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Short Communication

Expression Profiling of Development Related Genes in Rice Plants Ectopically Expressing *AtTOR*

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Abbreviations: *AtTOR*, *Arabidopsis thaliana Target of Rapamycin*; *OsFON*1, *Oryza sativa FLORAL ORGAN NUMBER* 1; *OsWOX*3, *Oryza sativa WUSCHEL*- like Homeobox 3; *OsKNOX*2, *Oryza sativa KNOTTED*2-like homeobox; *OsKNOX3*, *Oryza sativa KNOTTED*3-like homeobox; *OsOSE*2, *Oryza sativa ORGAN SPECIFIC ELEMENT* 2; *OsPIN*1c, *Oryza sativa PIN FORMED*-1c, SAM, Shoot Apical Meristem; TFs, Transcription factors; WT, Wild Type; DAG, Days After Germination

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Abstract

Expression analysis of genes associated with development at different growth stages such as shoot apical meristem (SAM), root apical meristem (RAM), shoot and root tissues 10 DAG, flowers and grains of two high expression transgenic lines of rice ectopically expressing *AtTOR* revealed the involvement of AtTOR in transcriptional regulation of these genes. We have observed that in the SAM of these two selected lines, TR-2.24 and TR-15.1, *OsFON*1 and *OsFON*4 (orthologs of *AtCLV*1 and *AtCLV*3, respectively), *OsKNOX*2, *OsKNOX*3 and *OsWOX*3 became up-regulated. The up-regulation of *OsFON*1 and *OsFON*4 is likely to be involved in the maintenance of effective meristem size of the inflorescence and phyllotaxis. The grains and spikes of transgenic plants exhibited enhanced transcript levels of *OsMADS*1, *OsMADS*6, and *OsMADS*29 further implicating the role of TOR in modulating the expression of the genes in rice grain formation and development. Moreover, the up-regulation of auxin transporter, *PIN*1c in RAM and roots derived from seedlings 10 DAG showed the involvement of TOR in root development. The seeds of two high expression lines also showed increased expression of *OSE*2 and *GAMYB* transcription factors involved in seed development. In summary, the present study, by heterologous expression of *AtTOR* in rice, demonstrated the involvement of TOR in regulating genes involved in various growth and developmental stages of rice plant and also in photosynthesis, productivity related functions and water-use efficiency.

TEXT

The conserved Ser/Thr protein kinase, Target of Rapamycin (TOR) regulates growth and development in all eukaryotes. The pivotal role of TOR in embryonic development has been demonstrated in Arabidopsis in various studies.[1] TOR expression had been predominantly reported in root and shoot meristems.[2] The *tor* knockout mutants or treatment of plants with TOR inhibitors exhibited reduced root meristem and leaves.[1,3] Transgenic Arabidopsis plants overexpressing *TOR* exhibited increased root and shoot growth with enhanced seed yield.[4] Also, the up-regulation of the rRNA transcripts was reported in *AtTOR* overexpression transgenic Arabidopsis.[1] In our previous report, the overexpression of *AtTOR* in *indica* rice plants lead to increased plant height, tillering, panicle length and seed yield.[5] The high yielding phenotypes in transgenic Arabidopsis and rice that exhibited overexpression of *AtTOR* highlighted the functions of activated TOR signaling in transgenic plants.[1,5] Additionally, the photosynthesis derived glucose and light mediated *TOR* signaling also activated cell proliferation in root and shoot meristems.[2,6] The *AtTOR* overexpressing transgenic rice plants in T_2 generation were separated into high, medium and low lines based on transcript levels of *AtTOR*. The high expression lines, TR-2.24 and TR15.1 had enhanced photosynthetic and water-use efficiency compared with the low expression line, TR5.1 and WT.[5] Based on the observed yield attributes, we had selected these two lines (TR2.24 and TR15.1) for exploring the expression of genes involved in rice development. Quantitative expression analysis of these developmental genes showed that TOR is also involved in the modulation of the expression of development related genes in rice.

In this study, we have selected eleven important genes involved in rice development such as *OsFON*1 (an ortholog of *AtCLV*1), *OsWOX*3, *OsKNOX*2, *OsKNOX*3, *OsFON*4 (an ortholog of *AtCLV*3), *OsMADS*1, *OsMADS*6, *OsMADS*29, *OsGAMYB*, *OsOSE*2 and *OsPIN*4 (presently named as *OsPIN*1c) in various developmental stages of rice. The *OsFON*1, *OsWOX*3, *OsKNOX*2 and *OsKNOX*3 are involved in proliferation of SAM. CLV3 functions as a peptide ligand for a receptor like kinase consisting of leucine rich repeats (LRR-RLK), CLV1. In Arabidopsis and also in grass species, CLV3 negatively regulates the expression of WUS to reduce the proliferation of stem cells in SAM.[7] The *CLV*3 overexpression completely eliminates stem cells resulting in meristem termination, whereas its loss of function causes over-proliferation of meristem.[8,9,10] The CLV3/CLV1 complex regulates the WUS activity in the organizing center of the apical meristem.[11] The *FLORAL ORGAN NUMBER* (*FON*) genes in rice are *CLV* orthologs and are involved in the meristem maintenance.[12,13] Rice *FON*4 (*FLORAL ORGAN NUMBER* 4) encodes an Arabidopsis CLV3 ortholog, containing similar functional CLE motif.[14] FON4 also regulates SAM development in rice and *fon*4 mutants in rice exhibited increased floral organ number and more than one primary rachis.[14,15] Similarly, FON1 in rice is expressed in all meristems regulating development of vegetative tissues and functions as AtCLV1.[12] The KNOX (KNOTTED1-like homeobox) transcription factors have a key role in SAM and leaf development.[16,17,18] The OsWOX3 (WUSCHEL-like homeobox 3) regulates leaf and flower development and is specifically expressed in leaf primordial and floral meristems. It also induces the expression of *KNOX* genes.[19] The other transcription factors like MADS-box and GAMYB are primarily associated with flower development.[20] GAMYB is highly expressed in aleurone tissue of germinating cereal seeds and also involved in seed development.[21] OsMADS6 regulates

the development of floral meristems and its loss of function mutants have been shown to exhibit altered floral organ identities.[22] The combination of OsMADS6 and OsMADS1 controls flower patterning in rice.[23] The OsMADS29 regulates early stages of seed developmental by regulating grain filling, grain weight and grain size.[24] A bZip transcription factor, OsOSE2 (organ-specific elements-2) has also been reported to regulate embryogenesis and other stages of development.[25] The PIN (PIN- FORMED) proteins are auxin efflux carriers, which facilitate auxin flow and its distribution toward root tips and growing meristems.[26] The *Arabidopsis thaliana PIN*1 mutants were first characterized for their pin-like inflorescence. The *OsPIN*1c is expressed in early stages of lateral root primordial development.[5]

Previously we have reported that two high *AtTOR* expression transgenic lines of rice, TR-2.24 and TR-15.1 displayed increased plant height, panicle length, increased number of tillers and increase in overall seed yield of the plant compared to WT rice along with enhanced water use efficiency.[5] This study suggested the involvement of *AtTOR* in the observed development associated phenotypes of these rice transgenic lines.

To get further insights into the underlying genetic factors, we have performed expression analysis of several development associated genes in high *AtTOR* expression lines, TR-2.24 and TR-15.1 in T_3 generation in the present study. We have used three biological replicates to isolate total RNA from different tissues of two lines (TR-2.24 and TR-15.1) along with WT controls. The RNAs used in the study include shoot and root tissues from 10 DAG seedlings, SAM, RAM (3-4 cm growing tips of seedlings), embryo, flowers, grains, seeds and spikes of panicles.

The seeds and embryonic tissues were collected after maturity and overnight incubation of sterilized transgenic and WT seeds in water, respectively. The 10 DAG transgenic and WT seedlings were transferred to pots in greenhouse for further growth and collection of the other tissues, such as flowers, which were collected 50 d after transfer of seedlings, whereas the grains and the spikes were collected after 65 d.

The total RNA was isolated from 100 mg tissues of transgenic and WT plants using Trizol (Sigma Aldrich, St. Louis, Missouri, US) method. The first strand cDNA was synthesized using 2 µg of total RNA and SMART^{TM} MMLV Reverse Transcriptase (Takara Bio, Clonetech, USA). The seven times diluted cDNA was used for qRT-PCR analysis of different developmental genes using SYBR Green ® Premix (Takara Bio, Clonetech, USA). The primers used in qRT-PCR have been listed in Table 1. The qRT PCR reaction conditions included an initial denaturation at 94°C for 2 min, followed by 40 cycles of 94°C for 15 sec, with annealing temperature ranging from 50-55°C for 25 sec and an extension at 72° C for 30 sec. The qRT PCR data of three biological and three technical replicates was analyzed according to the $\Delta\Delta C_T$ method.[27] The expression of rice *Actin1* was used as an endogenous positive control. The two selected high expression rice transgenic lines, TR-2.24 and TR-15.1 exhibited increased panicle length with the concurrent enhanced expression of *OsMADS*1, *OsMADS*6 and *OsMADS*29 in grains compared with WT (Fig. 1a, 1c & 1e). The enhanced expression of *OsMADS*1 and *OsMADS*6 was also observed in spikes of transgenic lines indicating the regulation of spike and seed development by TOR (Fig. 1b & 1d). The up-regulation of *OsFON*1, *OsKNOX*2, *OsWOX*3, *OsKNOX*3 and *OsFON*4 genes in SAM tissues of the two selected lines indicated the role of TOR and its associated signaling pathways in shoot development (Fig. 1g, 1h, 1i, 1j & 1k).

The *OsFON*4 was expressed up to 1.5 fold higher in transgenic lines compared with WT. The simultaneous up-regulation of both *FON*1 and *FON*4 genes in the SAM of high expression lines had no other detectable developmental defects on shoot meristem. These results suggest that although the FON4 is an ortholog of CLV3 of Arabidopsis, It has also been reported previously that the *fon1* mutants in rice showed enlarged floral meristem whereas, the vegetative meristem had normal development.[12,28] The exogenous application of FON4 peptide on rice RAM had not resulted in any perturbed phenotype suggesting the presence of other unknown receptors like FON1 receptor for FON4 in rice.[15] Also, the increased and similar expression of both *OsFON*1 and *OsFON*4 in transgenic lines might possibly be a reason for executing continuous activation of rice *WUS*-like genes and balancing the meristematic activity in shoot apex. The continuous but balanced WUS activity in SAM of *AtTOR* transgenic plants led to increased shoot growth. The bzip TF, *OsOSE*2 expression was also enhanced in embryos of *AtTOR* transgenic seeds suggesting the TOR mediated activation of genes involved in embryonic and seed/grain development (Fig. 1l).

The TOR activates nutrient and energy signaling at growing root apices and the ROS-TOR signaling mediates negative tropism in roots in order to avoid the light and salt stress.[2,29] The auxin efflux carrier, *OsPIN*1c was up-regulated in both RAM and roots of 10 DAG transgenic seedlings suggesting the involvement of TOR in root development (Fig. 1m&1n). In our previous report, we have shown that germination and growth of the high expression rice transgenic lines on MS medium with glucose as a supplement resulted in significant up-regulation of TOR transcripts with enhanced lateral root formation. This is possibly due to the TOR mediated activation of PIN1c to improve the auxin transport in root meristems.[5] The Myb

TFs such as OsGAMYB involved in gibberellic acid signaling regulates anther and pollen development, which is ultimately related to grain maturity and seed development.[30] The interaction of GAMYB TFs with other TFs has been reported in the activation of endosperm specific genes during seed development in Barley.[31] The *OsGAMYB* was up-regulated 3-fold in seeds of transgenic lines, whereas no significant expression of OsGAMYB was noticed in flowers (Fig. 1o & 1p). Although transgenic seeds exhibited increased transcript levels of *OsGAMYB*, there was no phenotypic distinction suggesting the post-transcriptional regulation of the OsGAMYB in the normal seed development.

Our present study explored the novel functions of AtTOR in regulating genes involved in meristem growth and overall development of rice. The key findings of this study suggest that targeting TOR signaling could potentially generate a novel tool for developing performance conferring phenotypes in rice. The recent research on plant TOR signaling has mainly focused on model plant Arabidopsis. Only limited reports are available on crop plants and much needs to be elucidated. The available literature on TOR suggests that it is a very effective gene for genetic manipulation in crop plants for enhanced productivity and abiotic stress tolerance.

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Figure Legend

Figure 1

Quantitative expression analysis of genes involved in development in rice

a, b) Expression of *OsMADS*1 in grains and spikes, c, d) Expression of *OsMADS*6 in grains and spikes, e, f) Expression of *OsMADS*29 in grains and spikes in high *AtTOR* expression transgenic lines, TR-2.24 and TR-15.1. Similarly g) *OsFON*1, h) *OsKNOX*2, i) *OsWox*3, j) *OsKNOX*3, and k) *OsFON*4, had increased transcript level in SAM of high *AtTOR* expression transgenic lines. The expression level of l) *OsOSE*2 in embryo, m, n) *OsPIN*1c in 10 day old root and root apical meristem, o, p) *OsGAMYB* in flower and seeds of high *AtTOR* expression transgenic lines. The expression data was analysed by $\Delta\Delta C_T$ method using mean of three biological and three technical replicates. The relative expression was considered statistically significant at *P* value <0.05 (represented with asterisks) based on one-way ANOVA in all the analyzed genes.

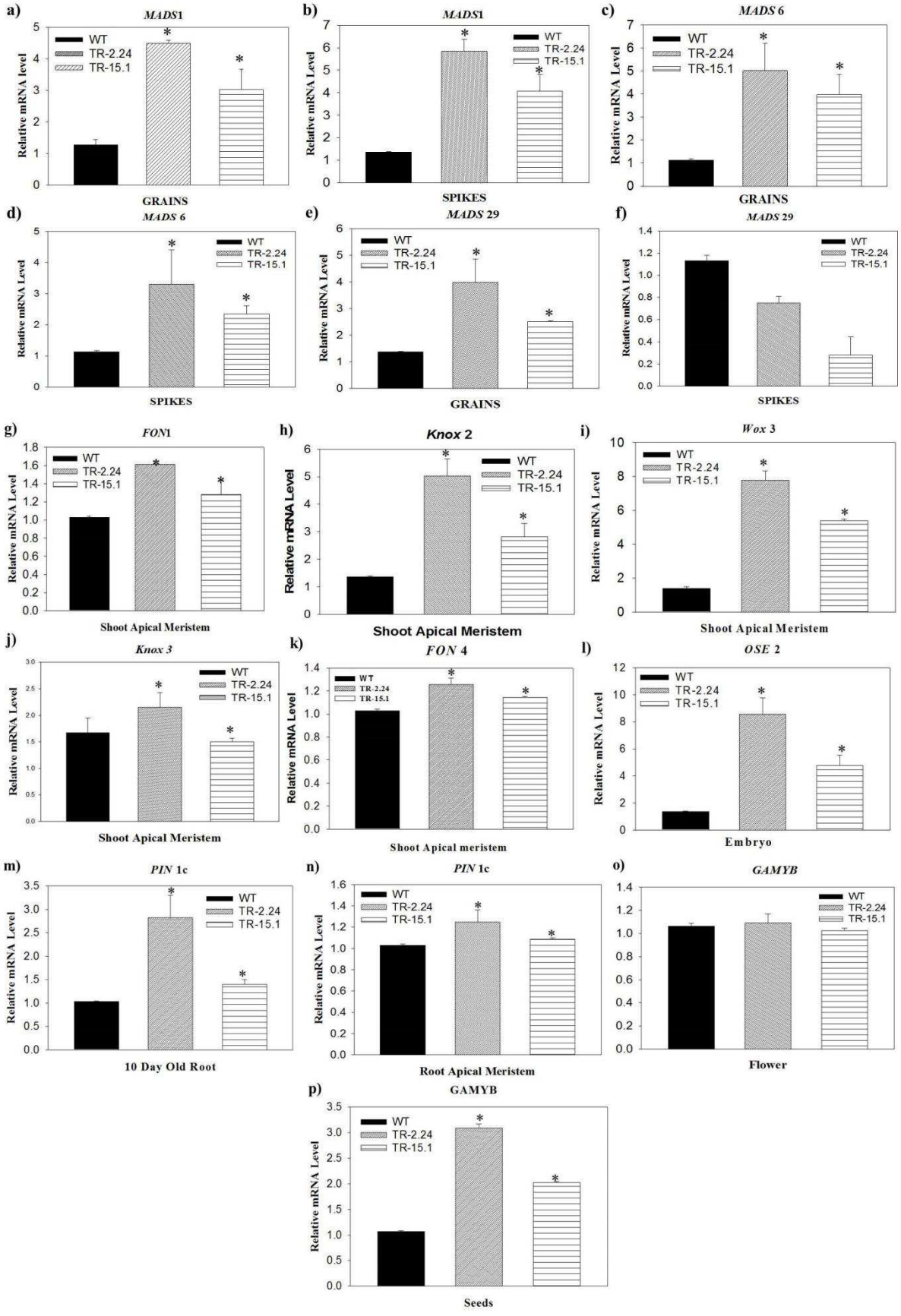

Table 1

List of qRT-PCR Primers used for expression analysis

S. No.	Primer name	Sequence 5'- 3'	Bases	Product size
1	*OsOSE2* FP	CTAGTTGCGGTGAATACATGAG	22	281
2	*OsOSE2* RP	CATATCAGCATACCTAGAGTCACC	24	
3	*OsWox3*FP	AGCTTACACCACCAGCTACTACT	23	101
4	*OsWox3*RP	CCTGGTTGTAGTGGAAGAGG	20	
7	*Osknox2*FP	TCTAGGACAGAGGGAGTGGTAT	22	294
8	*Osknox2*RP	GCACATCAGTAGCTGGAATAAG	22	
9	*Osknox3*FP	AAATCTCTCGTCTTCTCGTCTC	22	246
10	*Osknox3*RP	TAGCAGCTAGGCTCTCTCTCTT	22	
13	*OsMADS1*FP	GAGAGAGAGAGAGAGGAGAGGA	22	274
14	*OsMADS1*RP	CTGCATCCTGTGAGTTGTAGTT	22	
15	*OsMADS6*FP	ACTGATGATGGAACAAGTGGA	21	117
16	*OsMADS6*RP	ATGGCTCTGTAGTTGCTGGT	20	
17	*OsMADS29*FP	GGAGCTAGGAGTAACTTGGAGA	22	260
18	*OsMADS29*RP	CCAGTTCAGTAGTTCACACACC	22	
19	*OsGAMYB*FP	GTAAACCAGACAGGGATGCTAA	22	144
20	*OsGAMYB*RP	ATGGAGATAGTCAAAACCCACA	22	
25	*OsPIN1c*FP	CTTACAAGAAGTTGCAGGATG	21	208
26	*OsPIN1c*RP	GACTTAAATGGTGCGCTAGTA	21	
27	*OsFON1*FP	CCAATAGTGGTGACCTCCTC	20	159

28	*OsFON1*RP	GCAGTAGTAATCCGCCTGTT	21		
29	*OsFON4*FP	GCTTCAGTTCTGAGCCTTTC	20	253	
30	*OsFON4*RP	ACTCGATCCGGTAAACAGAG	20		
31	*OsActin*FP	CTCCCCCATGCTATCCTTCG	20		129
32	*OsActin*RP	CTTCATGTCCCTCACAATTT	20		

