Genetic analysis of the role of the *OsARF11* gene in rice development

by

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Abstract

Based on sequence similarity to the well-studied *Arabidopsis thaliana MP/ARF5* gene, we hypothesized that the *Oryza sativa Auxin Response Factor 11 (OsARF11)* gene is a prime candidate for auxin-signaling mediated development in rice. Here we describe characterizations of two independent insertion mutants in the *OsARF11* gene. Our results reveal that homozygous plants of both allelic mutants have reduced shoot and root growth and produce fewer seeds compared to wild type plants grown under the same growth conditions. In addition, the number of leaf veins per leaf and per unit leaf width is reduced, as is the width of leaf mid veins. Taken together, the results demonstrate for the first time that *OsARF11* contributes to plant growth, fecundity, and the regulation of leaf vein patterning in rice. The results also suggest that *OsARF11* may be a suitable target for breeding on these traits.

Keywords: Rice; Auxin signalling; *OsARF11*; Vein pattern; Height and Root growth; Fecundity.

Dedication

" The pursuit of knowledge is never-ending. The day you stop seeking knowledge is the day you stop growing"

(Brandon Travis Ciaccio)

I dedicate this thesis to my parents for their endless support

and being an inspiration throughout my life. Thank you for

nurturing me to appreciate the value of knowledge and

strength in life.

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Chapter 1.

1. Introduction

1.1. The role of genetics in the study of plant development and physiology

The application of molecular genetics has transformed our conceptual understanding of plant development and physiology (Howell 1998). Extensive mutant screens, analysis of numerous mutant phenotypes, followed by map-based cloning of corresponding genes have identified genes involved in the formation and patterning of flowers, stems, leaves and roots. Components of signal transduction pathways for light, hormones, biotic and abiotic stress have been identified in similar ways. In addition, the ability to create modified genes such as promoter-marker gene fusions and introduce them into plants by various transformation techniques allow detailed mapping of both gene expression patterns and gene interactions (Howell 1998; Jander *et* al., 2002; Dutt *et al*., 2014). Transformation also allows large-scale T-DNA and transposon insertional mutagenesis, which greatly facilitates the subsequent identification of mutated genes (Wang *et al*., 2011). Nevertheless, there is still much to learn and the understanding of many processes, both developmental and physiological, is limited (Itoh *et al*., 2005).

1.2. Overview of plant development

Higher plants are comprised of many specialized organs, tissues, differentiated and undifferentiated cells. Unlike animals, in which the developmental processes are mostly limited to embryogenesis, differentiation and development continues throughout the life cycle (Smyth *et al*., 1990; Itoh *et al*., 2005). All plants undergo three different phases of development: embryogenesis, vegetative development and reproductive development. In each phase, numerous events occur sequentially that result in the regulated proliferation of cells and eventually their recruitment into several tissues and organs (Taiz and Zeiger 2006).

Although, the basic body pattern forms during embryogenesis, the majority of tissues and organs form and differentiate during postembryonic growth. Vegetative development, usually the longest period of growth, occurs between germination and flowering phases (Telfer *et al*., 1997). Plant organs such as branches and leaves are also formed from shoot and root meristems during postembryonic development. Photosynthesis and accumulation of resources required for flowering and reproductive phases are carried out during the vegetative phase (Howell 1998; Asai *et al*., 2002; Itoh *et al*., 2005).

The study of lateral organ formation at the flanks of the shoot apical meristem has and continues to be a topic of great interest, in both dicots and monocots. In dicots such as *Arabidopsis thaliana,* (hereafter refer to as *A. thaliana*), the formation of auxin maxima in the margin of the shoot apical meristem triggers leaf primordia formation (Reinhardt *et al*., 2000). This process is followed by differentiation of leaf primordia into apical and basal regions, which results in the expansion of a leaf blade and elongation of

a petiole (Tsukaya 2013). Unlike dicot leaves, monocot leaves are wide along their entire length, and subdivided into blade and sheath structures with a junction that consists of an auricle, a small ear-like outgrowth formed at the base of the leaf, and a ligule, a hairy attachment at the junction of sheath and blade. Furthermore, leaf elongation and growth occur after primarily transverse cell divisions at the base of the leaf. As a result, a developmental gradient of the leaf can be seen from the mature tip to the immature base (Howell 1998; Taiz and Zeiger 2015).

A. thaliana is a small flowering plant that is widely used as a model to study development (Koornneef and Meinke 2010). *A. thaliana* is a dicotyledonous plant species; therefore, a general understanding of development in angiosperm requires the study of model species also in monocotyledons. Rice (*Oryza sativa*) together with maize (*Zea mays*) are currently the most researched model monocot plant species (Sasaki and Burr 2000). Many publications highlight developmental similarities and differences between dicots and monocots based on comparison of these species (summarized in Rashid 2009).

1.3. Auxin Biosynthesis, Transport and Signalling

1.3.1.   Auxin Biosynthesis

There are two major pathways for IAA biosynthesis in plant: the Tryptophan (Trp) independent and the Trp-dependent pathways (Mano and Nemoto, 2012). In the Trp dependent pathway, Tryptophan (Trp) is first converted to indole-3-pyruvic acid (IPA) by a Trp aminotransferase encoded by the *TAA1* gene (Tryptophan Aminotransferase of *A. thaliana*) (Tao *et al.,* 2008; Mockaitis and Estelle 2008). IPA produced by TAA1 proteins

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is converted to IAA by YUCCA proteins. *YUCCA* genes encode flavin monooxygenase-like enzymes that act in a separate branch of the tryptophan-dependent auxin biosynthetic pathway (Zhao *et al*., 2001; Mano and Nemoto, 2012). There are a total of 10 *YUCCA*-like genes identified in *A. thaliana* (Zhao *et al*., 2001). *YUCCA* genes redundantly regulate the final step of Trp-dependent biosynthesis of indole-3-acetic acid (IAA), during embryogenesis, leaf vasculature and inflorescence development (Zhao *et al*., 2001; Cheng *et al*., 2006; Won *et al*., 2011).

A combination of multiple *yucca* mutants show strong defects in leaf venation and decreased auxin levels in *A. thaliana* (Cheng *et al*., 2006; Baylis *et al*., 2013). Expression of the bacterial auxin biosynthesis gene *iaaM* results from the *YUC1* promoter in *yuc1 yuc4* double mutants rescues the mutant phenotype, providing evidence that the developmental defects seen in *yuc1 yuc4* double mutants are caused by an auxin deficiency (Cheng *et al*., 2006 and 2007). Additionally, *yuc1 yuc4* double mutants show a down-regulation in the expression of the auxin reporter gene DR5-GUS (Cheng *et al*., 2006). Reduction in vascular strand formation seen in *yucca* quadruple mutants emphasizes the role of auxin as a crucial factor in vascular initiation and differentiation (McSteen 2010).

Evidence for a role of *YUCCA* genes in auxin biosynthesis has been reported also in monocots (Yamamoto *et al*., 2007; Woo *et al*., 2007). The *YUCCA* gene family in rice consist of 13 members (McSteen 2010). Recently, seven *OsYUC* genes (*OsYUC1-7*) were found to encode YUC proteins similar to the *A. thaliana* YUC proteins (Yamamoto *et al*., 2007) and the other five genes were reported to be closely related to *AtYUC10* and *AtYUC11*, based on sequence similarity (Gallavotti *et al*., 2008). In rice, an increase in the activity of *OsYUC1* gene caused an increase in the level of endogenous

IAA and plants showed phenotypes similar to the auxin overproduction charactristics. For instance over-expression of *OsYUC1* gene reduces tiller number, while down regulation of *OsYUC1* gene results in dwarfism (Yamamoto *et al*., 2007), suggesting that *OsYUC1* is involved in auxin biosynthesis in rice.

In maize, loss-of-function of the *SPI1* gene, an ortholog of *OsYUC1* involved in the auxin biosynthesis pathway, causes defects in vegetative and reproductive development (Gallavottie *et al*., 2008). A similar phenotype to *spi1* is seen in *A. thaliana* with loss-of-function of four *YUC* genes simultaneously, indicating a redundant gene function among *YUC* genes in *A. thaliana* (Cheng *et al*., 2006) probably due to changes in the copy number of genes and expression patterns (Gallavotti *et al*., 2008).

1.3.2. Auxin Transport

Charles and Francis Darwin showed that bending of coleoptiles towards light depends on polar transport of a signal from the apex down the far side from the light (Darwin and Darwin 1880). Later, this substance, named auxin, was identified as indole-3-acetic acid (Thimann, 1937). Based on the chemical requirements of polar auxin transport, the chemi-osmotic hypothesis was formulated stating that protonated IAA enters the cell through passive diffusion, converts to anionic form of IAA$^-$ captured inside the cell and is exported out of the cell by active transport (Raven 1975; Goldsmith 1977). In line with the predictions of the chemio-osmotic hypothesis, auxin efflux proteins but also influx carrier proteins have been identified. The *AUX1/LAX* family of auxin influx carriers and the *PIN*-formed (*PIN*) family of auxin efflux carriers have been characterized primarily in *A. thaliana* (Bennett *et al*., 1996; Friml *et al*., 2002). *AUX1/LAX* is found to regulate cell patterning in the quiescent center and embryonic root establishment. For

example, mutation in the *AUX1/LAX* gene shows a larger radicle root cap resulting from an increase in the number of root cap cells. Multiple mutations in the *AUX/LAX* genes also results in plants with severe abnormality in radicle apex development (Ugartechea *et al*., 2009).

The most distinct characteristic of PIN proteins is that they localize asymmetrically in the membrane of auxin transport-competent cells (Vieten *et al*., 2007). The location correlates with the direction of auxin transport and with the accumulation of auxin in adjacent cells (Vanneste and Friml 2009), suggesting that the direction of intercellular auxin flow is determined by the sub-cellular localization of PIN protein polarity. Among the *PIN* family of genes, *PIN1* is the most studied. Mutation in the *PIN1* gene in *A. thaliana*, named as *pin*-formed1 mutant, develops knitting needle-like apices (Okada *et al*., 1991). An interruption in the *PIN1* gene, encoding auxin efflux carrier proteins, causes disrubtion in endogenous auxin flow in *pin1* mutants, resulting in abnormal development of shoot (Xu *et al*., 2005). Mutation in the *PIN* genes also includes defects in leaf formation and inflorescence outgrowth, defects in primary root growth, reported in triple *pin* mutant *pin1 pin3 pin4*, and severely defective embryos in quadruple mutation of *pin1 pin3 pin4 pin7* (Vernoux *et* al., 2000; Reinhardt *et al*., 2003; Benkova *et al*., 2003; Weijers and Jurgens 2005; Xu *et al*., 2005). In addition, *OsPIN1* expression in rice has been detected in vascular tissues of all major organs with relatively higher expression levels in leaf and flower, in a similar manner to *AtPIN1* expression pattern in *A. thaliana* (Galweiler *et al*., 1998; Xu *et al*., 2005).

Before the molecular basis of polar auxin transport was known, Tsvi Sachs carried out a large set of wounding and auxin application experiments to address the roles of auxin sources, tissue polarity as well as the influence that pre-existing auxin

sinks had on vascular strand formation (Sachs 1981 and 1989). Based on his observations, he formulated the canalization of signal flow model with auxin being the most likely candidate for the signal (Sachs 1981; Rolland-Lagan and Prusinkiewicz 2005). In a field of cells exposed to auxin, some cells will gradually improve their capacity to transport auxin through a positive feedback mechanism and in the process also drain auxin from the vicinity. Consequently, auxin flow is canalized into narrow strands of cells, which differentiate into vascular cells (Sachs 1981 and 2003; Taiz and Zeiger 2015). The direction of flow is also influenced by pre-existing vasculature acting as auxin sinks. The expression patterns of an auxin-induced marker gene, auxin influx and efflux proteins as well as the *MP/ARF5* gene largely supports the canalization of auxin flow hypothesis (Mattsson *et al*., 2003; Scarpella *et al*., 2006; Wenzel *et al*., 2007).

1.3.3.   Auxin signalling

Genome-wide transcription studies show that auxin treatment results in extensive changes in the steady-state levels of many transcripts, suggesting an effect of auxin on the expression of a large range of genes (Reviewed by Mochaitis and Estelle 2008). There are two groups of well-studied genes that mediate auxin-induced responses in plants: the *Aux/IAA* genes and *AUXIN RESPONSE FACTOR* genes (*ARFs*) (Teale *et al*., 2006). ARFs and Aux/IAA proteins are together required for transcriptional regulation of auxin response genes, thus playing a central role in auxin signalling (Guilfoyle *et al*., 1998; Guilfoyle and Hagen 2007).

The studies of Aux/IAA and ARF protein structures show that both proteins contains conserved domain near the C-terminal, domain II, that interacts with a component of the ubiquitin-proteasome protein (SCF-TIR1) degradation pathway

essential for auxin signalling (Ouellet *et al*., 2001; Tiwari *et al*., 2001; Song *et al*., 2009). Domains III and IV mediate *ARF-ARF*, *ARF-Aux/IAA* and *Aux/IAA-Aux/IAA* dimerization (Ouellet *et al*., 2001; Hardtke *et* al., 2004; Muto *et al*., 2006), of which the heterodimers are more stable than homodimers (Ulmasov *et al*., 1999; Muto *et al*., 2006). For example, yeast two hybrid assays have shown that ARF1 and MONOPTEROS (MP)/ARF5 heterodimerize with AUXIN RESISTANT 3 (AXR3)/IAA17 in *A. thaliana* (Ouellet *et al*., 2001; Shen *et al*., 2010). MP/ARF5 also interacts with BODENLOS (BDL)/IAA12 (Hardtke *et al*., 2004; Weijers *et al*., 2006). The yeast two-hybrid assay is an in-vivo technique used to identify protein-protein/DNA interactions. In this mechanism, the protein of interest binds to DNA-binding domain (DBD) and generates a complex called bait. The bait is used invitro to identify potential interacting proteins called prey (Bruckner *et al*., 2009).

ARFs play a key role in auxin-dependent signalling. At low concentration of auxin, Aux/IAAs interact with ARFs and prevent ARFs from binding to the promoter of auxin-reponsive target genes; thus, repressing the auxin response (Fig. 1.1). At high auxin concentration, auxin directly stimulates the interaction between Aux/IAA proteins and auxin receptor SCF-TIR1 E3 ubiquitin-ligase complexes, resulting in the degradation of Aux/IAA proteins (Tian *et al*., 2002; Dharmasiri *et al*., 2003; Kepinski and Leyser 2004). As a consequence, ARF transcription factors are released from inhibition and can regulate the expression of auxin-responsive target genes (Weijers and Jurgens 2005).

Twenty-three *ARF* genes have been identified in *A. thaliana* (Guilfoyle and Hagen 2007) and 25 *ARF* gene members have been found in rice (Jain *et* al., 2006; Wang *et* al., 2007; Song *et* al., 2009). ARF proteins bind to TGTCTC auxin response elements (AuxRE) in the promoters of auxin response genes and regulate responses to

the plant hormone auxin (Wang *et al*., 2007). Ulmasov demonstrated that the DNA binding domain (DBD) of the *ARF1* gene is required to interact with the, TGTCTC sequence (Ulmasov *et al*., 1999; Sato *et al*., 2001). Genes that are up-regulated or down- regulated by auxin contain AuxRE in their promoters, which facilitate binding to transcription factors of the *ARF* gene family (Guilfoyle and Hagen 2007; reviewed by Mockaitis and Estelle, 2008).

The *A. thaliana* ARF5/MP, ARF6, ARF7, ARF8 and ARF19 proteins are identified as transcriptional activators, whereas the other ARFs are characterized as repressors (Ulmasov *et al*., 1999; Tiwari *et al*., 2003; reviewed by Li *et al*., 2016). Genetic analyses have shown that individual ARFs control distinct developmental processes (Guilfoyle and Hagen 2007; Rademacher *et al*., 2012).

MONOPTEROS (*MP*)/*ARF5*, one of the well-studied *ARF* genes, is required for the specification of a group of cells at the early globular stage in embryogenesis that give rise to the hypocotyl and root. *A. thaliana mp/arf5* mutants fail to form hypocotyl and root structures (Berleth and Jurgens 1993; Przemeck *et al*., 1996; Hardtke and Berleth *et al*., 1998; Mattsson *et al*., 1999; Reviewed by Mochaitis and Estelle, 2008). *MP/ARF5* loss of function also causes strong defects in the formation of the vascular system in the embryo and later in leaves (Przemeck *et al*., 1996). With exception of defects in lateral organ formation on inflorescence meristems, described as *pin*-shaped inflorescence morphology with no or few incomplete flowers, *mp/arf5* mutants have a normal vegetative development (Przmeck *et al*., 1996; Schuetz *et al*., 2008).

Defects observed in *mp/arf5* mutants in *A. thaliana* provide evidence that auxin is essential and required in early embryogenesis and later for development of tissues and

organs (Berleth and Jurgens 1993; Hardtke and Berleth 1998; Weijers and Jurgens 2005). Furthermore, it highlights the role of *MP/ARF5* in plant development through the control of auxin signalling (Sato *et al*., 2001). Although quite a few members of the *ARF* genes have been functionally characterized in dicot plants such as *A. thaliana*, there is relatively limited information available about their functions in important crops such as rice. The *OsARF1* gene known as an auxin-induced gene is closely related to *AtARF1* and *AtARF2* in *A. thaliana* (Waller *et al*., 2002). Loss-of-function *Osarf1* mutants show defects in vegetative development, resulting in dwarfism with small curled leaves and defects in reproductive development such as sterility or failing to flower and delayed flowering (Waller *et al*., 2002; Ellis *et al*., 2005; Attia *et al*., 2009). These phenotypes are similar to the phenotypes seen in double *arf1 arf2* mutants in *A. thaliana* (Ellie *et al*., 2005; McSteen 2010).

Comparison of rice and *A. thaliana* ARF protein sequences suggests that one or two rice ARF proteins correspond to any given *A. thaliana* ARF proteins (Shen *et al*., 2010; Sato *et al*., 2001; Wang *et al*., 2007). Based on phylogenetic analysis, ARF proteins have been grouped in different classes of which a one-to-one (1:1) orthologous relationship has been found between AtARF2/OsARF24 and AtARF5(MP)/OsARF11 that also suggesting functional similarity between these ARF proteins (Wang *et al*., 2007). A one-to-two relationship has been reported between OsARF6a/OsARF6b/AtARF6 and OsARF7a/OsARF7b/AtARF7 NONPHOTOTROPIC HYPOCOTYL 4 (NPH4) proteins, indicating redundant gene function among *ARF* genes in rice (Sato *et al*., 2001). It is possible that the functions of ARF proteins are more precisely specialized in rice for more restricted organs or tissues or for specified stages (Sato *et al*., 2001).
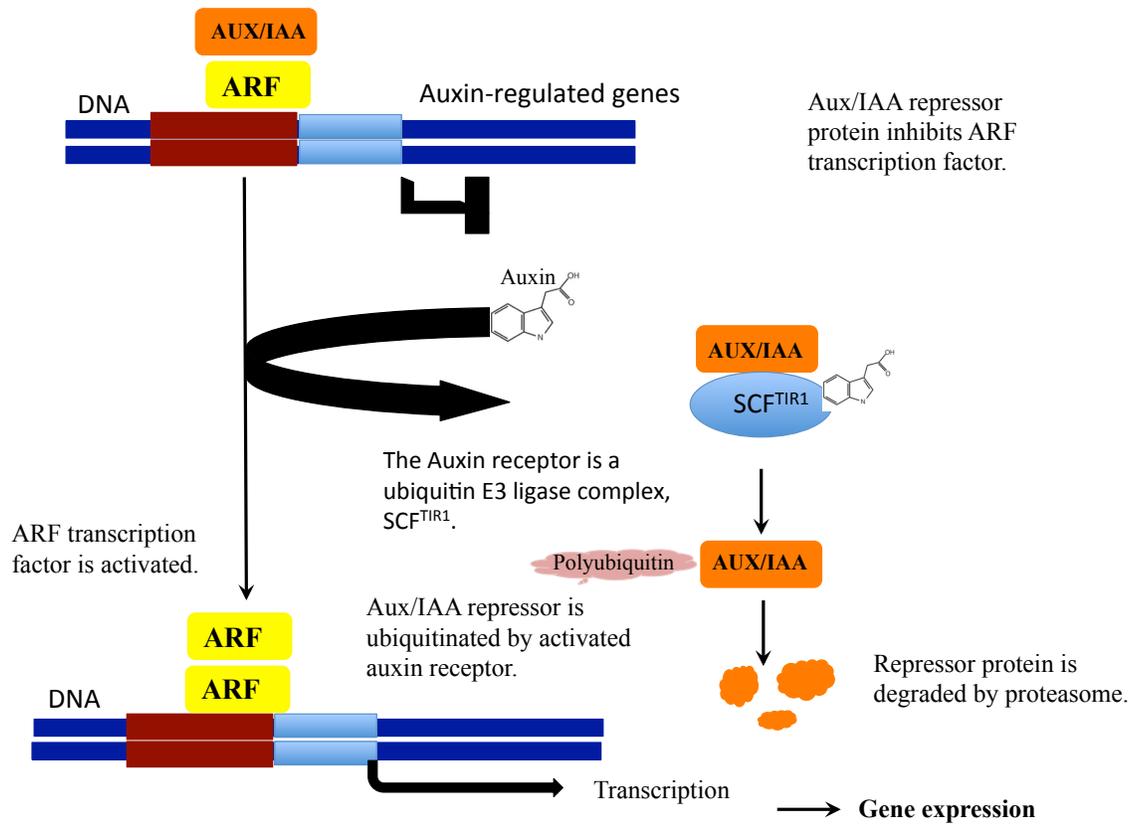
Figure 1.1. Schematic figure model of auxin signal transduction pathway in plants. The binding of auxin to its receptors SCF/TIR1 and Aux/IAA results in ubiquitination and degradation of the Aux/IAA protein and the release of bound ARF protein. The ARF protein binds to auxin response elements located in the promoters of auxin response genes such as *PIN1*. The drawing is adapted from figure 1. Teale *et al.,* 2006.
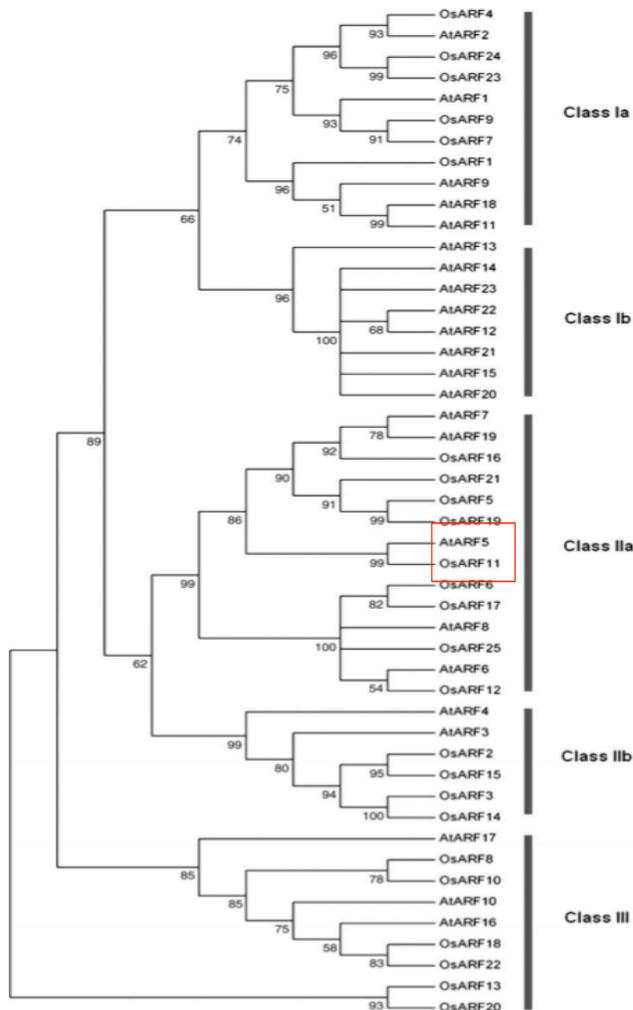
Figure 1. 2. Phylogenetic relationship of rice and *Arabidopsis ARF* proteins. Vertical bars show different classes generated by protein sequence similarity. Wang *et al*., 2007. Permission to reprint is provided by Elsevier (License number 3894361128512).

The *Aux/IAA* gene family consists of twenty-nine members in *A. thaliana* (Teale *et al*., 2006) and thirty-one members in rice (Jain *et al*., 2006; Song *et al*., 2009). One *Aux/IAA* gene of particular interest is the *A. thaliana BODENLOS* (*BDL*)/*IAA12* gene. *BDL* encodes one of the many *Aux/IAA* repressor proteins that interact with and repress *MP/ARF5* transcriptional activation (Hardtke *et* al., 2004; Weijers *et al*., 2005). It has been demonstrated that *A. thaliana BDL* gain-of-function mutants are resistant to auxin-induced degradation and suppress *MP/ARF5*'s activity through staying bound to *MP/ARF5,* generating a phenotype similar to *mp* mutants (Berleth *et al*., 2000).

1.4. Regulation of plant development

1.4.1. Factors involved in shoot growth

Shoot development in plants progresses through several discrete phases (Lawson and Poething 1995). The shoot apical meristem (SAM), surrounded by leaf primordia, is located at the apex of the stem and gives rise to the primary shoot, including stem, leaves, branches and flowers. Elongation of the shoot axis proceeds through a combination of cell division and cell elongation. In rice, as in other monocot plant species, internode elongation is caused by cell division in the intercalary meristem at the base of nodes in the stem, followed by cell elongation of those cells in the cell elongation zone (Hoshikawa 1989; Zou *et al*., 2005; Ishikawa *et al*., 2005). Defects in one or both of the two processes can result in dwarfism. Many investigations have been carried out to identify the source of factors regulating shoot growth and development. Hormone biosynthesis and the subsequent signalling regulating effect on downstream genes are the most important factors involved in these processes (Lawson and Poething 1995; Howell *et al*., 2003).

Exogenous application as well as mutant analysis have revealed that cytokinin (CK) biosynthesis and signalling plays a key role in shoot development. Ck biosynthesis defective mutants contain significantly fewer cells than wild type plants (Werner *et al*., 2003). In *A. thaliana*, a mutation in the *ALTERED MERISTEM PROGRAM* 1 (*Atamp1*) gene, encoding a putative carboxypeptidase, shows larger shoot apical meristem and dwarfism due to an increase in the level of endogenous cytokinin, which results in enhancing cell proliferation (Catterou *et al*., 2002; Howell *et al*., 2003; Huang *et al*., 2015). Furthermore, a reduction in cytokinin levels by overexpression of CK oxidase, involved in CK catabolism, results in a smaller shoot apical meristem, stunted shoot and much fewer leaves in tobacco (Werner *et al*., 2001). A loss of function mutation in the *CYTOKININ RESPONSE 1* gene, encoding a CK receptor in *A. thaliana*, reveals defects in shoot formation in tissue culture experiment (Inoue *et al*., 2001; Howell *et al*., 2003). In rice, a loss of function in the *LONELY GUY* (*LOG*) gene, involved in the last step of cytokinin biosynthesis, established the important role of cytokinin in modulating SAM development also in monocot (Kurakawa *et al* 2007; Zhao *et al*., 2008). The *Oslog* mutants develop smaller meristems because of a reduction in the transcripts of cytokinin-inducible *RESPONSE REGULATOR* genes (Kurakawa *et al* 2007).

Recent findings have also demonstrated that auxin (IAA) and Cytokinin (CKs) appear to co-ordinately regulating cell proliferation by adjusting activity of cyclin-dependent kinase 2 (CDC2). As auxin enhances the expression of CDC2 gene and Ck activating the CDC2 proteins (Swarup *et al.,* 2002).

Gibberllic acids (GAs) also play a key role in shoot development through the regulation of cell elongation in plants. Mutants defective in GA-biosynthesis and signalling have provided evidence of gibberillin contributions to shoot outgrowth in both

dicot and monocot plants (Richards *et al*., 2001). For example, *A. thaliana ga1-3* mutants show dwarfism because of mutation in the *GA1* gene that encodes an *ent*-CDP synthase enzyme, involved in an early step of gibberellin biosynthesis (Koornneef *et al*., 1980; Zeevaart and Talon 1992; Sun and Kamiya 1994). In addition, in rice a mutation in the *SD1* gene that encodes an oxidase enzyme involved in the biosynthesis of gibberellin results in plants with a dwarf phenotype (Sasaki *et al*., 2002). Rice plants with a mutation in the *DWARF1* gene, involved in GA signaling, also show dwarfism (Fujisawa *et al*., 1999; Richards *et al*., 2001). Meanwhile, OsGA20ox2 and OsGA3ox2 catalyze the production of bioactive GA(s) in rice and the loss of function OsGA3ox2 mutants display a severe dwarfism (Davies 2010).

Among several hormones in plants, auxin plays a fundamental role in shoot lateral organ initiation and development, which besides leaves gives rise to flowers in *A. thaliana*, florets and spikelets in grasses such as rice (McSteen 2009). Auxin also modulates cell expansion and cell division in collaboration with other hormones, in which their overlapping activities may act through the auxin responsive transcription factors (Hardtke et al. 2007). Studies on genes involved in auxin biosynthesis illustrate the effects of auxin overproduction on enhancing hypocotyl elongation. For instance, *A. thaliana* gain-of-function *YUC1* mutants have long hypocotyls similar to the phenotypes observed in auxin overproduction mutants such as the *superroot 1* and *2* (*Atsur1* and *Atsur2*) (Zhao *et al*., 2001; Zhao 2008). Loss of function mutants in the *Atsur1* and *Atsur2* genes, acting as C-S lyases in glucosinolate biosynthesis, exhibit high level of endogenous auxin (Boerjan *et al*., 1995; Delarue *et al.,* 1998; Mikkelsen *et al*., 2004). Similarly, the phenotypes of *YUC1* gain of function mutants are caused by auxin

overproduction as auxin-inducible genes and auxin reporter DR5-GUS are found up-regulated (Zhao 2008).

Screening for auxin signaling-defective mutants has also identified genes that function in cell elongation in shoots. For example, mutation in *AtARF7/NPH4* (non-phototropic hypocotyl 4) results in seedlings that do not bend, towards blue light as a consequence of impaired assymetric cell elongation, and defects in growth responses correlated with changes in auxin sensitivity (Harper *et al*., 2000; Wang *et al*., 2007). Another ARF, the *AtARF2* gene, also regulates hypocotyl bending through modulating a linkage between ethylene and auxin signaling pathways. Loss of function *AtARF2* mutants show elongated hypocotyls with enlarged cotyledons under various light conditions (Li *et al*., 2004; Okushima *et al*., 2005).

Tiller and plant height are agronomically important traits in the studies of shoot development in rice. The number of panicles is determined by number of tillers; thus, it affects productivity directly (Hedden 2003). Several lines of evidence demonstrate an important role of auxin and its signalling pathways in shoot development also in monocots such as rice (Xu *et al*., 2005). In rice, studies on mutant defective in auxin signalling have indicated the role of auxin signalling in determining plant height. For instance, a loss-of-function *Osarf1* mutant shows defects in vegetative development, resulting in dwarfism with small curled leaves (Ellis *et al*., 2005). These phenotypes are similar to the phenotypes seen in *arf1 arf2* double mutants in *A. thaliana* (Ellie *et al*., 2005; McSteen 2010). The *OsARF1* gene is an auxin-induced gene closely related to *AtARF1* and *AtARF2* in *A. thaliana* (Waller *et al*., 2002). Similar characteristics to *OsARF1 mutants* have been seen in transgenic plants over-expressing *OsIAA1*, a member of *Aux/IAA* family genes in rice. These plants exhibit distinctive morphological

changes such as dwarfism, suggesting that auxin signaling regulates plant height also in rice (Song *et al*., 2009). Furthermore, OsIAA1 interacts with OsARF1 in protein interaction assays. Recently, the *OsARF3* gene was reported to be involved in the regulation of shoot regeneration (Cheng *et al*., 2013) and the *OsARF19* gene is proposed to control tiller angles in rice (Zhang *et al*., 2015). Distribution of auxin is also found to be important in shoot outgrowth in rice. For example, a mutation in *Osarf16* causes dwarfism, alterations in the root system, reductions in photosynthesis and iron content (Shen *et al*., 2015). The *OsARF16* gene is reported to be included in the regulation of auxin distribution (Wang and Estelle 2014; Shen *et al*., 2015).

1.4.2. Factors involved in tillering and flower development

During inflorescence development in *A. thaliana*, the SAM gives rise to the inflorescence as well as lateral flowers (Long and Barton 2000; Grbic 2005; Reviewed by McSteen 2009). Secondary inflorescences arise from leaf axillary meristems. The development of influrescences in rice differs from *A. thaliana* in several ways. During vegetative development, axillary meristems give rise to tillers in grasses (McSteen *et al*., 2000; Reviewed by McSteen 2009). In rice, a tiller initiates from an axillary bud at the leaf axil, followed by outgrowth at the later stage independently of the main culm (stem). The panicle forms at the tip of each tiller; therefore, the tiller number determines grain yield in rice (Wang and Li 2011). Tillering and grain yield are complex traits regulated by multiple pathways, among which phytohormones play a critical role (Zuo and Li 2013).

The importance of phytohormone functions in the transmission phase from vegetative to reproductive stage has been studied in both dicot and monocot plants. Studies show that alteration in the level of endogenous cytokinin affects flower

development. Cytokinins are hormones that regulate cell division in meristems and are required for axillary meristem development. *A. thaliana* plants over expressing *AtCKX*, a member of the cytokinin oxidase/dehydrogenase family, shows a reduction in the level of endogenous cytokinin, reduced activity of the shoot apical meristem and flower development (Werner *et al*., 2003).

In rice, endogenous cytokinin levels influences panicle formation and therefore regulates grain yield. For example, cytokinin oxidase (OsCKX2/*Gn1a*) regulates rice yield by regulating the levels of cytokinins (Ashikari *et al*., 2005). A mutation in *OsDST*, a regulator of OsCKX2/*Gn1a* involved in the degradation of cytokinins, results in plants with an elevated level of cytokinins and increases grain number (Ashikari *et al*., 2005; Li *et al*., 2013). The *Osdst* mutants also show increased panicles number and decreased panicle length (Huang *et al*., 2009), indicating a wide range of physiological functions of the *OsDST* gene. The *LONELY GUY* (*LOG*) gene, encoding an cytokinin-activating enzyme, acts at the final step of cytokinin biosynthesis in rice. Mutation in the *Oslog* gene reduces the size of axillary meristem and consequently shows a severe reduction on the length of panicle and a decrease in the number of floral organ (Kurakawa *et al*., 2007).

The role of auxin biosynthesis, transport and signalling in inflorescence development and flower initiation has been highlighted in several studies of dicot and monocot plants. For example, in *A. thaliana*, mutations defective in auxin biosynthesis cause defect in flower initiation, characterized by pin-shaped inflorescences (Bennett *et al*., 1995; Przemeck *et al*., 1996; Cheng *et al*., 2006). Double mutation in *A. thaliana* *YUC1* and *YUC4* genes produces sterile flowers, and the *yuc2 yuc6* double mutants are defective in stamen development (Cheng *et al*., 2006), indicating a role of auxin

biosynthesis in flower development. Auxin biosynthesis also affects development of axillary shoots, or tillers in monocot plant such as rice and maize. For instance, an increase in the activity of the *OsYUC1* gene results in fewer tillers (Yamamoto *et al*., 2007). A similar phenotype, known as the barren inflorescence is also reported in the *spi1* mutants in maize due to defects in auxin biosynthesis (Gallavotti *et al*., 2008; Barazesh *et al*., 2009; McSteen 2010), suggesting that auxin biosynthesis is essential for inflorescence/tiller formation also in monocots. The *sparse inflorescence1* (*spi1*) mutants, a gene encodes a monocot-specific *YUC* gene family member required for auxin biosynthesis in maize, show fewer branches and subsequently fewer spikelet (Gallavotti *et al*., 2008). The *Zmspi1* phenotypes are more severe than the single *yuc* mutants of *A. thaliana*, indicating higher level of redundant function among the *YUC* gene family in *A. thaliana* (Cheng *et al*., 2006).

Auxin transport also regulates inflorescence development. For example, in *A. thaliana,* loss-of-function *pin1* mutants show a single pin-shaped stem with no flowers due to a reduction in auxin transport (Okada *et al.,* 1991). In support of this conclusion, auxin transport-inhibited tomato plants develop leaf-less stems, and application of auxin at the flank of the SAM results in the development of a leaf (Reinhardt *et al*., 2000). *Pin*-shaped inflorescence phenotype is also reported in the *pinoid* (*pid*) mutants*,* involved in the localization of PIN1 proteins in *A. thaliana* (Christensen et al., 2000; Friml et al., 2004; Michniewicz et al., 2007). The *PIN1* loci, involved in auxin transportation and distribution, has also been identified in maize and rice (Xu *et al*., 2005; Gallavotti *et al*., 2008). The maize *ZmPIN1a* rescues the *A. thaliana pin1* mutant, re-establishing its ability to make flowers, indicating homologous protein functions (Gallavotti et al., 2008). The *barren inflorescence2* (*bif2*) mutants, a gene that encodes a Ser/Thr protein kinase

that phosphorylates and affects ZmPIN1a protein localization, show similar phenotypes to the *pin* mutants of *A. thaliana* such as fewer branches and spikelets (McSteen and Hake 2001; McSteen *et al*., 2007). In addition, the *Ospin* antisense knockdown mutants support the conserved function of *PIN* genes as auxin transport regulators in rice (Xu et al., 2005). Further evidence on the role of auxin transport and distribution on rice tiller and panicle development is provided by functional analysis of the *OsPIN2* gene (Reviewed by Lu *et al*., 2015). Over-expression of the *OsPIN2* gene increases auxin transport capacities, resulting in an increase in tiller number with smaller panicles compared with the wild type plants (Chen et al., 2012). In addition, studies on the *OsPIN5b* gene, another auxin carrier in rice and potential homolog of *A. thaliana PIN5* gene, have suggested that the *OsPIN5b* gene functions in auxin transport and distribution with the implication of negatively regulating tiller formation and grain yield in rice (Lu *et al*., 2015). For instance, transgenic plants over-expressing the *OsPIN5b* gene show fewer tillers compared to wild type plants. In addition, pollen maturation is also defective, which explains reduced grain yield in the *OsPIN5b* transgenic plants. Down-regulation of the *OsPIN5b* gene, on the other hand, results in an increase in tiller number and an increased grain yield (Lu *et al*., 2015).

Several lines of evidence support the regulatory role of auxin response factors (ARFs genes) in inflorescence and flower development in *A. thaliana* and rice. For example, *A. thaliana arf2* mutants exhibit several developmental defects including thick inflorescence, delayed flowering and sterility in early flowers (Wang *et al*., 2007). Loss of function *ettin/arf3* mutants exhibits abnormal flowers with reduced number of stamen (Sessions *et al*., 1997; Nemhauser *et al*., 2000; Sato *et al*., 2001). Based on the rice and *A. thaliana* ARF proteins phylogeny analysis, the *OsETTIN1* and *OsETTIN2* genes of

rice are expected to have a similar function with the *ETTIN/ARF3* gene of *A. thaliana* (Sato *et al*., 2001)*.* In contrast, the *Osettin1* single mutation did not cause any defects in tiller and panicle formation, suggesting the *OsETTIN* genes act redundantly in rice (Sato *et al*., 2001).

The role of *ARF* genes in controlling growth and development of floral organs in dicots and monocots has been further investigated through studies on miRNA167 (Liu *et al*., 2014; reviewed by Li *et al*., 2016). In *A. thaliana*, miRNA167 has been found to regulate the expression of the *ARF6* and *ARF8* genes that in turn affect fertility (Ru *et al*., 2006; Wu *et al*., 2006). In rice, miRNA167 also plays a crucial role by negatively regulating the expression of the *OsARF6*, *OsARF12*, *OsARF17* and *OsARF25* genes involved in auxin signaling (Liu *et al*., 2012). Over-expression of miRNA167 in rice leads to down-regulation of four targeted-*ARF* genes expression, reducing the number of tillers and grain yield (Liu *et al*., 2012).

Grain production is a complex process of cell division and expansion occurring in a coordinated manner. Auxin signalling plays a crucial role in this process in dicotyledonous plant species. For example, in *A. thaliana*, a mutation in the *ARF8/FRUIT WITHOUT FERTILIZATION* (*FWF*) gene results in the formation of infertile flower, indicating the *ARF8* gene functions in seed development (Vivian-Smith *et al*., 2001; Goetz *et al*., 2006). Although mechanisms underlying seed formation have been studied in dicotyledonous plants, the mechanisms behind the formation of spikelet and grain in monocotyledonous plants are far from understood (Uchiumi and Okamoto 2010). In monocots, it is proposed that an increase in endogenous auxin level induces spikelet formation in a process that involves several *ARF* and *Aux/IAA* genes (Wang *et al*., 2005; Goetz *et al*., 2007; Uchiumi and Okamoto 2010). For example, the *OsIAA18* gene, a

transcriptional repressor regulates spikelet initiation by repressing auxin-responsive genes (*OsARFs*) required for spikelet development in rice (Woodward and Bartel 2005; Wang *et al*., 2005). In contrast, the *OsARF6* gene is a negative regulator of spikelet formation in rice (Uchiumi and Okamoto 2010).

Auxin transport is also involved in the regulation of seed development in rice. Grain yield in rice is determined by components such as number of grain per panicle and grain weight (Xing and Zhang 2010). Recently it has been reported that the *PLANT ARCHITECTURE AND YIELD 1* (*PAY1*) gene in rice controls grain yield through modulating polar auxin transport and distribution (Zhao *et al*., 2015). *PAY1* gene over-expressing plants show an increase in number of grain per panicle (Zhao et al., 2015). A similar effect of auxin transport contribution to seed development has been reported in other studies of the *OsCYP19-4* gene of rice. For example, over-expression of the *OsCYP19-4* gene, a putative homologue of *A. thaliana CYP19-4* gene involved in auxin polar transport in *A. thaliana*, results in an increase in grain weight (Michniewicz *et al*., 2007; Yoon *et al*., 2016).

1.4.3.   Factors involved in root growth

The plant root is responsible for nutrient and water acquisition, thus it is an important factor in determining traits previously discussed such as grain yield (Lu *et al*., 2015). Primary roots, lateral roots, and adventitious roots (also known as crown roots or secondary roots) comprise the root system in rice (Inukai *et al*., 2005). The formation of adventitious roots initiated from the stem, is a common feature among grasses (Reviewed by Zuo and Li 2013). Phytohormone plays a crucial role in the establishment

of root system including the initiation and elongation of primary and the development of adventitious and lateral roots in plants (Wu and Cheng 2014).

Cytokinin is found to act in the regulation of root system development through negatively modulating cell division in root apical meristems (RAMs) and is also known as a negative regulator of adventitious root initiation and lateral root formation (Mai *et al*., 2014). For example, exogenous application of cytokinin decreases primary root elongation by reducing the size of the root meristem (Beemster and Baskin 2000; Werner *et al*., 2003). Furthermore, exogenous application of cytokinin reduces the expression of the *PIN* genes and inhibits auxin flow, resulting in auxin maxima needed for lateral root initiation in *A. thaliana* (Laplaze *et al*., 2007; Kitumi *et al*., 2011). In contrast, mutants defective in cytokinin biosynthesis and signalling show a larger root meristem in *A. thaliana* (Dello *et al*., 2007). In rice, the *OsWOX11* gene, a *WUSCHEL*-related homeobox gene, is involved in the CK-regulated growth of crown root, providing evidence of the role of cytokinin also in monocot root development (Zhao *et al*., 2009; Wu and Cheng 2014). The *OsWOX11* gene is suggested to directly repress the *OsRR2* gene, a negative regulator of cytokinin signaling in rice, resulting in controlling cell proliferation during crown root development (Zhao *et al*., 2009).

Another hormone, auxin, is the most studied hormone in the regulation of root growth in plant species (Reviewed by Swarup *et al.,* 2002). Auxin acts as an accelerator of cell division in the root meristem, regulated by the concentration of endogenous IAA (Reviewed by Tanimoto 2005). For instance, exogenous application of auxin increases the size of the root meristem (Chapman and Estelle 2009). In contrast, size of the root meristem is reduced in auxin transport defective mutants (Dello *et al*., 2007; Moubayidin *et al*., 2009). In spite of the differences in the structure of root system in monocots and

dicots, recent studies indicate that auxin acts similarly in root development in both rice and *A. thaliana* (Hochholdinger and Zimmermann 2008).

Studies on mutants defective in auxin biosynthesis, transport and signalling support the role of auxin in the development of primary roots, adventitious roots and lateral roots in dicots and monocots. For instance, multiple mutations in the *YUC* genes indicate the importance of auxin biosynthesis in root growth in *A. thaliana*. The *yuc1 yuc4 yuc10 yuc11* quadruple mutants did not form a hypocotyl and root system (Cheng *et al*., 2007). In rice, over-expression of the *OsYUC1* gene, required for auxin biosynthesis, increases the number of lateral and crown roots in transgenic plants (Yamamoto *et al*., 2007; McSteen 2010).

PIN-dependent auxin transport mechanisms also act in root development (Reviewed by Teale *et al*., 2006). For instance, the *PIN* genes *PIN1*, *PIN2*, *PIN3* and *PIN4* act in root growth, lateral root initiation, and root gravitropism in *A. thaliana* plants (Friml *et al*., 2002; Teale *et al*., 2006). Localization of PIN proteins during this processes determine the direction of auxin flow (Wisniewska *et al*., 2006). For example, loss-of-function *PINOID* (*PID*) mutants, controlling *PIN* protein localization, results in reduced auxin concentration in the root meristem and subsequently it decreases RAM maintenance in *A. thaliana* (Christensen *et al*., 2000; Friml *et al*., 2004).

A role of polar auxin transport in the development of the root has also been reported in rice. For example, an application of polar auxin transport inhibitor (NPA) in root collars in wild type rice causes defects in crown and lateral root initiation and outgrowth (Zhoa *et al*., 2003; Xu *et al*., 2005; McSteen 2010). In addition, the *OsPIN1*, *OsPIN2* and *OsPIN3* genes contribute to crown root formation (Xu *et al*., 2005; Wang *et*

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al., 2009; Zhang et al., 2012). For instance, loss of function OsPIN1 mutants, an auxin efflux carrier and a homolog of A. thaliana PIN1 gene, results in fewer crown roots (Xu et al., 2005), similar to the phenotype observed in NPA treated rice plants (Xu et al., 2005). The OsAUX1 gene modulates lateral root initiation and growth through mediating polar auxin transport in rice (Yu et al., 2015; Zhao et al., 2015). Osaux1 mutants form shorter lateral roots due to a decreased in auxin transport, providing further evidence of the role of auxin transport in root development in rice (Yu et al., 2015).

Several lines of evidence also support the function of genes involved in auxin signaling in root growth of dicot and monocot plants (Teale et al., 2006). Studies on mutant defective in auxin signalling demonstrate the effects of auxin response factors (ARFs) in lateral root formation in A. thaliana (Reviewed by Mockaitis and Estelle 2008). For instance, the MONOPTEROS (MP)/ARF5 gene, an auxin response factor, plays an important role in the root formation (Hardtke and Berleth1998; Weijers et al., 2006). The mp/arf5 mutants show severe defects in embryonic root formation, which results in a rootless phenotype (Berleth and Jurgens 1993). Loss of function in AUXIN RESISTANT6 (AXR6), a member of the cullin/CDC53 family proteins and a subunit of the SCF ubiquitin ligase (Gray et al., 1999), displays a similar characteristic to that of mp mutants in A. thaliana (Hobbie et al., 2000). In addition, the A. thaliana ARF19 and ARF7 genes act redundantly in theformation of lateral roots (Okushima et al., 2005; Wilmoth et al., 2005; reviewed by Teale et al., 2006; Wang et al., 2007). For example, double mutation in the ARF7 and ARF19 genes reduce lateral root initiation (Okushima et al., 2007; reviewed by Mockaitis and Estelle 2008). The arf7 arf19 double mutants generate more severe auxin-related phenotypes than in the arf7 and arf19 single mutants (Okushima et al., 2007).

The role of auxin response factors in root establishment and growth has also been investigated in monocot such as rice. For example, the *OsARF1* gene, an ortholog of the *ARF1* gene in *A. thaliana*, is involved in auxin-mediated lateral root development (Attia *et* al., 2009; Smet *et al*., 2010 and 2011). Moreover, the *OsARF12* gene regulates primary root elongation (Wang *et al*., 2014). Also, loss-of-function *OsARF16* gene mutants display reduced lateral root formation (Shen *et al*., 2015).

Several lines of evidence support the role of miRNAs in mediating auxin signalling by restraining specific *ARFs* at the post-transcriptional level (Wu *et al*., 2006), subsequently controls plant developmental processes. In *A. thaliana*, the miR160 regulates the expression of the *ARF10*, *ARF16* and *ARF17* genes, involved in root development (Mallory *et al*. 2005; Wang *et al*. 2005). In addition, miR390 affect lateral root formation, a process modulated by the *ARF2*, *ARF3* and *ARF4* genes of *A. thaliana* (Williams *et al*. 2005; Yoon *et al*. 2010). miRNAs appear to regulate root development through regulating auxin response factors in rice (Liu *et al*., 2012). For instance, the miR167 is identified to regulate the *OsARF6* and *OsARF12* genes that regulate root growth (Qi *et al*., 2012; Liu *et al*., 2012).

Another interesting example of ARF-mediated root development has been reported in the study of the *CROWN ROOTLESS* 1 gene in rice. A loss of function mutation in the *CROWN ROOTLESS* 1 (*CRL1*) gene, modulated by *ARF* genes (Reviewed by Mai *et al*., 2014), shows a reduction in number of lateral roots (Inukai *et al*., 2005). The *crl4* mutants, also regulated by *ARF* genes, show defects in crown root formation (Kitomi et al., 2011).

1.4.4. Factors involved in leaf formation and vasculature

The plant vascular system is a network of interconnected cells that distributes water, minerals and photosynthesis assimilates throughout the plant. It is mainly comprised of interconnecting veins consisting of two main conducting tissue types, xylem and phloem, both of which generate vascular bundles (Taiz and Zeiger 2015).

Monocots and dicots show differences in terms of vascular ontogeny (Scarpella *et al.,* 2002). Venation patterns are divided into two wide categories: reticulate venation in dicots and parallel or striate venation in monocots (Nelson and Dengler 1997; Taiz and Zeiger 2015). In dicots, the stem vasculature extends into the leaf primordia and initiates the primary vein. Following that, secondary veins branch from the mid-vein to the margin of the leaf and connect with previously formed secondary veins (Aloni *et* al., 2003; Mattsson *et al.,* 2003; Reviewed by Scarpella and Helariutta 2010). There is also a third class of veins called tertiary veins that are accompanied by free-end veins within the loops (Nelson and Dengler 1997; Reviewed by Scarpella and Helariutta 2010). On the other hand, monocots show a different pattern of vein formation. Vasculature is comprised primarily of longitudinal veins. The mid-vein is the thickest vein and other smaller, secondary and tertiary longitudinal veins run parallel with the mid-vein throughout the leaf blade (Scarpella *et al*., 2002; Reviewed by Scarpella and Helariutta 2010). In addition, commissural veins connect adjacent longitudinal veins (Nelson and Dengler 1997; Howell 1998; Scarpella *et al*., 2002).

Similar to the developmental processes of plant organs, vascular development depends on many regulatory mechanisms, including hormone biosynthesis and signalling pathways. For example, it is reported that an exogenous application of

cytokinin in combination with auxin and gibberellin can result in ectopic formation of vascular cells (Aloni 1987). Studies on cytokinin-deficient plants have also shown its regulatory role in the regulation of cell proliferation and transition from undifferentiated cells to differentiated cells in the SAM (Werner *et al*., 2003). For instance, over-expression of the *AtCKX* gene, a member of the cytokinin oxidase/dehydrogenase in *A. thaliana*, shows a reduced cytokinin level and subsequently a reduction in cell division in the SAM, which results in decreasing the width of leaves (Werner *et al*., 2003). Furthermore evidence of the role of cytokinin in plant vasculature is provided by the loss-of-function *WOODEN LEG/CYTOKININ RESPONSE1* (*WOL/CRE1*) gene, which encodes a cytokinin receptor and is expressed in the procambium of the embryonic axis (Mähönen *et al*., 2000; Nishimura *et al*., 2004; Riefler *et al*., 2006). In *A. thaliana*, a mutation in the *wooden leg* (*wol*) gene causes impaired cell division in the procambium cells between xylem and phloem and consequently forms aberrant vascular formation (Scheres *et al*., 1995; Mähönen *et al*., 2000; reviewed by Scarpella *et al*., 2002). Moreover, multiple mutations in kinase-encoding genes (*AHKs*), involved in cytokinin signal transduction, reveal a phenotype of small and deformed rosette leaves with few veins (Nishimura *et al*., 2004).

Auxin can, under certain circumstances, induce trans-differentiation of cortex and mesophyll cells into vessel elements (Jacobs 1952). This trans-differentiation process serves multiple purposes in plants, including regeneration of severed vascular bundles after wounding (Jacobs 1952). Auxin also plays an important role in axillary meristem initiation (Woodward and Bartel 2005). In *A. thaliana*, during vegetative stage axillary meristems form leaf primordia at the sites of auxin maxima, in which high concentration of auxin influences the position of newly formed primordia (Reinhardt *et al*., 2003;

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Reviewed by McSteen 2009). In rice; however, axillary meristems grow out to produce tillers (Shimamoto and Kyozuka 2002).

Among plant hormones, auxin has frequently been reported to influence vascular development and patterning (Aloni *et al*., 2003; Fukuda 2004; Scarpella and Meijer 2004; Teale *et al*., 2006). Auxin is synthesised predominantly in young apical regions, such as leaf primordia and floral buds (Ljung *et al*., 2001). Discovery of auxin sources and auxin-related response genes such as *PIN1*, *MONOPTEROS* (*MP*), *BODENLOS* (*BDL*) and *AUXIN-RESISTANT6* (*AXR6*) provide strong evidence of roles of auxin signalling and transport in vein pattern formation (Sachs 1981; Mattsson *et al*., 1999; Mattsson *et al*., 2003; Berleth *et al*., 2000; Scarpella *et al*., 2006; reviewed by Aloni 2010). Although monocots are found to differently respond to exogenous auxin than dicots, most likely due to rapid degradation of exogenous auxin (Gauvrit and Gaillardon 1991; Reviewed by Fukuda 1997) and/or monocot's altered auxin perception (Kelley and Reichers 2007), the mechanisms of auxin biosynthesis, transport and signal transduction are conserved in both species (Zhao *et al*., 2003 and 2013).

Alterations in endogenous auxin levels in transgenic plants display severe effects on vascular development. For example, the reduction of auxin levels in transgenic tobacco, caused by over-expressing the *IAAL* gene that converts active IAA to an inactive form, decreases vascular strands formation (Romano *et al*., 1991; Howell 1998). Furthermore, YUC flavin monooxygenases, key enzymes in auxin biosynthesis, redundantly regulate vascular patterning (Zhao 2008). For instance, inactivation of single *YUC* gene results in no obvious defects in vascular development in *A. thaliana*. In contrast, *yuc1 yuc4* double mutants show strong defects in leaf vascular formation (Cheng *et al*., 2006). Furthermore, inactivation of the *YUC2* or *YUC6* in the *yuc1 yuc4*

double mutant background enhances the defects of *yuc1 yuc4* (Cheng *et al*., 2006).

Studies on vascular defective mutants have helped identify auxin-mediated response genes involved in the development of vasculature in both dicot and monocot. For instance, mutations in the *MONOPTEROS/ARF5* (*MP/ARF5*), *BODENLOS* (*BDL*) genes have been associated with altered vascular patterning caused by auxin signalling defects (Fig. 1.3), suggesting the importance of auxin perceptions in vascular development (Berleth and Jurgens 1993; Przemeck *et al*., 1996; Hamann *et al*., 2002; Weijers *et al*., 2005; Hardtke and Berleth 1998; Mattsson *et al*., 1999; Mattsson *et al*., 2003; Hardtke *et al*., 2004). *mp/arf5* mutants have reduced auxin sensitivity, showing that mutants are severely defective in auxin signalling (Mattsson *et al*., 2003). Gain-of-function mutation of the *BODENLOS* (*BDL*)/*IAA12* gene, encoding a member of the *Aux/IAA* family (*IAA12*) and an inhibitor of *MP/ARF5* gene transcriptional activity in *A. thaliana* (Hamann *et al*., 2002), results in defects in vascular development similar to that of *mp/arf5* mutants. The resulting effect is that enhanced expression of the *BDL* gene causes an *mp*-like phenotype (Hamann *et al*., 2002; Weijers *et al*., 2005). These observations support the impact of auxin responses on the development of vascular system.

WT   mp
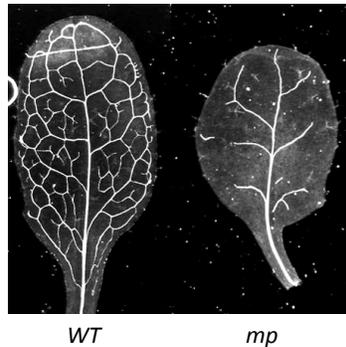
Figure 1.3. The *monopteros (mp)* mutant has reduced venation compared to wild type (WT) plants. Pictures from Jim Mattsson.

Recently it was reported that a gain-of-function delpetion of the domain III and IV in *MONOPTEROS*/*ARF5* (named *MPΔ*) perturbs the interaction between ARF and Aux/IAA proteins similar to the effect occurring in response to auxin in *A. thaliana* (Krogan *et al*., 2012). Although this *MPΔ* gain-of-function construct rescues *mp/arf5* loss-of-function defects such as re-establishing root formation in embryo and restoring flower fertility, the *MPΔ* mutants show narrow and pointed leaves with increased parallel vein formation at the center of leaf (Fig 1.4) (Krogan *et al*., 2012), indicating that domains III and IV are needed for normal MP/ARF5 function.

A similar effect has previously been reported in *A. thaliana* treated with auxin transport inhibitors (NPA) (Mattsson *et al*., 1999). It is postulated that in *MPΔ*, Aux/IAA proteins are unable to down-regulate the *MP/ARF5* gene activity, required for restricting the *PIN1* gene expression to narrow down to the position of vascular strands (Hardtke *et al.,* 2004; Scarpella *et al*., 2006; Wenzel *et al*., 2007). Thus, the resulting effect may be

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due to the stronger and wider expression of the *PIN1* in *MP∆* mutants that leads to expanded cell differentiation into the vascular strands (Scarpella *et* al., 2006; Scarpella and Helariutta 2010; Krogan *et al*., 2012). Auxin-mediated vascular patterning may be modulated in a similar manner in dicots and monocots (Scarpella *et al*., 2002; Qi *et al*., 2008). For example, the *RADICLELESS1* (*OsRAL1*) gene functions at the early stage of the vascular formation in rice (Scarpella *et al*., 2003). *Osral1* mutants show defects in vascular development such as reduced number of veins and a reduction in the distance between longitudinal veins due to defects in auxin response (Scarpella *et al*., 2003).
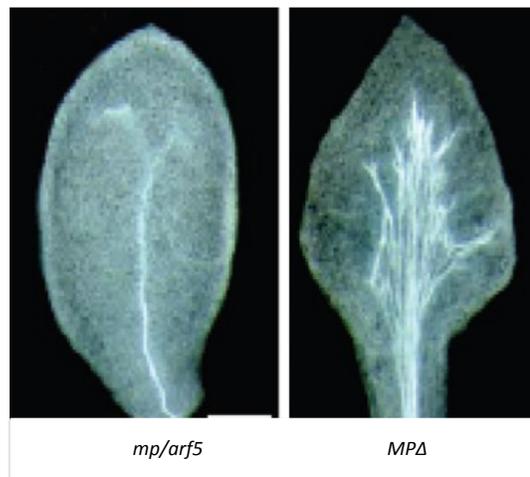

mp/arf5   MP∆

Fig 1.4. Vascular patterns in *A. thaliana* cotyledons. *MP∆* mutants develop more veins in the center of cotyledon than *mp/arf5* mutants. Permission to reprint is provided by John Wiley and Sons (License number 3896750140703).

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There is evidence that *MP*/ARF5 regulates in part *PIN1* gene expression (Wenzel *et al*., 2007; Schuetz *et al*., 2008). *mp/arf5 pin1* double mutants are unable to form leaves and instead develop a dome-shaped SAM (Schuetz *et al*., 2008). This synergistic effect was attributed to a complete loss of auxin maximum formation at the flanks of the SAM, which in turn is required for leaf formation (Schuetz *et al*., 2008).

Auxin distribution regulated by *PIN*-mediated auxin transport is required for the establishment of vein patterning in *A. thaliana* (Mattsson *et al*., 2003; Scarpella *et al*., 2006; Wenzel *et al*., 2007). For example, application of the N-1-naphthylphthalamic acid (NPA), an auxin transport inhibitor, results in wider midveins, more secondary veins, and extensive vascularization at the leaf margin (Mattsson et al., 1999; Sieburth 1999). The phenotype from low levels of NPA exposure is similar to that of *pin1* (Mattsson *et al*., 1999). These findings support the role of polar auxin transport in vascular tissue differentiation, vascular strand location in leaves.

In addition, studies of auxin transport-mediated vascular development also support the role of PAT in vascular pattern establishment in monocots. For example, the analysis of the expression pattern of the *ZmPIN1a* gene, a homolog of the *AtPIN1* gene, shows similar pattern to *AtPIN1* in maize, suggesting having a similar function in maize as in *A. thaliana* (Carraro *et al*., 2006; Lee *et al*., 2009). The supporting evidence is derived from an experiment in which the *Atpin1* mutant phenotypes can be rescued by introducing the *ZmPIN1a* gene (Gallavottie *et al*., 2008; reviewed by McSteen 2010). In rice, the *OsPIN1* has been suggested to act as an auxin efflux facilitator in rice similar to that of *PIN1* in *A. thaliana* (Xu *et al*., 2005). Unlike *ZmPIN1a*, *Ospin1* mutants have not revealed *pin1* specific mutant phenotypes, most likely due to redundant function among *OsPIN* gene family (Xu *et al*., 2005).

The size and shape of the leaf is also an important agronomic trait in crop plants. Anatomical studies revealed a constant relationship between the width of leaf blade and the number of longitudinal veins in monocots (Reviewed by Nelson and Dengler 1997). For example, mutation in the *NARROW LEAF1* (*NAL1*) gene, associated with auxin transport-mediated vascular patterning and leaf development in rice, exhibit a reduction in the width of leaf blade with decreased number of longitudinal veins (Qi *et al*., 2008; Reviewed by Lu *et al*., 2015). Meanwhile, it has recently been shown that over-expression of *THE NARROW LEAF* 2 and 3 (*NAL2*/3) genes of rice results in more expanded leaves and increased number of longitudinal veins (Ishiwata *et al*., 2013).

1.5. Research hypotheses and objectives

A large number of candidate genes that are potentially regulated by auxin and that may function in growth and developmental processes have been identified (Reviewed by Liu *et al*., 2014). Among these genes, members of the auxin response factors (ARFs) play a key role in auxin-mediated regulation of development and the control of the transcriptional activity of downstream genes during these processes in *A. thaliana* (Berleth and Jurgens 1993; Przemeck *et al*., 1996; Hardtke and Berleth *et al*., 1998; Mattsson *et al*., 1999; Scarpella *et al*., 2006; Wenzel *et al*., 2007). While our understanding of ARF proteins regulatory function, in particular that of *MP/ARF5*, and their role in growth and development has been increasingly improved in *A. thaliana*, little is known about the role of auxin signalling in monocots. Although bioinformatic analysis has helped to identify candidate genes associated auxin signalling and the regulatory effects on the establishment of monocot growth (Zhao *et al*., 2005; Gallavottie *et al*., 2008; Barbez *et al*., 2012; Ding *et al*., 2012; reviewed by McSteen 2010; Lu *et al*., 2015),

nothing has yet been revealed about the role of the *OsARF11* gene, a potential orthologue of *A. thaliana ARF5* (*MONOPTEROS*), in monocots.

The central objective of my work is to investigate whether the MP/ARF5-like OsARF11 also plays a central role in divergent processes of growth and development in rice, a monocot model species and an important food source. Therefore, my hypothesis is that *OsARF11* plays a role in developmental processes in rice such as vascular differentiation. I have tested my hypothesis by evaluating the phenotype of *Osarf11* loss-of-function mutants. Briefly, I have found that the rice *OsARF11* gene plays a role in the regulation of plant height, root growth, regulation of leaf vein density and seed fecundity in rice.

2. Materials and Methods

2.1. Plant materials and growth

Two mutants in the *OsARF11* gene (Accession no. AL606999) were obtained, one from TRIM database carrying T-DNA insertion (hereafter referred to as *OsARF11^{TRIM}*, M0030446) (Wang *et al*., 2007), and the other from TOS-17 database carrying Transposon insertion (hereafter referred to as *OsARF11^{Tos-17}*, NC2659) (Hirochika 2001). Both mutants are in the genetic background of Nipponbare (Hirochika 2001; Wang *et al*., 2007).

First the coat was removed from all seeds and seeds were sterilized in bleach solution (3% bleach + 0.05% Tween 20), immersed for more than 3 hours on shaker. Then, seeds were washed thoroughly with autoclaved water three times and were plated in jars with 1/2 MS. Seeds were incubated at ~28 degrees Celsius under 16/8 hour light/dark in growth chamber with high humidity (98%) for about ten days. 10 days old seedlings were floated on Styrofoam pieces with the roots in a fertilized water solution (20-20-20NPK, 2 g/L) for about a week and later planted in pots containing granulated clay (profile, anufactured by Rigby Taylor) according to Moulton *et al*. (2012). Plants were fertilized twice per week alternating between (20-20-20NPK, 2 g/L) and (20-0-0NPK, 0.2 g/L) and held in a growth chamber with 95-99% relative humidity under 14/10 hour light/dark at 28-32 °C as described (Moulton *et al*., 2008 and 2012).

2.2. Genotyping assays

DNA was extracted from 14 days old rice seedlings, using Plant Genomic DNA Purification reagent kit (Invitrogen). Genotyping assays were performed by the Polymerase Chain Reaction (PCR), using primer combination sets that were specific for wild type and mutant alleles to identify homozygous and heterozygous mutant plants. Homozygous mutants were identified for $OsARF11^{TRIM}$ rice using T-DNA primer; 5'-tcgcgatccagactgaatgc-3' in combination with $OsARF11$ forward primer; 5'-ggggactcccaagggtttga-3'. To double-check insertions, we used Hygromycin primers; 5'-gtctgctgctccatacaagc-3' and 5'- tgtcacgttgcaagacctgc-3' (Fig 3.1). Genotyping of Tos-17 transposon insertion allele of $OsARF11^{Tos-17}$ was done by using $OsARF11^{Tos-17}$ forward primer; 5'-cagaaatattcagtggggtg-3' paired with tail6 transposon specific primer; 5'-aggttgcaagttagttaaga-3'. The $OsPIN1$ wild type forward and reverse primers; 5'-ttctccctggcgacaatgct-3' and 5'-aaccatcatccgtcctattcaattc-3' were used as positive controls (Fig 3.2). Sanger sequencing confirmed the predicted identity of Amplified-PCR products.

2.3. Quantitative real time PCR (q-PCR)

RNA was extracted using Plant RNA Purification reagent kit (Invitrogen). One μg RNA, treated with DNAase (1 unit; Fermentase), was used to synthesize cDNA, using superscript III (Invitrogen) reverse transcriptase. The qPCR was set up according to the manufacturers' protocol (BIO-RAD and Roche) and SYBR Green master mix, in a volume of 20 ul. The qPCR amplification was performed on three biological replicates and three technical replicates for each biological replicate. $OsARF11^{TRIM}$ expression was analyzed using the primers designed for upstream region; 5'-gtgacatttggtcgatggtg-3' and

5'-tgtgtttctcccatgagctg-3', insertion site; 5'-agcaaggtacaacaaggcaa-3' and 5'-tcacacgctcaggtctttct-3', downstream region; 5'-gaaggccacaagagaaatgc-3' and 5'-ggaaatgcatcaaagcatga-3'. *OsARF11*[TOS-17] expression was analyzed using the primers designed for insertion site; 5'-acaacatcaccttgcatgcc-3' and 5'-ggaggttgcattgagtaatcca-3', downstream region; 5'-tggcatgcatgttttgaacg-3' and 5'-cttcttgcaaaagatgagcctt-3'.

The expression of *OsARF11* in both allelic mutants was normalized against the *Actin1* (*ACT1*) housekeeping gene using forward and reverse primers; 5'-ggtcttaauccattggtgctgagcgttt-3' and 5'-ggcattaaucgcagcttccattcctatgaa-3'. Relative gene expression was calculated using Cq values for Roche as described by Roche Life Science, and ΔΔCT method for BIO-RAD as described by *Livak and Schmittgen* (2001).

2.4. Venation pattern, height and number of seeds

Sections of second, third and forth leaves of two-three weeks old seedling were collected as well as first-leaf of first tiller and first-leaf of third tiller collected during tillering stage. Sections were chosen from the widest part of the leaf referred to as middle part. All sections were fixed at room temperature overnight in 100% ethanol:acetic acid (6:1, v/v). They were then washed once in 100% ethanol and again in 70% (v/v) ethanol followed by clearing in 100% lactic acid at 95 °C for three hours. All sections were mounted in lactic acid and observed using dark field microscopy.

The width of leaf and mid-vein was measured, using a Varnier caliper ruler, and number of veins were counted for both allelic mutants and wild type. Plant height and seed number were also recorded. Photographs were taken using a Canon digital EOS-1 5D camera attached to a Nikon Eclipse 600 microscope.

2.5. Root growth analysis

Wild type and homozygous mutant plants were germinated on ½ MS media for 4-5 days and later grown in hydroponic condition as described above in the growth chamber with 99% high humidity. We measured the length of the primary roots using a Varnier caliper, and the number of secondary roots and lateral roots after 10 days of growth in liquid media.

2.6. Vector construction

The complete protein-coding region was PCR amplified from wild type cDNA (Nipponbare), using primers 5'-caggctgaggtcttatgcagctgttcttgggtttc-3' and 5'-aatgctgaggcattatgtgcatttctcttgtggcc-3' for *OsARF11*. primers 5'-caggctgaggtcttactccctttccttgccttcct-3' and 5'-aatgctgaggcattatcactcttggctgttcaggt-3' for *OsARF4* and 5'-caggctgaggtcttaaatgtgttcgctgagttccg-3' and 5'-aatgctgaggcattacaccctctaatgcgcttgtc-3' for *OsARF15*. The underlined nucleotides of primers overlap with the cloning site in the pUCE/UBI:USER:NOS plasmid (Hebelstrup *et al*., 2010), in which the amplicons were cloned by In-Fusion technology as described by the provider (Clontech). Cloning mixes were transformed into One Shot Mach1™-T1^R chemically competent E.Coli cells (Invitrogen), as per provided instruction and plated on LB solid media containing 50μg/ml Spectinomycin. Purified plasmids were sent for Sanger confirmatory sequencing, using plasmid-specific primers 5'-gccctgccttcatacgctat-3' and 5'- agtctagaggagcatgcgac-3'.

The following internal primers were used to obtain sequence of the complete cDNA sequences: *OsARF11*; 5'- ttccaaattcccgcattccg-3' and 5'-ctcggttgcatccatactgc-3', *OsARF4*; 5'- cttccgtcacatctttcgcg-3' and 5'- ggtcgtctccaacaagcatc-3', *OsARF15*; 5'-ctcgcttgtcccggagaa-3' and 5'- gaccaggggagtgagacttc-3'.

2.7. Statistical analysis

All statistical analyses were performed from mean values using 6 to 14 plants per experiment and standard errors were calculated by Student's t-tests, using JMP 12 software (SAS institute Inc.). Graphs were created using Microsoft Excel.

3. Results

3.1. Identification of homozygous *Osarf11* mutants among two independent mutants population

Evaluation of phylogenetic relationship of rice and *A. thaliana* ARF proteins by Wang *et al*., (2007), also known as *AtARF5*/*MP* and *OsARF11* form a separate clade based on 99% amino acid sequence similarity (Fig 1.2).

We obtained two independent insertion mutants in the OsARF11 gene (see materials and methods). To confirm the insertion point in *OsARF11* indicated in the TRIM database (Hsing *et al.,* 2007), we amplified and sequenced a fragment spanning the insertion point (Fig 3.1). We also confirmed a second *OsARF11* mutant allele in a TOS17 transposon tagged population (Hereafter referred to as *OsARF11*[Tos-17] (Miyao *et al.,* 2003; http://tos.nias.affrc.go.jp/), with an insertion in the fifth exon (Fig 3.2).

We only observed mutant phenotypes in plants genotyped as being homozygous for mutant alleles (T-DNA and transposon insertion). We did not observe any obvious morphological defects in heterozygous mutants, indicating that this state does not result in semi-dominance or haploinsufficiency. Homozygous mutants showed distinct reduction in height (Fig 3.7 and Fig 3.8) facilitating the correlation of genotype with phenotype. In segregating populations, the ratio of plants with reduced height, genotyped as homozygous mutants, was approximately 1:3 to that of heterozygous mutant and wildtype plants (data not shown), which is consistent with monogenic

segregation of a recessive loss-of-function allele, and the insertion of a single T-DNA element in the OsARF11 gene.

3.2. Effects of insertion elements on the *OsARF11* gene expression

We used three primer pairs to test for the presence of different regions of the *OsARF11* transcript in cDNA generated from wild type and *Osarf11^{TRIM}* mutant plants. The primer pairs amplified from the second exon, the tenth exon spanning the T-DNA insertion point, and the 15th exon (Fig 3.3).

PCR resulted in amplified DNA products from all three primer-pairs from wild type cDNA, but not the primer pair spanning the insertion point from mutant cDNA, in line with a disruption of the transcript at the insertion point in the *OsARF11^{TRIM}* mutants (Fig 3.3). The q-PCR revealed 44% and 43% reduction of transcript levels using primers for second, and 15th exons respectively. The primer pair spanning the insertion point (the tenth exon) resulted in undetectable amplification after cycle 35 in *Osarf11^{TRIM}* homozygous mutant cDNA (Fig 3.4).

Similarly, the presence of *OsARF11* transcripts was tested in the *OsARF11^{Tos-17}* allele. Primers spanning the fifth exon (insertion site) resulted in PCR products in wild type plants, but not in mutant plants. Primers matching the 3'end (the 15th exon) of *OsARF11^{Tos-17}* generated PCR products for both wild type and mutant cDNA (Fig 3.5).

Taken together, the results show that OsARF11 transcripts are interrupted at the insertion point in both mutant alleles.

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3.3. Phenotype of *Osarf11* homozygous mutant plants

3.3.1. *Osarf11* homozygous mutant height, fecundity and root development

We measured height of both wild type and homozygous mutants after 3-4 months of growth. Plant height was reduced by 30% in *Osarf11*^{TRIM} homozygous mutants compared to wild type plants (Fig 3.6). The reduction in height was less pronounced at 15% in *Osarf11*$^{Tos-17}$ mutants (Fig 3.7). The average number of seeds produced per *Osarf11*^{TRIM} mutant was 30% of wild-type plants (Fig 3.8), and *Osarf11*$^{Tos-17}$ mutants generated no seeds, indicating complete sterility (not shown). The weight of seeds was also reduced in *Osarf11*^{TRIM} mutants (Fig 3.9).

To test if the *Osarf11* mutants also had reduced root growth, both wild type and mutant plants were germinated and grown on ½ MS media for 10 days before root growth was assessed. The results showed a reduction in the root growth in mutants compared to wild type plants (Fig 3.10). *Osarf11*^{TRIM} mutants show 18%, 28% and 73% reduction in the length of primary roots, number of secondary (adventitious) roots and number of lateral roots respectively compared to wild type plants. In addition, *Osarf11*$^{Tos-17}$ mutants show 21%, 5% and 37% reduction in in the length of primary roots, number of secondary (adventitious) roots and number of lateral roots respectively compared to wild type plants (Fig 3.10). There was no significant difference in number of tillers in wild type and mutant plants (data not shown).

3.3.2. Vein patterning of Os*arf11* homozygous mutants

A key component of the pleiotropic *mp/arf5* phenotype in *A. thaliana* is a reduced number of veins in leaves. Therefore, we counted the number of veins in segments

taken at the same position (see materials and methods) of the second, third and fourth leaves of the primary shoot for both allelic mutants. The difference between wild type and mutant was the largest in the second leaves, with a 35% reduction in the number of veins in $Osarf11^{TRIM}$ mutants. In the third leaves, the number of veins was reduced by 20%, and in the fourth leaves by 25% (Fig 3. 11). A similar reduction in vein number was seen in $Osarf11^{Tos-17}$ homozygous mutant plants. The number of veins was most reduced in second leaves of mutant at 35% compared to wild type plants. The third and fourth leaves of mutants showed a reduction in the number of veins by 23% and 25 % respectively compared to wild type (Fig 3.12).

We also assessed the number of veins in leaves of secondary shoots known as tillers. The first leaf of the first tiller from $Osarf11^{TRIM}$ mutants had a 18% reduction in vein numbers and the first leaf of the third tiller had a 22% reduction relative to corresponding wild type leaf (Fig 3.13). A reduction in number of veins was also observed at tillering stage in $Osarf11^{Tos-17}$ mutant leaves compared to wild type plants. The number of veins was reduced by 43% in the first leaf of the first tiller in homozygous mutants. The first leaf of the third tiller also showed a reduction of 33% in vein numbers (Fig 3.14). Statistical analyses show that a total number of veins have significantly been reduced in homozygous of both independent mutants during tillering stage.

We found no reduction in vein numbers of floral organs when comparing wild type and mutant flowers in $Osarf11^{TRIM}$ (data not shown). $Osarf11^{Tos-17}$ mutants produced no panicle or flowering organs; therefore, we could not assess the venation pattern of floral organs of $Osarf11^{Tos-17}$ mutants.

3.3.3. Vein density of Os*arf11* homozygous mutants

It is possible that the number of veins is reduced in *Osarf11* mutant leaves because the width of leaves is reduced or because the veins occur at a lower density. These options are also not mutually exclusive. To test these hypotheses, we measured leaf width and calculated leaf vein density as number of veins per mm of leaf width.

Although vein density was reduced in both allelic mutants, the reduction was more pronounced in *Osarf11*^{TRIM} mutants. Homozygous mutants of TRIM have shown a reduction in vein density, statistically significant for the second leaf, the first leaf of the first tiller and the first leaf of the third tiller (Fig 3. 15). The width of leaf was reduced in *Osarf11*^{TRIM} mutants compared to wild type (data not shown). Vein density in *Osarf11*$^{Tos-17}$ mutants was reduced at significant level for the first leaf of the first tiller. No significant reduction was observed in vein density for the second leaf and the first leaf of the third tiller (Fig 3.16).

In addition, we observed a slight reduction in the width of mid-vein in mutants relative to that of wild type. Thus, we measured the width of mid-vein in both allelic mutants at tillering stage. The results showed a marked decrease in the width of the mid-vein of mutant leaves, especially in *Osarf11*$^{Tos-17}$ mutants. The width was reduced by 60% in the first leaf of the first tiller and by 25% in the first leaf of the third tiller (Fig 3. 17). In *Osarf11*^{TRIM} mutants, the width of mid-vein was reduced by 56% in the first leaf of the third tiller and 25% in the first leaf of the first tiller (Fig 3.17).

3.4. Over-expression constructs

Three over-expression constructs were successfully made from cDNAs for *OsARF11*, *OsARF4* and *OsARF15*. Protein-coding regions were PCR amplified and fragments were cloned into a cloning site (USER) of a plasmid flanked by a Ubiquitin gene promoter (Ubi) on one side and a transcription terminator (NOS) on the other side, (Hebelstrup *et al*., 2010). Transformed plasmids were sent for confirmatory Sanger sequencing. The results confirmed the insertion of the complete coding sequence via sequencing of about 1000bp from both sides of the insertion, using a plasmid-specific primer pair. Furthermore, to sequence one step further in inserted fragment inside the plasmid, not covered in the first sequencing, we sequenced about 1000bp from both sides of inserted fragment, using a primer pair designed from the first sequencing results as described in materials and methods.

Confirmed plasmids will be sent to Taiwan for *Agrobacterium tumefaciens*-mediated transformation of rice cell cultures. Seeds from transgenic rice plants will be used to assess over-expression activity of gene and its potential effects on leaf vein formation and other organs development.

Here are constructs that have been made;

1. Ubi: OsARF11cDNA: NOS

2. Ubi: OsARF4 cDNA: NOS

3. Ubi: OsARF15 cDNA : NOS
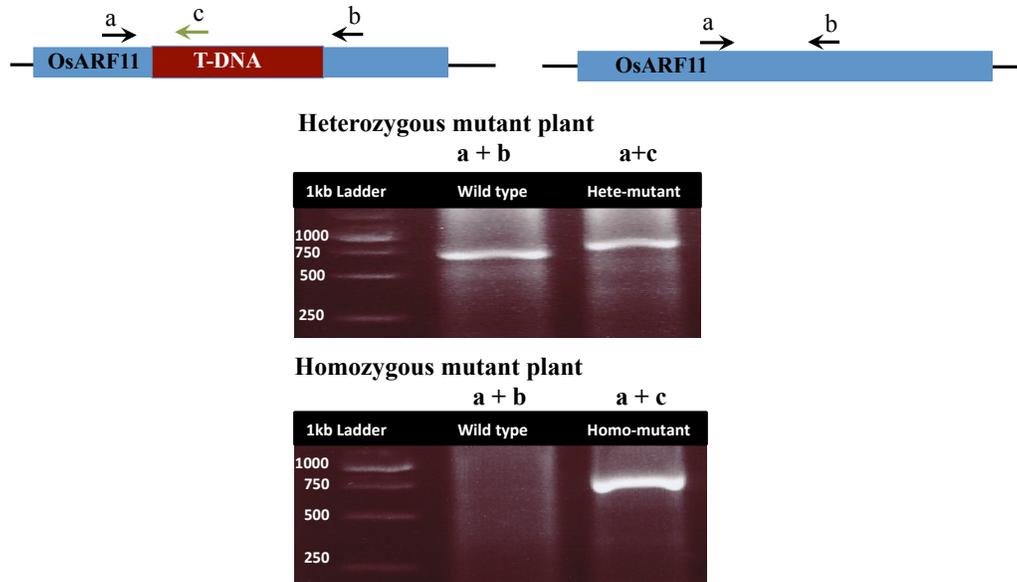
Fig 3.1. Identification of homozygous *Osarf11*^{TRIM} mutants. Drawing on top illustrates *OsARF11*^{TRIM} gene with and without T-DNA insertion and site of designed primers. wild type (*OsARF11*^{TRIM}) allele and T-DNA inserted (*Osarf11*^{TRIM}) allele in heterozygous mutant plant and wild type (*OsARF11*^{TRIM}) allele and T-DNA inserted (*Osarf11*^{TRIM}) allele in homozygous mutant plants. Homozyous mutants were identified by using wild-type forward primer (a), paired with T-DNA primer (c) for both wild type and mutant. Wild type forward primer (a) and wild type reverse primers (b) were applied to identify heterozygous mutant accordingly. 1Kb; DNA ladder (Fermentas) with size in basepairs indicated to the left.
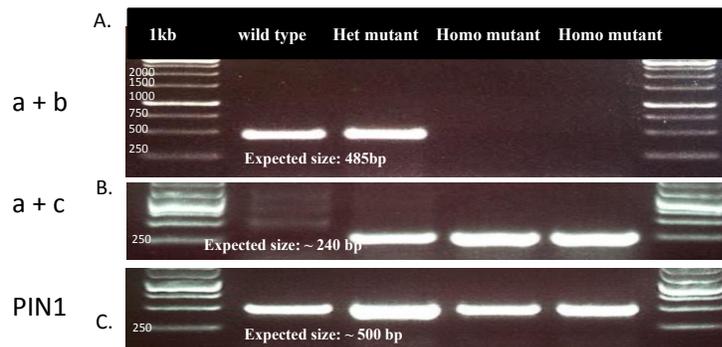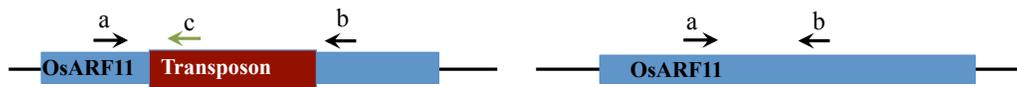
Fig 3.2. Identification of homozygous *Osarf11^{Tos-17}* mutants. Drawing on top illustrates *OsARF11^{TRIM}* gene with and without transposon insertion and site of designed primers. Wild type (*OsARF11^{TOS-17}*), heterozygous mutant (*OsARF11^{TOS-17}*), and homozygous mutants (*Osarf11^{TOS-17}*). Section A; wild type forward primer (a) and reverse primer (b) were applied for all four genotypes. Section B; forward primer paired with tail6 (c) (transposon specific primer), were used for all four genotypes. Section C; wild type primer pairs for *OsPIN1* gene were used as positive control. 1Kb: DNA ladder (Fermentas) with size in basepairs indicated to the left.
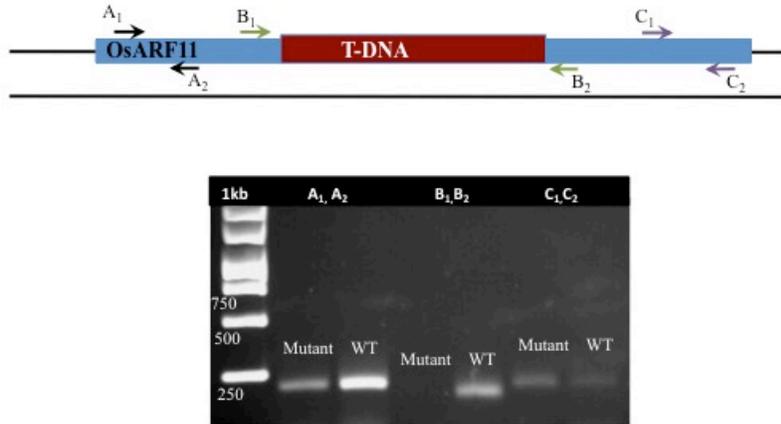
Fig 3.3. Drawing on top illustrates $OsARF11^{TRIM}$ cDNA with T-DNA insertion and approximate site of primer pairs used to assess the presence of transcripts. PCR results are shown, using three set combination primers. A_1,A_2; Primers designed for upstream region. B_1,B_2; Primers designed for insertion site. C_1,C_2; Primers designed for downstream region of OsARF11 cDNA. 1Kb; DNA ladder (Fermentas) with size in basepairs indicated to the left.
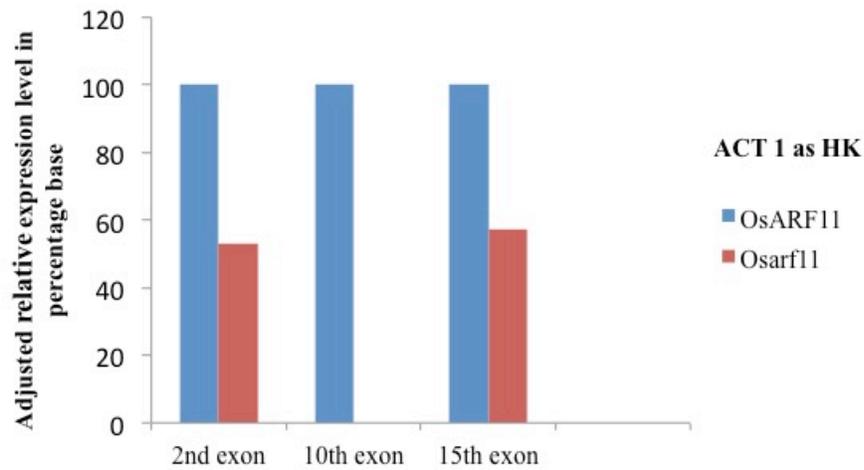
Fig 3.4. Analysis of the relative expression level of *OsARF11^{TRIM}* is shown. The expression level reduced and was undetectable after 35 cycles in mutant compared with wild type, using primer pair designed sorrounding insertion site (10th exon) in cDNA. Upstream (2nd exon) and downstream (15th exon) specific primers have shown reduction in expression level of OsARF11 in mutant by 44% and 43 % respectively. *ACT1* (*Actin* 1) was used as house keeping gene. qPCR was done using three biological replicates.
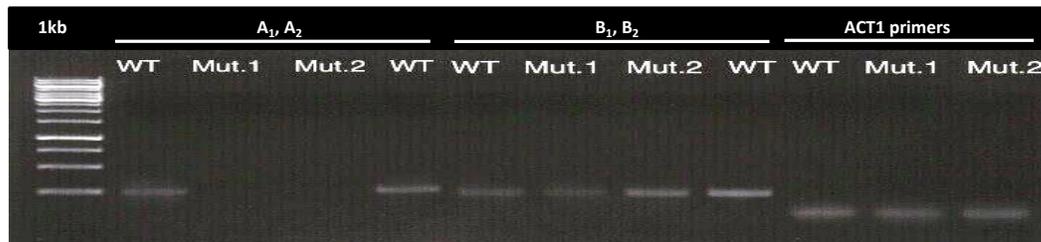
Fig 3.5. Drawing on top illustrates *OsARF11*[Tos-17] transcription unit with Transposon insertion and approximate site of primer pairs used to assess the presence of transcripts. PCR results are shown, using two set combination primers that are designed for insertion site (A_1, A_2) and down-stream (B_1, B_2) of *OsARF11*[Tos-17] cDNA, and ACT1 gene was used as a housekeeping gene. WT stands for Wild type and Mut stands for *OsARF11* mutants. 1Kb; DNA ladder (Fermentas) with size in basepairs indicated to the left.
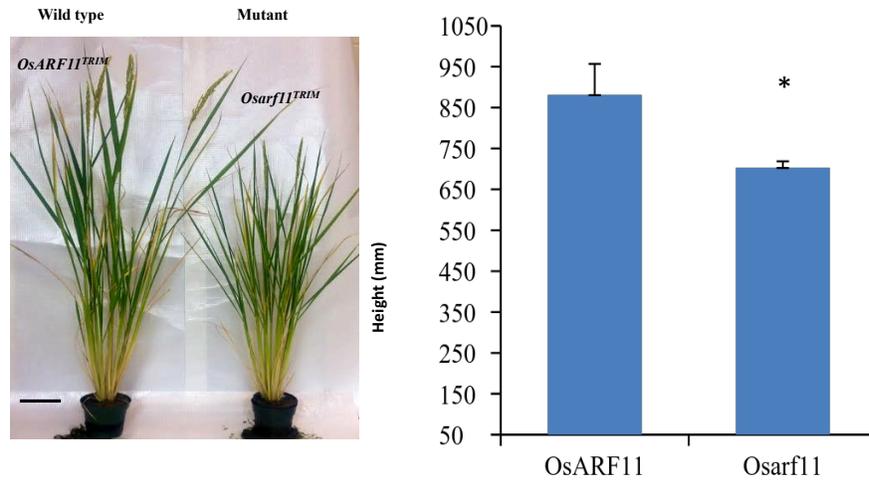
Fig 3.6. *Osarf11*^{TRIM} mutants were shorter than wild type plants *OsARF11*^{TRIM}. The values are the mean of 6 plants for each genotype in one experiment. Y axis shows plant height in mm. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Scale bar = 12cm.
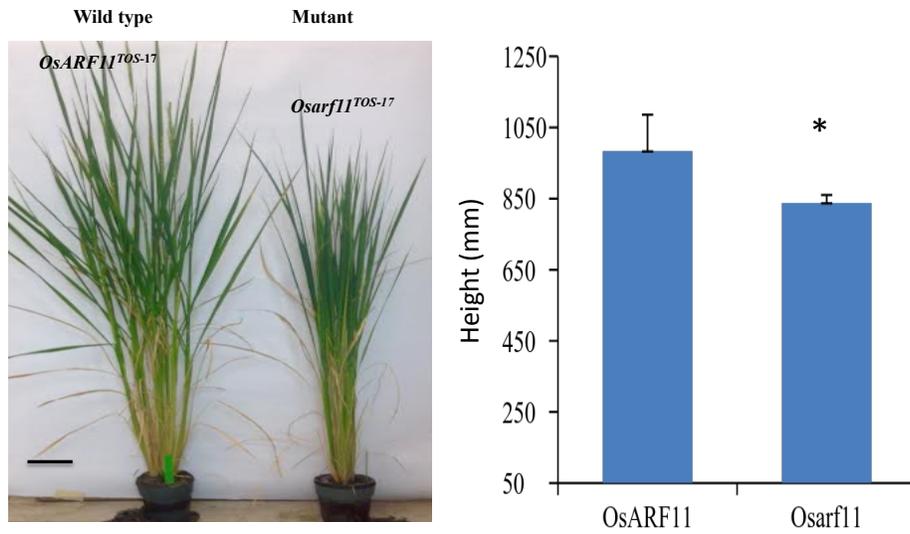
Fig 3.7. *Osarf11^{Tos-17}* mutants are shorter than wild type plants. The values are mean of 6 plants for each genotype in one experiment. Y axis shows plant height in mm. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Scale bar = 12cm
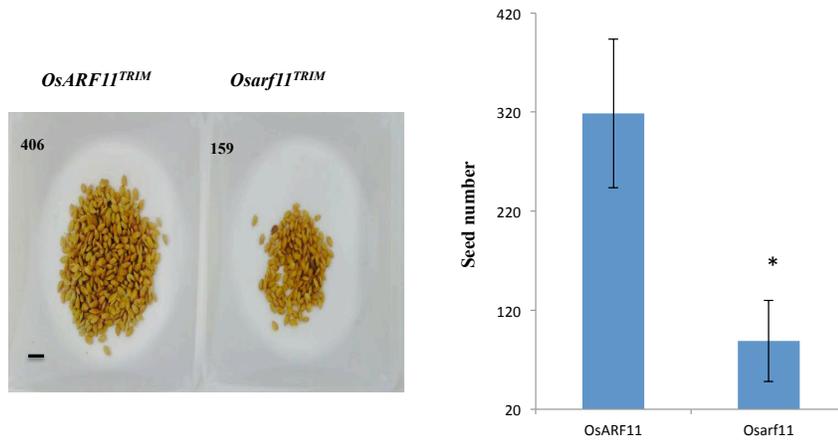
Fig 3.8. *Osarf11*^{TRIM} mutants showed semi-sterility compared to wild type plants (*OsARF11*^{TRIM}). The values are mean of 6 plants for each of genotype in one experiment. The number of seeds on the Y-axis. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Size bar = 1.2 mm
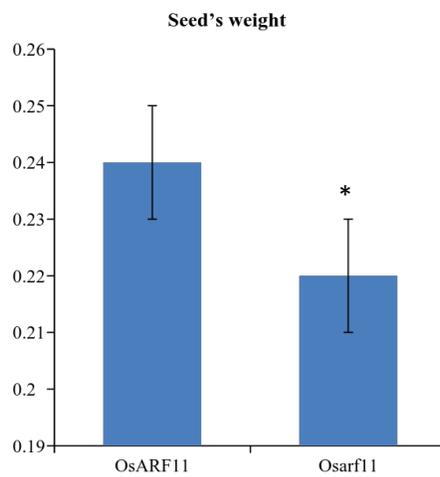
Seed's weight

Fig 3.9. The weight of seeds was reduced in *Osarf11*[TRIM] mutants compared to wild type (*OsARF11*[TRIM]). The values are mean of 10 seeds for each of genotype in 5 replicates in one experiment. Y axis shows seed weight in grams. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%.
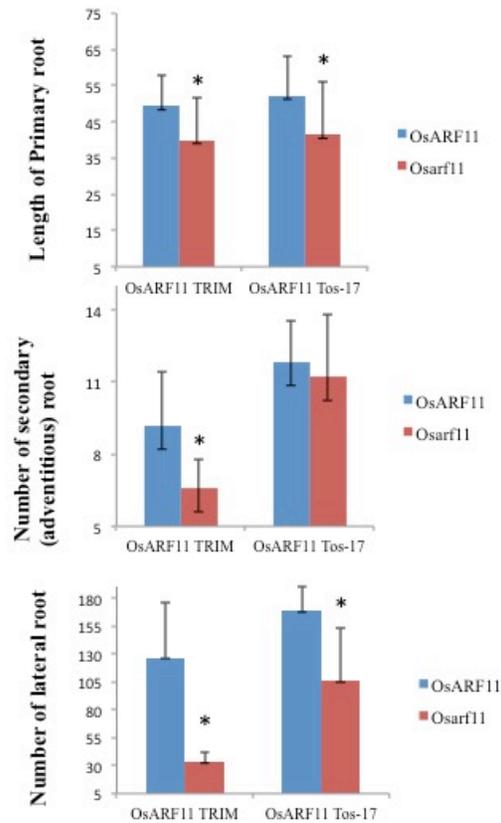
Fig 3.10. Mutants of both $Osarf11^{TRIM}$ and $Osarf11^{TOS-17}$ developed less root growth than wild type plants ($OsARF11^{TRIM}$ and $Osarf11^{Tos-17}$). The values are mean of 10 plants for each of genotype in one experiment. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%.
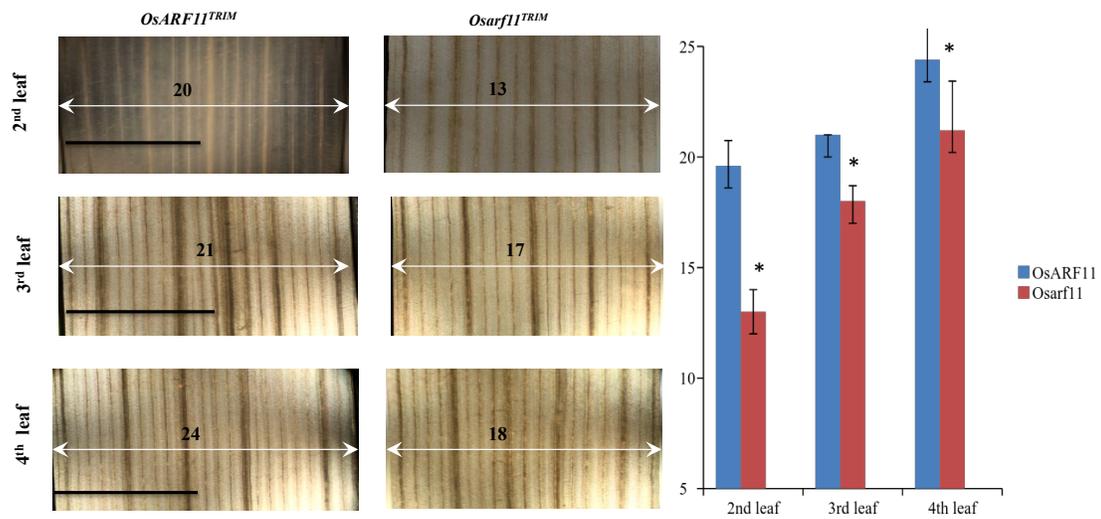
Fig 3.11. *Osarf11*[TRIM] mutant leaves have fewer veins during the early stage of development. *OsARF11*[TRIM] indicates wild type and *Osarf11*[TRIM] indicates homozygous mutant. The values on the Y-axis indicate number of veins and are mean of 6 plants for each of genotype in one experiment. Pictures on left provide examples of cleared leaves with the number of veins indicated for that leaf. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Size bar = 2 mm (2nd leaf Wild type), 3.52 mm (3rd leaf wild type) and 3.28 mm (4th leaf wild type).
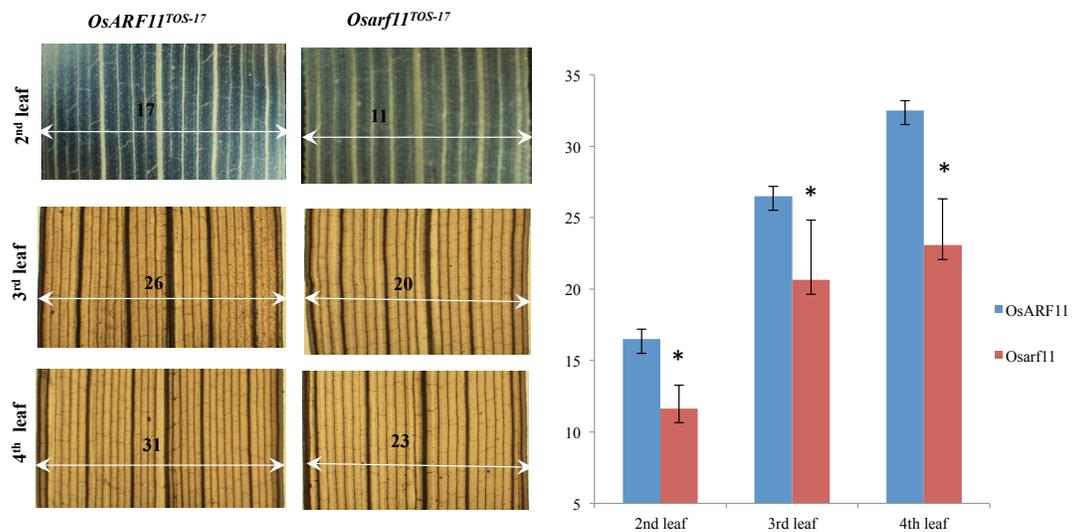
Fig 3.12. *Osarf11^{TOS-17}* mutant leaves have fewer veins during the early stage of development. *OsARF11^{TOS-17}* indicates wild type and *Osarf11^{TOS-17}* indicates homozygous mutant. The values on the Y-axis indicate number of veins and are mean of 6 plants for each of genotype in one experiment. Pictures on left provide examples of cleared leaves with the number of veins indicated for that leaf. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Size bar for previous figure can apply for this figure.
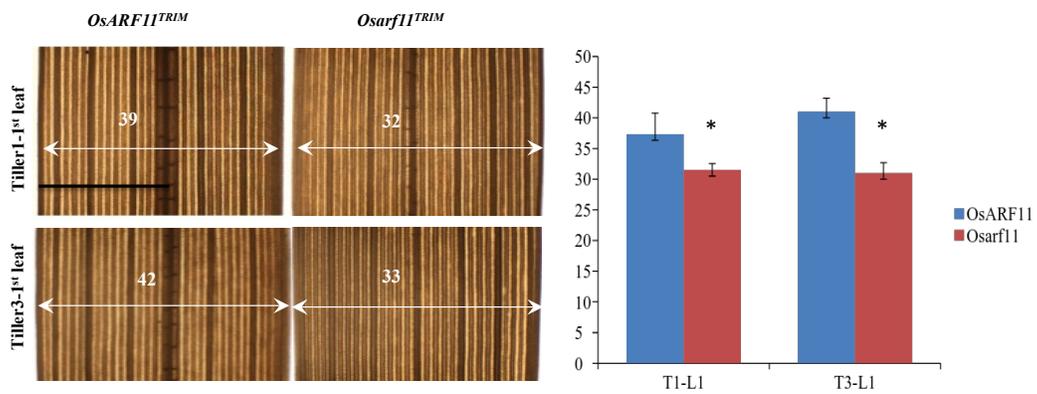
Fig 3.13. Tiller leaves have fewer veins in the *Osarf11*^{TRIM} mutants. *OsARF11*^{TRIM} indicates wild type and *Osarf11*^{TRIM} indicates homozygous mutant. The values on the Y-axis indicate number of veins and are mean of 6 plants for each of genotype in one experiment. Pictures on left provide examples of cleared leaves with the number of veins indicated for that leaf. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Size bar = 4.02 mm
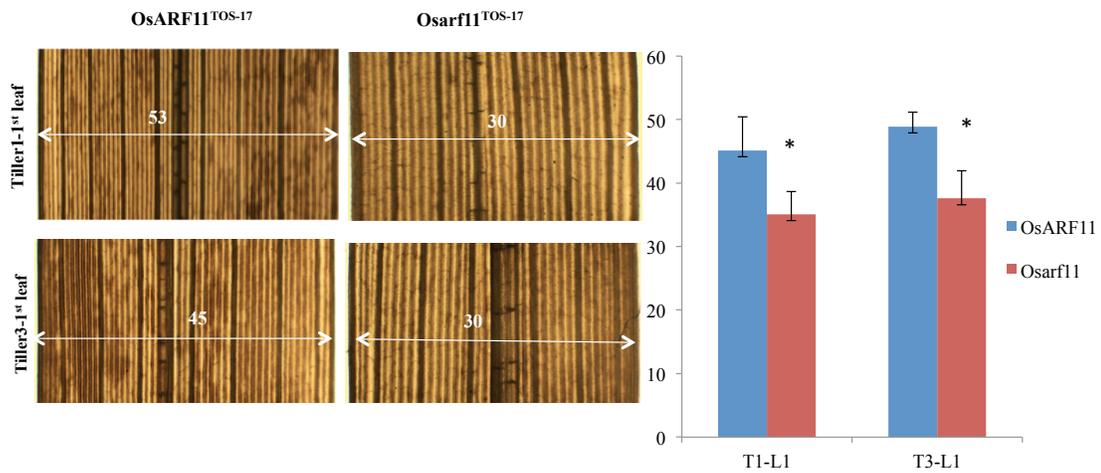
Fig 3.14. *Osarf11^{TOS-17}* mutant leaves have developed fewer veins at tillering stage. *OsARF11^{TOS-17}* indicates wild type and *Osarf11^{TOS-17}* indicates homozygous mutant. The values on the Y-axis indicate number of veins and are mean of 6 plants for each of genotype in one experiment. Pictures on left provide examples of cleared leaves with the number of veins indicated for that leaf. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%. Size bar for previous figure can apply for this figure.
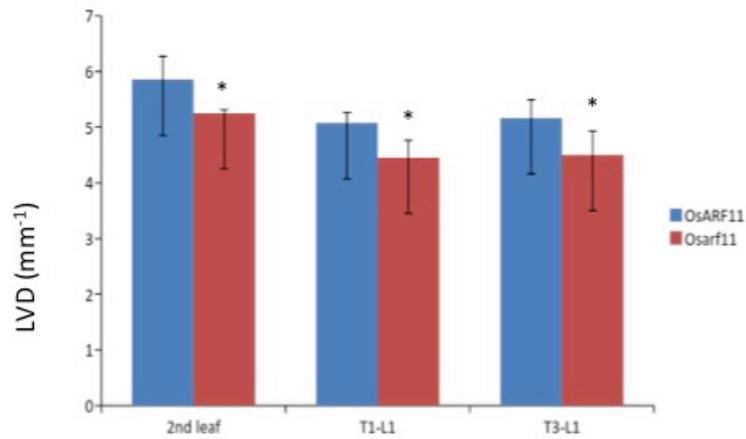
Vein density		Lower Dif
	OsARF11TRIM	
2nd leaf	Osarf11TRIM	0.0164*
T1-L1	Osarf11TRIM	0.0017**
T3-L1	Osarf11TRIM	0.008**

Fig 3.15. Vein density was decreased in *Osarf11TRIM* mutants. Vein density was calculated by dividing the total number of veins by the width of the leaf. Vein density was reduced in the 2nd leaf, T1-L1 and T3-L1 leaves, at significant level, of mutants compared with wild type. 2nd leaf; second leaf, T1-L1; the first leaf of the first tiller and T3-L1; the first leaf of the third tiller. n = 6 for each of genotype. The difference in LVD between wildtype and mutant plants is significant at a confidence level of > 95% (*) and >99% (**).
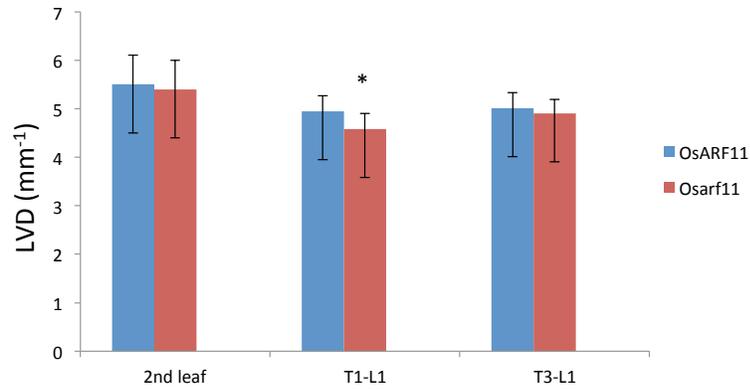
Vein density		Lower Dif
2nd leaf	**Osarf11**[TOS-17]	**0.23**
T1-L1	**Osarf11**[TOS-17]	**0.0272***
T3-L1	**Osarf11**[TOS-17]	**0.64**

Fig 3.16. Vein density was decreased in *Osarf11*[TOS-17] mutants. Vein density was calculated by dividing the total number of veins to the width of leaf. Vein density was only reduced in T1-L1 leaf of mutants, at significant level, compared to wild type. 2nd leaf; second leaf, T1-L1; the first leaf of the first tiller and T3-L1; the first leaf of the third tiller. n (replicates) = 6 for each of genotype. The difference in LVD between wildtype and mutant plants is significant at a confidence level of > 95% (*).
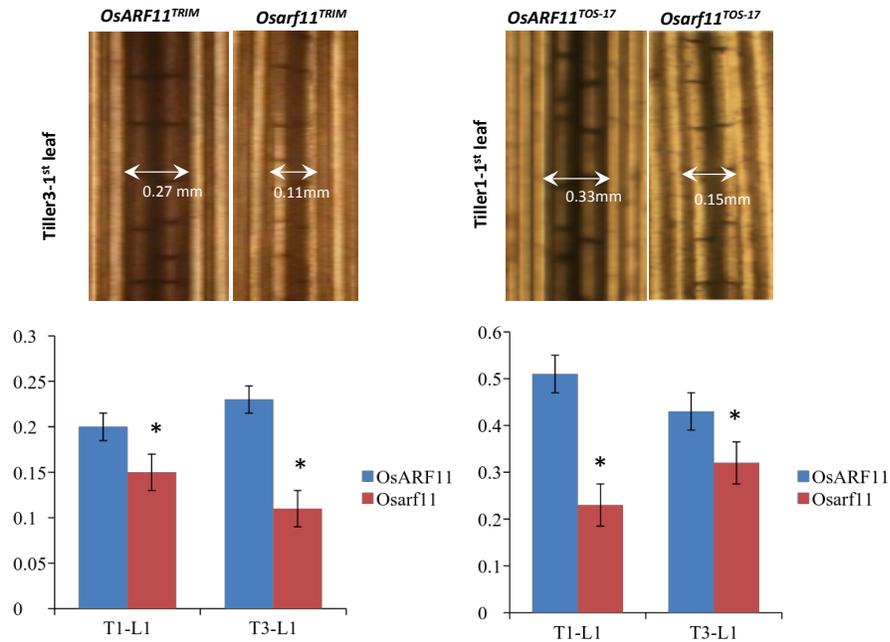
Fig 3.17. The width of the mid-vein was reduced in both allelic mutants. On the right is a picture of the midvein region of an $Osarf11^{Tos-17}$ mutant leaf and on the left is a picture of the midvein region of an $Osarf11^{TRIM}$ mutant leaf $OsARF11^{TOS-17}$ and $OsARF11^{TRIM}$ indicate wild type and $Osarf11^{TOS-17}$ and $Osarf11^{TRIM}$ indicate homozygous mutants. The values are mean of 6 plants for each of wild type and homozygous mutants. Y-axis shows leaf width in mm. Asterisk indicates that the difference between averages of wildtype and mutant plants is significant at a confidence level of > 99%.

4. Discussion

Auxin regulates various growth and developmental processes through controlling the expression of auxin-response genes (Ulmasov *et al*., 1997). Auxin response factors (*ARFs*) are key transcription factors in the early auxin response. They bind specifically to the auxin response elements (AuxREs) and modulate the transcription of the early auxin response genes (Guilfoyle and Hagen 2007). There is limited information available about auxin signalling in rice (Sato *et al.,* 2001; waller *et al*., 2002; Scarpella *et al*., 2005; Scarpella and Meijer 2004; Donner *et* al., 2006; Wang *et al*., 2007; Attia *et al*., 2009).

Numerous studies on *A. thaliana* reveal that the *AtARF5/MP* gene contributes crucially to developmental processes by regulating auxin signalling (Berleth and Jurgens 1993; Przemeck *et al*., 1996; Hardtke and Berleth 1998; Mattsson *et al*., 1999; Mattsson *et al*., 2003; Hamann *et al*., 2002; Hardtke *et al*., 2004; McKown *et al*., 2010; Wenzel *et al*., 2007; Krogan *et al*., 2012). Although mutants defective in auxin response factors (*ARFs*) have been identified and their corresponding functions in many aspects of rice growth have been investigated, there has not been any information reported about the regulatory role of an ortholog of the *AtARF5/MP* in rice. Based on sequence similarity, the *OsARF11* gene is the most likely rice ortholog of the *AtARF5/MP* gene (Wang *et al*., 2007; Shen *et al*., 2010).

The focus of this thesis is to identify potential developmental defects in *Osarf11* mutants. We also investigate the presence of full-length *OsARF11* transcripts in both

wild type and homozygous mutants. We use two allelic mutants, which have insertions

in different sites of *OsARF11* gene to provide independent mutation in *OsARF11*.

The results provide parallel phenotypes in both mutant alleles, thereby providing

independent and therefore strong evidence that the phenotypes can be attributed to

mutations in the *OsARF11* gene. The results presented in this thesis indicate that

OsARF11 plays an important role in controlling root and shoot growth, seed productivity

and leaf vascular development in rice. On a molecular level, we show that insertional-

mutation interferes with the production of full-length transcript of *OsARF11*.

4.1. Transcriptional activity of the *OsARF11* gene

Analysis of the relative expression levels of the *OsARF11* gene shows that both

allelic mutants (*Osarf11^{TRIM}* and *Osarf11^{TOS-17}*) were unable to generate full-length

transcripts. However, amplified products were seen for the 5' and 3' end of transcription

units. We speculate that the 5' end transcripts result from the still intact *OsARF11*

promoter. Similarly, promoters and enhancers in the T-DNA and transposon tags may

contribute to transcription of both 5' and 3' regions of the *OsARF11* gene.

Auxin response factors contain two domains that are conserved among members

of the protein family and that corresponds to important functionalities – an N-terminal

DNA-binding domain (DBD), and a C-terminal protein-protein dimerization domain

(motifs III and IV). Both mutant alleles described here harbor large insertions that occur

between the DNA-binding and dimerization domains. Since both domains are required

for DNA-binding and the dimerization domain is required for auxin-mediated post-

translational activation of ARF protein transcription factor activity (Guilfoyle and Hagen

2007) both mutant alleles are likely to result in complete loss of protein function. It has been reported that a transgenic construct known as *mpΔ/ARF5* with truncated domains III and IV (MPΔ), but otherwise intact protein takes on a novel gain-of-function activity (Krogan *et al*., 2012) but we did not observe any of the *mpΔ/ARF5* phenotypes nor did we observe dominance or semi –dominance expected of a gain-of-function allele.

4.2. The role of the *OsARF11* gene in rice shoot (tiller) growth and fecundity

Our results show a reduction in plant height and fecundity in both *Osarf11* allelic mutants. In that regard, several examples of mutants defective in auxin signalling show the regulatory role of auxin response factors in hypocotyl elongation and inflorescence development in *A. thaliana* (Harper *et al*., 2000; Li *et al.,* 2004; Waller *et al.,* 2002; Shen *et al*., 2015). Similarly, it has been reported that mutation in auxin response factors results in auxin-related defective phenotypes associated with important features such as height and fertility in rice (Sato *et al*., 2001; Ellis *et al*., 2005; Okushima *et al*., 2005; Wang *et al*., 2007; Attia *et al*., 2009; reviewed by Liu *et al*., 2014). In our study, the *Osarf11* mutants show reduced height (dwarfism) similar to the phenotypes reported in the *Osarf1* and *Osarf16* mutants of rice (Song *et al*., 2009; Shen *et al*., 2015; Reviewed by Li *et al.,* 2016). These publications conclude that the dwarf phenotype seen in *Osarf1 and 16* mutants are most likely due to defects in auxin signalling. We suggest that the *OsARF11* gene may not be essential for shoot initiation but it is required for a normal rate of shoot elongation.

Numerous studies have established the role of auxin in apical dominance and inflorescence development by mutants with defects in auxin signalling and biosynthesis.

For instance, *auxin-resistant1* (*axr1*) mutants in *A. thaliana* are bushy (Lincoln *et al*., 1990). Reduction of auxin biosynthesis by multiple *yuc* mutants causes reduced apical dominance (Cheng *et al*., 2006). In rice a negative correlation between number of tillers, associated with apical dominance, and plant height has been reported (Iwata *et al*., 1995; Yan *et al*., 1998; Li *et al*., 2003). For example, *Ospin1* mutants have an increased number of tillers compared to wild type plants (Zhou *et al*., 2003; Xu *et al*., 2005). Although mutation in *OsARF11* causes reduce plant height, *Osarf11* mutant does not affect apical dominance or number of tillers (data not shown), suggesting that the *Osarf11* mutation alone is not sufficient to affect these traits.

Mutants defective in auxin synthesis, transport and signalling that fail to establish floral organs in *A. thaliana* have also provided evidence of the role of auxin-mediated response in flower development. (Okada *et al*., 1991; Przemeck *et al*., 1996; Vernoux *et al*., 2000; Reinhardt *et al*., 2003; Cheng *et al*., 2006;). Given that, it is expected that the *OsARF11* gene also plays a role in flower development in rice. We, however, did not observe any flower defects in *Osarf11* mutant. On the other hand, both *Osarf11* mutants have reduced fertility. Recently, it was shown that an increase in endogenous auxin levels following pollination induces seed formation (Uchiumi and Okamoto 2010). At the same time, the expression level of the *OsARF11* gene was 32 fold up-regulated during flowering (Uchiumi and Okamoto 2010). Furthermore, treatment with 2,4-D has also shown to increase the transcriptional activity of *ARF* genes such as the *OsARF1* and *OsARF11* (Uchiumi and Okamoto 2010). The *OsARF1* gene has been proposed to play a key role in both shoot (tiller) outgrowth and fertility in rice (Waller *et al*., 2002; Attia *et al*., 2009). For instance, loss-of-function *Osarf1* mutants show dwarfism and sterility

(Attia *et al*., 2009). In this study, we provide for the first time evidence that the *OsARF11* gene plays a role in seed production as well.

In addition, functional redundancy among *ARF* genes associated with flower development has been reported in *A. thaliana*. For instance, *AtARF6* and At*ARF8* genes are found to act redundantly in flower maturation in *A. thaliana* (Finet *et al*., 2010). *A. thaliana ARF1* and *ARF2* genes also redundantly control floral organ abscission (Ellis *et al*., 2005; Wang *et al*., 2007). Redundant function has also been suggested among *ARF* genes in rice (Wang *et al.,* 2007). Thus, it is possible that the *OsARF11* gene may act redundantly with other *ARFs* in regulating flower development in rice.

Seed weight is also reduced in *Osarf11^{TRIM}* mutants, indicating a role of the *OsARF11* gene in rice seed endosperm development. Our result is consistent with the finding recently reported that the *Big Grain* 1 (*OsBG1*) gene*,* involved in auxin response and transport, contribute to the regulation of seed weight in rice (Liu *et al*., 2015). Over-expression of the OsBG1 protein leads to an increase seed weight and subsequently grain yield (Liu *et al*., 2015). It is possible that OsARF and OsBG1 acts in the same auxin signaling pathway contributing to seed development.

During the process of crop domestication and improvement, selection for larger seeds and higher number of seeds have naturally been the main criteria for obtaining high-yielding varieties. Rice is one of the most important crops in the world and improving rice grain yield has been considered as a crucial issue due to rapid growth of the world's population (Zou *et al*., 2005). We show for the first time that the OsARF11 gene affects both seed size and number, thereby providing a new potential target for breeding of these traits.

4.3. The role of the *OsARF11* gene in rice root growth

We observed defects in root development of both *Osarf11* mutants. Both mutant alleles resulted in a reduced length of primary roots, number of crown (adventitious) roots and lateral roots, implicating this gene in both initiation and elongation of roots. Auxin is well known to play a role in the regulation of root formation as well as growth in higher plants, including rice (Reviewed by Wu and Cheng 2014). In addition, the role of auxin response factors in root development is known for both *A. thaliana* and rice. For example, mutation in the *MP/ARF5* gene of *A. thaliana* shows defect in body axis patterns and results in seedlings lacking hypocotyl and the root. *mp/arf5* mutants can be induced to form adventitious roots through exogenous application of auxin, but at a much reduced rate relative to wildtype plants and also relative to the highly auxin insensitive mutant axr1, indicating drastically reduced perception of auxin in *mp/arf5* mutants (Mattsson *et al*., 2003). In addition, double mutation in the *arf10 arf16* genes results in the absence of lateral root formation in *A. thaliana* (Reviewed by Wang *et al*., 2005). In rice, several *ARF* genes have been demonstrated to contribute to the development of root system. For instance, the *OsARF1* gene is associated with auxin-mediated lateral root formation (Attia *et al*., 2009), the *OsARF12* gene is reported to regulate root elongation (Qi *et al*., 2012) and the *OsARF16* gene is found to be involved in lateral root growth (Shen *et al*., 2015).

Furthermore, a reduction of the number of crown (adventitious) roots and lateral roots is often shown in other auxin-related mutants in rice and *A. thaliana* (Tian *et al*., 2004; Inukai *et al*., 2005; Liu *et al*., 2005; Okushima *et al*., 2007; Kitomi *et al*., 2008, Liu *et al*., 2009). For example, in *A. thaliana*, the *LBD16/ASL18* and *LBD29/ASL16* were identified to function in auxin-mediated lateral root development in which their

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expression is regulated by ARFs proteins (Okushima *et al*., 2007). In rice, the *OsPIN1* gene, expressed in a similar pattern to *AtPIN1*, plays an important role in auxin-dependent crown root initiation (Xu *et al*., 2005). The *CROWN ROOTLESS* (*CRL1*) is involved in auxin-related root development in rice and is a direct target of ARFs protein such as OsARF1 based on the presence of the Aux-RE in the promoter. The *crown rootless1* (*crl1*) mutant is defective in crown (adventitious) root formation; however, the CRL1 gene is not needed for the initiation of lateral roots, indicating that the initiation of crown roots and lateral roots differ (Inukai *et al*., 2005). In our study, the number of crown (adventitious) roots was significantly reduced in $Osarf11^{TRIM}$ mutants. Also, the number of lateral roots was significantly reduced in both *Osarf11* mutants, suggesting that a reduced root growth seen in the *Osarf11* mutants may result from defect in auxin signalling.

Lateral roots and adventitious roots are crucial for water and nutrition acquisition in rice (Liu *et al*., 2009). A positive correlation between the percentage of filled grain and root activity during the reproductive stage has been described in rice (Inukai *et al*., 2005). We see a similar correlation in *Osarf11* mutants, suggesting that reduced shoot and seed growth may, at least in part be a secondary effect of reduced root growth. However, we have quantified root growth only in young seedlings growing in liquid medium and do not know if older Osarf11 mutants also have reduced root systems. The major genetic approach for root-related traits is Quantitative Trait Loci (QTL) mapping (Reviewed by Gowda *et al*., 2011) and it is possible that such QTLs map to intervals that contain the OsARF11 gene.

4.4. The role of the *OsARF11* gene in leaf vasculature

In our study, the total number of veins was reduced in leaves of both *Osarf11* allelic mutants during early and later stages of growth. It is well known that auxin is a central regulator of vascular development in plants (McSteen and Leyser 2005). Auxin is produced mainly in the shoot apex and young leaves and is transported basipetally through the plant in a stream known as polar auxin transport (Reviewed by McSteen 2009). Studies on the *monopteros (mp/arf5*) mutant, defective in vein patterning, supports a role for *MP/ARF5* gene in regulating vascular development (Berleth and Jurgens 1993; Przemeck *et al*., 1996; Mattsson *et al*., 1999; Schuetz *et al.,* 2008)*.* As reported, venation is dramatically reduced in the *monopteros (mp/arf5)* mutant, caused by defects in auxin signalling and transport (Przemeck *et al*., 1996). In our study, the *Osarf11* mutants show reduced total vein number in young leaves (The second, third and fourth living leaves of adult plants) as well as tiller-leaves. Therefore, we propose that OsARF11 contributes to auxin-mediated vascular development in rice. Although the *mp/arf5* mutants show severe vascular defects in *A. thaliana*, the phenotypes seen in the *Osarf11* mutants are not as strong as the *mp*/*arf5.* This discrepancy may be because the *OsARF11* gene may act redundantly with other *OsARF* genes in response to auxin signals in vein formation in rice as described before for many ARF genes. Alternatively, it is also possible that auxin signaling plays a much smaller role in the formation of veins in monocots than in dicots.

With an increase in the size of the human population, higher yield has again become an important target of rice breeding. Photosynthesis, occurring mostly in leaves, is the primary source of grain yield in rice (Chen *et al*., 1999). In that regards, the size/shape of leaves and vein patterning influence the photosynthetic efficiency and

thereby grain yield (Yue *et al*., 2006). Rice mutants with narrow-leaf phenotypes often have abnormal arrangements of vascular bundles in the leaves. For example, *the narrow leaf1* (*nal1*) mutants, involved in polar auxin transport, exhibit a reduction in the width of leaf blade and in the number of longitudinal veins (Qi *et al*., 2008). *The narrow leaf7* (*nal7*) mutants also display a considerable reduction in leaf blade width (Fujino *et al*., 2008). Our data also show a reduction at a significant level in the width of leaf and reduced vein number and vein density in *Osarf11* mutant leaves, suggesting that the *OsARF11* gene affects leaf width and subsequently vein arrangement in rice.

Based on the phenotype of the described *Osarf11* loss of function mutants, it is possible that enhanced expression of this gene may have the opposite effect, i.e. result in enlarged shoots, roots, seeds and seed number. To investigate this hypothesis, over-expression constructs generated in this study will be used to produce transgenic rice. Further assessments will be conducted afterwards. This study provides evaluated function of the *OsARF11* gene for the first time in rice developmental processes and growth.

5. Future perspectives

Although our study reveals important evidence about the *OsARF11* potential role in rice developmental processes, more detailed-morphological assessments in the *Osarf11* mutants, both anatomical and molecular, will be required to reveal the regulatory role of *OsARF11* in auxin signalling and transport in rice. As mentioned before *MP/ARF5* plays a crucial role at early stage of embryogenesis as well as the formation of leaf veins in *A. thaliana*. Therefore, further investigation on the expression of *OsARF11* during embryo and leaf development will be important to establish whether *OsARF11* is active during these processes. Also, the cause of reduced number of seeds in *Osarf11* mutants is unknown and may potentially be linked to any or several defects in male and female organs and gamete formation.

The function of all ARF proteins is still unknown presumably due to extensive genetic redundancy within *ARF* gene family. Redundancy among the *AtARF* genes has been reported repeatedly in several studies on *A. thaliana* (Ellis *et al*., 2005; Pekker *et al*., 2005; Wilmoth *et al*., 2005; Wang *et al*., 2005; Hunter *et al*., 2006; Finet *et al*., 2010; Kelley *et al*., 2012; reviewed by Li *et al*., 2016). It is also worthwhile to investigate the redundancy among the *OsARF* genes in rice. Thus, the generation of double mutants between *Osarf11 mutants* and mutants in the two most similar genes *OsARF16* and *OsARF21* as such mutants become available may reveal if such overlaps in gene function exists and what their combined functions may be.

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Appendix.

The effects of increased local auxin concentration on vein formation

The plant vascular system is a network of interconnected cells that distributes water, minerals and photosynthesis-derived sugars throughout the plant. Vascular bundles, known as veins in leaves, are derived from procambial cells. Once elongated and interconnected, procambial cells continue to proliferate to give rise to several tissue types, including phloem, xylem, fibers, and bundle sheath cells. Phloem and Xylem, the most familiar types of vascular tissues, provide pathways for transporting photosynthetic assimilates and minerals along the whole body of plants (Mckown and Dengler, 2010; Turner and Sieburth, 2003; Mattsson *et al.,* 1999).

In the past 15 years, the establishment of vein patterning in *A. thaliana* leaves has been extensively studied. Numerous studies including quantitative and developmental approaches reveal that the whole process of vein formation and patterning is complex, and involving interaction between molecular and genetic pathways during the leaf developmental processes (Mckown and Dengler, 2010; Michniewicz *et al*., 2007).

The plant hormone auxin is a key regulator of many plant growth processes. Auxin via carefully regulated transport appears to provide positional and inductive

information for the formation of the embryo proper, the root apical mersitem (RAM) and lateral roots, lateral shoot organs such as cotyledon, leaf and flower (reviewed by Friml et al., 2004). A common theme in these processes is the formation of an auxin maxima via directed polar auxin transport mediated primarily by auxin efflux carriers of the PIN-FORMED (PIN) family. Accumulated auxin then activates position-dependent growth programs that result in the development of the correct structure. Therefore, auxin is a crucial component of the plant development (Tsukaya, 2003; Michniewicz, 2007).

It has been known for some time that auxin can under certain circumstances induce trans-differentiation of cortex and mesophyll cells into vessel elements (Jacobs 1952). Auxin treatment of zinnia leaf mesophyll cell cultures cells can induce large-scale and rather synchronous trans-differentiation into vessel elements (Fukuda 2010 or 2004). This trans-differentiation process serves multiple purposes in plants, including regeneration of severed vascular bundles after wounding (Jacobs, 1952), and formation of transfusion tracheid in conifer leaves that presumably facilitate transpiration (Aloni *et al.,* 2013). The formation of continuous bundles depends on polar auxin transport, ensuring not only that a string of cells trans-differentiate to form a functional vessel, but to some extent elongate along this axis (Jacobs, 1952). These observations led Tsvi Sachs to carry out a large set of experiments to address the roles of auxin sources, tissue polarity as well as the influence that pre-existing auxin sinks had on vascular strand formation (Sachs, 1981 and 1989). Based on his observations, he formulated the canalization of signal flow model, with auxin being the most likely candidate for the signal.

There are now multiple levels of support for canalization of auxin flow model in the development of leaf veins in *A. thaliana*. Pharmacological inhibition of PAT in developing leaves leads to enhance leaf venation and defective connections between vessel elements, in line with reduced canalization of auxin flow (Mattsson *et al*., 1999; Sieburth *et al*., 1999). The changes in vein distribution are preceded by similar changes in expression of an auxin response marker, providing visual evidence for correlations between auxin responses and vascular development (Mattsson *et al*., 2003). In addition, gradual refinement of both the expression and sub-cellular protein localization of the auxin efflux carrier PIN-FORMED1 (PIN1) into sites of vein formation provides visual support of the canalization of signal flow hypothesis (Scarpella *et al*., 2006; Wenzel *et al*., 2007). Local auxin biosynthesis may also contribute to leaf vein development. The *A. thaliana* YUCCA gene family of 11 members encodes flavin monooxygenases some of which carry out the final rate-limiting step of Tryptophan-dependent biosynthesis of the main auxin, indole-3-acetic acid. *A. thaliana YUCCA* members have unique expression patterns in young leaves and combinations of multiple yucca mutants result in plants with reduced leaf venation and reduced auxin levels implicating also local auxin biosynthesis in the regulation of vein patterning and density. While vein patterning and the role of auxin signalling is increasingly well understood in *A. thaliana*, and similar observations have been made in other dicot plants, next to nothing is known about the role of local auxin production in regulating the extent of veins in both dicot and monocot plants. We hypothesize that the strength of local auxin sources influence the pattern and extent of veins in both di- and monocot plants.

To test this hypothesis a former undergraduate student, Lorenz Nierves, has generated gene fusions between a fragment with four multiples of the 35S CaMV enhancer and fused that to genomic fragments of some *A. thaliana* genes that contain putative promoter, transcript encoding region and 3 terminator region. It has previously been shown that 35S enhancers enhance the expression of nearby genes, maintaining spatio-temporal aspects of the endogenous gene but providing overall higher levels of expression. An increased number of veins would verify the hypothesis, while an unaltered venation density would falsify the hypothesis (provided the gene is found to be upregulated and result in higher levels of auxin).

The experiments that have been done to test this hypothesis:

1. Specific constructs for the target genes were made and used for the transformation into the *A. thaliana* plants by using the floral dipping method.
2. Seven constructs including: Yucca 1+ 0.5KB control, Yucca1 + 0.5KB 4*35S, Yucca 1+ 1 KB control, Yucca 1+ 1KB 4*35S, Yucca 2+ 2KB 4*35S, Yucca 4+ 1KB 4*35S, Yucca 4+ 0.5 KB 4*35S, were transformed to *A. thaliana* plants successfully.
3. Select for transformed plants on Kanamycin-containing medium and grow to maturity.

4. Collect T2 seeds from individual plants, plate on Km medium and record segregation of sensitive versus resistant plants to identify plants that have one T-DNA insertion based on a 3:1 resistant to sensitive segregation ratio.

5. Grow Km resistant plants, allow to self-fertilize, and collect seeds from individual plants.

6. Test aliquots of T3 seeds from individual plants to identify populations that contain only homozygous mutant plants.

7. Evaluate venation of cotyledon, first and third leaf by microscopy of cleared leaves.

8. Compare wildtype control of the same genetic background to that of homozygous mutants

Results:

Transformed plants showed altered shapes of the cotyledons, but no significant change in pattern and extent of veins. Therefore, this avenue of investigation was not extended further.