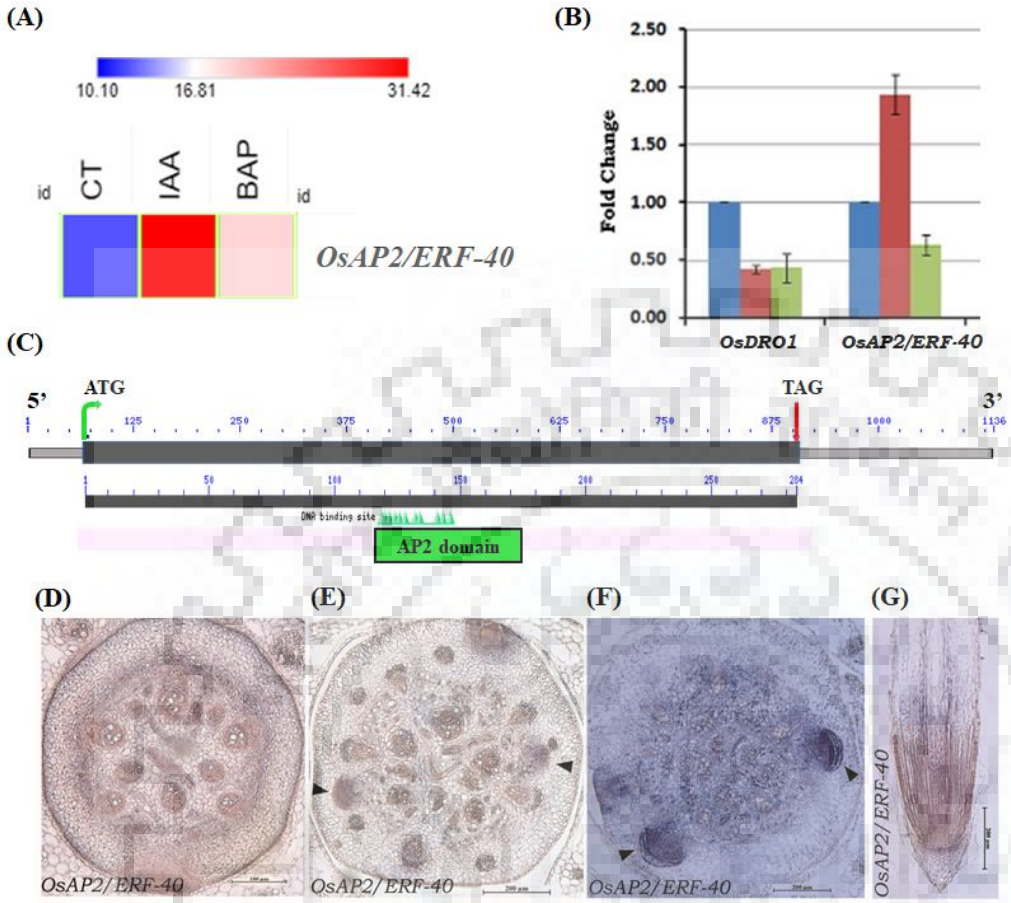

Figure 3:1 Effect of auxin treatment on the expression level *OsAP2/ERF-40* and its crown tissue-specific expression pattern. (A) Heat-map for de-regulated genes upon IAA and BAP treatment as compared to mock treatment (log2 fold change ≥1.0; Neogy et al., in press). **(B)** Auxin induced expression of *OsAP2/ERF-40*, analyzed by qRT-PCR. The expression is not affected upon cytokinin treatment. *DRO1* was used control for auxin treatment. **(C)** Gene map for *OsAP2/ERF-40* containing 60 amino acid long single AP2 domain extended from 116 to 176 on the protein map below the c-DNA map. **(D)** Prior to CR primordia establishment, lower expression in the tissues peripheral to vascular cylinder. **(E, F)** Crown primordia specific induced expression is visible in the cross sections during later stages of development. **(G)** In emerged crown roots, *OsAP2/ERF-40*expression is limited to the root meristem. Arrow heads mark developing CR primordia specific expression, scale bars: (D- G) 20 µm.

To get thorough information about the gene, we retrieved genomic, c-DNA and protein sequences for *OsAP2/ERF-40* whose expression was down-regulated in *OsCRL1* (Coudert et al., 2015) from NCBI sequence database, RiceXPro and Gamene databse. The sequence analysis predicted locus on sense strand of chromosome 4 without any intron. The 1136 bp long mRNA has a 64 bp 5' UTR and 217 bp 3' UTR. The ORF (65-919) encodes a predicted 285 amino acids long protein with 60 AAs (116-176) comprising the conserved AP2 DNA binding domain, found in transcription regulators *OsAP2/ERF-40* in plants such as *APETALA2* and *EREBP* family of genes (Figure 3:1 C). The sequence alignment of predicted OsAP2/ERF-40 protein sequence identity with ERF/AP2 transcription factor family of *Arabidopsis thaliana*. The gene having high expression in root compared to the shoot tissues at vegetative stage of the plant. Considering higher degree of genetic redundancy among the members of AP2/ERF gene family, we took mis-expression based approach to study function of *OsAP2/ERF-40*. To functionally characterize *OsAP2/ERF-40*, our complementary approach was to down-regulate in rice.

3.3.3 Cloning of constructs for functional characterization of *OsAP2/ERF-40* and generating transgenic rice plants

We generated a construct for ectopic over-expression of *OsAP2/ERF-40* into a pCAMBIA based plant specific binary vector. A 1136bp full-length cDNA of *OsAP2/ERF-40*, was PCR amplified using cDNA made from crown tissues which has higher level of the transcript. After few sub-cloning steps full length cDNA fragment was cloned in the rice expression vector, pUN under maize Ubiquitin promoter (Figure 3:2 A) (Prasad et al., 2001) and sense orientation cDNA is validated through several restriction digestions. Rice embryogenic calli was co-cultivated by *Agrobacterium* mediated transformation for raising transgenic rice and several lines were regenerated for further phenotyping and to decipher genetic regulation. About 45 transgenic lines were generated with pUbi-*OsAP2/ERF-40*-nosT construct with a range of phenotypic severity (Figure 3:2 B). Only few transgenic lines attained reproductive phase in our growth condition and produced very few seeds. The phenotypic severity of these transgenic lines is corroborated with the extent of over-expression of *OsAP2/ERF-40*, the transgenic lines expressing high level of transgene display stronger phenotypes as compared to weak lines (Figure 3:2 B, C). To down-regulate, *OsAP2/ERF-40*, the endogenous expression of *OsAP2/ERF-40* is curtailed using double stranded RNAi (dsRNAi). A unique region including the 3'UTR of *OsAP2/ERF-40* cDNA has been targeted for RNA interference by ubiquitous

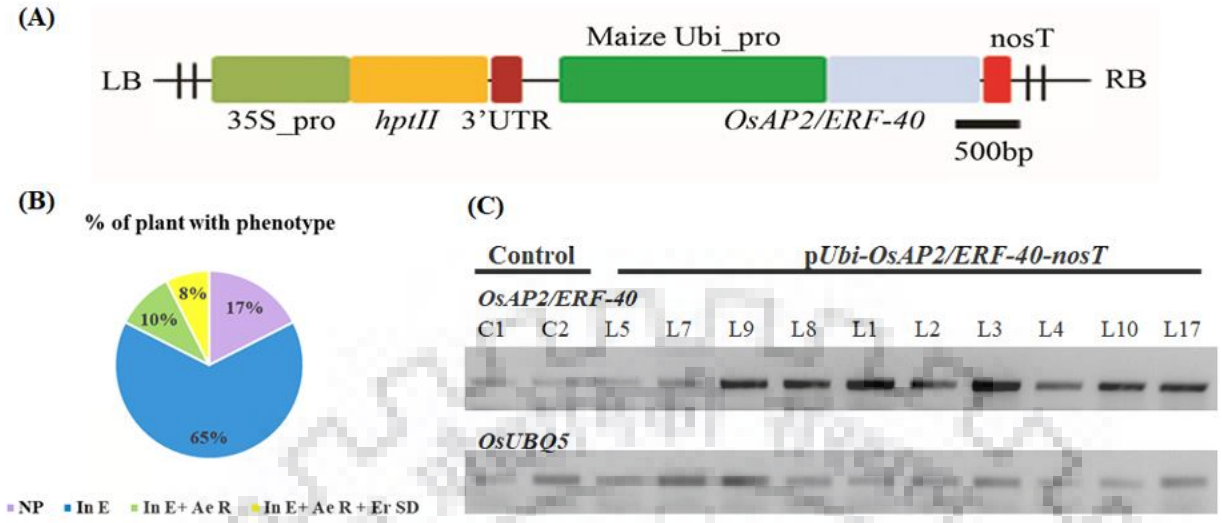

Figure 3:2. Range of phenotypic severity in transgenic lines with pUbi-*OsAP2/ERF-40*-nosT construct. (A) Schematic representation of T-DNA of *OsAP2/ERF-40* over-expression construct. **(B)** Pie chart representation of the range of *OsAP2/ERF-40* over-expression phenotypes observed in multiple independent transgenic lines. NP: null phenotype, In E: internode elongation, In E+ Ae R: internode elongation plus aerial roots, In E+ Ae R+ Er SD: internode elongation with aerial roots and early side-tillers. n= 45. **(C)** RT-PCR analysis showing over-expression of *OsAP2/ERF-40* in shoot tissues of ten independent transgenic lines. C1 and C2 are control lines.

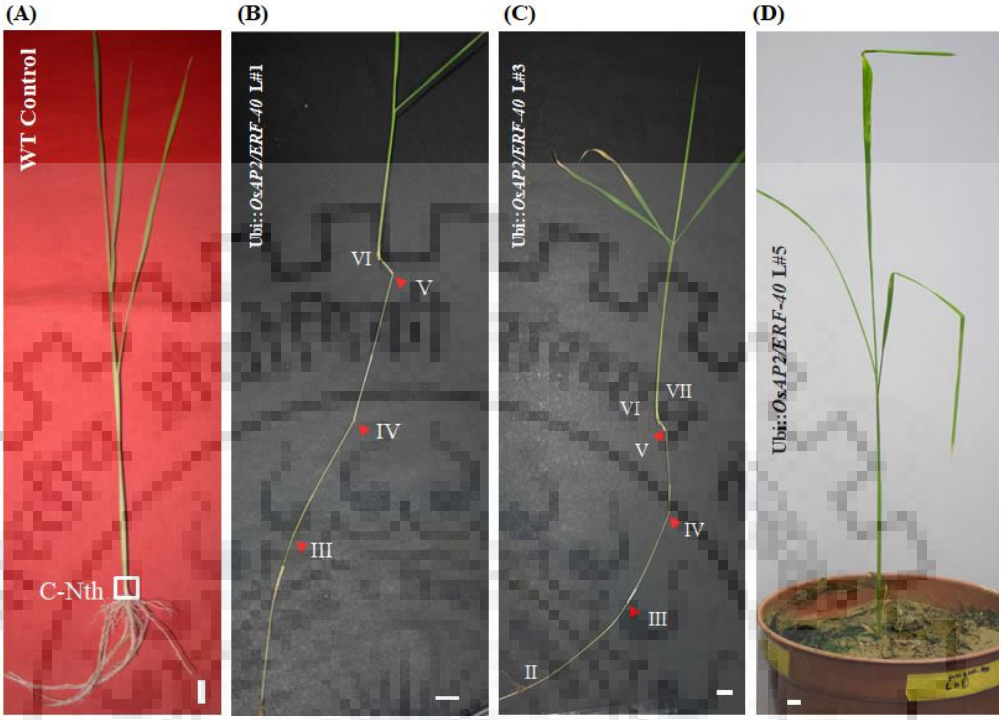

Figure 3:3. Effect of *OsAP2/ERF-40* over-expression in transgenic rice lines (A) In vector control plant square highlighting stem base (crown region) in that contains several nodes with un-elongated internodes. **(B, C)** Two independent *OsAP2/ERF-40* over-expression lines showing multiple nodes with significantly elongated internodes. Elongated internodes (red arrowheads) are seen only in the stronger lines (B, C) not in weak line L#5 (D). C, coleoptilar, nodes are numbered in roman. (A- D) Scale bars: 1 cm.

expression of transgenic hairpin RNAs against endogenous *OsAP2/ERF-40* mRNAs (Figure 3:7). In dsRNAi construct we targeted a gene-specific 410bp nucleotide fragment comprising the 217bp 3'UTR and 193bp allied segment from the coding sequence of the mRNA. The same 410 bp fragment is assembled in both antisense and sense orientation around the GUS linker to a final construct pBSSKds_AP2 (antisense) (sense) #7 (Figure 3:7 A). The *OsAP2/ERF-40* hairpin was then sub cloned in pUN plant expression vector in between maize ubiquitin promoter and nos-terminator to generate pUN::ds*OsAP2/ERF*-40-nosT. to be carried to the same LBA4404 strain of *Agrobacterium.* Rice transformation for raising transgenic was done to generate multiple independent lines harbouring RNAi construct.

3.3.4 Ectopic Over-expression of *OsAP2/ERF-40* promotes aerial shoot-borne root formation and acts synergistically with polar auxin transport machinery

Next, we analysed *OsAP2/ERF-40* over-expression lines as compared to control plants and observed phenotypes in about 65% transgenic lines (Figure 3:2 B). In stronger over-expression lines, stem internodes were significantly elongated, resulting in upward shift of the crown nodes (Figure 3:3 B, C). The crown region of wild-type rice has several compressed un-elongated internodes (Figure 3:3 A) which were elongated by several folds upon *OsAP2/ERF-40* over-expression (Figure 3:3 B, C). In addition to internode elongation, over-expression of *OsAP2/ERF-40* also induced roots from the aerial nodes in stronger transgenic lines (Figure 3:4 B- D). In the wild-type (Figure 3:4 A) and weak over-expression plants (Figure 3:3 D), roots are not usually developed from the aerial nodes whereas several roots were emerged at the aerial node of elongated internodes in strong *OsAP2/ERF-40* over-expression lines under same environmental conditions (Figure 3:4 B- D). Strikingly, we observed aerial root formation from all nodes, including the node of uppermost internode, just beneath the panicle node (Figure 3:4 G, I). In wild-type plants, internode elongation occurs after transition from vegetative to reproductive phase, but root formation was not observed at the uppermost node under our growth condition, consistent with previous reports (Itoh, et al., 2005; Yadav et. al., 2011). These aerial roots when penetrate the soil, support plant growth and development (Figure 3:4 C), suggesting that they are functional roots. These observations suggest that *OsAP2/ERF-40* is sufficient to induce root-specific developmental program at aerial nodes. Similar root phenotypes are also seen when active pool of auxin is increased, either by over-expression of auxin biosynthesis genes, *OsYUCCA* (Yamamoto et al., 2007; Zhang et al., 2018) or down-regulation of auxin-inactivating gene, *OsMGH3* (Yadav et al., 2011). The phenotypic similarities of these lines with *OsAP2/ERF-40* ever-expression lines further support that

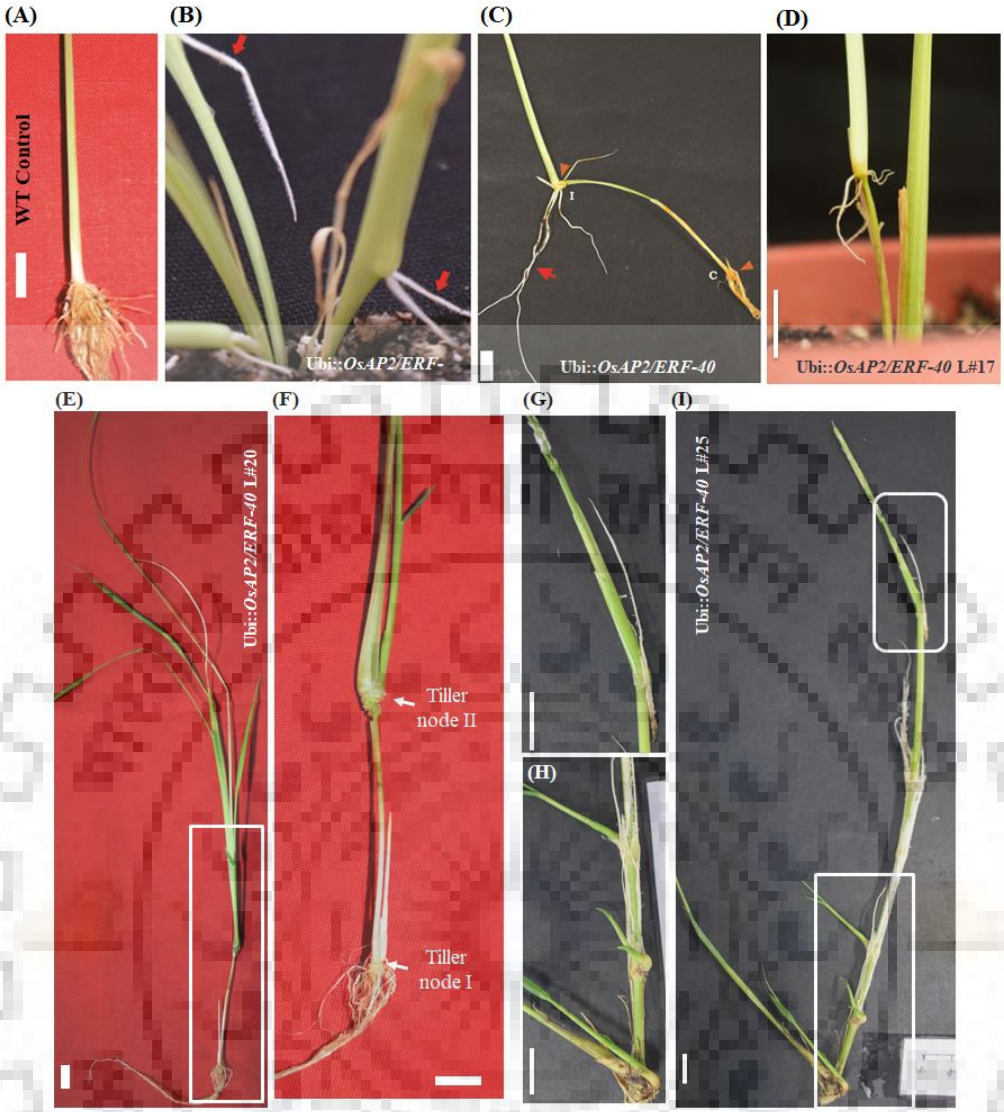

Figure 3:4. Aerial root and side shoot development in *OsAP2/ERF-40* over-expression lines. (A- D) Adventitious root formation from aerial nodes in two independent transgenic lines at various stages of plant growth, red arrows mark aerial roots; crown region of vector control plant (A) that contains several nodes with un-elongated internodes from where the crown roots have been emerged. **(C)** Two consecutive nodes (c and i) of elongated internode are highlighted by arrow heads. Adventitious roots at node i of (C) elongate and support plant after penetrating the soil.**(E- H)** In few other lines aerial roots are emerged at every nodes including uppermost nodes just beneath the panicle node marked by rounded corner white rectangle (**G, H**) and side tillers are also appeared at the aerial nodes highlighted in white rectangular boxes **(E, I**) and enlarged in (**F, H**). White arrows indicate early side tiller formation at aerial nodes. scale bars: (**A- H**) 1cm.

OsAP2/ERF-40 controls auxin mediated signaling in rice. Apart from aerial roots, we also noticed side tillers originating from those elongated aerial nodes in few of the transgenic lines (Figure 3:2 B, Figure 3:4 E, F, H, I). Generally side tillers are observed as an allude to the reproductive stage of rice but in some of the over expression lines, tillers developed prematurely (Figure 3:4 E, F).

We next examined if *OsAP2-ERF-40* over-expression acts synergistically with polar auxin transport machinery. Towards this, we pharmacologically inhibited the polar auxin transport by treating transgenic lines with sub-optimal concentration of polar auxin transport inhibitor, 1-N-Naphthylphthalamic acid (NPA). Treatment of wild-type seedling with various concentration of NPA suggested that 50 nM NPA does not significantly affect root numbers or growth (Figure 3:5 A). We, therefore, germinated wild-type and *OsAP2-ERF-40* over-expression lines on media supplemented with 50 nM of NPA (Figure 3:5 B- E). Transgenic plants from overexpression line L#9 used in NPA experiment were genotyped using transgene specific primers, band was absent in wild type for primer set 1 with TOC168 FPIII and nosT RP (Figure 3:6 A). Primer set 2 i.e. Toc 168 FPIII and Toc 168 RP is taken as a positive control and OsUBQ5 is a constitutive internal control (Figure 3:6 A). Quantitative analysis shows almost ten-fold over expression of *OsAP2/ERF-40* transcripts in transgenic plants of L#9 (Figure 3:6 B) and up to 2.5-fold over expression in the plants of L#23 (Figure 3:6 C). Unlike in wild-type plants (Figure 3:5 B, C), root growth was strongly inhibited by NPA in over-expression lines (Figure 3:5 D, E), suggesting that *OsAP2-ERF-40* over-expression acts synergistically with polar auxin transport machinery to control CR development.

3.3.5 *OsAP2/ERF-40* function is necessary for rice crown root development

Our ectopic over-expression based gain-of-function study suggest that *OsAP2/ERF-40* is sufficient to promote adventitious root development. Next, we studied if its function is necessary for crown root development in rice. For this purpose, RNA interference (RNAi) based the loss-of-function approach was taken for better understanding of *OsAP2/ERF-40* role in crown root development. Several independent transgenic lines were generated with RNAi construct pUN::ds*OsAP2*/*ERF*-40-nosT. We screened these lines in T1 generation for down-regulation of endogenous *OsAP2/ERF-40* to study the consequence of its down-regulation during crown root development. We germinated these transgenic lines in ½ MS media supplemented with hygromycin to analyse segregation pattern of the transgene. We observed approximately 3:1 Mendelian segregation ratio in some of the lines (L#1, 5, 6, 8, 9, 10) on hygromycin, suggesting that these lines harbour single transgene (Figure 3:8 A, B). These lines

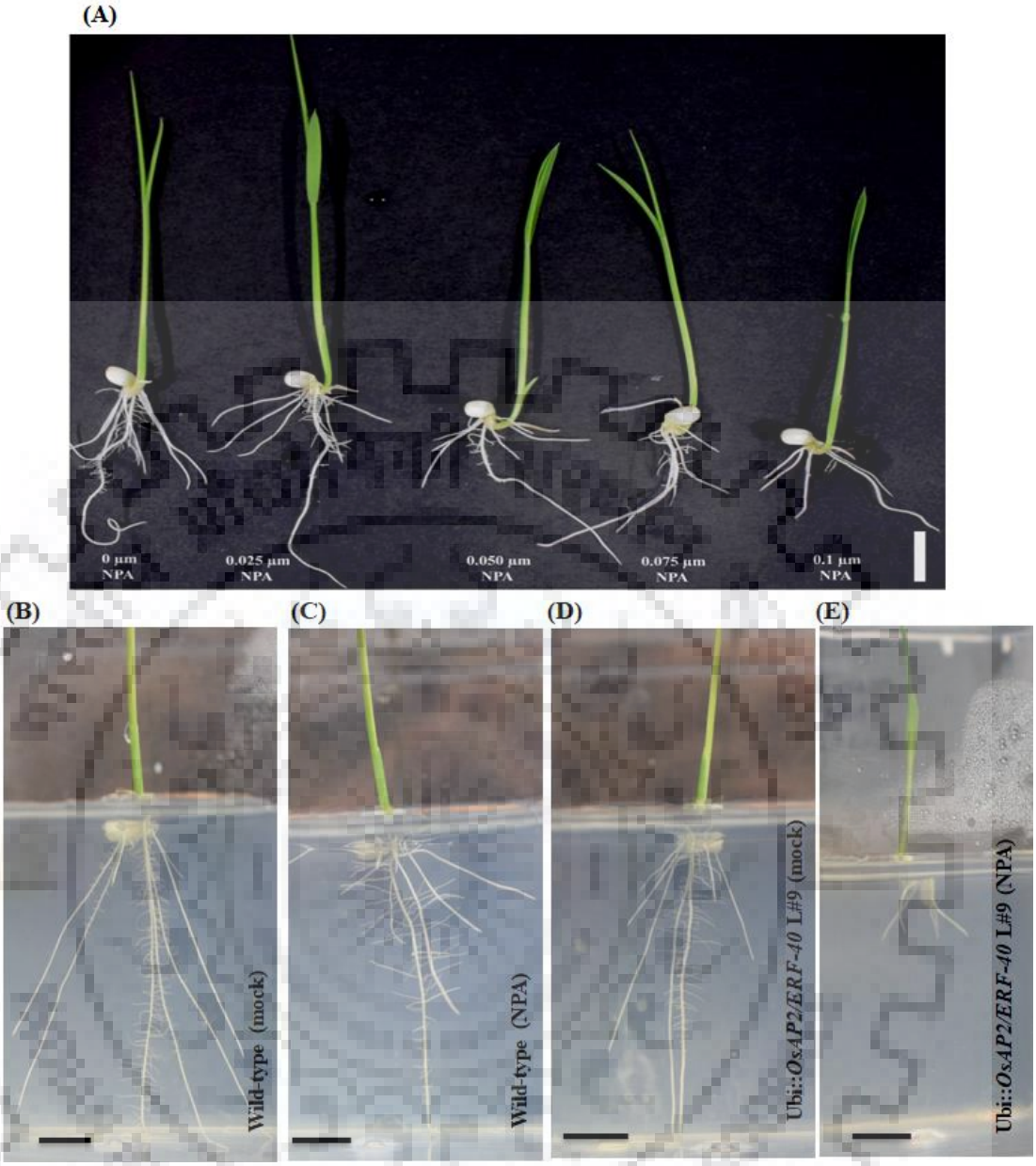

Figure 3:5. Effect of sub-optimal NPA treatment on Ubi-*OsAP2/ERF-40* plants. (A) Rice plants were grown in ½ MS media supplemented with various concentration of auxin inhibitor, NPA for 7 days. 50nM concentration was not showing any visible effects on wild type root growth. **(B, E)** Effects of NPA treatment (50 nM) on 7-d old wild-type (B, C) and transgenic line Ubi-*OsAP2/ERF-40* L#9 (D, E). Scale Bars: 1cm (A- E).

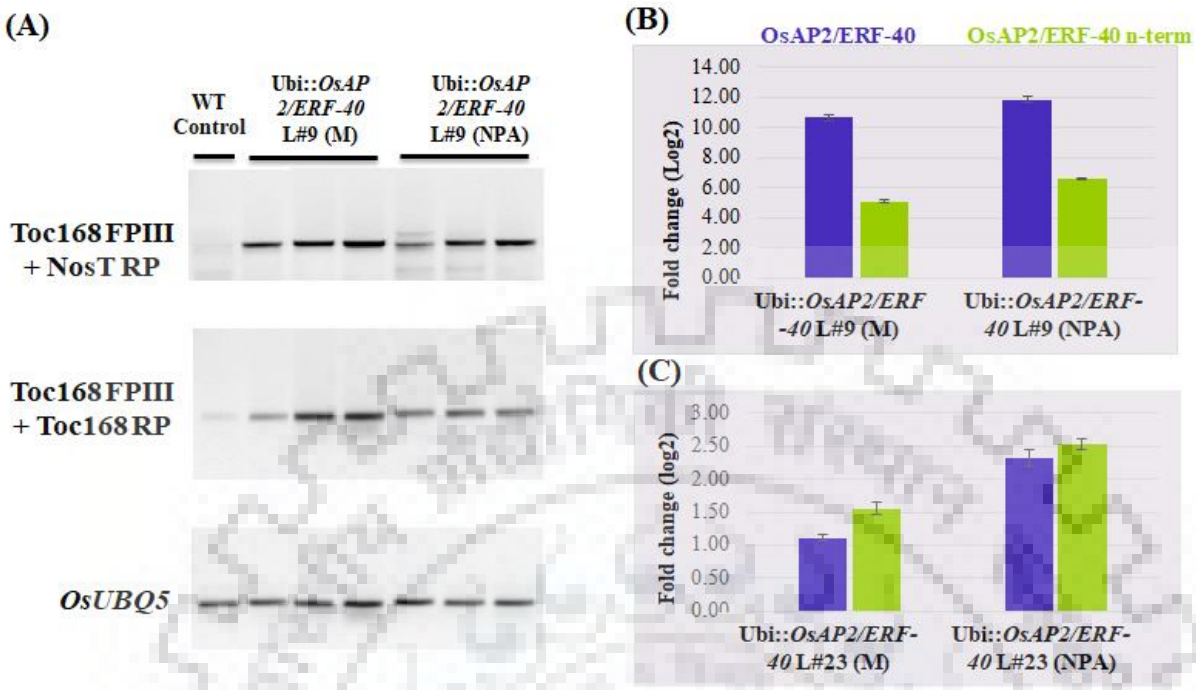

Figure 3:6. Molecular characterization of over expression lines in T1 generation. (A) PCR-based genotyping of overexpression line L#9 used in NPA experiment showing integration of transgene in the genome of transgenic plants of L#9, band was absent in wild-type for construct-specific primers, TOC168 FPIII and nosT RP. *OsUBQ5* and TOC168 FPIII and TOC168 RP primer combinations were used as internal PCR control. **(B, C)** qRT-PCR analysis shows over expression of *OsAP2/ERF-40* transcripts in transgenic plants of L#9 (B) and L#23 (C).

were also PCR genotyped using a gene specific primer (RP_TOC168) in combination with a primer placed in stuffer GUS fragment (RP GUS) on the genomic DNA extracted from leaf tissues of these lines. Wild-type rice genomic DNA was used as a negative control for the PCR. Our PCR genotype shows that these lines contain transgene copy in their genome (Figure 3:8 C). Consistent with the presence of transgene construct, these transgenic plants display defects in the root system architecture (Figure 3:8 A, B).

Next, we analysed expression level of endogenous transcript of *OsAP2/ERF-40* using gene-specific primers by qRT-PCR and observed about 70% reduction in the expression levels in line RL#1. We proceeded with RL#1 for detailed analysis and observed a prominent defect in root development. When we germinated line LR#1 on ½ MS media, we observed a segregation in the root phenotype, one plant grew as comparable to wild type but the rest of the two plants show severely reduced root growth on 7th day of germination (Figure 3:8 D). Next we genotyped these segregating plants using primer combination specific to hygromycin resistant gene, *hptII* (Figure 3:9 E). We compared a plant with transgene (RL#1-7) with RL#1-5 which does not show presence of transgene for root development. We observed that primary root growth was significantly slower in the RL#1-7 as compared with RL#1-5 in 7d old plants (Figure 3:9 A, B). Apart from the primary root growth, number and growth of crown roots are also decreased in RL#1-7 as compared to RL#1-5, as visible in 7d and 10d old plants (Figure 3:9 A- D). Combining these observations with the phenotype observed upon over-expression of *OsAP2/ERF-40* suggest that *OsAP2/ERF-40* is a positive regulator of crown initiation and emergence.

3.3.6 Regulatory relationship of *OsAP2/ERF-40* with *OsERF3-OsWOX11-OsRR2* pathway in crown root tissues

The phenotypes of *OsAP2/ERF-40* over-expression indicate that auxin signalling might be affected in the over-expression lines. To study this, we analysed effect of *OsAP2/ERF-40* over-expression on expression levels of various components of auxin signalling pathway. We determined expression levels of auxin biosynthetic gene *OsYUCCA6*, and early auxin responsive genes (*OsIAA1* and *OsGH3*.8) in *OsAP2/ERF-40* over-expression lines. Our qRT-PCR analysis revealed that the expression of *OsYUCCA6*, *OsIAA1* and *OsGH3.8* was induced in *OsAP2/ERF-40* over-expression lines (Figure 3:10). We have observed that extent of induction of these genes depends on expression level of transgene, weak line #L7 has lower induction than strong lines (Figure 3:10 A). In ds *OsAP2/ERF-40* lines also we checked

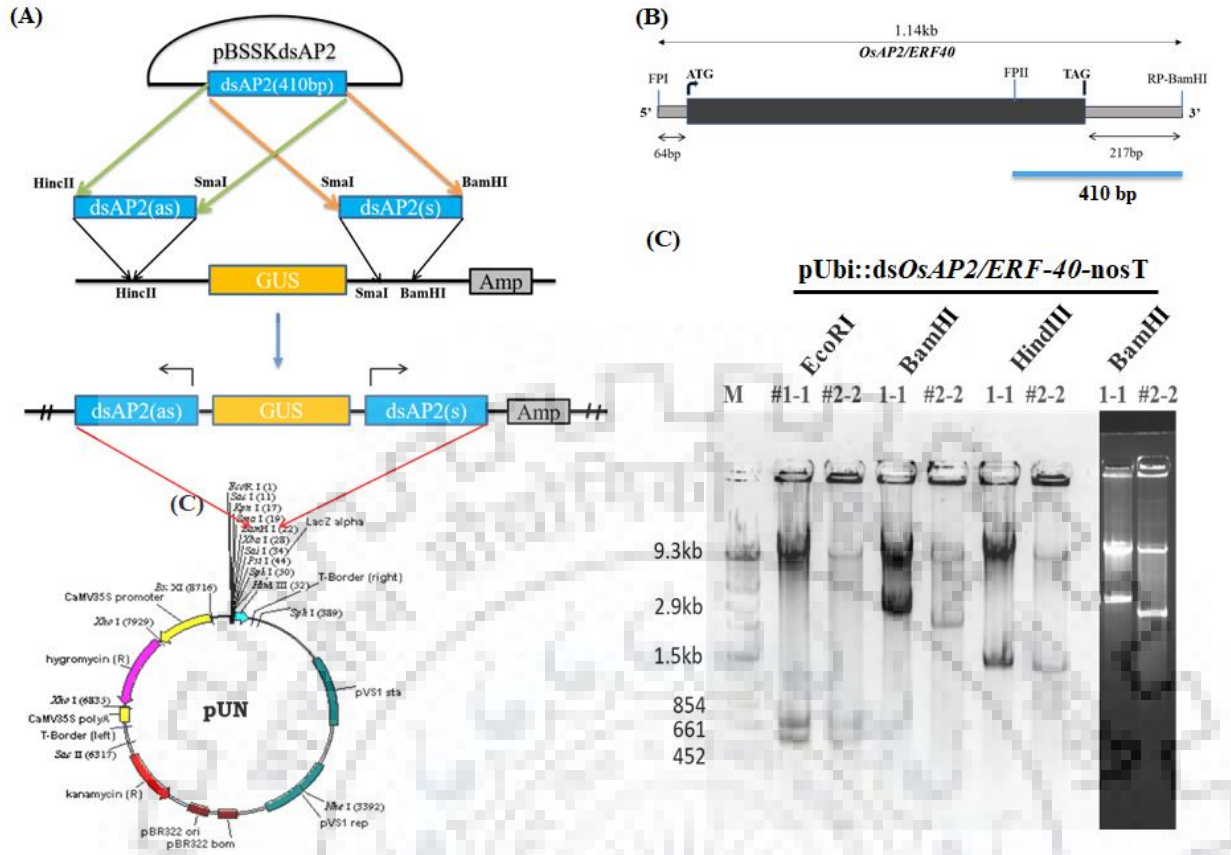

Figure 3:7. Transgene construct to down-regulate the expression of endogenous *OsAP2/ERF-40*. (A) Stepwise assembly of antisense RNA in pBSSk+ vector then to pUN to generate hairpin loop RNAs to silence *OsAP2/ERF-40*. **(B)** Schematic diagram of *OsAP2/ERF-40*cDNA showing 5'UTR, ORF and 3'UTR. 410 bp region indicated by blue line below the cDNA were targeted for RNA interference in the pUbi::ds*OsAP2/ERF-40*-nosT construct. (**C**) Validation of two clones with pUbi::ds*OsAP2/ERF-40*-nosT construct using several RE. Clone #1-1 has antisense sequence-linker-sense sequence whereas clone #2-2 has sense-linker-antisense and differentiated by *Bam*HI restriction digestion.

expression for the same genes and found down regulation of auxin biosynthetic gene *OsYUCCA6* suggesting that they might be the indirect targets of *OsAP2/ERF-40.* Other auxin responsive genes such as *OsIAA1* and *OsMGH3* were unaltered in their expression (Figure 3:10 A). Thus, these data together suggest that *OsAP2/ERF-40* regulates several steps of auxin signalling pathway (e.g. auxin biosynthesis and its responses) in a dose-dependent manner.

In rice, physical and regulatory interactions among *OsWOX11*, *OsERF3* and *OsRR2* ensure crown root initiation and elongation (Zhao et al., 2015, 2009b). *OsERF3* induces expression of *OsRR2* during crown root initiation whereas represses *OsRR2* expression together with *OsWOX11* at later stage of CR elongation (Zhao et al., 2015). We, therefore, studied regulatory interaction of *OsAP2/ERF-40* with OsERF3-OsWOX11-OsRR2 pathways. We analysed expression levels of *OsERF3*, *OsWOX11* and *OsRR2* in shoot tissues of a weak and two strong *OsAP2/ERF-40* over-expression lines by qRT-PCR analysis. We observed induced expression of *OsERF3* in all three lines to a similar level whereas expression of *OsWOX11* was induced to at lower extent in the weak transgenic lines whereas its expression was strongly induced in strong lines (Figure 3:10 B). On the other hand, the expression level of *OsRR2* was not affected in the weak lines but was significantly repressed in the strong lines. These observations suggest that a strong induction of *OsWOX11* is needed to repress the expression of *OsRR2* in shoot tissues and *OsAP2/ERF-40* is inducing root formation through *OsERF3-OsWOX11-OsRR2* pathway.

Our previous analysis indicates that auxin signaling pathway and *OsERF3-OsWOX11-OsRR2* pathway is affected in over-expression line, we therefore analysed expression level of these genes in RNAi line. This was estimated by qRT PCT analysis of RNA isolated from 1cm stem base region of ds*OsAP2/ERF-40* L#1 and wild type plants. The relative expression of *OsAP2/ERF-40* transcripts were decreased in the stem base of transgenic line harbouring the ds*OsAP2/ERF-40* construct compared to the stem base of similar staged wild type plants. We observed that expression of *OsYUC6* is marginally down regulated in the RNAi line suggesting a marginal effect on the auxin signaling pathway. However, we observed expression of *OsERF3* and *OsWOX11* was reduced in the down-regulation line (Figure 3:10 B), suggesting that *OsAP2/ERF-40* is a positive regulator of *OsERF3* and *OsWOX11* during crown root development. Importantly, these effects are opposite to the effects seen in the over-expression line, confirming the regulatory relationship.

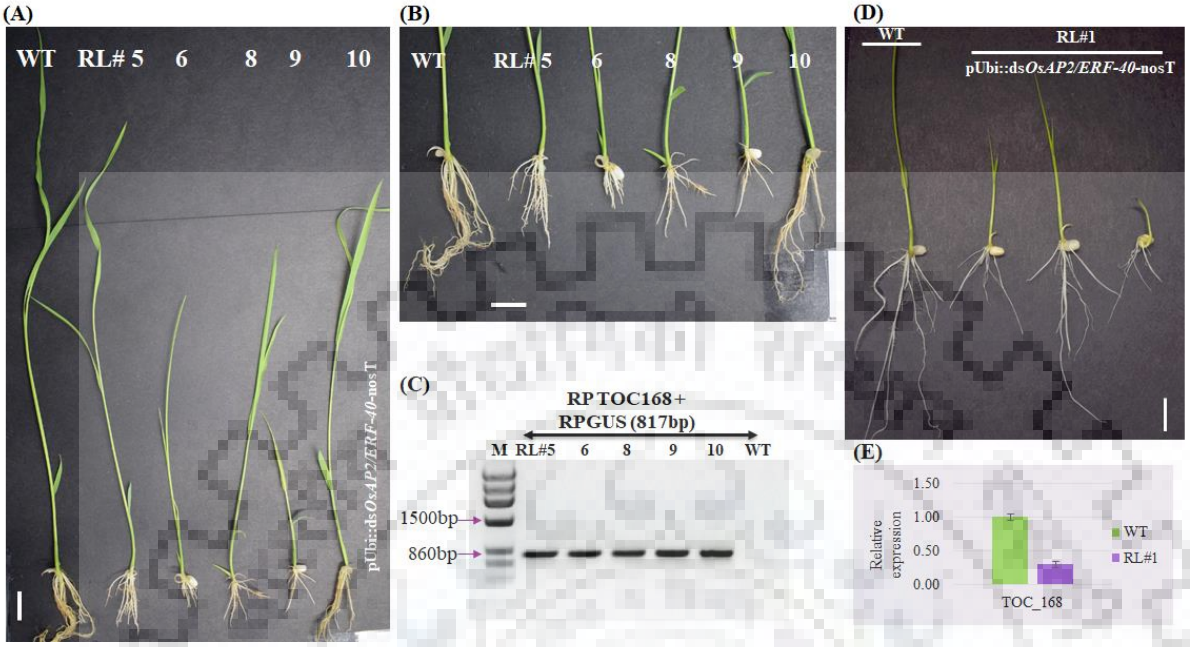

Figure 3:8. Phenotypic consequences of pUbi::ds *OsAP2/ERF-40* transgenic plants. (A- B) Five transgenic RNAi lines (RL#5, 6, 8, 9, 10) grown on ½ MS media containing hygromycin for phenotyping as well as genotyping, lines with retarded root growth. **(C)** Genotyping of the RNAi lines through PCR showing integration of transgene. **(D)** Another independent transgenic line RL#1 grown on plate showing 3:1 Mendelian segregation. **(E)** qRT analysis of RL#1 show reduction of *OsAP2/ERF-40* transcripts compared to WT. Scale bars:1cm (A, B, D).

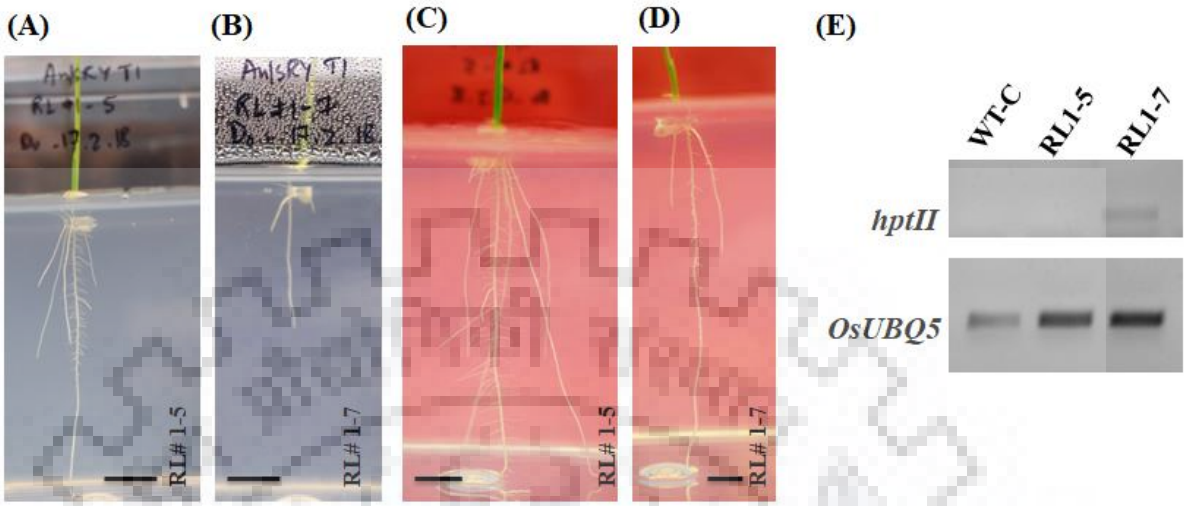

Figure 3:9. Phenotyping with respect to root growth of selected RNAi line. (A, B) Seven-day old RL#1 (B) show lower PR length and no visible LR, compared to the WT (A) from the segregating population. **(C, D)** 10 days of germination the length of PR could reach comparable length of the WT, but lateral roots were smaller and still lagging in numbers. **(E)** PCR genotyping of two plants of RL#1-5 and RL1-7 using hygromycin specific primers shows presence of RNAi transgene in RL#1-7 but absent in both RL#1-5 and wild type negative control. Scale bars: 1cm (A- D).

3.4 DISCUSSION

The regulatory networks operating during rice CR specification and differentiation are largely conserved but some species-specific divergence also exists (Coudert et al., 2010; Orman-Ligeza et al., 2013). Modulation of auxin levels or mutation in the components or upstream regulators of auxin signaling pathway (e.g. *OsYUCCA1, OsIAA23, OsGH3.8, OsCRL6, OsCRL4/OsGNOM1 or OsCAND1*) displays defects in CR patterning and development (Yamamoto et al. 2007, Ding et al. 2008, Kitomi et al. 2008, Liu et al. 2009, Jun et al., 2011, Yadav et al. 2011; Wang et al. 2011, Wang et al. 2016; Zhang et al., 2018). These together suggest that proper auxin biosynthesis, distribution, activation and signaling is essentially required for CR primordia initiation and emergence. Several short root mutants of rice, the monocot model plant, have been isolated and genetically characterized (Yao et al. 2002; Jiang et al. 2005; Li et al. 2006). Apart from auxin, cytokinin also plays crucial role in root development. In Arabidopsis, cytokinin suppresses LR initiation directly by acting at LR founder cells (Laplaze et al., 2007). Alteration in cytokinin metabolism also affects CR development in rice. For example, in a dominant mutant root enhancer1 (ren1-D) of *OsCKX4* gene, CR numbers are increased (Gao et al. 2014). Cytokinin-regulated genes have been recently identified in shoots and roots of rice (Raines et. al. 2016).

Individual and a cross-talk between auxin and cytokinin signalling pathways also regulate expression of several TFs such as *OsCRL1, OsCRL5, OsWOX11, OsQHB* and *OsERF3* during CR development (Kamiya et al. 2003a, Kitomi et al. 2011a, Inukai et al. 2005, Zhao et al. 2009, 2015, Orman-Ligeza et al. 2013, Mai et al. 2014). The expression of rice *OsCRL1* is positively regulated by ARF protein (Inukai et al., 2005). In Arabidopsis, ARF7 and 19 regulate expression of OsARL1/OsCRL1 related genes, LBD16/ASL18 and LBD29/ASL16 during LR development (Okushima et al. 2007). Some of these TFs also integrate auxin and cytokinin signaling pathways. The expression of *OsWOX11* and *OsERF3* is induced by both, auxin and cytokinin that in turn regulates expression of several auxin and cytokinin responsive genes (Zhao et al. 2009, 2015). *OsCRL5, OsWOX11* and *OsERF3* activate expression of type-A cytokinin response regulators which are negative regulators of cytokinin pathway and over-expression of *OsRR1* partially complements *crl5* mutants (Kitomi et al. 2011a, Zhao et al. 2009, 2015).

Most of the CR regulators act downstream of auxin signaling pathway. A LOB-domain TF, *OsCRL1* is essential for auxin-mediated CR development as *crl1* mutants do not develop CR, display decreased LRs and impaired root gravitropism (Inukai et al. 2005). Also, a member of

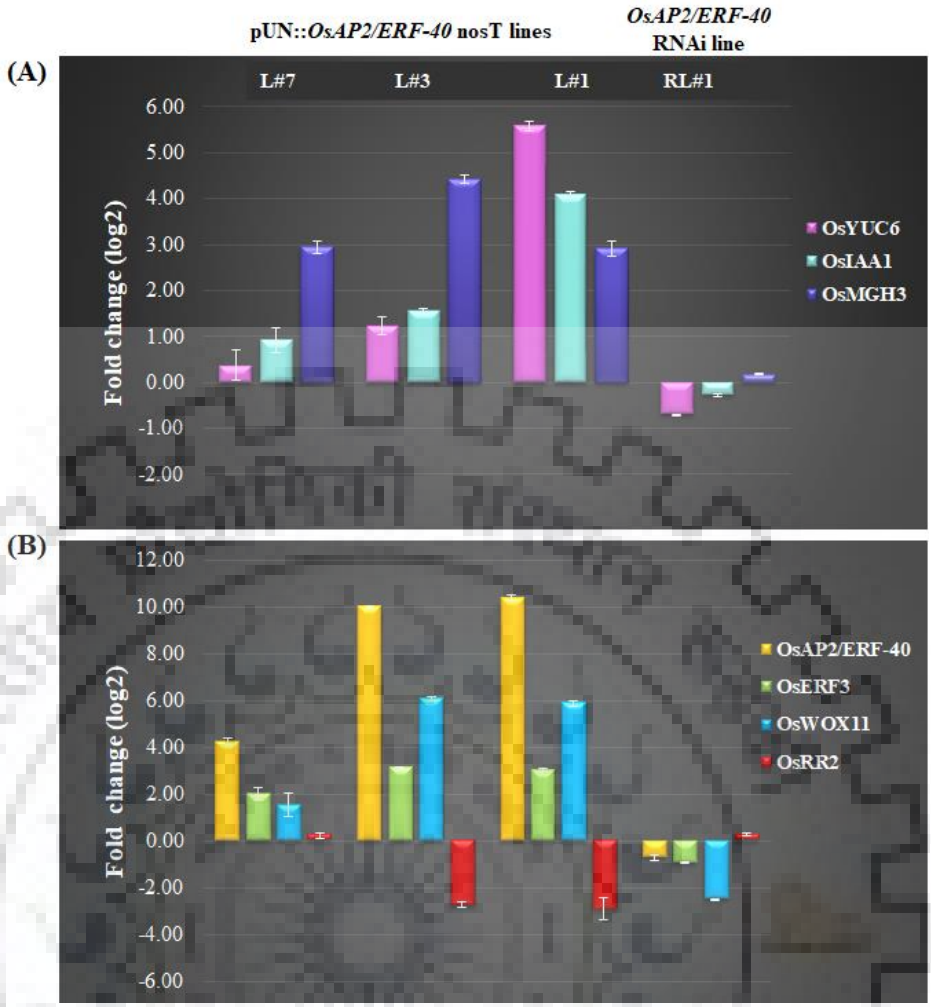

Figure 3:10. Regulatory relationship of *OsAP2/ERF-40* with auxin and *ERF3-WOX11-RR2* pathways. (A) Real-time qRT-PCR analysis of expression levels of *OsYUCCA6*, *OsIAA1* and *OsGH3.8* in shoot tissues of a weak (L#7) and two strong over-expression lines (L#3 and L#1) and stem base of a RNAi line, RL#1. (B) Quantitative expression levels of *OsAP2/ERF-40, OsWOX11*, *OsERF3* and *OsRR2* in shoot tissues of over-expression lines (L#7, L#3 and L#1) and RNAi line, RL#1. *OsUBQ5* was used as an internal reference gene. Three technical replicates were measured to calculate log2 fold change ±SD by normalizing with vector control lines.

AP2/ERF TF family, *OsCRL5* when mutated, develops fewer CRs (Kitomi et al. 2011a). Additive phenotypes of *crl1 crl5* double mutant revealed that they function in different genetic pathways during CR initiation, suggesting roles of auxin in multiple genetic pathways in CRs (Kitomi et al. 2011a). Furthermore, WUSCHEL-related Homeobox TF, *OsWOX11* is necessary and sufficient to activate crown root-specific developmental program (Zhao et al. 2009). Loss-of-function *wox11* mutant or *OsWOX11* down-regulated rice lines develop fewer CRs with decreased root length whereas over-expression of *OsWOX11* promotes ectopic root formation with increased root biomass in rice (Zhao et al. 2009). Another AP2/ERF gene, *OsERF3* also regulates CR development and functions in cooperation with *OsWOX11* (Zhao et al. 2015). Similar to *OsWOX11*, CR number is reduced in *OsERF3* down regulated and increased in *OsERF3* over-expression transgenic rice lines (Zhao et al. 2015). Our study provides function of yet another AP2/ERF gene family, *OsAP2/ERF-40* in promoting crown root development in rice. We show that *OsAP2/ERF-40* acts downstream of auxin signaling pathway in crown tissues and is specifically expressed in developing CR primordia, implicating its role during CR development. Transgenic rice lines with down regulated *OsAP2/ERF-40* expression level, showed defects in adventitious root development with a lesser number of crown roots and decreased root growth, suggesting that it is necessary for crown root development and growth. Complementarily, ectopic over expression of *OsAP2/ERF-40* was sufficient to trigger the emergence of adventitious root from aerial nodes. The aerial adventitious roots in *OsAP2/ERF-40* over-expression lines are fully developed and functional, indicating that *OsAP2/ERF-40* could regulate proper developmental program during outgrowth of adventitious roots. These data together demonstrate that *OsAP2/ERF-40* is a positive regulator of crown root development in rice.

Our mechanistic analysis shows that *OsAP2/ERF-40* not only acts downstream of auxin signaling but can also regulate expression of the regulator of auxin signalling (*OsCRL4*/*OsGNOM1*), auxin biosynthetic genes (*OsYUCCA1* and *OsYUCCA6*) and auxin responsive genes (*OsIAA1* and *OsGH3*.8) suggesting a possible regulatory feedback loop between auxin signaling and *OsAP2/ERF-40* activity. Our studies on pharmacological inhibition of polar auxin transport in *OsAP2/ERF-40* over-expressing rice plants further revealed a synergistic interaction between *OsAP2/ERF-40* and polar auxin transport machinery. Taken together our studies provide compelling evidence for multi-layered intricate regulatory interactions between crown root-specific transcription factor *OsAP2/ERF-40* and auxin signaling which is instrumental to shape up the architecture of CR development in rice.

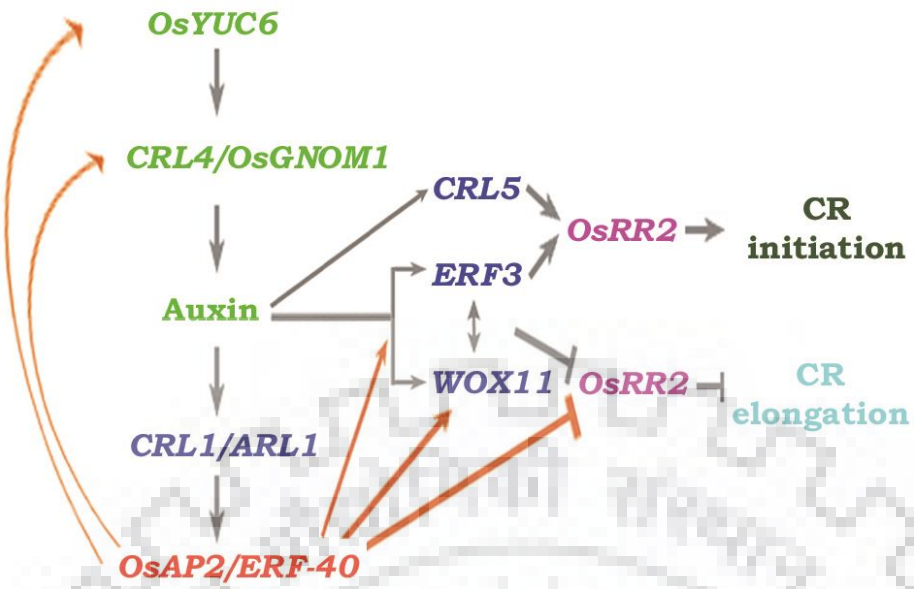

Figure 3:11. Schematic diagram demonstrating plausible regulatory interactions of *OsAP2/ERF-40* with other regulators of crown root development. The interactions highlighted by dashed arrows in saffron colour is from this study.

Interestingly, *OsCRL5* and *OsWOX11* functions at different developmental stages through type-A cytokinin response regulators. Activation of *OsRR2* expression by *OsCRL5* during CR initiation and repression of the same regulator by *OsWOX11* during CR emergence and growth suggests stage-specific differential effects of cytokinin signalling pathway in CRs (Kitomi et al. 2011a, Zhao et al. 2009). *OsERF3* is essential for CR development and functions differentially during CR primordia initiation and elongation through cytokinin signaling. It directly induces expression of *OsRR2* during primordia initiation, whereas during primordia elongation, *OsERF3* interacts with *OsWOX11* and represses expression of *OsRR2* (Zhao et al. 2015). Our expression analysis of *OsERF3*, *OsWOX11* and *OsRR2* in *OsAP2/ERF-40* over-expression and down-regulated lines suggests a possible mechanism wherein *OsAP2/ERF-40* mediated induction of *OsERF3-OsWOX11-OsRR2* regulatory module could contribute to primordia elongation and emergence (Figure 3:11).

CHAPTER 4

ROLE OF AUXIN-INDEPENDENT TRANSCRIPTION FACTOR, *OSAP2/PLT3* IN RICE

4.1 INTRODUCTION

Indeterminate growth is a characteristic feature of higher plants which occurs due to activities of pluripotent plant stem cells located in a specified domain, called meristem. Post-embryonic development along the longitudinal axis acquires cells from stem cell niche (SCN) positioned in the shoot apical meristem (SAM) and the root apical meristem (RAM), respectively. Apart from these primary meristems, plants also develop some secondary meristems such as the cambium and branch meristems for radial and lateral growth and development. In shoot, the stem cells are located in the central zone (CZ) of the SAM and instruction to maintain pluripotency is received from underlying organizing center (OC). Once stem cells divide, their daughter cells are displaced in the peripheral zone (PZ) of the SAM (Gaillochet and Lohmann, 2015). Higher cell division rate in the PZ continuously produced cells that enter in the process of cell differentiation during lateral organ development. The RAM is placed at the tip of the roots and is source for root tissue development. The root organizing center is composed of few mitotically less-active cells, also called as the quiescent center (QC). Unlike the SAM, the QCs are surrounded by stem cells (also known as 'initials'). After division of the initial cells, the daughter cell which remains in the direct proximity of the QC, retains stem cell property whereas the distal cell acquires new cell identity depending on its relative position in the meristem.

In model dicot plant, *Arabidopsis thaliana,* organization and maintenance of SAM and RAM have been extensively studied and the underlying mechanism is revealed. Strikingly, a largely similar mechanism is involved in the regulation of stem cell activities and the key regulator has been shown to be functionally interchangeable between SAM and RAM (Sarkar et al., 2007). In the SAM, homeodomain transcription factor *WUSCHEL* (*WUS*) and small signaling peptide *CLAVATA3* (*CLV3*) based regulatory module is required for the stem cell activity and similar *WOX/CLE* module involving *WUSCHEL-RELATED HOMEOBOX 5* (*WOX5*) and *CLAVATA3/ESR-RELATED 40* (*CLE40*) peptide controls RAM functions. Phytohormones such as auxin and cytokinin regulate stem cell activities and integrate local transcriptional networks and SAM and RAM. Other key regulators of RAM and SAM activities in plants are members of PLETHORA (PLT) family, encoding AP2-domain transcription factors (Galinha et al., 2007).

Expression of PLT/AINTEGUMENTA-LIKE (AIL) genes, is confined to developing organs such as leaves or lateral roots to control their outgrowth (Hofhuis et al., 2013; Prasad et al., 2011). To date, PLTs/AILs are well characterized transcription factor family which is known to be comprised of a subclade of six members each of them possess two AP2/ERF domains

(APETALA2/ETHYLENE RESPONSE FACTOR) (Aida et al., 2004; Horstman et al., 2014; Mähönen et al., 2014). Four PLT members, *PLT1*, *PLT2*, *PLT3*, and *BBM*/*PLT4*, have their overlapping transcriptional domains in the root meristem. These genes maintain meristematic cell division but preclude cell differentiation at RM redundantly (Galinha et al., 2007). They are also required for the expression of SHORT-ROOT (SHR) and SCARECROW (SCR), the QC markers within the stem cell niche (Aida et al., 2004), for proper radial tissue patterning (Helariutta et al., 2000; Nakajima et al., 2001; Sabatini et al., 2003). Other members of PLETHORA, PLT3, 5 and 7 are expressed in LR founder cells and in the SAM to act redundantly in the positioning of lateral organs (Hofhuis et al., 2013; Prasad et al., 2011). In addition, PLT2, BBM/PLT4, and PLT5 have the remarkable capacity to ectopically initiate the formation of specific organs and somatic embryos (Galinha et al., 2007). *PLT1/AIL3* and *PLT2/AIL4* genes are essential for QC specification and stem cell activity and act in parallel with SCR- and SHR-mediated patterning input to define QC and stem cell position and specification. Exogenous auxin application couldn't rescue reduced root growth of *plt1 plt2* double mutants. Thus, indicating that auxin is not enough to bypass the PLT gene necessity (Aida et al., 2004). The gradient PLT transcription factors activity apart from controlling meristem identity steers proper cell expansion and differentiation. Auxin induces PLT expression (Aida et al., 2004; Mähönen et al., 2014), and in turn PLT regulatory feedbacks aggravate polar auxin transport and auxin biosynthesis genes, increasing auxin levels. Apart from typical phytohormone auxin, the Tyr-sulfated RGF peptide hormones via their receptors induce and stabilize PLTs (Matsuzaki et al., 2010; Ou et al., 2016), while these genes through feedback regulations are targets of PLT2. Triple mutant *plt1plt2plt3* does not develop any roots, and the *plt1plt2plt3bbm* quadruple mutant entirely lacks root and hypocotyls (Galinha et al., 2007). Like, *PLT2* and *PLT4* have the intriguing capability to establish pluripotency in different developmental frameworks (Kareem et al., 2015b). Many such genes which plays active part in organ regeneration as ARF5/MP (Chang et al., 2013; Hamann et al., 2002; Su et al., 2011), SHR (Du and Scheres, 2017; Möller et al., 2017), and FEZ (Willemsen et al., 2008) are directly regulated by auxin and *PLT2* (Horstman et al., 2014).

In rice, although different root types origin from diverse tissues, the meristem at the root tip remains crucial during root development. Studies have revealed a central role of PLTs in root growth in *Arabidopsis*, the functions of PLTs in monocot rice remains to be elucidated. Ten PLT family members are identified in rice through BLAST analysis. It could now provide the basis to characterize their physiological functions. In *Arabidopsis*, PLT1, 2, 3 and *AtBBM/PLT4* are predominantly expressed in root. The rice homologs of *AtPLT* share similarity were

designated as OsPLT1 to OsPLT10, located in different chromosomes of rice genome. Most of the members have been interpreted as transcription factor *BABY-BOOM*, AP2-like ethylene-responsive TF *AINTEGUMENTA*, and *PLETHORA2*. *OsPLT* family consists of more members than that of *Arabidopsis* probably because of genome duplication events and it is evident from the chromosomal location of them. Among eight *Arabidopsis* six PLTs, PLT1/2/3, *AtBBM*, *AIL5*, and *AIL7* are grouped with six rice *PLTs* (*OsPLT1* to 6) proteins in Group A. Rest *AtPLTs* (ANT and AIL1) and *OsPLTs* (OsPLT7 to 10) were clustered in Group B. Rice PLT member, *OsPLT8* (also called *OsCRL5*) regulates CR development (Kitomi et al., 2011; Li and Xue, 2011). The expression analysis classifies the PLT family of rice into three types. The root-preferential family (OsPLT1 to 5), stem-preferential family has three members OsPLT7 to 9, and rest two OsPLT6 and OsPLT10 are seed-preferential. OsPLT2 to 5 showed high transcription level in PR, CR and stem base. As we already know that rice RSA is a dense fibrous root system made up of numerous crown roots from stem base and dominant in the root system of all types of cereals. The high expression of many *OsPLTs* indicate that there must be a crucial role of them in root development. A member of AP2/ERF TF family, *OsPLT8/OsCRL5* is already reported to be involved in CR formation as crl5 mutants develop fewer CRs (Kitomi et al., 2011). Additive phenotypes of *crl1 crl5* double mutant revealed that they function in different genetic pathways during CR initiation through auxin mediated response (Kitomi et al., 2011). The presence of rice *PLT* genes in the initial stages of the crown root primordia manifests their important roles in the development of the crown root. We have selected *OsAP2/PLT3* which functions downstream of rice *CRL1* for functional characterization. Using gain-of-function based approach, we show that ectopic over-expression of *OsAP2/PLT3* in transgenic rice causes altered root architecture, stem thickness, leaf blade angle, floral transition and floret fertility.

4.2 MATERIALS AND METHODS

4.2.1 Plasmid Construction

For generating ectopic over-expression construct for *OsAP2/PLT3*, 2.56 kb full-length gene was PCR amplified in two fragments on rice genomic DNA using two sets of overlapping primers. First 1427bp fragment was amplified using primers Os_AP2-AK-O/E-070FP and Os_AP2-AK-O/E-070RP1 and was cloned in pBluescript SK+ at EcoRV site as blunt to generate pBSSk-*OsAP2*/*PLT3* (SetI). Second fragment which was 1289bp was PCR amplified using primers Os_AP2-AK-O/E-070FP1(s) and PLT2-RP and was cloned in pBluescript SK+ at EcoRV site as blunt to generate pBSSk-*OsAP2/PLT3* (SetII). Both setI and setII fragments were sequenced in pBSSk vector backbone. Next, pBSSk-*OsAP2*/*PLT3* (SetI) is digested with NdeI and SmaI to be used as the vector. Second fragment of the gene was taken as NdeI and SmaI RE digestion of pBSSk-*OsAP2/PLT3* (SetII) to assemble the complete 2.56kb cDNA. Assembled clone was designated as pBSSk-O/E *OsAP2/PLT3* #5 which was validated using several RE like EcoRI, PstI, PvuII, BamHI. *OsAP2/ERF-40* was sub-cloned into the BamHI site of plant expression vector pUN under maize *Ubiquitin* promoter to generate pUbi-*OsAP2/PLT3*-nosT (Fig. 4.2 B). Then is validated by restriction digestion pattern by several enzymes (Fig. 4.2 C).

4.2.2 Rice transformation

Over-expression construct was mobilized to *Agrobacterium tumefaciens* LBA4404 and used to raise transgenic rice lines as described by Prasad et. al. (Prasad et al., 2001). Briefly, embryogenic calli of rice japonica var. TP309 was co-cultivated with *Agrobacterium* harbouring the construct. Co-cultivation was done for 2-3 days in dark (at 26°C) on NB6-AS medium supplemented with 100 mM acetosyringone. Transformed calli were selected on NB6-SEL medium containing 50 mg/L hygromycin. Actively proliferating calli were further transferred to NB6-RM media (50 mg/L hygromycin, 3 mg/L BAP and 0.5mg/L NAA) for shoot regeneration. Regenerated shoots were subsequently transferred to rooting media (1/2 MS with 25 mg/L hygromycin, 0.05 mg/L NAA). Hardening of regenerated plantlets was done in soilrite before transferring to clay for completing the life cycle. Since *OsAP2/PLT3* over-expression lines with strong transgene expression did not produce fertile seeds, multiple independent stronger lines were phenotyped in T0 generation for their overall growth and development.

4.2.3 RNA extraction and RT-PCR

Total RNA from leaf tissue of the transgenics were isolated using TRI reagent (SIGMA). 10ug RNA were treated with DNase I (NEB) and precipitated after phenol and chloroform treatment. For expression analysis, through semi quantitative RT, cDNA was synthesized from 1 µg of total RNA using iScript cDNA synthesis kit (Bio-rad) and PCR was performed using Taq-Polymerase (NEB). A total of 10 ng cDNA was used for semi quantitative real-time PCR (qRT-PCR) using 250 nM of gene-specific primers. Actin or *UBQ5* transcript levels were used as endogenous normalization control. A list of primer sequences is provided as Appendix G.

4.2.4 Plant Growth and Microtome Sectioning for Microscopy

Seeds of (Oryza sativa var TP309) wild-type and were de-husked, surface sterilized and germinated on ½ MS media with 1% sucrose and 0.3% phytagel (Sigma) and grown vertically in sterile square petri dishes (Himedia; 12 cm × 12 cm), at 26 °C in 16/8-hour light/dark period for 6 days. For histological and RNA in situ hybridization analysis, approximately 2mm rice stem base region was dissected from each wild-type and transgenic plants and fixed in FAA (10% formaldehyde, 5% glacial acetic acid and 50% ethanol). Ethanol series was performed to dehydrate the tissues and finally embedded in paraffin (SIGMA) blocks. After solidification and reshaping, materials were cut into 6-8 µm thin sections using microtome (Thermo Scientific) and sections were taken on poly-L-lysine coated glass slides.

4.2.5 RNA-RNA *in-situ* hybridization

For preparing DIG-UTP-labelled 380 bp anti-sense probe for *OsAP2/PLT3* was generated using NruI linearized pBluescript SK+ clones transcribed with T7 RNA Polymerase (Sigma). Hybridization was performed on cross sections as described by Prasad et al, (Prasad et al., 2005). The signal was developed using alkaline phosphatase conjugated anti-DIG antibodies (Sigma) and NBT/BCIP substrate (Sigma). Sections were mounted in Entellan (Merck-Millipore).

4.3 RESULTS

4.3.1 Identification of auxin-independent transcription factor in rice crown tissue

Auxin has been shown as a key regulator of various stages of rice crown root development starting from primordia initiation, primordia emergence and growth of the emerged roots. This is also supported by the fact that the expression of a large number of key regulators of crown root development is regulated by auxin signaling, directly or indirectly. We therefore analyzed our RNA sequencing data obtained upon auxin and cytokinin treated rice crown tissues to identify a putative transcription factor with putative functions during rice crown root development. We observed that member of a rice AP2-domain containing PLETHORA gene family, *OsPLT3* (renamed as *OsAP2/PLT3*) has an auxin independent expression in the crown tissues as its expression is not significantly altered upon auxin or cytokinin treatment as compared to mock treatment (Figure 4:1 A). Next, we validated its expression upon hormonal treatments by reverse transcription. Our qRT-PCR analysis using *OsAP2/PLT3* specific primer sets confirms that similar to RNA sequencing data, the expression of *OsAP2/PLT3* is not significantly changed in the rice stem base upon treatment with IAA or BAP (Figure 4:1 B). Rice *DRO1* was used as control to ensure that auxin treatment was robust and we observed that expression of *DRO1* was reduced upon auxin treatment, consistent with the previous report (Figure 4:1 B) (Uga et al., 2013).

In *Arabidopsis PLETHORA* (*PLT*) genes encoding double AP2-domains, are key developmental regulators of plant regeneration, embryogenesis, root meristem establishment and maintenance, shoot apical meristem function, floral organ patterning and lateral root outgrowth in *Arabidopsis* (Aida et al., 2004; Du and Scheres, 2017; Galinha et al., 2007; Hofhuis et al., 2013; Horstman et al., 2014; Kareem et al., 2015a; Mudunkothge and Krizek, 2012). *Arabidopsis PLT* genes are regulated by auxin and function largely in genetically redundant manner (Krizek, 2011; Mähönen et al., 2014; Pinon et al., 2013; Santuari et al., 2016). Importantly, *OsPLT8* (also called *CRL5*), a member of rice *PLT* gene family, regulates crown root development (Kitomi et al., 2011; Li and Xue, 2011).The expression of *OsAP2/PLT3* gene is not affected by auxin treatment in rice seedling as well and it is strongly expressed in the crown root primordia (Li and Xue, 2011). The phylogenetic analysis of rice PLT genes divides them into group A and B. *OsAP2/PLT3* and *OsPLT4* seems paralogous genes that might be a result of chromosomal duplication between chromosome 2 and 4 and are clustered with two other rice members (OsPLT2 and OsPLT5) in the same clade with a single

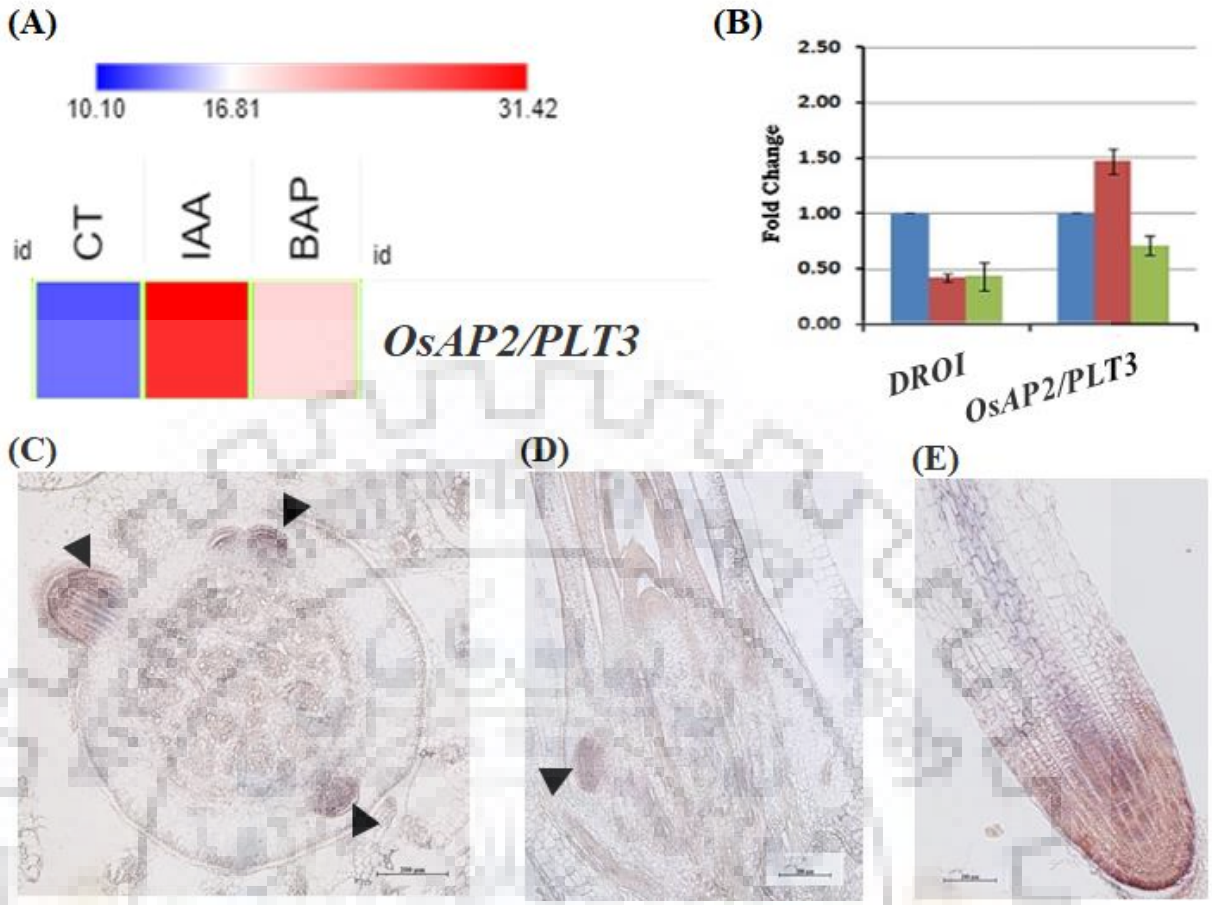

Figure 4:1. Effect of auxin treatment on the expression level of *OsAP2/PLT3* gene. (A) Heat-map for *OsAP2/PLT3* upon IAA and BAP treatment as compared to mock treatment (log2 fold change ≥1.0). **(B)** Auxin insensitive expression of *OsAP2/PLT3*, real-time quantitative RT-PCR (qRT-PCR). *DRO1* was control for auxin treatment. **(C, E)** Tissue-specific expression pattern of *OsAP2/PLT3* during CR development. Expression is seen in developing crown root primordia in transverse section (C) and longitudinal section (D), the root tip (E), in the elongation zone of the extruded root, the expression is visible in the central vascular tissues (E). (C- E) Scale bar: 20 uM

Arabidopsis member, *AIL5/PLT5* of Group A (Li and Xue, 2011). Moreover, *OsAP2/PLT3* is a putative downstream target of an important regulator of crown root development, *OsCRL1* (Coudert et al., 2011). Taken together all these observations, we selected *OsAP2/PLT3* for detailed expression analysis and functional study in rice.

4.3.2 Epression analysis of *OsAP2/PLT3* during crown root development in rice

OsAP2/PLT3 was first analysed *in silico* using RiceXPro (http://ricexpro.dna.affrc.go.jp) and RiceSRTFDB (http://www.nipgr.res.in/RiceSRTFDB.html) online databases and is identified as double AP2-domain containing transcription factor (Rashid et al., 2012). RiceXPro suggest that *OsAP2/PLT3* (also called *BBM2*, LOC_Os02g40070) has higher expression in the roots as compared to the shoot tissues during vegetative stage of the plant. OsAP2/PLT3(BBM2) is also reported to strongly expressed in the embryo along with *BBM1* and *BBM3* (Khanday et al., 2019).

Next, we analysed detailed spatial expression of *OsAP2/PLT3* in the developing rice crown root primordia. For this purpose, we collected stem base and emerged crown root from 6-day old rice plants and processed for RNA *in situ* hybridization. DIG-labelled anti-sense RNA probes against gene-specific region of *OsAP2/PLT3* were hybridized on 8 µm cross sections of wild-type rice stem base containing CR primordia at different stages and whole mount of emerged crown root. Anti-sense RNA probe was designed from the 380 bp region towards the 3' UTR of the mRNA out of the two conserved AP2 domain. We observed strong expression of *OsAP2/PLT* in developing crown root primordia (Figure 4:1 C & D). In the growing crown roots after primordia emergence, the expression is largely restricted to the root tip, particularly in the meristematic region and stem cell niche (Figure 4:1 E). However, in the elongation zone of the growing root, the expression is mostly observed in the central vascular tissues (Figure 4:1 E). This spatial expression pattern further suggest that *OsAP2/PLT3* could be a putative regulator of rice crown root development. After identification of key genetic regulator(s) expressing in crown root development, next step will be to functionally characterize them to understand their specific roles during crown root development in rice. From this study we have identified crown-root expressed auxin non-responsive gene *OsAP2/PLT3* for functional characterization.

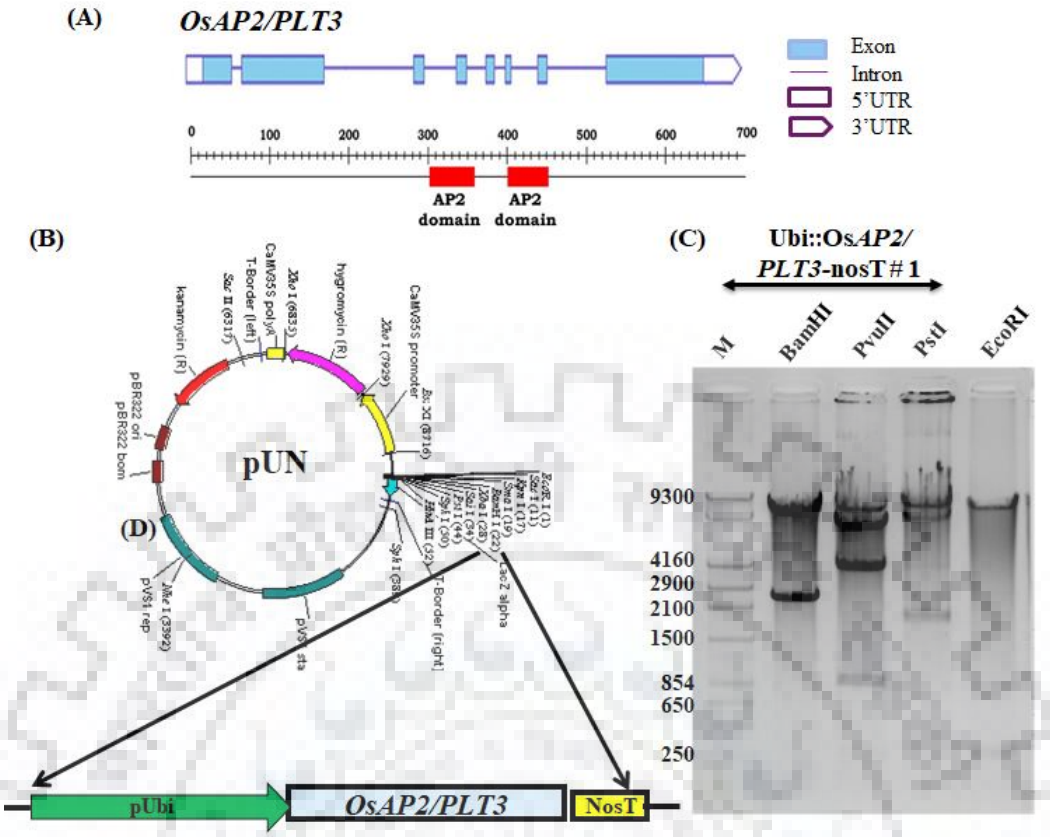

Figure 4:2. Generation of constructs for ectopic over-expression of *OsAP2/PLT3*. (A) Gene map for *OsAP2/PLT3* having two AP2 domains extended from 116 to 176 on the protein map below the c-DNA map. **(B)** Schematic representation of *OsAP2/PLT3* over-expression construct, full length*OsAP2/PLT3*cDNA cloned under the control of maize ubiquitin promoter and nos-terminator. **(C)** Agarose gel (0.8%) image showing validation of final over-expression clone in pUN vector, pUbi-*OsAP2/PLT3*-nosT#1 with several RE.

4.3.3 Gain-of-function study of *OsAP2/PLT3* in transgenic rice

Similar to *Arabidopsis,* genetic redundancy is also evident in the rice *PLT* gene family. Recent study has shown that double mutants of *bbm1 bbm3 and bbm2 bbm3* produced fertile plants (Khanday et al., 2019). Similarly, when rice *OsPLT3/BBM2* along with *OsPLT5/BBM3* were knocked out, there was no visible phenotype in the plants (Khanday et al., 2019). Strong genetic redundancy forced one to use mis-expression analysis for such genes as over-expression of *BBM1* produced ectopic somatic embryos from the leaves, indicating its role in rice somatic embryogenesis (Khanday et al., 2019). To bypass the problem of higher degree of genetic redundancy among the members of PLT gene family, we also took mis-expression based approach to shed light on the role of *OsAP2/PLT3* in crown root development.

We have generated a construct to study the consequence of its over-expression in ectopic tissues. For that purpose, a 2565bp full- length cDNA of *OsAP2/PLT3* was PCR amplified using cDNA made from crown tissues and the PCR fragment was cloned in a plant specific expression vector, pUN (Figure 4:2 B) (Prasad et al., 2001) to generate Ubi::*OsAP2/PLT3*-nosT. Sense orientation cDNA was validated through several restriction digestions (Figure 4:2 C). The construct has been mobilized to the LBA4404 strain of *Agrobacterium.* Rice embryogenic calli was co-cultivated with the *Agrobacterium* containing over-expression. We generated 11 independent transgenic rice lines. We collected leaf tissues from few of those lines for further molecular characterization. Semiquantitative RT-PCR analysis show over-expression of the transgene in Ubi::*OsAP2/PLT3* lines L# 1, L#4, L#5 (Figure 4:4 A). We also performed real-time PCR on the same lines to find almost 5 to 7-fold accumulation of *OsAP2/PLT3* transcripts in the over expression lines with L#1 being the highest and shows slightly more than seven-fold over expression (Figure 4:4 B).

4.3.4 Vegetative phenotypes due to over-expression of *OsAP2/PLT3* in rice

Phenotypic analysis of transgenic rice ectopically over-expressing *OsAP2/PLT* shows multiple effects during plant growth and development of rice plants. We observed that almost all these lines with higher expression of *OsAP2/PLT* had robust shoot organs without a major change in the overall morphology during early vegetative growth phase (Fig 4.3 A- D). However, during later stage of vegetative development, the stem was thicker, and base was enlarged in diameter in transgenic lines (Figure 4:3 C, D) as compared to the wild type (Figure 4:3 A, B). Constitutive overexpression of *ANT*, results in larger flowers than normal with larger organs

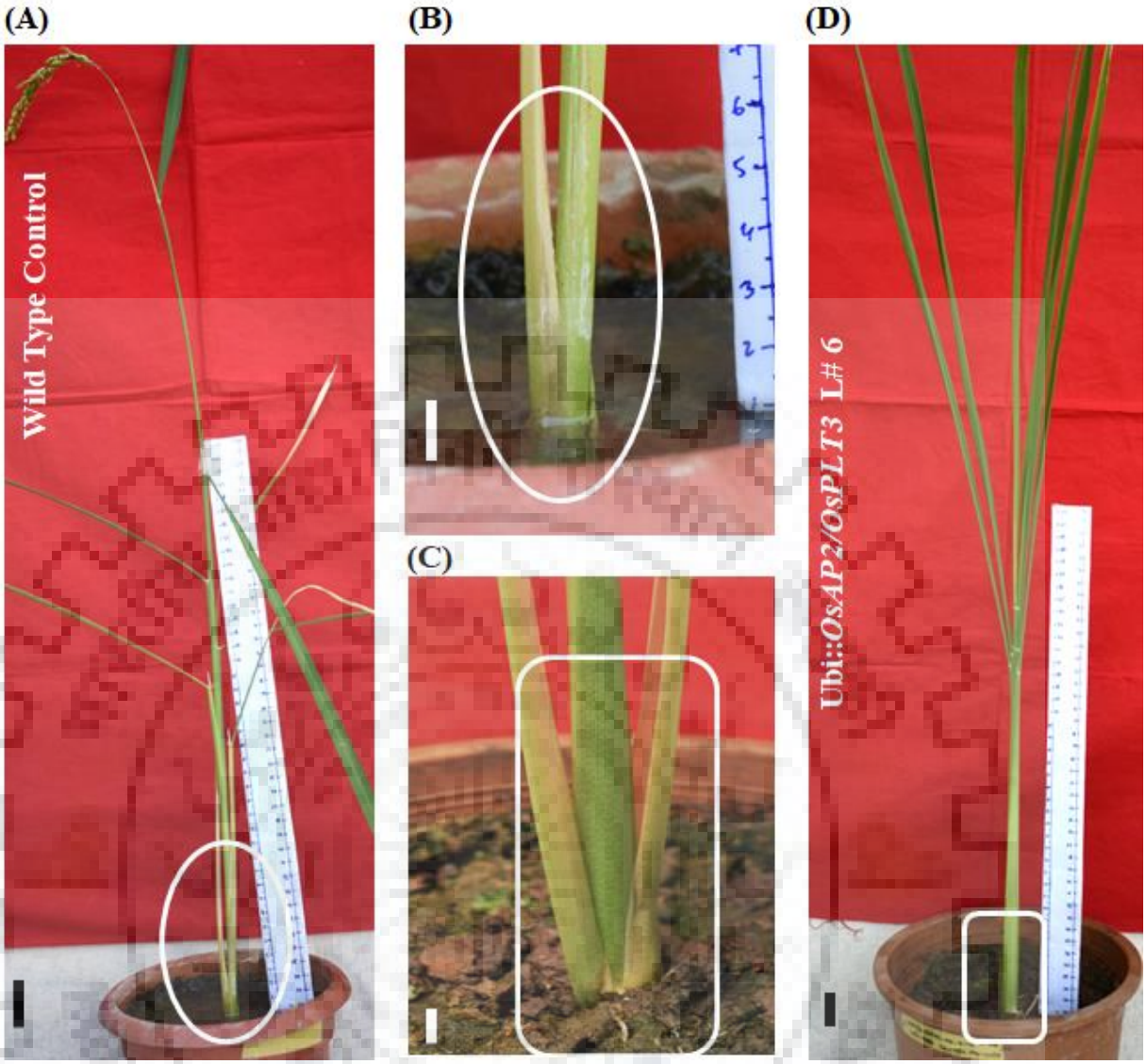

Figure 4:3. Vegetative phenotypes due to over-expression of *OsAP2/PLT3* in rice. (A- D) *OsAP2/PLT3*overexpressing rice plant (D) during early vegetative growth phase displays robust shoot organs compared to wild type control (A); increased stem girth of the transgenic is enlarged and highlighted in rectangular box (C, D), compared with wild type stem highlighted in oval shape (A, B). Scale bar- (A, D): 3cm, (B, C): 1cm.

with no change in either meristem size or cell cycle activity, but larger cells which proves ANT activity in cell size regulation (Krizek, 1999). *OsAP2/PLT3* over expressers show similar phenotype suggests it may regulate genes involved in cell division and/or expansion. Regulation of *ANT* and cell cycle gene *CYCD3* during lateral organ growth of Arabidopsis (Randall et al., 2015) and secondary thickening of poplar stems (Immanen et al., 2016), represents a species specific conserved regulatory mechanism of PLETHORAs in angiosperms. Although *OsAP2/PLT3* doesn't belong to *ANT* group of TFs but it is placed in the same clade with *Arabidopsis AIL5* and might regulate cell ANT like function in rice. The redundant role of many *PLT* and *ANT/AIL* genes (Elliott et al., 1996; Galinha et al., 2007) might reflect an ancestral function of the ANT clade in plant growth processes (Prasad et al., 2011). In addition, *OsAP2/PLT3* over expression lines seems to form a greater number of crown roots (Figure 4:5 A- C). This suggests that *OsAP2/PLT* might activate more cell proliferation in a radial manner that results in secondary growth-related phenotypes in the stem. In *Arabidopsis ANT* is reported to regulate cell division phase and is both necessary and sufficient for growth of the lateral organs (Scheres and Krizek, 2018). Though ectopic over-expression of *PLT2* in *Arabidopsis* induces ectopic root formation, we did not observe any ectopic root formation in rice (Figure 4:5 A- C). On one hand the combined action of *PLT3*, *PLT5*, and *PLT7* ensures proper LRP placement and promotes their emergence but else ways the same PLTHORAs define shoot organ spacing hints at the resemblances in the mechanisms operating to shape the whole plant architecture (Hofhuis et al., 2013; Prasad et al., 2011). Both *BBM* and *ANT*, shows sequence similarity in their DNA binding domains, and promote cell proliferation as well when ubiquitously overexpressed (Boutilier et al., 2002). The effect of *OsAP2/PLT3* on overall plant growth tempts one speculate the presence of such a mechanism in monocots as well.

4.3.5 Over-expression of *OsAP2/PLT3* alters leaf angle and floral transition

Apart from the stem thickness and root phenotypes, we also observed a defect in the leaf angle in the *OsAP2/PLT3* over-expression lines. The leaf blade is attached at the node by the leaf sheath, which encircles the stem. At the junction of leaf blade and sheath a pair of claw-like appendages are present, called the auricles. The leaves of all transgenics were oddly upright in nature with decreased angle between the leaf sheath and the leaf blade. Comparing the leaf angles, all wild-type leaves had angles of over 30° (Figure 4:6 A), whereas all *OsAP2/PLT3* leaves had angles much less than 30° (Figure 4:6 B- D). The pattern of leaf initiation and arrangement at the shoot axis is termed phyllotaxis which displays symmetries. *PLT3, PLT5*, and *PLT7* triple mutants show changes in phyllotaxis and largely attributable to PIN1 mis-

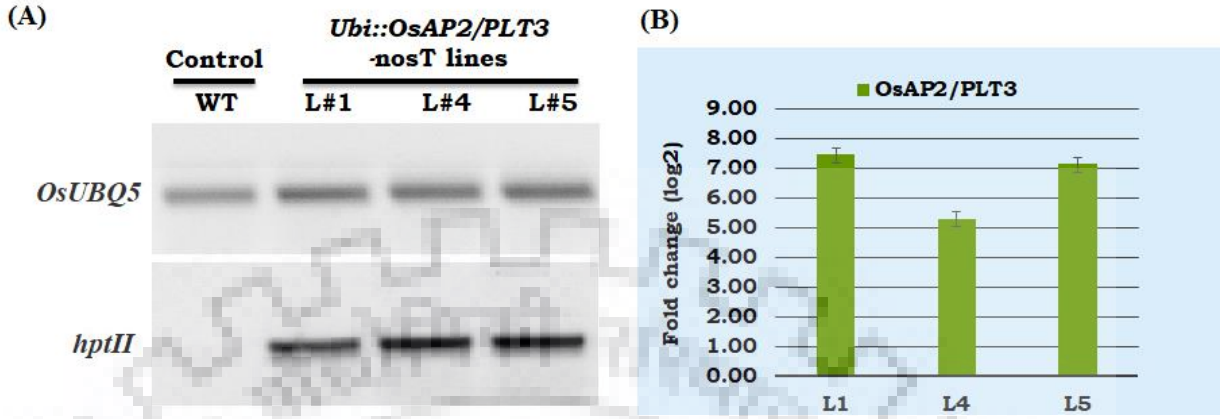

Figure 4:4. Molecular characterization of few independent transgenic lines. (A) Semiquantitative RT-PCR analysis show over-expression of the transgene in Ubi::*OsAP2/PLT3* lines L# 1, L#4, L#5. **(B)** qRT PCR on the same lines shows transcript accumulation of 7.5-fold in L#1, 5.3-fold in L#4 and 7.1-fold in L#5. Three technical replicates were measured to calculate log2 fold change ±SD by normalizing with vector control lines.

regulation. Thus, control of phyllotaxis is regulated by changes auxin transport though the PLT proteins (Prasad et al., 2011). An angular leaf arrangement is advantageous because the surface area exposed to the light is broadened. *OsAP2/PLT3* like *AtPLT3* may act to maintain the phyllotaxis which is necessary for rice to acquire the maximum light energy possible for photosynthesis.

At the reproductive stage, SAMs begin to extend and transition into inflorescence meristems. We observed strong delay in the flowering in all these *OsAP2/PLT3* over-expression lines. Rice inflorescence (also called Panicle) is established after vegetative to reproductive phase transition which later on emerged out from the leaf sheath in 2-3-months-old wild-type plants (Figure 4:7 A). The panicle did not emerge in these over-expression lines even in 8-10 months, suggesting a strong suppression of panicle emergence upon over-expression of *OsAP2/PLT3*. In few lines we observed panicle initiation, but it did not emerge out from leaf sheath. Upon dissecting the leaf sheath, we could observe the inflorescence in which the spikelets were deformed but did not produced fertile seeds, suggesting defect in the floral fertility (Figure 4:7 B- D). The mis expression-based approach helped us shed some lights on function of *OsAP2/PLT3* in overall plant growth and development. *OsAP2/PLT3* has been shown to play major task in vegetative development and might play critical direct or indirect role in transition to flowering in rice.

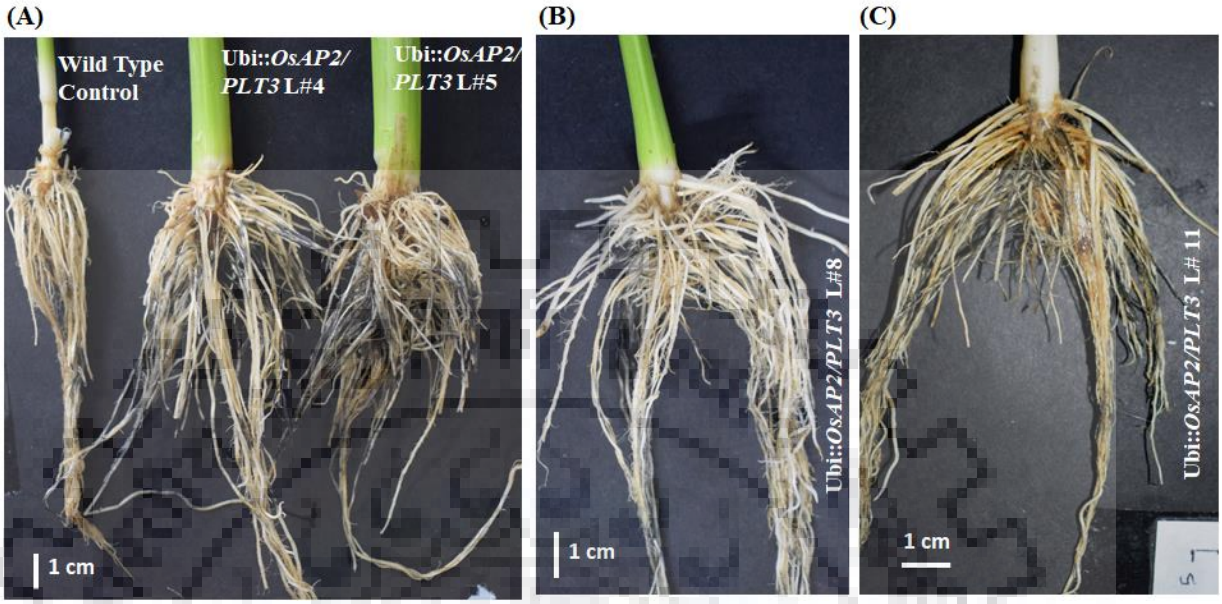

Figure 4:5. Adventitious root phenotype in Ubi::*OsAP2/PLT3* expressing rice lines. (A- C) Four independent over-expression lines with Ubi::*OsAP2/PLT3*, L#4, L#5 (A) L#8 (B), L#11 (C) shows vigorous root growth compared with wild type control lines in the extreme left in panel (A), increase in the girth of the stem provides more space for adventitious root formation at the stem base. Scale Bars: 1cm (A- C).

4.4 DISCUSSION

AINTEGUMENTA (ANT), BABY BOOM (BBM) and PLETHORA (PLT) genes, are key developmental regulators of plant regeneration, embryogenesis, root meristem establishment and maintenance, shoot apical meristem function, floral organ patterning and LR outgrowth in Arabidopsis (Aida et al., 2004; Du and Scheres, 2017; Galinha et al., 2007; Hofhuis et al., 2013; Horstman et al., 2014; Krizek, 2011; Krizek and Eaddy, 2012). Members of PLETHORA gene family encode double AP2-domain-containing plant-specific transcription factors. In *Arabidopsis, plt1plt2plt3* triple mutant has severe defects in growth and development of primary and lateral roots and in the *plt1plt2plt3bbm* quadruple mutant plants completely lacks roots (Galinha, et al., 2007). The expression of PLT genes is regulated by auxin and they are functioning in genetically redundant manner (Pinon et al. 2013, Mähönen et al. 2014, Santuari et al. 2016). *Arabidopsis ant* mutant have a smaller flower size than the wild type, whereas flower size increases in plants constitutively expressing *ANT* (Krizek, 2009; Mizukami and Fischer, 2000). ANT, AIL5, AIL6/PLT3 and AIL7 show partially overlapping, but distinct, expression patterns within inflorescence meristem and developing flowers (Nole-Wilson et al., 2005), and at least ANT and AIL6/PLT3 act redundantly to regulate floral growth and patterning in *Arabidopsis* (Krizek, 2009).

In this study, we demonstrated pleiotropic effects of ectopic over-expression of *OsAP2/PLT3* on the stem thickness, leaf angle, root architecture and flowering time and fertility. *Arabidopsis PLT* genes have been shown to regulate root growth and development. Constitutive ectopic over-expression of *AtPLT2* induces ectopic root development on the shoot apex (Galinha et al., 2007). Unlike *AtPLT2,* we could not observe any ectopic root phenotypes upon constitutive over-expression of *OsAP2/PLT3* in rice, suggesting that *OsAP2/PLT3* is not sufficient to promote roots in the ectopic rice tissues. Additionally, *PLTs* has also been shown to regulate de novo organogenesis during lateral root formation and rhizotaxis establishment in *Arabidopsis*, suggesting a role of *PLTs* gene in regulating root architecture (Hofhuis et al., 2013; Du and Scheres, 2017). Our data also suggest a role of *OsAP2/PLT3* in regulation of leaf blade angle. We observed altered angle at the junction of leaf sheath and the blade upon over-expression of *OsAP2/PLT3* in rice. In *Arabidopsis,* phyllotaxis (shoot organ positioning) is also regulated by *PLT* genes as the *plt3plt5plt7* triple mutants result in non-random and metastable changes in the phylotaxis (Prasad et al., 2011). This function is largely because of regulation of local auxin biosynthesis and polar auxin transport in *Arabidopsis* (Prasad et al., 2011; Pinon et al., 2013). Another phenotype which we observed upon *OsAP2/PLT3* over-expression was stem thickness. This could be because of more cambial activity in the stem. *AINTEGUMENTA* (*ANT*) and D-

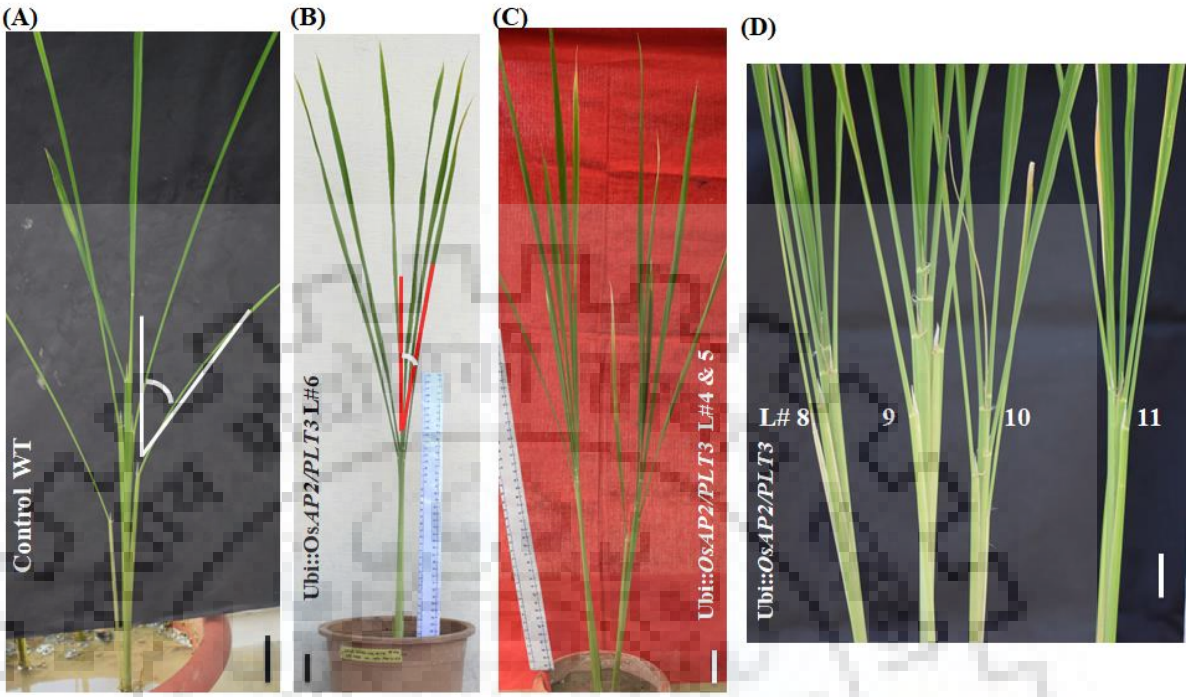

Figure 4:6. Upright plant morphology in Ubi::*OsAP2/PLT3* expressing rice lines. (A- C) Seven independent over-expression lines with Ubi::*OsAP2/PLT3*, L#6 (B), L#4 & 5 (C) L# 8, 9, 10 & 12 (C) shows reduced leaf angle compared with wild type control lines (A). Scale Bars: 1cm (A- D). Leaf angle of wild type marked in white angular lines (A) and in red angular lines in transgenic (B). Scale Bars: 4 cm (A- C), 1cm (D).

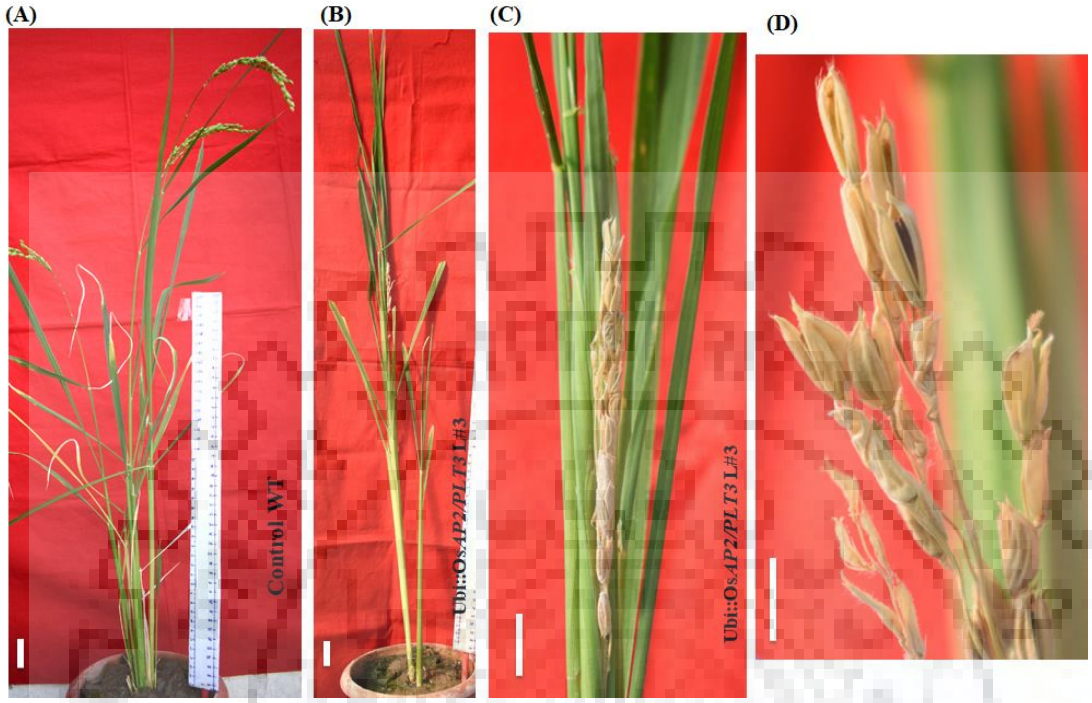

Figure 4:7. Floral morphology in *OsAP2/PLT3* over expression rice lines. (A- D) Over-expression of *OsAP2/PLT3*, caused delay in flowering in L#3 with non-emerged panicle and deformed spikelets while wild type control line has normal panicle growth (A). Scale Bars: 4 cm (A- B), 1cm (C, D).

type cyclin *CYCD3;1* are known to express in the vascular cambium of *Arabidopsis* roots and is required for the secondary thickening of the roots down-stream of cytokinin signalling pathway (Randall et al., 2015). Similar regulation of *ANT* and *CYCD3* by cytokinin is also observed during secondary thickening of poplar stems, suggesting possibility of a conserved regulatory mechanism during secondary growth (Randall et al., 2015).

Yet another strong phenotype observed upon *OsAP2/PLT3* over-expression was delayed flowering. In these transgenic lines, flowering was extremely delayed and panicle never emerged out. *LFY* of *Arabidopsis* is a key gene to regulate vegetative-to-reproductive phase transition. Auxin induces *LFY* expression via direct regulation of *LFY* by *AUXIN RESPONSE FACTOR5/MONOPTEROS* (*ARF5/MP*) (Yamaguchi et al., 2013). *ANT* and *AINTEGUMENTA-LIKE6/PLETHORA3* (*AIL6/PLT3*), has also been found to regulate expression of *LFY*, in parallel with MP in response to auxin and in *ant ail6* mutants, the meristem identity transition and *LFY* induction were delayed (Yamaguchi et al., 2016). The role of *ANT* and *AIL6/PLT3* as a regulator of *LFY* is also supported by the fact that they are expressed prior to *LFY* expression and bind to the *LFY* promoter. In *ant ail6* double mutants have been shown to affect growth of floral organs, defect in their positioning and identity indicating contribution of *ANT* genes floral growth and patterning (Krizek, 2009). *RFL*, the rice homolog of *LFY* when up regulated causes early transition to flower and there is delay in transition to flower down regulation in expression. *OsAP2/PLT3* overexpression causes delay in flowering which is in contrary to the function of *RFL* which positively regulates *OsSOC1*/*OsMADS50* (Lee et al., 2004), an activator of flowering (Rao et al., 2008). Along with other vegetative phenotypes like narrow leaves, *WOX3A* mutants produce rice plants with opened spikelets (Yoo et al., 2013). Knock down of *SUPERMAN* gene were vegetatively normal, but flower formation was defective (Nandi et al., 2000). With ubiquitous expression of *OsAP2/PLT3*, we also find normal plant growth and development but defect in flower formation which is in contrast with the *SUPERMAN* over expression phenotype. The delay in flowering and deformed floral organ in *BBM2* or *OsAP2/PLT3* over expressing plants suggests its influence in floral organ development. The mis expression-based approach helped us shed some lights on function of *OsAP2/PLT3* in overall plant growth and development. *OsAP2/PLT3* has been shown to play major task in vegetative development and might play critical direct or indirect role in transition to flowering in rice.

CHAPTER 5

SUMMARY AND CONCLUSIONS

Rice has become a model monocotyledons plant and cereal crop to elucidate the developmental programs occur throughout the life cycle. The developmental life cycle of an angiosperm is roughly divisible into three stages, the embryonic, the vegetative and the reproductive phase. Some of the processes such as development of leaf, root and spikelet have been categorized into substages based entirely on the morphological and anatomical viewpoints. Growing genetic and molecular data with the anatomical illustrations has given logic behind all the stages of development. One lustrous example is the development of flower which consists of four whorls sepal petal, stamen and carpel. The developmental genetic regulation of the four whorls have been well characterized since the 1980s, through study of numerous mutant phenotypes and spatiotemporal expression pattern of the causal genes that facilitated the postulation of the classic ABC model of flower development (Coen and Meyerowitz, 1991). During the embryogenesis body axis is established by positioning two distinct apical meristems, the shoot apical meristem (SAM) and the root apical meristem (RAM). The motto of our developmental studies is to answer the identity, origin and genetic regulation of rice root organs originating from the RAM.

Higher plants exhibit an amazing diversity of root architectures at both the systems and anatomical level. The rice root system consists of seminal, crown and lateral roots, among which we are focusing our study to the development of stem-borne crown roots. Except from auxins and cytokinins, the major plant hormones are abscisic acid (ABA), gibberellins (GA), ethylene and more recently discovered salicylic acid, jasmonates, brassinosteroids and strigolactones. Most of these hormones play role in abiotic and biotic stress avoidance. Apart from auxin and cytokinin, few others such as ethylene, ABA, GA and strigolactones are also reported to play discrete role in plant development through transcriptional regulators (Abbas and Chattopadhyay, 2014; Kushwaha et al., 2008; Sun et al., 2015). Evidence from many studies highlights the central role of auxins, endogenously as well as in mediating environmental stimuli in shaping the final root architecture. Our study focused towards responses and function of auxin and cytokinin signalling, they are the key hormones identified to mould the crown root development. Yet their dynamic responses and functional relevance of their interaction has not been studied in rice. Recent studies have been exploring root development in monocots, as several molecular markers expressed in root specific tissues of few of the cereals like rice, barley, maize and wheat have been reported. In this thesis, we have described the domains of hormonal response during crown root development of the rice plant, and then describe in detail the role of transcription factors in shaping the morphology of CR.

Also, for the facility of future studies, we propose a regulatory mechanism down stream of those transcription factors involved in adventitious root formation.

Studying auxin and cytokinin responses & function during crown root development

Auxin and cytokinin signalling pathway regulate expression of some key developmental regulators including transcription factors in during lateral and adventitious root development (Orman-Ligeza et al., 2013; Yadav et al., 2010). Auxin distribution in the roots was examined using the synthetic promoter DR5 and a variant of this named DR5rev and fused reporter proteins (Friml et al., 2003). Cytokinin distribution also has many essential roles in postembryonic growth and development. To visualize the signalling output in vivo, a universal synthetic promoter reporter system TCS::GFP/GUS has been profoundly used in Arabidopsis (Hwang et al., 2012). Auxin and cytokinin signaling pathways function through auxin response factors (ARFs) and cytokinin response regulators (RRs), respectively, to regulate their down-stream genes including TFs and often crosstalk during plant development (El-Showk et al., 2013; Lavy and Estelle, 2016). *OsCKX4* controls cytokinin homeostasis and integrates auxin and cytokinin signaling pathways in CRs (Gao et al. 2014).

Plant hormones interact with each other and those interactions can be antagonistic or synergistic. We were also able to show that two major phytohormones, cytokinin and auxin, display chiefly dissimilar yet partially overlapping responses across the crown root primordia. Auxin is concentrated in actively dividing stem cell niche, while cytokinin is found to be concentrated in the stele. By connecting these hormonal domains with the developmental stages of the crown root, our work matches recent studies of domain-specific auxin and cytokinin profiles of Arabidopsis lateral root development (Antoniadi et al., 2015; Bielach et al., 2012; Dello Ioio et al., 2008). Extended analysis of spatiotemporal cytokinin response which was absent in the periclinal layer before CRP initiation, contradicts cytokinin action prior to the first division of periclinal cells to inhibit LR formation (Bielach et al., 2012; Chang et al., 2015). The outcome of these hormonal signalling is dependent on different developmental context, allowing the same hormones to play versatile role in diverse plant species. Identification of auxin-cytokinin response domains helped us addressing the interconnected nature of their distributions. To better understand the contribution of the active auxin pool in the interconnected domains of auxin and cytokinin we strategically removed auxin in those domains. *OsMGH3* was driven by synthetic TCSn promoter and construct used to deplete auxin in anatomical regions of CRP where both auxin and cytokinin is present. Transgenics with such construct show very similar phenotype as *OsMGH3* over expression lines such as dwarfism and

loss of apical dominance. In few weak lines in T1 generation after supply of exogenous auxin, we could observe redeem of root phenotype. Not so surprising, although the domains of interaction in CRP were not so broad, auxin proves its necessity even in the border lines of different CRP tissues. The interplay of auxin and cytokinin will enable us to precisely identify crown root specific auxin-cytokinin modules during various stages of adventitious root development in plant.

Function of auxin-regulated transcription factor, *OsAP2/ERF-40* in CR formation

The mechanisms by which plant hormone signaling pathways control the architecture of adventitious root remains largely unknown. So, in next objective, we studied the modulations in global genes activity in developing rice adventitious root to external auxin and cytokinin signaling cues to filter out key developmental regulators (Neogy et al., 2019). Transcription factors (TFs) are master regulators for developmental program in plants. Therefore, we aimed to identify crucial auxin responsive TFs regulating crown root development in order to understand the transcriptional networks of crown roots. An auxin-induced transcription factor containing AP2/ERF domain, *OsAP2/ERF-40* was selected from the global transcriptome analysis. A rice LOB-domain containing TFs, CRL1 specifically controls crown root development as crl1 mutants do not develop crown roots. *OsAP2/ERF-40* was also identified as a downstream target of *OsCRL1* through transcript profiling of crl1 mutants (Coudert et al., 2015).

Many AP2-family genes are involved in shoot and root organogenesis in eudicot as well as monocot. An auxin regulated AP2/EREBP domain containing transcription factor PUCHI is required for early morphogenesis of lateral root formation in Arabidopsis thaliana (Hirota et al., 2007). In this objective we studied the consequence of *OsAP2/ERF-40* knock-down and ectopic over-expression during adventitious root development. Our study demonstrates its necessity and sufficiency to confer the adventitious root fate. The ability to trigger the root developmental program is largely attributed to *OsAP2/ERF-40* mediated dose dependent transcriptional activation of genes that can facilitate generating effective auxin response, and OsERF3-OsWOX11-OsRR2 pathway. Our studies reveal gene regulatory network operating in response to hormone signaling pathways and identify a novel transcription factor regulating adventitious root developmental program, a key agronomically important quantitative trait, upstream of OsERF3-OsWOX11-OsRR2 pathway (Neogy et al., 2019). AP2 family transcription factors also influence several other hormone signalling pathways such as ethylene- jasmonic acid signalling, ABA response in Arabidopsis (Gautam and Nandi, 2018;

Sanyal et al., 2017). An important single AP2 domain containing gene APD1, a positive regulator that functions upstream of salicylic acid mediated plant defence as well as ethylene-JA mediated plant growth (Giri et al., 2014). The chosen TF, *OsAP2/ERF-40*, is categorized in DREB1F subgroup in Group-IV of AP2 family by sequence homology (Rashid et al., 2012). Dehydration-responsive element-binding protein (DREB) sub family is well known in Arabidopsis, rice, maize, rape seed, and tobacco plants for their response to drought stress. In future it will be intriguing to consider how crown root development is modified in drought condition through endogenous factors. The study may consolidate endogenous regulation to exogenous factors mediating root development.

Role of auxin-independent transcription factor, OsAP2/PLT3 in rice

AP2-domain containing *AINTEGUMENTA-LIKE* (*AIL*) gene family contains eight genes in Arabidopsis including *BABY BOOM, AINTEGUMENTA*, and the *PLETHORA* (Horstman et al., 2014). We selected a member of rice PLETHORA gene family, OsAP2/PLT3 for functional study as an auxin non-responsive putative target of *CRL1/ARL1* (Coudert et al., 2011; Li and Xue, 2011). Although it did not show any response to auxin treatment, we found strong expression of the gene in developing crown root primordia and in the extruding crown roots. So, it was provoking towards identification an auxin-independent regulation of CR development. We functionally characterized *OsAP2/PLT3* by taking ectopic over expression approach and showed a pleotropic effect upon its over-expression. Over the years many transcription factors have been identified that promote cell proliferation and meristem size, the important ones being *PLETHORA (PLT)* or *AINTEGUMENTA (ANT)* family of redundant TFs. In accordance with many plethora genes studied *OsAP2/PLT3* over expression gave morphological pleotropic effect in rice plants. Vegetative organs were robust in nature though plant height remained the same. Such phenotypic possibilities can be the result of needless cell divisional activities or cell expansion events. Transcription factors from the AP2/ERF family, in rice, *FRIZZY PANICLE (FZP)* and in maize, *BRANCHED SILKLESS1 (BD1)* genes are known to regulate the transition from the BM to the SM and respectively (Chuck et al., 2002; Komatsu et al., 2003b). We have observed no flowering in most of the cases or delay in flowering in few transgenics suggesting its role in transition from vegetative to reproductive stage suggesting critical role of *OsAP2/PLT3*. In summary, this thesis has provided an insight towards auxin and cytokinin responses during crown root development and delineates function and mechanism of action of *OsAP2/ERF-40* in promoting crown root development. It has also provided significant leads towards future works, particularly in delineating specific functions of *OsAP2/PLT3* in adventitious root formation.

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Publications:

(A) Research articles

1. Neogy, A., Garg, T., Kumar, A., Dwivedi, A.K., Singh, H., Singh, U., Singh, Z., Prasad, K., Jain, M., Yadav, S.R., 2019. Genome-Wide Transcript Profiling Reveals an Auxin-Responsive Transcription Factor, OsAP2/ERF-40, Promoting Rice Adventitious Root Development. Plant Cell Physiol. doi:10.1093/pcp/pcz132

2. Garg, A., Singhania, T., Singh, A., Sharma, S., Rani, S., Neogy, A., Yadav, S.R., Sangal, V.K., Garg, N., (2019). Photocatalytic Degradation of Bisphenol-A using N, Co-doped TiO2 Catalyst under Solar Light. Sci. Rep. 9, 765. doi:10.1038/s41598-018-38358-w.

(B) Review

3. Yadav, S.R., Kumar, A.*, Neogy A.* and Garg T.* (2017). OsMADS1/OsLHS1: Diversified Regulatory Functions in Ensuring Transition and Completion of Sexual Reproduction in Rice. Proc Indian Natn Sci Acad, 83, 2017, 67-79. *Equal contribution.

(C) Conference Paper/Poster

4. Ananya Neogy, Zeenu Singh, Khrang K. Mushahray, Nikita Yadav, Shri Ram Yadav. (2018). Dynamic response and functional significance of hormones during rice adventitious root development. Auxin and Cytokinin in Plant Development-2018, Prague, Czech Republic, Europe [poster presentation, P-03-23, page-101].

5. Ananya Neogy, Anil Kumar, Gopal Guguloth, Shri Ram Yadav. (2015). Functional genomics of root system architecture. Mcb75: From molecules to organisms, IISc., Bangalore, India. [poster presentation].

(D) Manuscript under preparation

6. Ananya Neogy, Zeenu Singh, Khrang K. Mushahary, Shri Ram Yadav, Auxin removal in the cytokinin responsive domains reveals their synergistic interaction during rice crown root development (Manuscript).

7. Ananya Neogy, Anil Kumar, Tushar Garg, Harshita Singh, Shri Ram Yadav, Over-expression of OsAP2/PLT3 results pleiotropic effects during rice growth and development (Manuscript).