Running Title: Functional characterization of OsbZIP48 in rice

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OsbZIP48, a HY5 transcription factor ortholog, exerts pleiotropic effects in light-regulated development

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Author contribution
NB designed and performed the experiments, analysed the data and wrote the manuscript; AB helped in performing some of the experiments; JPK supervised the research work and finalised the manuscript.

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Summary
The *OsbZIP48* gene from rice can complement *hy5* mutant of *Arabidopsis* but exerts pleiotropic effects and causes semi-dwarfism when over-expressed in rice and its mutant/RNAi lines are seedling lethal.

Abstract
Plants have evolved an intricate network of sensory photoreceptors and signalling components to regulate their development. Among the light signalling components identified till date, HY5, a bZIP transcription factor, has been investigated extensively. However, most of the work on HY5
has been carried out in *Arabidopsis*, a dicot. In this study, based on homology search and phylogenetic analysis, we could identify three homologs of AtHY5 in monocots; however, AtHYH (HY5 homolog) homologs are absent in monocots analysed. Out of the three homologs identified in rice, we have functionally characterized OsbZIP48. OsbZIP48 was able to complement Athy5 mutant. OsbZIP48 protein levels are developmentally regulated in rice. Moreover, OsbZIP48 protein does not degrade in dark grown rice and Athy5 seedlings complemented with OsbZIP48, which is in striking contrast to AtHY5. In comparison to AtHY5, which does not cause any change in hypocotyl length when over-expressed in *Arabidopsis*, the over-expression of full-length OsbZIP48 in rice transgenics reduced the plant height considerably. Microarray analysis revealed that OsKO2, which encodes ent-kaurene oxidase 2 of GA biosynthesis pathway, is down-regulated in OsbZIP48<sup>OE</sup> and up-regulated in OsbZIP48<sup>KO</sup> transgenics as compared to the wild type. Electrophoretic mobility shift assay showed that OsbZIP48 directly binds to the OsKO2 promoter. The RNAi lines and T-DNA insertional mutant of OsbZIP48 showed seedling lethal phenotype despite the fact that roots were more proliferative during early stages of development. These data provide credible evidences that OsbZIP48 performs more diverse functions in a monocot system like rice in comparison to its *Arabidopsis* ortholog, HY5.

**Introduction**

Light signalling network is one of the most extensively studied network in plants. The light signal transduction can be divided into sensory photoreceptors, early signalling factors, central integrators and downstream effectors (Chory, 2010). The photoreceptors like phytochromes, cryptochromes, phototropins, UVR8 and zeitlupe are involved in the perception of light signal. They perceive the light signal and transmit it to early signalling factors like HFR1, FAR1, LAF1, PIFs and EID1. The central integrators of COP/DET/FUS class regulate other proteins involved in this pathway by targeting them for degradation (Chory, 2010). Downstream effectors like HY5 regulate the expression of an innumerable number of genes associated with photomorphogenesis. The *hy5* mutant (long hypocotyl 5) was one of the five *Arabidopsis* mutants isolated originally by Koornneef and co-workers that showed a long hypocotyl phenotype even when grown in light (Koornneef et al., 1980); the wild type seedlings characteristically developed short hypocotyl. Molecular genetic analysis revealed that *HY5* codes for a basic leucine zipper (bZIP) transcription factor that serves as a positive regulator of photomorphogenesis in *Arabidopsis* (Oyama et al., 1997). Subsequently, it was found that HY5 is constitutively nuclear localized and acts downstream of phytochromes, cryptochromes and
UVR8, indicating that it promotes photomorphogenesis under a broad spectrum of wavelengths, including far-red (FR), red (R), blue (B) and UV-B (Koornneef et al., 1980; Oyama et al., 1997; Osterlund et al., 2000; Ulm et al., 2004; Jiao et al., 2007; Huang et al., 2012; Jiang et al., 2012; Ram and Chattopadhyay, 2013; Zheng et al., 2013). Detailed characterization of hy5 mutant showed that apart from elongated hypocotyl, the light-grown hy5 mutant seedlings also develop more number of lateral roots and exhibit altered gravitropic and touch response in roots (Oyama et al., 1997). In addition, secondary thickening was reduced and the number of lignified xylem vessels and fibre elements were less. The chlorophyll and anthocyanin accumulation in the hy5 mutant seedlings was considerably reduced as compared to the wild type, although the hy5 mutant seedlings had slightly larger cotyledons than the wild type (Oyama et al., 1997; Sibout et al., 2006). Chip-chip analysis revealed that HY5 binds to the promoters of more than 3000 genes (Zhang et al., 2011) indicating that it is a master regulator that binds to an array of genes associated with multiple regulatory circuits and metabolic pathways. Among these genes, 1173 genes showed HY5-dependent expression indicating that it probably co-regulates many target genes through integrated sub-programs; some of those genes were positively and others were negatively regulated by HY5 indicating that it may act as both transcriptional activator and repressor (Zhang et al., 2011).

Although HY5 has been characterized extensively from Arabidopsis, it has been a subject of investigation in other organisms like Physcomitrella patens, Lotus japonica and Pisum sativum (Nishimura et al., 2002; Weller et al., 2009; Yamawaki et al., 2011). But none of the homologs of HY5 in monocots has been characterized so far. In our previous publication on an overall analysis of the bZIP family in rice, we had identified three putative homologs in rice, which form part of the same clade of bZIPS as AthY5 and AthYH (Nijhawan et al., 2008). In the present study, we have functionally characterized OsbZIP48, one of the three AthY5 homologs in rice, with the aim to find out if it can functionally complement Athy5 mutant and whether it performs any unique functions in a monocot system like rice.

**Results**

**Monocots have three homologs of AthY5**

The phylogenetic and pairwise distance analysis of homologs of AthY5 showed that there are three homologs of AthY5 in monocots while HYH homologs are represented in the genomes of only dicots and gymnosperms (Figure 1). The HY5/HYH phylogenetic tree in figure 1 suggests that out of the three HY5 homologs, the clade of two homologs of HY5 in monocots is closer to the eudicots clade of HY5 while there is no HYH homolog in monocots. However, we were able
to retrieve HYH homolog from *Picea*, a gymnosperm. Moreover, a sub-clade of monocot HY5 homologs was found to be present as a sister clade of the HY5/HYH homologs found in lower plants along with HY5/HYH clade. Therefore, pairwise distance analysis of the bZIP domain of these proteins was performed ([Supplementary Table 1](#)), which showed that this sub-clade is closer to AtHY5 than AtHYH. The pairwise distance analysis results indicate that the HY5/HYH homologs in lower organisms like moss and lycopod are closer to HY5 than HYH. Thus, HYH homologs might be absent in lower plants and monocots but are present in gymnosperms and dicots. Although, gymnosperms diverged before the divergence of monocots and dicots, the presence of AtHYH homolog in gymnosperms but its absence in monocots is intriguing. A detailed evolutionary analysis of AtHYH would be possible only when more genomic data on gymnosperms become available.

The alignment of protein sequences of all HY5 homologs showed that COP1-interaction motif (as described by Holm et al., 2002) is present in most of the homologs suggesting that it might be regulated by COP1 in these organisms as well ([Supplementary figure 1](#)). A consensus casein kinase II (CKII) phosphorylation site (ESDEE) is conserved in most of the HY5 homologs and in one homolog of *Chlamydomonas reinhardtii* indicating that if the COP1-interaction motif is functional in other organisms, HY5 activity and its binding ability to COP1 is likely to be modulated by phosphorylation. The conserved VP pair of this motif is important for interaction of HY5 with COP1 (Holm et al., 2001). The COP1-interaction motif in green algae lacks this conserved VP pair and has many insertions in COP1-binding motif. Therefore, interaction studies need to be done in order to find whether HY5 homologs of green algae are able to interact with its COP1 counterpart.

**Expression analysis of OsbZIP48 in different tissues of rice**

The microarray based expression of *OsbZIP48* was first checked in Affymetrix meta-analysis data and rice atlas data sets (GSE6893 and GSE14298) using rice oligonucleotide array database; this also includes our own microarray data on whole genome expression analysis at various stages of panicle and seed development in rice (Sharma et al., 2012). Although *OsbZIP48* expression was maximum in stigma followed by ovary, its transcripts could be detected in the plumule, coleoptile, shoot, leaf, flag leaf, spikelet, lemma/palea and embryo sac of the rice plant. Among different tissues and at various developmental stages of rice examined, *OsbZIP48* expression was found to be significantly high in second leaf and during tiller initiation, P5 panicle and S1 stage of seed development. Apart from these tissues, *OsbZIP48* expression was found to be high in the gynoecium during pollination and fertilization ([Supplementary
Real time PCR analysis was carried out to validate the microarray based expression data of OsbZIP48 at different stages of development in rice (Figure 2A). The expression of OsbZIP48 was found to be maximum at the P6 stage of rice panicle development, which was slightly different from the microarray data which showed maximum expression in S1 stage as compared to P6; these stages are however in succession and thus closely related. The expression of OsbZIP48 was examined at different stages of pollen development and in P6 ovary, P6 lemma, P6 palea, pre-pollinated anther, pre-pollinated ovary, pre-pollinated stigma, 0-DAP (Days after pollination) ovary, 0-DAP stigma, 1-DAP ovary and 1-DAP stigma. The real-time PCR analysis revealed that OsbZIP48 expression was maximum in 1 DAP stigma followed by P6 palea and pre-pollinated stigma. Some basal level expression could be detected in 0 DAP ovary, 0 DAP stigma and P6 lemma. High level of expression in 1 DAP stigma and pre-pollinated stigma (Figure 2B) indicates that it might have some role to play in pollination and post-pollination events of panicle development.

The over-expression of gene encoding AtHY5 lacking COP1-binding domain is known to cause reduction in hypocotyl length in Arabidopsis when grown in light. On the other hand, no change in hypocotyl length was observed between the wild type and transgenics overexpressing AtHY5 without COP1-binding domain when grown in dark (Ang et al., 1998). The structure of mature rice stem differs from that of Arabidopsis as it is hollow and consists of nodes and internodes. Therefore, expression of OsbZIP48 was checked in the internodes of the mature rice plant just after the whole panicle has emerged. The expression pattern of OsbZIP48 showed a decline from the bottom to top internodes with the first and the bottom most internode (which does not have shoot borne roots) having the maximum expression and the second last and topmost (the last internode), which bears the panicle, having the least (Figure 2C). This correlates well with the fact that the bottommost internode is shortest while the second last and last internode are the longest in a rice plant.

Since AtHY5 is known to be involved in light signalling, the expression of its ortholog in rice, OsbZIP48, was checked in rice seedlings grown in dark and light (75 μmol/m²/s) for 3 to 7 days (Figure 2D). The expression of OsbZIP48 was of basal level in 3- and 4-day-old dark and light grown seedlings and then reached its peak on the 5th day in both dark and light grown seedlings and then began to decline in 6- and 7-day-old seedlings. When expression was checked in 5-day-old shoot and root, OsbZIP48 transcript levels were higher in shoots of the 5-day-old light grown seedlings than the roots (Figure 2E).
OsbZIP48 protein levels are not light regulated

To determine whether OsbZIP48 protein levels are developmentally regulated, the protein extracts from light as well as dark grown rice seedlings were processed for western analysis. In light grown seedlings, OsbZIP48 protein levels increased gradually in 3- to 5-day-old seedlings and were maintained in 7-day-old seedlings but decreased in 10-day-old seedlings (Figure 3A). However, in dark grown seedlings the OsbZIP48 protein level was less in 3-day-old seedling as compared to 5-day-old seedling but thereafter it remained constant till the 10th day (Figure 3B). OsbZIP48 levels were also examined during light to dark transition and they were found to be constant even after 20 h of light to dark and dark to light transition, indicating that OsbZIP48 protein levels are not light regulated in rice (Figure 3C, D). In order to investigate whether rice OsbZIP48 followed a similar pattern of accumulation when expressed in Arabidopsis, light to dark transition experiments were performed. The Athy5 mutant seedlings complemented with OsbZIP48 were used to examine the level of OsbZIP48 in Arabidopsis. Its levels were found to be constant during light to dark transition suggesting that it is not degraded in Arabidopsis as well (Figure 3F).

OsbZIP48 protein levels were checked during different stages of panicle and seed development. During panicle development, the protein levels were found to be maximum in P3 and P4 stages while during seed development the protein levels were found to be high initially, i.e. during S1 and S2 stages, and then decreased gradually (Figure 3G,H). Among the vegetative tissues examined, the level of OsbZIP48 protein was found to be more in the mature root than mature leaf (Figure 3I).

The HY5 protein in Arabidopsis exists in two forms, i.e. phosphorylated and unphosphorylated (Hardtke et al., 2000). In the present study too, two bands could be recognised by anti-OsbZIP48 antibodies in the western blots, where the higher molecular weight form may represent phosphorylated form of OsbZIP48; although it remains to be validated experimentally. It has been shown that the unphosphorylated form of HY5 is more active than phosphorylated form and is the preferred substrate for COP1 mediated degradation (Hardtke et al., 2000). In case of OsbZIP48, the phosphorylated form of protein (the upper band in western blots) gradually increased till it attained maximum level in 5 to 7-day-old light grown seedlings and thereafter it declined and was virtually undetectable in 10-day-old seedlings (Figure 3A). It is interesting to note that in 5 to 7-day-old light grown seedlings, the phosphorylated and unphosphorylated forms of protein were almost equal while in 5 to 10-day-old dark grown
seedlings the abundance of phosphorylated form was low than the unphosphorylated form of OsbZIP48 (Figure 3A, B).

**OsbZIP48 is nuclear localized and forms a homodimer**

The bZIP proteins are transcription factors, therefore, to elucidate the intra-cellular localization of OsbZIP48, particle bombardment of onion peel cells using gold particles as macro-carriers was done using pSite3CA-OsbZIP48 as the construct. DAPI was used as a control for nucleus staining while empty pSite3CA was used as a vector control. The YFP-OsbZIP48 fusion protein was found to be localized in the nucleus (Figure 4A) as predicted by ProtComp 9.0, an online program to predict sub-cellular localization of plant proteins. The bZIP proteins are known to bind to the DNA as homo- or hetero-dimers. This dimerization is mediated by the leucine zipper region of the bZIP domain (Landschulz et al., 1988). Therefore, homodimerization of OsbZIP48 was checked by Fluorescence Resonance Energy Transfer (FRET) and Bimolecular Fluorescence Complementation (BiFC) analysis (Figure 4B-D). The FRET efficiency of OsbZIP48 was 28% indicating that it can form a homodimer. BiFC analysis using onion peel cells confirmed that OsbZIP48 homodimerizes exclusively in the nucleus.

**OsbZIP48 lacks trans-activation domain**

Many bZIP transcription factors like OsbZIP16 are known to have trans-activation domain through which they can activate the transcription machinery of the cell (Chen et al., 2012). However, there is a debate on whether AtHY5 can act as a transcriptional activator or repressor. AtHY5 in fact does not show transcription activation in yeast cells (Ang et al., 1998) but Chip-chip analysis of AtHY5 suggested that it can act both as an activator and a repressor of a rather large number of genes (Zhang et al., 2011). Therefore, trans-activation analysis of OsbZIP48 in yeast was carried out (Figure 4E). This assay showed that while the yeast cells containing the positive control grew on SD-HW media, the cells containing OsbZIP48-pGBK construct did not grow on SD-HW media, indicating that OsbZIP48 does not have a functional trans-activation domain.

**OsbZIP48 is a functional ortholog of AtHY5**

To find out whether OsbZIP48 is functionally similar to Arabidopsis HY5, complementation of Arabidopsis hy5 mutant was carried out by over-expressing OsbZIP48 in the hy5 mutant. The homozygous transgenic lines were checked for the expression of OsbZIP48 by real time PCR and for the presence of hygromycin (hptII) gene by PCR (Supplementary Figure 3). Since the Arabidopsis hy5 mutant showed elongated hypocotyl phenotype in light, the homozygous
transgenic lines of OsbZIP48/hy5 (mutant background) were checked for hypocotyl length in 3- and 6-day-old white light grown seedlings. As shown in figure 5A and B, the average hypocotyl length of a 3-day-old WT, hy5 mutant and OsbZIP48 over-expressed in hy5 mutant background (OsbZIP48;hy5) were approximately 1 mm, 3.7 mm and 1.2 mm, respectively, indicating that OsbZIP48 can compensate for the loss-of-function of HY5 in Athy5 mutant. Arabidopsis transgenics over-expressing OsbZIP48 in Col-0 (WT) background (OsbZIP48OE) were generated and their hypocotyl length measured under white light. While the average 3-day-old wild type hypocotyl was approximately 1 mm, the average OsbZIP48OE hypocotyl length was approximately 0.86 mm (Figure 5B). Since Deng and co-workers had done most of the hypocotyl length measurements in 6-day-old seedlings (Holm et al., 2002), the hypocotyl length of the transgenics was also measured in 6-day-old seedlings (Figure 5C). While the average hypocotyl length of the wild type and hy5 mutant was approximately 1.4 and 6.6 mm, respectively, the average hypocotyl length of OsbZIP48OE and OsbZIP48;hy5 seedlings was approximately 1.2 and 1.6 mm, respectively (Figure 5C). No significant difference in hypocotyl length was observed in the dark grown seedlings (data not shown).

**OsbZIP48 is able to rescue the agravitropic response of hy5 mutant**

The hy5 mutant seedlings are known to lack proper gravitropic response (Oyama et al., 1997; Sibout et al., 2006). In order to check whether OsbZIP48 is able to alter the gravitropic response of hy5 mutant and make it respond in a manner similar to the wild type, vertical growth index (VGI) was used for the quantitative analysis of root morphology (Figure 5D, E). VGI can be defined as the ratio between a vertical projection of the base-to-tip chord and the root length (Vicente-Agullo et al., 2004). On measuring VGI of 3-day-old wild type, hy5 mutant and OsbZIP48;hy5 seedlings, it was found that while the average VGI of the wild type was 0.82, VGI of hy5 mutant and OsbZIP48;hy5 was 0.65 and 0.76, respectively. In fact, some of the transgenics showed VGI similar to wild type indicating that OsbZIP48 over-expression is able to rescue the gravitropic response in hy5 mutant roots to normal wild-type phenotype. Interestingly, the root hairs of hy5 mutant were found to be agravitropic and OsbZIP48 was able to rescue this response as well (Figure 5D). The cotyledon angle of hy5 mutant seedlings was also less as compared to the wild type and OsbZIP48 was able to complement the loss of AtHY5 in the transgenics in this respect too (Figure 5F, G).

In addition to altered root gravitropic response, we found that gravitropic set angle of siliques in hy5 plants was different from that of the wild type (Figure 5H), which probably did not come to the notice of previous researchers. In mature plants, the lateral organs generally do not remain
parallel to the gravity vector but tend to exist at a particular angle with respect to the vertical growth axis (Wei et al., 2010). The angle between the pedicel and the inflorescence stem influences both the architecture and the yield potential of the plants (Wang and Li, 2008). This stem-pedicel angle of the siliques was larger in hy5 mutant as compared to the wild type. The stem-pedicel angle of OsbZIP48;hy5 plants was similar to the wild type.

**OsbZIP48 complements hy5 mutant in restoring anthocyanin and chlorophyll content**

HY5 is known to be involved in anthocyanin and chlorophyll biosynthesis and hy5 mutant seedlings show reduced greening in the middle and lower parts of the hypocotyl (Oyama et al., 1997; Chattopadhyay et al., 1998). Therefore, anthocyanin and chlorophyll content of WT, hy5 mutant, OsbZIP48\(^\text{OE}\) and OsbZIP48;hy5 Arabidopsis seedlings were estimated. (Supplementary Figure 4). The anthocyanin content of 3-day- and 6-day-old seedlings of OsbZIP48\(^\text{OE}\) was similar to the wild type indicating that over-expression of full-length OsbZIP48 does not cause any increase in anthocyanin content in Arabidopsis seedlings (Supplementary figure 4B and C). The chlorophyll content of 6-day-old OsbZIP48\(^\text{OE}\) transgenics was similar to the wild type (Supplementary figure 4D). In 3-day-old OsbZIP48;hy5 seedlings, although the anthocyanin content was more than the hy5 mutant seedlings, it was comparatively less than the WT (Supplementary figure 4B). However, the anthocyanin content of 6-day-old OsbZIP48;hy5 seedlings was almost similar to the WT in three of the five transgenic lines analysed (Supplementary figure 4C). The chlorophyll content of 6-day-old OsbZIP48;hy5 seedlings was more than the hy5 mutant but was comparatively less than the WT (Supplementary figure 4D).

**OsbZIP48\(^\text{OE}\) rice transgenics display semi-dwarf phenotype**

In addition to functional complementation of hy5 mutant of Arabidopsis by its rice ortholog OsbZIP48, as described in preceding pages, it was imperative to generate rice transgenics to decipher if OsbZIP48 performs any additional or unique functions in a monocot system like rice. Thus, over-expression transgenics of OsbZIP48 were raised in rice. The level of expression of OsbZIP48 in the leaves of mature plants for over-expression lines was checked and was indeed significantly higher than the wild type (Supplementary figure 5). Southern blot analysis was performed using hygromycin ( \textit{hptII} ) gene as the probe. The genomic DNA of the wild type, vector control and transgenics was digested by EcoR1 restriction enzyme which is a non-cutter of hygromycin gene. The probe was made using hygromycin ( \textit{hptII} ) gene as the template. As a result, the bands in the Southern blot correspond to the hygromycin ( \textit{hptII} ) gene, which in turn indicate the number of inserts present in the transgenic line. As is evident from the
supplementary figure 5, no bands could be seen in the wild type lane of the Southern blot while three bands are visible in the vector control (pB4NU vector), indicating that there are three inserts in the vector control. Line 1 has one, line 38 has two and line 39 has three bands, indicating that there may be as many corresponding insertions of OsbZIP48 CDS in these three lines. Since, no two transgenics have the bands corresponding to the transgene in the same position(s), one can conclude that each transgenic line represents independent event(s) (Supplementary figure 5).

The rice OsbZIP48OE transgenic seedlings displayed a semi-dwarf phenotype and were distinctly shorter than the wild type seedlings (Figure 6A, B). Even the adult OsbZIP48OE vegetative plants and those bearing panicles were shorter in height as compared to the wild type (Figure 6C, D). The over-expression transgenics were also greener for a slightly longer time than the wild type (Figure 6D). The over-expression of OsbZIP48 resulted in reduction of the internode length as well as the panicle length of the rice plants (Supplementary Figure 6B,C). The reduction in plant height was accompanied by reduction in the stem width as the stem of mature OsbZIP48OE transgenics was thinner than that of the wild type (Figure 7A-E). Quantitative analysis of the width of the stem of WT and OsbZIP48OE transgenics and of cell length and cell area showed that there was an overall reduction in the stem width and cell size in over-expression transgenics (Figure 7F-J; Supplementary Figure 7). The transgenics showed smaller vascular bundles and less secondary cell wall thickening as compared to the wild type (Figure 7F-G, 8). Since OsbZIP48OE transgenics showed a reduction in internode length and panicle length, a detailed morphometric analysis of these transgenics was performed and parameters like total plant height, culm length, flag leaf length, panicle length, total number of florets and total number of fertile florets were considered and measured according to IRRI guidelines (Supplementary Figure 8, 9). While the average plant height of the wild type was approximately 82 cm, the average plant height of OsbZIP48OE transgenics was approximately 59 cm; about 32% reduction. Similarly, the culm of OsbZIP48OE showed 36% reduction as compared to the wild type; with the wild type and OsbZIP48OE having an average culm length of approximately 51 cm and 32 cm, respectively. There was no change in the flag leaf length of most of the transgenics as compared to the wild type but the transgenics showed 19% reduction in the length of their panicle, on an average, as compared to the wild type. This indicated that OsbZIP48 over-expression is mainly affecting the length of the stem and panicle. However, there was close to 38% reduction in the total number of florets in the panicle with the average number of florets per panicle in the wild type plants and transgenics being 73 and 45,
respectively. The lines which had much higher expression of OsbZIP48 (Line 38 and Line 39) had a high degree of sterility. While the fertility percentage of the wild type plants was 76%, the average fertility percentage in these two transgenics was 47%. However, one transgenic event (line 1 with single insert), which had only 2.4-fold increase in OsbZIP48 expression as compared to the wild type, had fertility percentage nearly similar to WT.

Whether the decrease in height (semi-dwarfism) of OsbZIP48OE transgenics is because of uniform reduction of all internodes or some particular internode(s), the length of internodes was measured. Although there was no major change in the number of nodes in most of the transgenics as compared to the wild type, however, a reduction of 48% was recorded in the the length of second last internode followed by the last internode (30% reduction) in the transgenics as compared to the wild type. The first internode showed the least reduction (Supplementary Figure 9). There was not much difference in the total number of tillers as the average tiller number of wild type plants and of transgenics varied between 3 to 4, under the greenhouse conditions maintained (Supplementary Figure 9). The over-expression transgenics appeared to be greener than the wild type and, therefore, their chlorophyll content was estimated. The chlorophyll content in over-expression transgenics was found to be more than the wild type (Supplementary Figure 10). The over-expression of OsbZIP48 also resulted in reduced secondary cell wall thickenings as revealed by the scanning electron microscopy. All the three transgenic lines showed reduced secondary cell wall thickenings in sclerenchyma cell walls as compared to the wild type indicating that cell wall composition might be altered (Figure 8).

OsbZIP48KD rice transgenics are lethal
RNA interference technique was used to create OsbZIP48 knock-down (OsbZIP48KD) lines. The PCR-based confirmation of these transgenics and the real time PCR analysis was carried out to measure the degree of silencing in white light grown transgenic seedlings (Supplementary Figure 5). The T1 OsbZIP48KD transgenics showed profuse root growth at the seedling stage and the plants were slightly taller than the wild type in the contained greenhouse environment (Figure 9A-C). However, in T2 generation onwards, two types of seedlings were seen segregating among the populations of RNAi transgenics. One set of seedlings (referred as ‘Hh’) showed similar morphological characters as the wild type but were hygromycin positive. These seedlings showed growth pattern similar to the wild type when they were shifted to greenhouse. The other set of seedlings (referred as ‘hh’) showed retarded growth, less chlorophyll content and perished within one month on MS basal medium (Figure 9D & E); these seedlings were hygromycin positive as they were PCR positive for hptII gene. The wild type seedlings died...
within five to ten days of transfer to hygromycin containing MS basal medium. Also, the OsbZIP48 transcript levels were drastically reduced in the RNAi transgenic seedlings that perished than the ones that were similar to the wild type (Supplementary Figure 5). Seedlings which resembled wild type seedlings in the T2 generation, in the next generation, also showed the similar two-phenotype segregating pattern; even after reaching to the T4 generation, same segregation pattern was observed. The chlorophyll content in 20-day-old OsbZIP48KD transgenics (showing retarded growth) was considerably less as compared to the wild type (Supplementary Figure 10).

Phenotypic analysis of OsbZIP48 T-DNA insertional mutant
In order to further evaluate the functional role of OsbZIP48, analysis of a T-DNA insertional mutant obtained from Rice Functional Genomic Express (RiceGE) database was carried out. When grown on MS basal medium containing hygromycin, they showed a growth pattern similar to the T2 RNAi transgenic lines (OsbZIP48KD). Two types of seedlings were obtained, one which were similar to the wild type seedlings and the other type exhibited lethal phenotype. It lacked any internode, showed bleached spots on the leaves and profuse root growth (Figure 9F, G). However, it was able to grow to a limited extent on MS basal medium containing sucrose but was not able to survive at all on the medium which lacked sucrose, i.e. the rice growth medium used in this study for raising transgenics. The phenotypic analysis of the T-DNA insertional mutant and OsbZIP48KD plants suggests that OsbZIP48 is crucial for the proper seedling development and its transition to the adult plant and its drastic reduction or total absence can be lethal.

Microarray analysis of OsbZIP48 over-expression and RNAi transgenics
Since OsbZIP48 is a transcription factor, its major mode of action would be to alter the expression of downstream genes. In order to get an insight into the mechanism of action of OsbZIP48 through which it regulates such an array of developmental events, whole-genome microarray analysis was performed for the over-expression as well as RNAi transgenics along with respective vector controls (pB4NU for over-expression and pANDA for RNAi). Microarray analysis of both types of RNAi transgenics was performed and those which resembled wild type seedlings in phenotype were labelled as 'Hh' and those which showed lethal phenotype were labelled as 'hh'.

OsbZIP48OE alters expression of hormone biosynthesis pathway genes
Microarray analysis of 10-day-old white light (75 μmol/m²/s) grown over-expression transgenic seedlings showed that close to 1741 genes were differentially expressed as compared to pB4NU vector control (VC), with 251 genes up-regulated and 1490 genes down-regulated. The over-expression of OsbZIP48 caused repression of genes associated with gibberellin, jasmonic acid, brassinosteroid and ethylene biosynthesis pathways (Figure 10; Supplementary Figure 11). However, no significant change in expression of genes involved in ABA and auxin biosynthesis/signalling pathways was detected in our analysis. The down-regulation of cytokinin glucosylation was also observed in OsbZIP48<sup>OE</sup> transgenics as compared to the vector control. Cytokinins can be glucosylated to form O-glucosides and N-glucosides, which are inactive and involved in hormone homeostasis (Hou et al., 2004). This pathway was down-regulated in OsbZIP48<sup>OE</sup> transgenics and may have resulted in more accumulation of free cytokinins (Supplementary Figure 12). This might explain the slightly delayed senescence observed in OsbZIP48<sup>OE</sup> transgenic plants as compared to the wild type, as stated earlier. Strikingly, among two of the homologs of OsbZIP48 in rice, OsbZIP01 (another AtHY5 homolog in rice) was found to be up-regulated by 9-fold in OsbZIP48<sup>OE</sup> transgenics as compared to the vector control, whereas OsbZIP18 (another AtHY5 homolog in rice) was found to be downregulated by 2.7-fold (Figure 11).

**Altered expression of genes involved in GA biosynthesis and signalling in OsbZIP48<sup>OE</sup> transgenics**

Microarray analysis and real time PCR showed that OsCPS (LOC_Os02g36210) and OsKO2 (LOC_Os06g37300) were downregulated in OsbZIP48<sup>OE</sup> transgenics (Figure 10 and Figure 11). Literature search was carried out to identify rice genes that are known to be involved in controlling plant height in rice. The list of genes involved in controlling rice plant height and their expression in over-expression transgenics is provided in table 1. Expression of some of them, including OsNAC2, OsGDD1, OsPIL1 and OsEATB, was checked (Li et al., 2011; Qi et al., 2011; Todaka et al., 2012; Chen et al., 2015) The expression of OsNAC2 was high but OsPIL1, OsGDD1 and OsEATB were downregulated in these transgenics (Figure 11).

The real time PCR analysis revealed that the expression of OsGA20ox2, OsGA2ox1, KO-like genes -- OsKOL4 and OsKOL5 -- in OsbZIP48<sup>OE</sup> transgenics was downregulated (Figure 11). OsCYP714B1, which encodes gibberellin 13-oxidase, was also significantly downregulated. The expression of genes that are involved in GA signalling, like OsSLR1, OsSLRL, OsGID1 and OsGID2, was also checked. OsSLR1 and OsSLRL1 are central repressors of GA signalling while OsGID1 and OSGID2 are positive regulators of GA signalling (Hauvermale et al., 2012).
The expression of OsSLR1, OsSLRL1, OsGID1 and OsGID2 was downregulated. Essentially similar trend was also observed with regard to the expression of OsEXPANSIN S1, OsEXPANSIN S2, OsEXPA2 and OsEXPA4 genes (Figure 11).

Changes in gene expression in RNAi transgenics

Microarray analysis of OsbZIP48 RNAi transgenics (OsbZIP48\textsuperscript{KD}) showed altered expression of 1857 genes with 856 and 1001 genes showing up-regulation and down-regulation, respectively. The heatmap of 10-day-old hh, Hh and pANDA vector control seedlings grown in white light (75 μmol/m\textsuperscript{2}/s) showed that the degree of differential expression of these genes was greater in ‘hh’ seedlings as compared to ‘Hh’ seedlings (Supplementary Figure 13). This also corresponds to the phenotype which these two seedlings show; ‘hh’ seedlings correspond to the RNAi seedlings which show lethal phenotype and, therefore, exhibit greater degree of differential expression. ‘Hh’ seedlings are phenotypically similar to the wild type and, therefore, do not show any drastic difference in their expression pattern as compared to the wild type. However, the expression of two other homologs of OsbZIP48, i.e. OsbZIP01 and OsbZIP18, was not significantly altered in OsbZIP48\textsuperscript{KD} transgenics as compared to the vector control (Supplementary Figure 16).

In OsbZIP48\textsuperscript{KD} lines, the activation of GA biosynthesis, jasmonic acid and IAA biosynthesis pathways must have occurred as genes encoding for enzymes involved in these pathways showed higher expression as compared to the vector control (Figure 10; Supplementary Figure 14). In relation to GA\textsubscript{12} biosynthesis, while two genes (LOC_Os02g36210 and LOC_Os06g37300) were down regulated in OsbZIP48\textsuperscript{OE} transgenics, only one of them (LOC_Os06g37300 or OsKO2) was up-regulated in OsbZIP48\textsuperscript{KD} transgenics. With respect to jasmonic acid biosynthesis, four genes encoding for the enzymes involved in this pathway showed altered expression in comparison to the vector control. Only one gene (LOC_Os08g39850) is common in OsbZIP48\textsuperscript{OE} and OsbZIP48\textsuperscript{KD} transgenics and it showed decreased expression in OsbZIP48\textsuperscript{OE} transgenics and its expression was high in OsbZIP48\textsuperscript{KD} transgenics. For brassinosteroid biosynthesis pathway, one gene (LOC_Os01g01650) showed reduced expression in OsbZIP48\textsuperscript{KD} transgenics as compared to the vector control and this gene is different from the genes altered in OsbZIP48\textsuperscript{OE} transgenics but catalyse the same step. IAA biosynthesis pathway genes were not altered in OsbZIP48\textsuperscript{OE} transgenics but were up-regulated in OsbZIP48\textsuperscript{KD} transgenics. Thus, down-regulation of OsbZIP48 does indeed alter the homeostasis of major hormonal pathways. Real-time PCR confirmation of the changes in expression of genes involved in hormonal biosynthesis pathway and showing differential
expression in over-expression and RNAi transgenics by microarray was also done and data are presented in supplementary figure 15 and 16.

**OsbZIP48 directly binds to the promoter of OsKO2 through a G-box element**

Since OsKO2 was found to be downregulated in OsbZIP48\(^{OE}\) and up-regulated in OsbZIP48\(^{KD}\) (Figure 10A), promoter analysis of OsKO2 was performed manually as it could be a direct target of OsbZIP48. The analysis revealed that there are three G-box binding elements (CACGTG) within 1 kb region upstream of the transcription start site of OsKO2 gene (Figure 10B). Electrophoretic mobility shift assay showed that OsbZIP48 binds to the G-Box at -504 bp (G-box II) from transcription start site (Figure 10C) and not to the other two G-box elements. In the same assay we were able to demonstrate that OsbZIP48 is able to bind to the labelled probe containing this G-box but not the mutated version (G-mut II) where the G-box had been disrupted (Figure 10D). This indicates that OsbZIP48 directly binds to the promoter of OsKO2 and may regulate its expression.

**Discussion**

**AtHY5 has three homologs in monocots**

Phylogenetic tree and pairwise distance analysis of AtHY5 and AtHYH homologs across different plant species shows that while there are three AtHY5 homologs in monocots, AtHYH homologs are present only in gymnosperm and dicots. Also the homologs in moss and lycopod seem to be closer to AtHY5 than AtHYH indicating that HYH might have evolved more recently as compared to HY5. OsbZIP48 represents one of the three homologs of AtHY5 in rice and has been functionally characterized in this study. The other two homologs of OsbZIP48 are OsbZIP1 and OsbZIP18 (Nijhawan et al., 2008) that have a different expression profile in vegetative and reproductive tissues examined and may have undergone neofunctionalization; their functional validation will throw more light on the specific and/or overlapping functions they perform vis-à-vis OsbZIP48.

**OsbZIP48 has expression profile and molecular characteristics essentially similar to AtHY5**

OsbZIP48 was found to have maximum expression in 1 DAP stigma, although it expressed in pre-pollination stigma, P6 panicle stage and the first internode of rice plant. In Arabidopsis too, the maximum expression of AtHY5 has been reported in the floral organs and stem of the Arabidopsis plant (Oyama et al., 1997) indicating that, in terms of expression pattern, OsbZIP48 resembles AtHY5 to a large extent. Gibberellins are known to be required for seed development...
and pollen tube growth in *Arabidopsis* (Singh et al., 2002) and HY5 is known to regulate gibberellin levels (Weller et al., 2009). In our analysis, we have found that there is high level of expression of OsbZIP48 in the stigma. The genes involved in gibberellin biosynthesis are downregulated in OsbZIP48\textsuperscript{OE} transgenics. This indicates that OsbZIP48 might play a role in pollen tube elongation and fertilisation by modulating gibberellin levels. The expression of OsbZIP48 was found to be high in 5-day-old dark and light grown seedlings indicating that the expression of OsbZIP48 is developmentally regulated instead of being regulated by light. OsbZIP48 is nuclear localized and lacks transactivation activity as assayed using yeast system; this is akin to the observation made for AtHY5 too (Ang et al., 1998; Chattopadhyay et al., 1998; Stracke et al., 2010).

**OsbZIP48 protein accumulation is developmentally regulated but it is not light labile**

Western blot analysis of total protein extracts from light and dark grown rice seedlings as well as from different developmental stages of rice showed that anti-OsbZIP48 antibody recognises a protein band running at an apparent molecular weight of 30 kDa, which is similar to what was observed in case of HY5 protein in *Arabidopsis* (Osterlund et al., 2000). OsbZIP48 was expressed in the bacterial system and the protein purified was used as positive control in order to confirm its migration in SDS-PAGE.

AtHY5 protein levels were found to be maximum 2-3 days after seed germination and after that they declined gradually (Hardtke et al., 2000). In contrast, OsbZIP48 protein levels in rice were found to be maximum 5 to 7 days after seed germination and, like AtHY5, declined gradually thereafter. In 3- to 10-day-old dark grown rice seedlings, levels of OsbZIP48 were rather high. It is worth mentioning here that as compared to photomorphogenesis in dicots like *Arabidopsis*, monocots undergo partial photomorphogenesis in dark. In monocots like rice first and second leaf emergence takes place in dark (Zhang et al., 2006). It is possible that accumulation of OsbZIP48 protein might play a role in partial photomorphogenesis in dark during rice seedling development as its levels increase in 5-day-old seedling as compared to 3-day-old seedlings grown in dark.

HY5 protein levels change within 5 hours of light to dark and dark to light transitions in *Arabidopsis* with its levels drastically decreasing within 20 hours of light to dark transition and, similarly, increasing within 20 hours of dark to light transition, which shows that HY5 levels are light regulated (Osterlund et al., 2000). However, unlike AtHY5, light to dark and dark to light
transition experiments wherein the 4-day-old rice seedlings grown in continuous light or dark conditions were transferred to opposite light/dark conditions for 5, 10, 15 and 20 hours, showed no drastic change in OsbZIP48 protein levels; the OsbZIP48 protein levels were found to be unaffected by the presence or absence of light. OsbZIP48 protein levels did not change even when hy5 mutant seedlings complemented with OsbZIP48 were subjected to light to dark transition, implying that OsbZIP48 is not degraded in dark by AtCOP1 as well. This might be due to the slightly different amino acid composition of the conserved COP1 binding domain of OsbZIP48 as compared to that of AtHY5. Despite being an ortholog of AtHY5 and having a COP1 binding domain, it is intriguing that OsbZIP48 is not degraded in dark. COP1 plays a major role in the light regulation of HY5 in Arabidopsis. In dark, COP1 localizes in the nucleus and binds to HY5 thus, causing proteasome mediated degradation of HY5 in the nucleus (Oyama et al., 1997; Ang et al., 1998; Osterlund et al., 2000), whereas in light, it localizes to the cytoplasm (von Amim and Deng, 1994). It is likely that the mechanism of regulation of HY5 ortholog in rice might be different from that in Arabidopsis.

OsbZIP48 protein levels were also checked during different developmental stages of rice and it was found to be present in tissues representing all the stages of panicle development starting from P1 to P6. The protein levels were high in P3 and P4 stages while the real time PCR analysis showed its transcript levels to be maximum in P6 stage. This indicates that OsbZIP48 levels are regulated at both transcriptional and translational levels during panicle development; however, what is the significance of these changes in expression of OsbZIP48 during various stages of panicle development remains to be elucidated. Interestingly, the protein levels were found to vary during seed development, being high in the initial stages of seed development (S1-S2) and declining gradually in the later stages (S3-S4) and were hardly detectable in the last stage of seed development (S5). This shows that OsbZIP48 accumulation starts after seed germination and peaks during early seedling development after which it gradually declines and its accumulation resumes during panicle development and continues till early stages of seed development. This correlates with the transcript profile of OsbZIP48 checked by real-time PCR. To a large extent, it appears that the protein profile of OsbZIP48 is similar to AtHY5, which is also known to accumulate in the inflorescences of Arabidopsis and is hardly detectable in the leaves, shoots and siliques (Hardtke et al., 2000).

**OsbZIP48 can functionally complement hy5 mutant**

When over-expressed in hy5 mutant of Arabidopsis, OsbZIP48 was able to complement hy5 mutant in respect of hypocotyl elongation growth in light, indicating that it is a functional ortholog
of AtHY5. However, over-expression of OsbZIP48 in Arabidopsis Col-0 wild type showed no significant effect on hypocotyl elongation growth, anthocyanin and chlorophyll content in comparison to the wild type. Morphometric analysis of Arabidopsis hy5 mutant seedlings showed that AtHY5 might be involved in root hair gravitropic response and silique gravitropic set angle (observation made in the present study), in addition to the agravitropic root response that has already been reported previously (Oyama et al., 1997; Sibout et al., 2006). It is surprising that alteration in positioning of root hairs and silique angle in hy5 mutant plants has escaped notice in previous studies. OsbZIP48 over-expression could functionally complement these traits as well. Arabidopsis hy5 mutant roots are known to show reduced basipetal auxin transport, which is consistent with their agravitropic response (Sibout et al., 2006). However, the genes involved in the agravitropic response of hy5 mutant and the mode of action through which AtHY5 controls gravitropic response has not been completely elucidated. Auxin is known to play an important role in regulating root gravitropism and involvement of the polar auxin transport is well documented in this response. The polar auxin transport is mainly mediated by membrane localized auxin influx and efflux carriers like AUX1/LAX proteins, PIN-formed proteins and the multidrug resistance/p-glycoprotein (PGP) class of ATP-binding cassette auxin transporters (Petrásek et al., 2006; Bandyopadhyay et al., 2007; Swarup et al., 2008; Zhang et al., 2011). Among the components of polar auxin transport, AUX1, PIN2, PIN3, PIP5K (phosphatidylinositol monophosphate 5-kinase) and PINOID (PID) proteins have been found to be involved in root gravitropic responses (Bennett et al., 1996; Müller et al., 1998; Friml et al., 2002; Sukumar et al., 2009; Mei et al., 2012). Chip-chip analysis showed that AtHY5 binds to the promoters of AUX1, PIN3, PIP5K and PID (Lee et al., 2007). Therefore, it is quite possible that AtHY5 might regulate root gravitropic response by altering the expression of these genes which, in turn, might alter the polar auxin transport machinery of the plant.

OsbZIP48 over-expression does not alter anthocyanin and chlorophyll content in Arabidopsis

In an earlier study, although over-expression of the full-length AtHY5 did not cause any increase in anthocyanin content in Arabidopsis but the partial CDS of AtHY5 (HY5-ΔN77) that lacked COP1-binding domain, caused increased anthocyanin accumulation in the upper region of the hypocotyl (Ang et al., 1998). HY5-ΔN77 transgenics showed more chlorophyll accumulation and precocious chloroplast development (Ang et al., 1998). In the present study, the chlorophyll and anthocyanin content in Arabidopsis transgenics over-expressing OsbZIP48 were not affected
significantly as compared to the wild type, although it could complement the hy5 mutant practically in all aspects of photomorphogenesis studied.

**OsbZIP48 controls plant height in rice**

The over-expression of OsbZIP48 in rice caused a decrease in plant height resulting in a semi-dwarf phenotype of the transgenics. This was caused due to reduction in cell size and maximum reduction was visible in the second last and last internodes. In wild type rice plants, the expression of OsbZIP48 is least in the second last internode followed by last internode and is maximum in the first internode. This is in accordance with the observation that in wild type plants the last internode (which bears the panicle) is longest followed by second last internode while the first internode is shortest in length. Therefore, the ectopic expression of OsbZIP48 caused maximum compression in the last and second last internodes as the natural expression of OsbZIP48 is normally less in these two internodes. This observation is quite different from what is known for AtHY5 as over-expression of full length AtHY5 does not show any significant difference in hypocotyl length of light grown Arabidopsis seedlings as compared to the wild type, whereas the over-expression of truncated AtHY5, which lacks COP1-binding domain, can shorten the hypocotyl (Ang et al., 1998). It is intriguing that only partial HY5 CDS lacking the COP1-binding domain can cause hypocotyl reduction, and increase in chlorophyll and anthocyanin accumulation in Arabidopsis while in rice, full length CDS of its ortholog, OsbZIP48, can cause reduction in height and increase in chlorophyll accumulation. Moreover, both the rice T-DNA insertional mutant and RNAi lines of OsbZIP48 resulted in lethal phenotype, although root proliferation in early stages of rice seedling development was profuse, a phenotype essentially similar to hy5 mutant seedlings. AtHY5 orthologs have been characterized in Physcomitrella patens, Lotus japonica and Pisum sativum and its role defined by analysing the mutants defective in this gene. However, the over-expression transgenics in the wild type Arabidopsis have been raised only for PpHY5a lacking COP1 binding domain and, like truncated AtHY5, the hypocotyl length was shorter than the wild type. In fact, besides Arabidopsis, the over-expression transgenics of the full length AtHY5 orthologs in the same organism have not been raised and that makes it difficult for us to conclude whether full length HY5 over-expression causes semi-dwarfism only in rice (present study) or in other organisms too.

Several plant hormone pathways are altered in OsbZIP48OE and OsbZIP48KD transgenics of rice
AtHY5 is known to be involved in auxin, gibberellin, cytokinin, ABA, ethylene and strigolactone signalling; while it represses ethylene, gibberellin and auxin signalling, it promotes ABA signalling (Cluis et al., 2004; Vandenbussche et al., 2007; Alabadi et al., 2008; Chen and Xiong, 2008; Li et al., 2011; Yu et al., 2013). In the present study too, the expression of genes associated with biosynthesis of gibberellin, jasmonic acid, ethylene and brassinosteroid, and cytokinin glucosylation was altered in OsbZIP48\textsuperscript{OE} transgenics. The over-expression of OsbZIP48 caused repression of gibberellin and ethylene biosynthesis related genes. The HY5 of pea was shown to bind to the promoter of GA2ox2 and increase its expression (Weller et al., 2009). In Arabidopsis also, chip-seq analysis showed that ATHY5 binds to the promoter of AtGA2ox2 (Lee et al., 2007). GA2ox2 converts bioactive GA\textsubscript{1} into bio-inactive GA\textsubscript{8}. Thus, evidently, HY5 reduces the amount of active GA\textsubscript{1} and that consequently may cause decrease in elongation of the hypocotyl. AtHY5 has been shown to bind to the promoter of AtERF11 and induce its expression which, in turn, represses the ethylene biosynthesis pathway (Li et al., 2011). In the microarray analysis carried out in the present study, we found that the transcript levels of rice homologs of these two Arabidopsis genes were elevated in OsbZIP48\textsuperscript{OE} transgenics but their fold change was less than 2-fold cut-off used in our analysis. We found that genes involved in GA\textsubscript{12} biosynthesis were repressed by more than 2-fold. GA\textsubscript{12} is the precursor for the synthesis of active GAs in rice. In addition to repression of genes involved in GA biosynthesis, genes involved in GA signalling like OsSLR1 and OsSLR1\textsubscript{L} were also repressed. The expression of OsPIL1 was significantly reduced in OsbZIP48\textsuperscript{OE} transgenics. OsPIL1 is known to be a positive regulator of internode elongation and causes internode elongation by increasing the cell size (Todaka et al., 2012). OsPIL1 does not alter the GA levels but up-regulates the expression of cell-wall related genes like OsEXPANSIN S1, OsEXPANSIN S2, OsEXPA2 and OsEXPA4. In OsbZIP48\textsuperscript{OE} transgenics too, the expression of OsEXPANSIN S1, OsEXPANSIN S2, OsEXPA2 and OsEXPA4 was reduced. Thus, OsbZIP48 might be regulating plant height in two ways. First, it decreases the biosynthesis of active GA by inhibiting the expression of GA biosynthesis genes or stimulating genes involved in its catabolism and, second, it down-regulates the expression of OsPIL1 which, in turn, decreases the expression of cell-wall related genes coding for expansins, which then leads to decrease in cell elongation. In addition, the expression of OsbZIP01 and OsbZIP18, which are the other two AtHY5 homologs in rice, was found to be up-regulated and down-regulated, respectively, in OsbZIP48\textsuperscript{OE} transgenics. The significance of this observation will be realized only when these two genes are functionally characterized.
OsbZIP48 over-expression results in down-regulation of OsGDD1. The gdd1 mutant also shows phenotype similar to OsbZIP48\textsuperscript{OE} transgenics. The gdd1 mutant is defective in gibberellin biosynthesis and has reduced secondary cell wall thickenings (Zhang et al., 2010; Li et al., 2011). Therefore, the reduction in secondary cell wall thickening in OsbZIP48\textsuperscript{OE} might be because of reduction of OsGDD1 expression.

In ethylene biosynthesis, the major role of OsbZIP48 could be to reduce the expression of genes encoding tryosine transaminase enzyme as the expression of OsERF11 was only slightly increased as compared to the vector control. However, no significant change in expression of genes associated with ABA and auxin signalling pathway was detected in our analysis, although genes associated with jasmionic acid and brassinosteroid biosynthesis pathways were repressed. In Arabidopsis, the hy5 mutant seedlings are known to be hypersensitive to brassinosteroid and it is known that AtHY5 plays a role in brassinosteroid signalling by altering the expression of AtMSBP1 and by interacting with AtBZR1 (Shi et al., 2011; Li and He, 2015). AtHY5 has been shown to bind to the promoter of Arabidopsis Membrane Steroid Binding Protein 1 (MSBP1) and trigger its expression. AtMSBP1 is a negative regulator of brassinosteroid signalling and its expression is less in hy5 mutant seedlings, causing hypersensitive response of hy5 mutant seedlings to brassinosteroid (Shi et al., 2011). We were not able to find any putative homolog of AtMSBP1 in rice through TIGR ortholog finder. The genes which code for enzymes like 3-beta hydroxysteroid dehydrogenase/isomerase, leucoanthocyanidin reductase, NAD-dependent epimerase/dehydratase, which are involved in brassinosteroid biosynthesis, showed more than 2-fold repression in OsbZIP48\textsuperscript{OE} transgenics, which could possibly result in the repression of brassinosteroid biosynthesis. Brassinosteroids are known to affect cell expansion and division, reproductive development, tissue differentiation and stress resistance (Wang and Irving, 2011). Therefore, reduction in brassinosteroid biosynthesis may also be involved in the semi-dwarf phenotype of OsbZIP48\textsuperscript{OE} transgenics raised in this study.

AtHY5 is known to work in a synergistic manner with AtHY1 to control jasmonic acid (JA) responsiveness in Arabidopsis (Prasad et al., 2012). However, role of AtHY5 in JA biosynthesis is yet to be elucidated. In rice, JA is known to play diverse roles in various growth and developmental stages and is involved in defense and environmental responses. It positively regulates spikelet development, senescence, photomorphogenesis, defense against microbes and nematodes but negatively regulates germination, shoot and root growth and gravitropism (Liu et al., 2015). We found that OsbZIP48 down-regulates genes encoding lipoxygenase

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enzyme involved in JA biosynthesis. Jasmonic acid is known to be involved in plant defense and insufficient jasmonic acid results in sterile floral organs. It plays a central role in biosynthesis of secondary metabolites which protect plants from biotic and abiotic stresses (Wang and Irving, 2011). Therefore, it will be interesting to study the role of OsbZIP48 in biotic and abiotic stresses.

The genes associated with cytokinin glucosylation were down-regulated in OsbZIP48\(^{OE}\) transgenics as compared to the vector control. Cytokinins can be glucosylated to form O-glucosides and N-glucosides, which are inactive and are involved in hormone homeostasis (Hou et al., 2004). The down regulation of this pathway in OsbZIP48\(^{OE}\) transgenics could result in greater accumulation of free cytokinins. Cytokinins are involved in plastid development and are known to delay senescence. Thus, the accumulation of elevated levels of free (active) cytokinins could account for prolonged greening in the OsbZIP48\(^{OE}\) plants.

The knock-down of OsbZIP48 could have resulted in activation of GA, jasmonic acid and IAA biosynthesis pathways as genes encoding for enzymes involved in these pathways showed higher expression as compared to the vector control. IAA biosynthesis pathway was not altered in OsbZIP48\(^{OE}\) transgenics but was up-regulated in OsbZIP48\(^{KD}\) transgenics. In Arabidopsis also, hy5 mutant has higher auxin levels than the wild type. AtHY5 binds to the promoters of AXR2 and SLR/IAA14 genes and increases their expression which, in turn, inhibits auxin signalling (Cluis et al., 2004); these genes encode for Aux/IAA proteins that act as negative regulators of auxin signalling. We were not able to find any putative homologs of AXR2/IAA7 and SLR/IAA14 through TIGR ortholog finder. Therefore, the mode of action of OsbZIP48 might be slightly different than AtHY5 or else it may stimulate the expression of another set of Aux/IAA genes. Ethylene biosynthesis pathway genes were, however, not altered in their expression in OsbZIP48\(^{KD}\) transgenics as compared to the vector control.

**OsbZIP48 may regulate OsKO2 expression by directly binding to its promoter**

We found that OsbZIP48 directly binds to the G-box II (at -504 bp) present in the promoter upstream region of OsKO2. Chip seq analysis of AtHY5, however, did not show the promoter of AtKO2 to be a binding site of AtHY5 (Lee et al., 2007). OsNAC2 is known to repress the expression of OsKO2 by directly binding to its promoter as shown by chip-seq analysis (Chen et al., 2015). In OsNAC2 over-expression transgenics, the expression of OsbZIP48 was shown to increase by four-fold (Chen et al., 2015). We also found that the expression of OsNAC2 is enhanced in OsbZIP48\(^{OE}\) transgenics. Therefore, it will be interesting to see whether OsbZIP48...
directly binds to the promoter of OsKO2 as a homodimer or as a heterodimer with OsNAC2 or they act independently.

To conclude, the results obtained in this study provide evidence that in monocot systems like rice there are at least three homologs of AtHY5, the bZIP transcription factor that plays a central role in regulating photomorphogenesis in plants. Although only one of them (OsbZIP48) has been functionally characterized in this study but their differential expression profile (unpublished data) indicates that they may have evolved to regulate at least some unique functions. It is striking that the knock down of OsbZIP48, either by insertional mutagenesis or RNAi, results in arresting growth at early stage of development leading to lethality; however, like Arabidopsis hy5 mutant, these OsbZIP48\textsuperscript{KD} lines did display excessive root proliferation. Its ectopic expression caused semi-dwarfism in rice but, like AtHY5, it had no significant effect in altering seedling height when expressed ectopically in the wild type Arabidopsis. It thus raises a question why both rice OsbZIP48 and Arabidopsis HY5 are ineffective in reducing seedling height when over-expressed in the wild type Arabidopsis, whereas hy5 mutant develops an elongated hypocotyl when grown in light; moreover, it can be complemented functionally by both AtHY5 and OsbZIP48, reducing its height that is fairly comparable to wild type seedlings. OsbZIP48 from rice could complement Arabidopsis hy5 mutant with respect to agravitropic responses displayed by root and root hairs and gravitropic set angle of the silique. That the semi-dwarf plants of rice over-expressing OsbZIP48 are compromised in its secondary cell wall thickenings is unusual; rather than providing strength to the semi-dwarf plant, these transgenics are fragile. OsbZIP48 appears to have a role in regulating panicle development and seed set since its ectopic expression caused partial sterility. Although this study has unravelled some novel functions the AtHY5 ortholog OsbZIP48 performs in rice, more work on HY5 homologs in both monocots and dicots is required to elucidate the specific and redundant functions they perform in regulating plant development. Whether the three AtHY5 homologs present in rice (and other monocots) have undergone neofunctionalization is another aspect that is presently under investigation in our laboratory.

**Materials and Methods**

**Plant material and growth conditions**

Rice (Oryza sativa indica) seeds (varieties PB1 and IR64) were obtained from the Indian Agricultural Research Institute (IARI), New Delhi. These seeds were surface sterilized by treating with 0.1% HgCl\textsubscript{2} for 10 min and then washed repeatedly with autoclaved MQ water and then kept at 28±1\degree C for 16 h for imbibition. Seeds were allowed to germinate and seedlings
grown hydroponically for seven days on Yoshida medium in a culture room maintained at 28±1°C for white light grown seedlings and in incubators maintained at 28±1°C in a dark room for dark grown seedlings. Light was provided by fluorescent lamps with the fluence rate of 200 μm/m²/s for Arabidopsis and 75 μm/m²/s for rice. For RNA isolation, the tissues were harvested every day from 3rd day onwards till 7th day, frozen in liquid nitrogen and kept at -70°C for long-term storage and used when required. White light was supplied from a bank of Cool Daylight fluorescent lamps (Philips, TL 5800°K, Philips, India). For harvesting of tissues belonging to different developmental stages, plants were grown in the rice fields of IARI, New Delhi, and the tissue was harvested as per the requirement. Tissue samples were frozen in liquid nitrogen and kept at -80°C until use.

**Real-time PCR analysis**
Rice tissues of different developmental stages, harvested in liquid N₂ and stored at -80°C, was used for RNA extraction. Total RNA was isolated using TRIZol Reagent (Invitrogen, USA) as per manufacturer’s instructions. The primers for real time-PCR were designed by using the Primer Express 2.0 (PE Applied Biosystems, USA). The cDNA synthesis was carried out with random hexamer primers as per manufacturer’s instructions using High Capacity cDNA Archive kit (Applied Biosystems, USA). Each sample with two biological replicates and three technical replicates was used for real-time PCR analysis in the LightCycler© 480II Real Time system (Roche, Germany) as per manufacturer’s instructions. The relative mRNA levels of OsbZIP48 in different samples were computed with respect to the internal standard Ubiquitin 5 (UQB5) for Oryza sativa (Jain et al., 2006). The conditions used for real time-PCR cycles were: pre-incubation for 10 min at 95°C, followed by 45 cycles of 10 s at 95°C, 20 s at 60°C and 10 s at 72°C, then cooling at 40°C for 30 s. The relative mRNA levels corresponding to different genes in different RNA samples were measured by ΔΔCt method (relative mRNA level = 2^(ΔΔCt)). The heatmap representing the expression profile of OsbZIP48 was generated using rice oligonucleotide array database (Cao et al., 2012).

**Western blot analysis**
Rice seedlings were grown hydroponically in rice growth medium at 28°C. For light treatment, seedlings were grown under white light at an intensity of 100 μmol/m²/s in a culture room (16 h light/8 h dark). For dark treatment, seedlings were grown in complete darkness. Seedlings were harvested 3, 5, 7 and 10 days after germination. For light shift experiments, the rice seedlings were grown for four days in continuous light or darkness under conditions as described above and on the fifth day they were transferred to the opposite light conditions for the designated
duration. In case of light to dark transition, 5-day old seedlings grown in continuous light were used as control and similarly in case of dark to light transition, 5-day-old seedlings grown in continuous darkness were used as control. In case of *Arabidopsis*, the transgenic line overexpressing *OsbZIP48* in *hy5* mutant background was used. The transgenic seeds were surface sterilized and plated on half-strength MS basal medium followed by 72 hours stratification at 4°C and then transferred to continuous light conditions (100 µmol/m²/s) at 22°C for 4 days. These 4-day-old seedlings were then transferred to dark for desired duration, harvested at different time points and processed for western analysis.

Panicles and seeds were harvested at different stages of development P1; 3–5 cm, P2; 5–10 cm, P3; 10–15 cm, P4; 15–22 cm, P5; 22–30 cm, P6) and seed (0–2 DAP, S1; 3–4 DAP, S2; 5–10 DAP, S3; 11–20 DAP, S4; 21–29 DAP, S5) (Jain et al. 2007) from rice plants grown under field conditions. Frozen seedlings and different stages of panicle and seed development were ground in a buffer (200 mM Tris, pH 8.0, 100 mM NaCl, 10 mM EDTA, 10 mM DTT, 5% glycerol, 0.05% tween 20, protease inhibitor cocktail (Sigma)) and the extracts were centrifuged at 13,000 rpm, 4°C. The supernatant was centrifuged again at 13,000 rpm, 4°C and the concentration of proteins was estimated by Bradford Assay (Bradford, M.M. 1976). Equal concentration of proteins were loaded on SDS-PAGE gels and the protein extracts were subjected to western blotting (Sharma et al. 2014). For detection of OsbZIP48 protein, peptide antibody synthesis was done. Actin was used as a loading control and its levels were detected by using ThermoFisher Scientific actin monoclonal antibody (catalog #: MA1-744).

**Phylogenetic analysis**

Homologs of AtHY5 were identified by using reverse best hit approach (using BlastP and an e-value cut off of 10⁻⁵). For *Cyanidioschyzon merolae* and *Ostreococcus tauri*, KEGG database (http://www.genome.jp/kegg/kegg1.html) while for other genomes Phytozome database ver 9 (http://www.phytozome.net/) was used (Kanehisa and Goto, 2000; Goodstein et al., 2012; Kanehisa et al., 2014). For retrieving gymnosperm sequences, NCBI blast tool was used. For reciprocal hit in *Arabidopsis* genome, wu-blast tool of TAIR was used (Huala et al., 2001). Since there is a large variation in the degree and quality of annotation of genomic information available across species, RBH was performed manually and if no homologue was identified, a tblastN approach was used to manually annotate the genes in those species using FGENESH+ (http://www.softberry.com/berry.phtml?topic=fgenes_plus&group=programs&subgroup=gfs), a gene predicting software, which uses similar protein support. Effort was made to reannotate the incomplete proteins using this software. The sequences of the reannotated proteins is available...
on request. The genes retrieved were then checked for the presence of the requisite domains using Interproscan (http://www.ebi.ac.uk/Tools/pfa/iprscan/) (Quevillon et al., 2005; Jones et al., 2014; Mitchell et al., 2015). EST evidence was also checked using tblastN in NCBI database for the particular species in which no ortholog was identified by the above method. ESTs were considered only if they had the requisite domain. The protein phylogenetic tree was made using conserved bZIP domain. Three phylogenetic trees were prepared using phylml, maximum parsimony and neighbor-joining method using Phylip package and then a consensus tree was generated. Pairwise distance between protein sequence was calculated using MEGA6 (Tamura et al., 2013).

Amplification of OsbZIP48

OsbZIP48 CDS is predicted to be of 552 bp but KOME clone of OsbZIP48 (AK241558) shows a truncated protein. Therefore, we amplified a 552 bp amplicon as predicted by TIGR from the cDNA derived from P6 stage panicle tissue and cloned it in a primary vector.

Generation of Rice and Arabidopsis transgenics

Arabidopsis transgenics were raised using floral dip method (Clough and Bent, 1998) while rice transgenics were raised using the protocol described by Toki et al. (2006) with some modifications. Seeds of indica rice variety PB1 were grown under light on NB medium at 32°C. The 7-day-old calli were co-cultivated with EHA105 strain of Agrobacterium tumefaciens. The calli were washed on the third day after co-cultivation and kept in selection medium with appropriate antibiotics. The positive calli were then transferred to regeneration medium with appropriate antibiotics till they formed plantlets. They were then transferred to rooting medium and then rice growth medium. The Arabidopsis hy5 mutant (SALK_096651C) were obtained from ABRC (https://abrc.osu.edu/) while the rice OsbZIP48 mutant (PFG_3A-07378.R) were procured from RiceGE database (http://signal.salk.edu/cgi-bin/RiceGE). Genotyping of T-DNA insertional mutant was done as described in Jung et. al. (2008).

Southern analysis

Southern analysis was carried out as described in Sharma et al. (2014) with some modifications. A 10 µg aliquot of genomic DNA from each transgenic line, vector control and the wild type plant was restriction digested using EcoRI (Roche Molecular Bio Labs, Indianapolis, USA) and probed with hptII gene cassette (850 bp). PCR amplified and gel eluted hptII gene was used as positive control.
Particle bombardment and transactivation assay

In-silico prediction for intra-cellular localization was done using ProtComp 9.0 (http://www.softberry.com/berry.phtml?topic=protcompl&group=programs&subgroup=proloc). Particle bombardment for BiFC, FRET and intracellular localization in onion epidermal peel cells was carried out using Biolistic PDS-1000/He particle delivery system (Bio-Rad, USA) according to the protocol described earlier (Thakur et al., 2005; Giri et al., 2011). The spring onion peels were kept on MS basal plates and particle bombardment carried out using the following parameters: 27 mm Hg vacuum, 1100 psi He pressure and target distance of 6 cm. The plates were then kept at 28°C in dark for 16 h. Confocal microscope (Leica TCS, SP5) was used to observe the onion peels for BiFC, FRET and intracellular localization. For FRET and BiFC, the experiment was repeated thrice.

For transactivation assay, full length OsbZIP48 CDS was cloned in pDEST-GBKTT7 (CD3-764) obtained from TAIR (https://www.arabidopsis.org) and transformed in AH109 yeast strain (Clonetech). The cloned construct was transformed into yeast as described in the Yeastmaker™ Yeast Transformation System 2 User Manual (Clonetech). Serially diluted transformed colonies were dropped on –Tryp/-His SD (synthetic dropout) selection media. The sealed plates were incubated at 30°C for 3-6 days.

Arabidopsis morphometric analyses

1. Hypocotyl length measurement

The Arabidopsis seeds were surface sterilised, washed with sterile autoclaved RO water and plated on Murashige and Skoog (1962) medium supplemented with 0.8% agar and 1% sucrose. The plates with seeds were then kept at 4°C for 3-4 days (for stratification) and transferred to light chambers maintained at 22°C with 200 μmol/m²/s of white light for 3 or 6 days and the hypocotyl length measured using ImageJ1.41 software (National Institutes of Health, Bethesda, MD, USA).

2. Chlorophyll and anthocyanin estimation

Chlorophyll a/b was estimated by overnight incubation of 20 Arabidopsis seedlings in 500 μl of DMSO at 65°C in dark as described by Hiscox and Israelstam (1979) with some modifications. Absorbance was recorded at 645 and 663 nm in a Beckman DUTM 640B (Beckman Instruments Inc., Fullerton, CA, USA) spectrophotometer. Chlorophyll a/b contents were calculated according to the following formulae:

\[ \text{Chl a} = \frac{([12.3 \ A_{663} - 0.86 \ A_{645}] \times V)}{x1000 \times n} \]

\[ \text{Chl b} = \frac{([19.3 \ A_{645} - 0.86 \ A_{663}] \times V)}{x1000 \times n} \]
where $V = \text{volume of DMSO in mL}$; $X = \text{path length, 1 cm}$; $n = \text{No. of seedlings}$.

Anthocyanin was estimated by overnight incubation of 20 *Arabidopsis* seedlings in 150 μl of 1% HCl in methanol at room temperature in dark. After addition of 100 μl of MQ water, an equal volume (i.e. 250 μl) of chloroform was added to remove chlorophyll. The quantity of anthocyanins was determined by spectrophotometric measurements of the aqueous phase ($A_{530}$-$A_{657}$) (Neff and Chory, 1998).

3. **Cotyledon angle measurement**

Cotyledon angle represented the angle between a straight line drawn between the tip of the cotyledon and the cotyledon blade/cotyledon petiole junction and a straight line drawn through the cotyledon petiole. An angle of 180° reflected a fully folded cotyledon (Christians et al., 2012). The cotyledon angles were measured using ImageJ1.41 software (National Institutes of Health, Bethesda, MD, USA).

4. **Root gravitropic response**

Root gravitropic response was measured as described by Vicente-Agullo et al. (2004) and Grabov et al. (2005). To analyse root geometry, plates with seedlings were photographed with Leica® S8AP0 stereo microscope. The obtained images were analysed using Image J 1.41 software (National Institutes of Health, Bethesda, MD, USA). The vertical growth index (VGI) was calculated using the following formula: $VGI = CHα/RL$, where $CHα$ is a projection of the base-to-tip chord $CH$ on vertical axis and $RL$ is total root length.

**Rice morphometric analysis**

The morphometric analysis of rice transgenics were carried out according to the IRRI guidelines as described in the book ‘Descriptors for Rice’ written by IBPGR-IRRI Rice Advisory Committee (http://books.irri.org/getpdf.htm?book=971104000X). The internode closest to the roots was considered as the first internode while the panicle bearing internode was considered to be the last internode.

**Microarray**

Microarray analysis was done with transgenic plants expressing transgene(s) constitutively and showing the desired phenotype. Both the vector control (VC) and transgenic lines were grown on half-strength MS medium with antibiotics for 10 days under white light (100 μmol/m²/s). Seedlings were gently pulled out of the medium and immediately frozen in liquid nitrogen. Total RNA was isolated (Trizol method) as described by Chomczynski and Sacchi (1987). The isolated RNA (500 ng) was used for microarray experiments and subsequent analysis carried out as per manufacturer’s protocol (Affymetrix) (Jain et al., 2007). The pathway analysis was
carried out using Plant MetGenMAP (http://bioinfo.bti.cornell.edu/cgi-bin/MetGenMAP/home.cgi) (Joung et al., 2009). The metabolic pathways were reconstructed using Adobe Illustrator® software. The microarray data have been submitted in GEO with accession no. GSE90472.

Electrophoretic mobility assay

The G-box element (CACGTG) was manually searched in 1kb promoter of OsKO2 gene. The probe for G-box element was designed and the sequence is given in the primer list. For EMSA, OsbZIP48 cDNA was cloned in pET28A vector and 6X his-tagged OsbZIP48 was induced using 0.5 mM isopropylthio-β-galactoside and overexpressed in Escherichia coli. The overexpressed 6X his-OsbZIP48 was affinity purified according to the manufacturer’s protocol (Qiagen). All DNA binding reactions were carried out in 15 mM HEPES-KOH; pH7.5, 35 mM KCl, 1 mM EDTA; pH 8.0, 6% glycerol, 1 mM dithiothreitol, 1 mM MgCl₂, 1 µg/µl poly (dl-dC). Gel shift assays were performed as described in Jain et al. (2009).

Sectioning and SEM analysis

The second last internode of the wild type and over-expression transgenics were fixed in fixative [100 mM Pipes (pH 7.2), 10% formaldehyde (37%) and 80% RO water] by vacuum infiltration and kept overnight at 4°C. The tissue was then dehydrated in a graded ethanol series (70%, 80%, 90%, 100%) followed by a tertiary butanol series (25%, 50% and 100%), before placing in paraplast plus (Sigma Aldrich, USA). Paraplast embedded internodes were sectioned by using a Leica RM2245 rotary microtome producing 50-100 µm thick sections that were placed on Poly-L-Lysine coated slides (Polysciences Inc.). Quantitative analysis of cell size was done using ImageJ 1.41 software (National Institutes of Health, Bethesda, MD, USA). Cell area is cell base area which is similar to the base area of a cylinder and was calculated using ImageJ 1.41 software. For SEM analysis, the second last internode of over-expression transgenics and the wild type were harvested in Trumps 4F:1G fixative, vacuum infiltrated and sent for fixation and gold coating to University Science Instrumentation Centre (USIC), Delhi University, New Delhi, India. Scanning of coated samples was carried out by using JEOL scanning electron microscope (JSM 6610LV).

Supplemental data

Supplemental figure S1. Protein alignment of HY5 orthologs showing presence of COP1-interaction motif in most of the homologs.
Supplemental figure S2. Heat maps showing expression of OsbZIP48 in different anatomical and developmental stages of rice.

Supplemental figure S3. PCR confirmation and real time PCR analysis of Arabidopsis transgenics.


Supplemental figure S5. Confirmation of rice transgenics through PCR, real time and southern analysis.

Supplemental figure S6. OsbZIP48 over-expression reduces panicle and internodal length in rice.

Supplemental figure S7. Longitudinal section of wild type and OsbZIP48OE transgenic stem, respectively, showing difference in cell length.

Supplemental figure S8. Morphometric analysis of wild type and OsbZIP48OE transgenics.

Supplemental figure S9. Morphometric analysis of wild type and OsbZIP48OE transgenics.

Supplemental figure S10. Chlorophyll content of rice transgenic seedlings.

Supplemental figure S11. Schematic representation of different hormone pathways showing altered gene expression in OsbZIP48OE transgenics.

Supplemental figure S12. Schematic representation of cytokinin glucosylation pathways showing altered gene expression in OsbZIP48OE transgenics.

Supplemental figure S13. Heatmap showing differentially expressed genes in RNAi transgenics (Hh, hh) and pANDA vector control.

Supplemental figure S14. Schematic representation of different hormone pathways showing altered gene expression in OsbZIP48KD transgenics in rice.
Supplemental figure S15. Real time PCR of genes of different hormonal biosynthetic pathway which were shown to be altered by microarray in OsbZIP48\textsuperscript{OE}.

Supplemental figure S16. Real time PCR of genes of different hormonal biosynthetic pathway which were shown to be altered by microarray in OsbZIP48\textsuperscript{KD} (RNAi).

Supplemental Table S1. Pairwise distance result of protein sequences of HY5/HYH homologs.

Supplemental table S2. Primers used for qRT-PCR analysis.

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Accession numbers
GEO accession number for the microarray data is GSE90472.

Legends
Figure 1: Phylogenetic tree of HY5 and HYH homologous proteins from across the species (* represents manually re-annotated proteins, † represents incomplete proteins even after manual re-annotation but having the bZIP domains). The consensus tree was generated after merging the individual trees generated by phyml, neighbor-joining and maximum parsimony approach. The numbering at the node represents the number of trees (generated by three different methods) which have the same topology as the consensus tree.
Figure 2: Expression profile of OsbZIP48 in various tissues and at different stages of development. (A) Expression of OsbZIP48 in vegetative (seedling, Y-leaf, mature leaf and SAM), panicle and seed stages of development in rice variety IR64 as analysed by real-time PCR. (Panicle: P1-1- 0.5 to 2 mm, P1-2- 2 to 5 mm, P1-3- 5 to 10 mm, P1- 0 to 3 cm, P2– 3 to 5 cm, P3– 5 to 10 cm, P4– 10 to 15 cm, P5– 15 to 22 cm, P6– 22 to 30 cm; Seed stages: S1- 0 to 2 DAP, S2– 3 to 4 DAP, S3– 5 to 10 DAP, S4– 11 to 20 DAP, S5– 21 to 29 DAP). (B) Real-time PCR analysis of OsbZIP48 using different organs of the inflorescence; PMA – Pre-meiotic Anther, MA – Meiotic Anther, SCP – Single Cell Pollen, BCP – Bi-cellular Pollen, TPA – Tri-nucleate Pollen Anther, DAP- Days after pollination. (C) Real-time PCR analysis to check the expression of OsbZIP48 in different internodes of the mature rice stem. (D) Expression analysis of OsbZIP48 using real time PCR in 3-day- to 7-day-old light and dark grown rice seedlings. (E) Expression analysis of OsbZIP48 root and shoot of 5-day-old light grown seedlings using real time PCR. Data shown are mean ± SE. The expression data presented are relative to UBQ5.

Figure 3: Western blots showing OsbZIP48 protein expression levels in different tissues of rice plant and Arabidopsis hy5 mutant complemented with OsbZIP48. (A) OsbZIP48 protein levels in 3, 5, 7 and 10-day-old light grown rice seedlings (100 μmol/m²/s). (B) OsbZIP48 protein levels in 3, 5, 7 and 10-day old dark grown rice seedlings. (C) OsbZIP48 protein levels in seedlings grown in continuous light for 4 days and then transferred to dark for 5, 10, 15 and 20 h; control is 5-day-old seedlings grown in continuous light. (D) OsbZIP48 protein levels in seedlings grown in continuous dark for 4 days and then transferred to the light for 5, 10, 15 and 20 h; 5-day-old seedlings grown in continuous dark were used as control. (E) Western blot using OsbZIP48 antibodies shows no cross-reactivity with Athy5 mutant protein extracts. (F) OsbZIP48 protein levels in Arabidopsis hy5 mutant seedlings complemented with OsbZIP48, grown in continuous light for 4 days and then transferred to dark for 5, 10, 15 and 20 h; control represents 5-day-old seedlings grown in continuous light. (G) Changes in OsbZIP48 protein levels during various stages of panicle development in rice. (H) OsbZIP48 protein levels during seed development (S1 to S5) stages in rice. (I)
OsbZIP48 protein levels in mature leaf and root in rice. Positive control in (E), (F), (G) & (H) is bacterially expressed 6X His-tagged OsbZIP48 protein.

**Figure 4:** OsbZIP48 is localized in the nucleus, forms homodimer and lacks transactivation activity. (A) Particle bombardment of the YFP-OsbZIP48 construct in onion cells. The first column shows pictures taken in dark-field and the second column shows merged picture of dark-field and bright-field captured using Leica microscope. The first row (YFP control vector) shows localisation of only YFP protein, the second row (OsbZIP48-YFP) shows localisation of OsbZIP48 tagged to YFP protein and the third row (DAPI) shows DAPI stained nucleus. (B) Pre- and post-bleach images showing bleaching of YFP-OsbZIP48 for FRET analysis. (C) Histogram showing FRET efficiency of CFP-OsbZIP48 and YFP-OsbZIP48 interaction as compared to the controls. Data shown are mean ± SE; n = 10 (D) BiFC analysis using onion peel cells showing the homo-dimerization of nEYFPC1-OsbZIP48 and cEYFPC1-OsbZIP48 in the nucleus. (E) Trans-activation assay of OsbZIP48 in yeast cells. OsbZIP48 lacks transactivation activity as the yeast cells containing OsbZIP48-pGBKT construct were unable to grow on SD-HW medium.

**Figure 5:** Phenotypic analyses of Arabidopsis hy5 mutant seedlings/plants over-expressing OsbZIP48. (A) Phenotype of 3-day-old white light grown wild type, hy5, OsbZIP48\(^{OE}\) and OsbZIP48;hy5 seedlings. (B, C) Hypocotyl length of 3-day-old and 6-day-old white light (200 μmol/m\(^2\)/s) grown wild type (WT), hy5 mutant, OsbZIP48\(^{OE}\) and OsbZIP48;hy5 mutant seedlings, respectively. (D and E) Vertical Growth Index of roots of 3-day-old wild type (WT), hy5 mutant and OsbZIP48;hy5 seedlings. (F and G) Cotyledon opening angle in response to white light. (H) Altered gravitropic set angle in siliques of Arabidopsis hy5 mutant plants. Data presented are mean ± SE, n=15 plants in each case. Statistically significant differences (P > 0.05 represented by * and P>0.005 represented by **) were identified by Dunnett’s test using WT as control for overexpression transgenics and hy5 mutant as control for OsbZIP48;hy5 transgenics in (B) & (C) and hy5 mutant as control for (E) & (G).

**Figure 6:** Phenotype of OsbZIP48\(^{OE}\) rice transgenics at different developmental stages. (A) Photograph of 10-day-old seedlings of wild type (WT), pB4NU vector control (VC)
and OsbZIP48\textsuperscript{OE} transgenics grown in white light (75 μmoles/m\textsuperscript{2}/s). (B) Photograph of 30-day-old seedlings grown in white light (75 μmoles/m\textsuperscript{2}/s). (C) Photograph of plants at the vegetative phase of life. (D) Photograph of plants grown in greenhouse at the reproductive stage.

**Figure 7:** Phenotypic comparison of the stem of wild type and OsbZIP48\textsuperscript{OE} rice transgenic plants. (A) Photograph showing difference in the stem diameter of mature green plants. (B, C) Scanning electron microscopic pictures showing difference in the diameter of the stem of wild type and OsbZIP48\textsuperscript{OE} transgenic plants, respectively. (D, E) Methylene blue stained transverse section of wild type and OsbZIP48\textsuperscript{OE} transgenic stem showing difference in the diameter of the stem taken at 2.5X magnification. (F, G) Methylene blue stained transverse section of wild type and OsbZIP48\textsuperscript{OE} transgenic stem with red arrows showing difference in the size of vascular bundle, parenchyma cells and the secondary cell wall thickening of the sclerenchyma cells, respectively. (H-J) Histogram showing difference in stem diameter, cell length and cell area of wild type and overexpression transgenics respectively. Cortical cells were used to measure cell length and cell area. Data presented are mean ± SE, n=10 in each case. Statistically significant differences (P > 0.05 represented by * and P>0.005 represented by **) were identified by Student’s t-test.

**Figure 8:** Scanning electron microscopic pictures of second last internode of wild type and OsbZIP48\textsuperscript{OE} transgenic plants. (A, B) SEM of second last internode of wild type and OsbZIP48\textsuperscript{OE} transgenic plants at different magnifications showing size of vascular bundles and secondary cell wall thickening. In B, yellow arrow shows the thickness of secondary cell wall thickenings in WT and OsbZIP48\textsuperscript{OE} transgenics, respectively.

**Figure 9:** Phenotype of OsbZIP48\textsuperscript{KD} lines and T-DNA insertion mutant of OsbZIP48. (A) T1 OsbZIP48\textsuperscript{KD} lines showing profuse root growth at seedling stage as compared to wild type. (B, C) T1 OsbZIP48\textsuperscript{KD} plants at the vegetative stage showing increased height at two different magnifications; note elongated stem of OsbZIP48\textsuperscript{KD} plants in ‘B’. (D) T2 OsbZIP48\textsuperscript{KD} transgenics showing two type of plants; seedlings of 10-day-old wild type (WT), pANDA vector control (VC) and T2 OsbZIP48\textsuperscript{KD} transgenics grown in white light (75 μmoles/m\textsuperscript{2}/s). (E) T2 OsbZIP48\textsuperscript{KD} 10-day-old transgenics showing lethal
phenotype in multiple lines. (F) Photograph of 15-day-old wild type and OsbZIP48 mutant lines showing their phenotype. (G) T-DNA insertional mutant plants showing profuse rooting (at higher magnification).

**Figure 10:** (A) Schematic representation of gibberellin biosynthesis pathway showing altered gene expression in OsbZIP48\textsuperscript{OE} and OsbZIP48\textsuperscript{KD} transgenics. Color bar at the base represents log\textsubscript{2} expression values, with blue color representing low-level expression, black representing medium level and yellow signifying high-level expression. The numbers written in red in the pathway corresponds to the serial number of the locus ID in the heatmap, i.e. the gene with the serial number performs in the step where it is mentioned in the pathway. (B) Schematic representation of OsKO2 1 kb promoter showing location of three G-box motifs. In the diagram the sequence of the probe is given with G-box sequence (CACGTG) highlighted in red. G-mut II probe sequence shows the mutated G-box sequence and is highlighted in red. These probes were labelled with \textsuperscript{32}P for Electrophoretic Mobility Shift Assay (EMSA). (C) OsbZIP48 binds in-vitro to G-box II in the OsKO2 promoter in EMSA. (D) EMSA gel showing OsbZIP48 in-vitro binding to G-box II in the OsKO2 promoter with proper controls. For the assay, the radiolabelled probes were incubated with OsbZIP48 protein. Cold (unlabelled) probe (100x or 50X), G-box II probe and G-mut II probe (G-boxII mutated) were used as indicated.

**Figure 11:** Real-time PCR based expression analysis of genes known to be involved in regulating plant height in OsbZIP48\textsuperscript{OE} seedlings. The last histogram shows the transcript levels of OsbZIP1 and OsbZIP18 in OsbZIP48\textsuperscript{KD} line. Data shown are mean ± SE.

**Tables**

**Table 1:** List of genes known to be involved in regulating plant height in rice.

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Pathway involved</th>
<th>Function</th>
<th>Expression level in OsbZIP48\textsuperscript{OE}</th>
<th>Literature</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPS</td>
<td>GA biosynthesis</td>
<td>Take part in the early steps of GA biosynthesis</td>
<td>Downregulated</td>
<td>Yamaguchi, 2008</td>
</tr>
<tr>
<td>KO2</td>
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<td>OsKOL4</td>
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<td>Function</td>
<td>Protein or Gene</td>
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<td>-----------------------</td>
<td>--------------</td>
<td>---------------------------------</td>
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<tr>
<td>OsGA20ox2</td>
<td>GA biosynthesis</td>
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<td>Yamaguchi, 2008</td>
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<tr>
<td>OsGA20ox1</td>
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<td>Yamaguchi, 2008</td>
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<tr>
<td>OsGA13ox</td>
<td>(OsCYP714B1) GA biosynthesis</td>
<td>Converts GA$_2$ to GA$_3$, which is then converted to GA$_4$, by OsGA20ox</td>
<td>Downregulated</td>
<td>Yamaguchi, 2008</td>
</tr>
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<td>OsGA2ox1</td>
<td>GA biosynthesis</td>
<td>Negative regulator of plant height. Binds to the promoter of OsKO2 and represses its expression.</td>
<td>Uprregulated</td>
<td>Chen et al., 2015</td>
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<td>OsNAC2</td>
<td>Transcription factor</td>
<td>Positive regulator of plant height. Binds to the promoter of OsKO2 and promotes its expression.</td>
<td>Downregulated</td>
<td>Li et al., 2011</td>
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<tr>
<td>OsGDD1</td>
<td>Kinesin-Like Protein</td>
<td>Positive regulator of plant height. Binds to the promoter of OsKO2 and promotes its expression.</td>
<td>Downregulated</td>
<td>Li et al., 2011</td>
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<td>OsPIL1</td>
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<td>Downregulated</td>
<td>Todaka et al., 2012</td>
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<td>OsEATB</td>
<td>Rice ethylene-response AP2/ERF factor</td>
<td>Restricts the ethylene induced enhancement of gibberellin responsiveness by downregulating the gibberellin biosynthetic gene, ent-kaurene synthase A.</td>
<td>Downregulated</td>
<td>Qi et al., 2011</td>
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<tr>
<td>OsEXPANSIN S1</td>
<td>Cell wall-related genes</td>
<td>Regulate cell elongation by cell wall expansion, which is caused by acid-induced cell wall relaxation</td>
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<td>Todaka et al., 2012</td>
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<td>OsSLR1</td>
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<td>Positive regulator of GA signalling</td>
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<td>Positive regulator of GA signalling</td>
<td>Downregulated</td>
<td>Hauvermale et al., 2012</td>
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**Figure 1:** Phylogenetic tree of HY5 and HYH homologous proteins from across the species (* represents manually re-annotated proteins, † represents incomplete proteins even after manual re-annotation but having the bZIP domains). The consensus tree was generated after merging the individual trees generated by phylm, neighbor-joining and maximum parsimony approach. The numbering at the node represents the number of trees (generated by three different methods) which have the same topology as the consensus tree.
Figure 2: Expression profile of OsbZIP48 in various tissues and at different stages of development. (A) Expression of OsbZIP48 in vegetative (seedling, Y-leaf, mature leaf and SAM), panicle and seed stages of development in rice variety IR64 as analysed by real-time PCR. (Panicle: P1-1- 0.5 to 2 mm, P1-2- 2 to 5 mm, P1-3- 5 to 10 mm, P1-0- 0 to 3 cm, P2- 3 to 5 cm, P3- 5 to 10 cm, P4- 10 to 15 cm, P5- 15 to 22 cm, P6- 22 to 30 cm; Seed stages: S1- 0 to 2 DAP, S2- 3 to 4 DAP, S3- 5 to 10 DAP, S4- 11 to 20 DAP, S5- 21 to 29 DAP). (B) Real-time PCR analysis of OsbZIP48 using different organs of the inflorescence; PMA – Pre-meiotic Anther, MA – Meiotic Anther, SCP – Single Cell Pollen, BCP – Bi-cellular Pollen, TPA – Tri-nucleate Pollen Anther, DAP-Days after pollination. (C) Real-time PCR analysis to check the expression of OsbZIP48 in different internodes of the mature rice stem. (D) Expression analysis of OsbZIP48 using real time PCR in 3-day- to 7-day-old light and dark grown rice seedlings. (E) Expression analysis of OsbZIP48 root and shoot of 5-day-old light grown seedling using real time PCR. Data shown are mean ± SE. The expression data presented are relative to UBQ5.
Figure 3: Western blots showing OsbZIP48 protein expression levels in different tissues of rice plant and Arabidopsis hy5 mutant complemented with OsbZIP48. (A) OsbZIP48 protein levels in 3, 5, 7, and 10-day-old light grown rice seedlings (100 μmol/m²/s). (B) OsbZIP48 protein levels in 3, 5, 7, and 10-day-old dark grown rice seedlings. (C) OsbZIP48 protein levels in seedlings grown in continuous light for 4 days and then transferred to dark for 5, 10, 15, and 20 h; control is 5-day-old seedlings grown in continuous light. (D) OsbZIP48 protein levels in seedlings grown in continuous dark for 4 days and then transferred to the light for 5, 10, 15, and 20 h; 5-day-old seedlings grown in continuous dark were used as control. (E) Western blot using OsbZIP48 antibodies shows no cross-reactivity with Athy5 mutant protein extracts. (F) OsbZIP48 protein levels in Arabidopsis hy5 mutant seedlings complemented with OsbZIP48, grown in continuous light for 4 days and then transferred to dark for 5, 10, 15, and 20 h; control represents 5-day-old seedlings grown in continuous light. (G) Changes in OsbZIP48 protein levels during various stages of panicle development in rice. (H) OsbZIP48 protein levels during seed development (S1 to S5) stages in rice. (I) OsbZIP48 protein levels in mature leaf and root in rice. Positive control in (E), (F), (G) & (H) is bacterially expressed 6X His-tagged OsbZIP48 protein.
Figure 4: OsbZIP48 is localized in the nucleus, forms homodimer and lacks transactivation activity. (A) Particle bombardment of the YFP-OsbZIP48 construct in onion cells. The first column shows pictures taken in dark-field and the second column shows merged picture of dark-field and bright-field captured using Leica microscope. The first row (YFP control vector) shows localization of only YFP protein, the second row (OsbZIP48-YFP) shows localization of OsbZIP48 tagged to YFP protein and the third row (DAPI) shows DAPI stained nucleus. (B) Pre- and post-bleach images showing bleaching of YFP-OsbZIP48 for FRET analysis. (C) Histogram showing FRET efficiency of CFP-OsbZIP48 and YFP-OsbZIP48 interaction as compared to the controls. Data shown are mean ± SE; n = 10 (D) BiFC analysis using onion peel cells showing the homo-dimerization of nEYFP-C1 and eYFP-C1-OsbZIP48 in the nucleus. (E) Trans-activation assay of OsbZIP48 in yeast cells. OsbZIP48 lacks transactivation activity as the yeast cells containing OsbZIP48-pGBK construct were unable to grow on SD-HW medium.
Figure 5: Phenotypic analyses of *Arabidopsis hy5* mutant seedlings/plants over-expressing OsbZIP48. (A) Phenotype of 3-day-old white light grown wild type, *hy5*, OsbZIP48OE and OsbZIP48;hy5 seedlings. (B, C) Hypocotyl length of 3-day-old and 6-day-old white light (200 μmol/m²/s) grown wild type (WT), *hy5* mutant, OsbZIP48OE and OsbZIP48;hy5 mutant seedlings, respectively. (D and E) Vertical Growth Index of roots of 3-day-old wild type (WT), *hy5* mutant and OsbZIP48;hy5 seedlings. (F and G) Cotyledon opening angle in response to white light. (H) Altered gravitropic set angle in siliques of *Arabidopsis hy5* mutant plants. Data presented are mean ± SE, n=15 plants in each case. Statistically significant differences (P > 0.05 represented by * and P>0.005 represented by **) were identified by Dunnell’s test using WT as control for overexpression transgenics and *hy5* mutant as control for OsbZIP48;hy5 transgenics in (B) & (C) and *hy5* mutant as control for (E) & (G).
Figure 6: Phenotype of OsbZIP48OE rice transgenics at different developmental stages. (A) Photograph of 10-day-old seedlings of wild type (WT), pB4NU vector control (VC) and OsbZIP48OE transgenics grown in white light (75 μmoles/m²/s). (B) Photograph of 30-day-old seedlings grown in white light (75 μmoles/m²/s). (C) Photograph of plants at the vegetative phase of life. (D) Photograph of plants grown in greenhouse at the reproductive stage.
Figure 7: Phenotypic comparison of the stem of wild type and OsbZIP48OE rice transgenic plants. (A) Photograph showing difference in the stem diameter of mature green plants. (B, C) Scanning electron microscopic pictures showing difference in the diameter of the stem of wild type and OsbZIP48OE transgenic plants, respectively. (D, E) Methylene blue stained transverse section of wild type and OsbZIP48OE transgenic stem showing difference in the diameter of the stem taken at 2.5X magnification. (F, G) Methylene blue stained transverse section of wild type and OsbZIP48OE transgenic stem with red arrows showing difference in the size of vascular bundle, parenchyma cells and the secondary cell wall thickening of the sclerenchyma cells, respectively. (H-J) Histogram showing difference in stem diameter, cell length and cell area of wild type and overexpression transgenics respectively. Cortical cells were used to measure cell length and cell area. Data presented are mean ± SE, n=10 in each case. Statistically significant differences (P > 0.05 represented by * and P<0.005 represented by **) were identified by Student's t-test.
Figure 8: Scanning electron microscopic pictures of second last internode of wild type and OsbZIP48OE transgenic plants. (A, B) SEM of second last internode of wild type and OsbZIP48OE transgenic plants at different magnifications showing size of vascular bundles and secondary cell wall thickening. In B, yellow arrow shows the thickness of secondary cell wall thickening in WT and OsbZIP48OE transgenics, respectively.
Figure 9: Phenotype of OsZIP48\textsuperscript{KD} lines and T-DNA insertion mutant of OsZIP48. (A) T1 OsZIP48\textsuperscript{KD} lines showing profuse root growth at seedling stage as compared to wild type. (B, C) T1 OsZIP48\textsuperscript{KD} plants at the vegetative stage showing increased height at two different magnifications; note elongated stem of OsZIP48\textsuperscript{KD} plants in ‘B’. (D) T2 OsZIP48\textsuperscript{KD} transgenics showing two type of plants; seedlings of 10-day-old wild type (WT), pANDA vector control (VC) and T2 OsZIP48\textsuperscript{KD} transgenics grown in white light (75 umoles/m²/s). (E) T2 OsZIP48\textsuperscript{KD} 10-day-old transgenics showing lethal phenotype in multiple lines. (F) Photograph of 15-day-old wild type and OsZIP48 mutant lines showing their phenotype. (G) T-DNA insertional mutant plants showing profuse rooting (at higher magnification).
Figure 10: (A) Schematic representation of gibberellin biosynthesis pathway showing altered gene expression in OsbZIP48OE and OsbZIP48KD transgenics. Color bar at the base represents log2 expression values, with blue color representing low-level expression, black representing medium level and yellow signifying high-level expression. The numbers written in red in the pathway corresponds to the serial number of the locus ID in the heatmap, i.e. the gene with the serial number performs in the step where it is mentioned in the pathway. (B) Schematic representation of OsKO2 1 kb promoter showing location of three G-box motifs. In the diagram the sequence of the probe is given with G-box sequence (CACGTG) highlighted in red. G-mut II probe sequence shows the mutated G-box sequence and is highlighted in red. These probes were labelled with 32P for Electrophoretic Mobility Shift Assay (EMSA). (C) OsbZIP48 binds in-vitro to G-box II in the OsKO2 promoter in EMSA. (D) EMSA gel showing OsbZIP48 in-vitro binding to G-box II in the OsKO2 1 kb promoter with proper controls. For the assay, the radiolabelled probes were incubated with OsbZIP48 protein. Cold (unlabelled) probe (100x or 50X), G-box II probe and G-mut II probe (G-box II mutated) were used as indicated.
Figure 11: Real time PCR based expression analysis of genes known to be involved in regulating rice plant height in OsbZIP48\textsuperscript{OE} seedlings. Data shown are mean ± SE.


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