A Drought Senescence NAC Gene, *OMTN4*, Confers Seed Development Defects in Rice

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Abstract

Transcription factors are important for gene regulation system in both animals and plants as these factors control various development processes. The NAC (NAM, ATAF, and CUC2) transcription factors play an important role in vital diverse processes and responses to stress. In rice, six NAC transcription factors targeted by *miR164* were designated as *OMTN1-6*. Among them, *OMTN4* (*LOC_Os06g46270*) plays a role in causing negative effects on drought resistance. Bioinformatic information revealed that *OMTN4* is a rice *NAC1/ANAC021* analog gene. In this study, we conducted the gain-of-function of *OMTN4* overexpression and expression analysis. The results suggested that *OMTN4* plays a negative role in inflorescence fertility. β-glucuronidase (GUS) histochemical study revealed that *OMTN4* was expressed in leaf, seed husk, stigma, and root. *OMTN4* expression profile was in correspondence with GUS histochemical result. Subcellular localization demonstrated that *OMTN4* was localized in the nucleus, which was consistent with the other NAC TFs. Partial seed sterile phenotype and expression profile suggested that, other than the negative effect on drought stress, *OMTN4* plays a negative role on inflorescence development.

Keywords: NAC Transcription Factors; *OMTN4*; *Oryza Sativa*; Stigma Development

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Introduction

Cereals are world's most important staple food. Among them, rice is one of the most important food crops. Grain size, grain number, and yield are determining factors of rice production. Seed setting rate is fluctuating, in which, several factors such as pollen viable rate, have influences. Transcription factor plays a role as a regulator for the transcription process. NAC (**N**AM/**A**TAF/**C**UC) transcription factors are one of the largest plant-specific transcription factors. In NAC members, approximately 150 amino acids are conserved on N terminal while C terminal is usually divergent. Further, NAC transcription factors participate in several plant development cycles [1]. NAC and *miR164* cooperate to fine-tuning leaf senescence. To date, *miR164* is reported to negatively regulate NAC targeted *ORESARA1* (*ORE1* also known as *ANAC092*) for age-dependent cell death in *Arabidopsis*. ORE1/ANAC092 positively regulates leaf senescence, notably *ore1/anac092* loss-of-function mutation leads to delay leaf senescence and overexpression of *miR164B* led to mRNA of *ORE1* cleavage, while *ORE1* is negatively regulated by the *EIN2*, providing a *miR164* example in negative leaf senescence regulation [2]. Another report clarified that *EIN3* promotes age-dependent leaf senescence and *EIN3* also acts as *EIN2* downstream by directly binding to the *miR164* promoter and suppressing *miR164* transcription. Moreover, *EIN3* expression level is increased during senescence [3], these results imply the regulatory system for fine-tuning senescence regulation gene network. Therefore, it is assumed that, in rice, NAC genes probably targeted by *miR164* system are present. In Arabidopsis, *miR164* is coded by *miR164A/B/C* and targeting five NAC transcription factors [3] - [4].

In contrast to Arabidopsis, rice genome consists of six genes coding for *miR164* [5] - [6]. Notably, a previous study conducted by Fang, Y., Xie, K., and Xiong, L. [5] identified six NAC transcription factors targeted by *miR164*, which designated as *OMTN1-6*. It should be noted that overexpression of *OMTN4* induces sensitive drought stress during reproductive stage. Other than leaf senescence-related manner, *miR164* target No Apical Meristem (NAM) also plays a crucial role in the fusion process of carpel margins in *Arabidopsis thaliana* and *Medicago truncatula*. NAM expression level is reduced or non-detectable during carpel margin fusion and may repressed via *miR164* [7]. *NAM/miR164* function related to the reproductive organ development is conserved among monocot species including rice [8].

In this study, we performed *OMTN4* function analysis via overexpression approach and spatio-temporal analysis. This study suggests that other than the negative effect on drought resistance function revealed by Fang, Y., Xie, K., and Xiong, L. [5], *OMTN4* also plays a negative role in inflorescence development in rice.

Materials and methods

1. Plant materials

The japonica rice cv. Nipponbare was used as wild type, three transgenic lines of *OMTN4* overexpression (ox) (line 3, 5, 7) and *OMTN4*$_{pro}$*::β-glucuronidase (GUS)* plants were grown under controlled conditions of 14 h. day and 10 h. night.

2. RNA isolation and real-time PCR

The total RNA from leaf tissues were extracted using RNAprep pure Kit (For Plant) [TIANGEN Biotech (Beijing) Co., Ltd., China] following the manufacturer's instructions. First strand cDNA was synthesized using ReverTra Ace quantitative PCR RT Master Mix Kit with gDNA remover (Toyobo, Japan) according to the manufacturer's instructions. Quantitative real-time PCR (qRT-PCR) reaction was performed with Light cycler 480 System (Roche, Germany) using SYBR Premix Ex Taq II (Takara, Japan) according to the manufacturer's instructions.

3. Overexpression construct

To generate *OMTN4*-overexpression (ox) construct, confirmed cDNA fragments were used for gene specific primers design and containing In-Fusion® adapter (*PstI* and *SpeI* for forward and reverse primer, respectively). Purified fragment was then inserted into pCUbi1390 vector driven by ubiquitin promoter. Primer list is given in Table 1. Construct were verified for its accurate information without discrepancy base and the verified construct was transformed into rice cv. Nipponbare mediated by *Agrobacterium* method [9].

Table 1 list of primers used in this study. Underlined indicates engineered sequence for In-fusion using Clontech™ In-fusion® kit

Construct name	Forward primers (5'-3')	Reverse primers (5'-3')
Primers for gene construction amplification		
OMTN4-ox	TCTGCACTAGGTACCTGCAGATGAGCGGGATGAATTCGCT	TAGCGTTAACACTAGTTCAACTGAGTGAGTTCCACAT
OMTN4$_{pro}$*::GUS*	CCATGATTACGAATTCCGATCGAGGTTGTACGTGGT	CTCAGATCTACCATGGGCCCACCCCCTCCTCCTCGA
OMTN4-GFP	GCCCAGATCAACTAGTATGAGCGGGATGAATTCGCT	TCGAGACGTCTCTAGAACTGAGTGAGTTCCACATTTG

4. β-glucuronidase (GUS) histochemical study

To generate *OMTN4*$_{pro}$*::GUS* construct ~2-kb sequence prior *OMTN4* start codon was confirmed by PCR and the gene specific primers, containing In-Fusion® adapter (*Eco*RI and *Nco*I for forward and reverse primer, respectively), were designed and used for fragment amplification. Purified fragment was inserted into pCAMBIA 1305.1 vector. Primer details are listed in Table 1. Construct was verified for its accurate information without discrepancy base and the verified construct was transformed into rice cv. Nipponbare mediated by *Agrobacterium* method [9]. Various tissues (leaf, root, seed, and seedling) from $OMTN4_{pro}$::GUS transgenic line were incubated in GUS solution as previously described [10]. The incubated tissues were photographed using Stereo microscope model SMZ1500 (Nikon, Japan).

5. Transient expression in rice protoplast

In order to establish *OMTN4*-GFP, the full-cDNA sequence without stop codon was amplified by PCR using gene specific primer pairs (Table 1). PCR product containing engineered restriction enzyme cutting site was subcloned into pCAMBIA1305-GFP using In-Fusion® HD Cloning Kit (Clontech) where the transcription was controlled by 35S promoter. *OMTN4*-GFP and *OsMADS3*-mCherry were co-transformed by polyethylene glycol (PEG) method as describe previously by Yoo, S., Cho, Y., and Sheen, J. [11] with minor modification. Photographs were taken using a confocal laser scanning microscope (Zeiss, Germany). *OsMADS3*-mCherry was used as a nuclear marker [12].

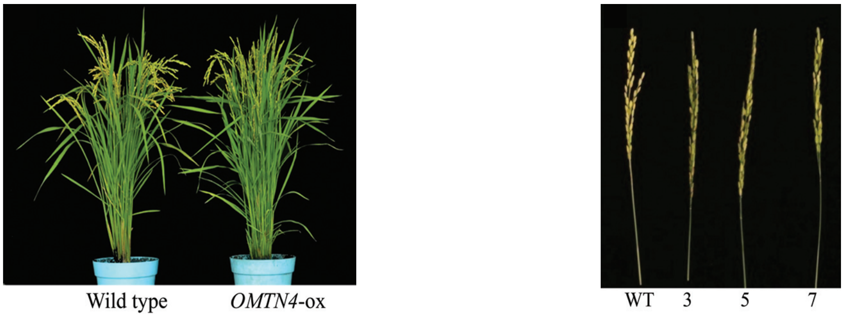
Results

1. Putative Gene identification

NAC1/ANAC021 (AT1g56010) encoded a transcription factor that played a role in shoot apical meristem formation and auxin-mediated lateral root formation [13]. Moreover, *NAC1/ANAC021* was up-regulated during senescence process, and targeted by *miR164* [14] - [15]. MSU rice annotation bioinformatic database showed that *OMTN4* was *NAC1/ANAC021* analog gene. Moreover, both *NAC1/ANAC021* and *OMTN4* were targeted by *miR164* [5], [13]. Since leaf senescence mediated analogous gene of *NAC1/ANAC021* in rice is *OMTN4*, we, therefore, selected *OMTN4* for further study. Together, FPKM of *OMTN4* (*LOC_Os06g46270*) in two inflorescence organs, Post-emergence inflorescence and anther, was 9.25059 and 11.9226, respectively, which was higher compared to the other organs. It is expected that expression of *OMTN4* in inflorescence organs could possess function other than leaf senescence. Protein multiple alignment sequence suggested that NAC1.1, NAC1.2 and OMTN4 contained highly conserved region on N-terminal (Supplemental S1).

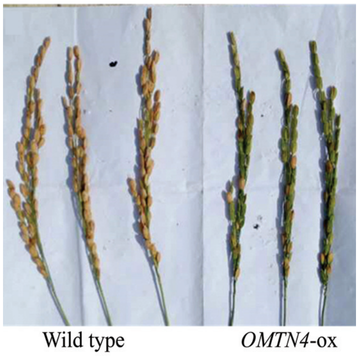
2. *OMTN4*-ox transgenic plant appearance

OMTN4 gene encoded 304 amino acid residues containing NAC conserved domain on its N-terminal region. Phenotype between the wild type (cv. Nipponbare) and the *OMTN4*-ox was not significantly different since seed sowing date. However, at 90 days after sowing date, the difference of phenotype of wild type and *OMTN4*-ox was displayed. *OMTN4*-ox leaves exhibited normalcy as well as plant height with slightly higher tiller (Figure 1(a)), all independent transgenic lines exhibited similar morphology. Panicle amount in *OMTN4*-ox was also higher due to higher tiller number. Interestingly, when the spikelet was considered, grain from wild type was filled up and exhibited normal green-brown color at ~90 days after sowing but the panicles from *OMTN4*-ox lines exhibited partial sterile green color with seed setting rate less than 50% in both T_0 and T_1 (Figure 1(b) and (c)) implying that semi-sterility was probably controlled by higher expression level of *OMTN4*. These results indicate that *OMTN4* possesses negative function in controlling grain filling.



(a) Panicle characteristics of wild type and T_1. WT, wild type; 3, *OMTN4*-ox line 3; 5, *OMTN4*-ox line 5; 7, *OMTN4*-ox line 7

(b) Wild type and T_0 panicle characteristics. WT, wild type; 3, *OMTN4*-ox line 3; 5, *OMTN4*-ox line 5; 7, *OMTN4*-ox line 7

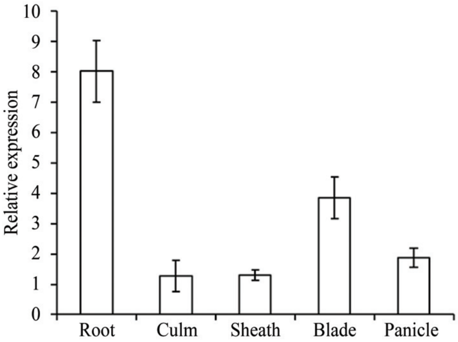


(c) Panicle characteristics of wild type and T_1

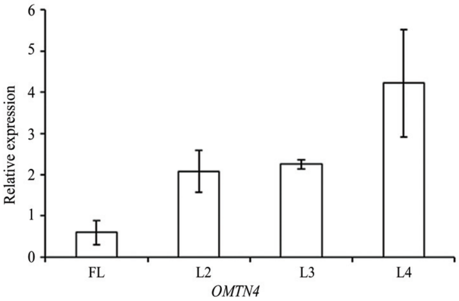
Figure 1 Phenotype of wild type and *OMTN4*-ox transgenic line

2. *OMTN4* expression levels

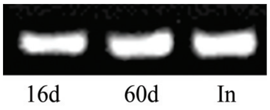
To investigate the spatio-temporal *OMTN4* expression pattern, qRT-PCR was employed to clarify transcript levels in various tissues and leaf age. The result resembled a previous report that *OMTN4* was highly expressed in root and blade with lower expression in culm and panicle (Figure 2(a)) [5]. *OMTN4* expression had an age-dependent pattern with lower expression level in younger leaf and slightly higher expression level in aged leaf. The expression was lower in flag leaf and slightly increased in 2nd leaf from top, 3rd leaf from top and finally higher expressed in 4th leaf from top (Figure 2(b)). In line with previous study, it suggested that *OMTN4* plays an important role in leaf senescence. Semi-quantitative RT-PCR analysis showed that *OMTN4* expressed in leaf and inflorescent (Figure 2(c)).



(a) Spatial *OMTN4* transcription in different rice organs



(b) *OMTN4* transcription level in different leaves. FL, flag leaf; L2, 2nd leaf from top; L3, 3rd leaf from top; L4, 4th leaf from top

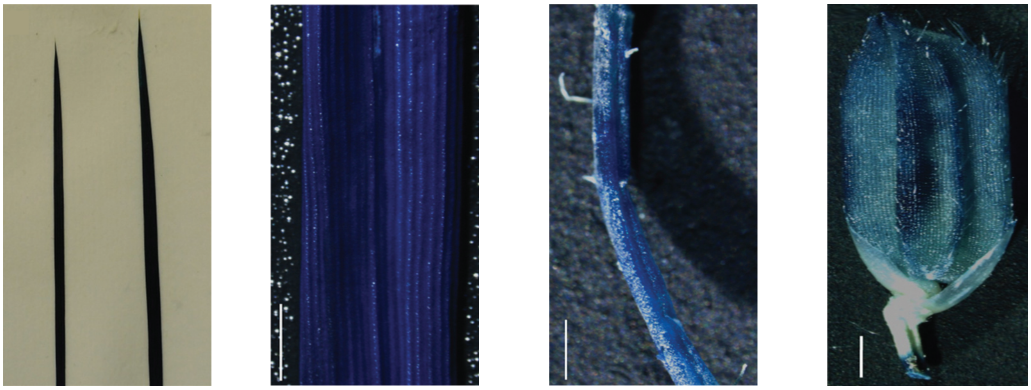


(c) Semi-quantitative RT-PCR analysis. 16d, Leaf tip at 16 days after planting; 60d, Leaf tip at 60 days after planting; In, Inflorescence

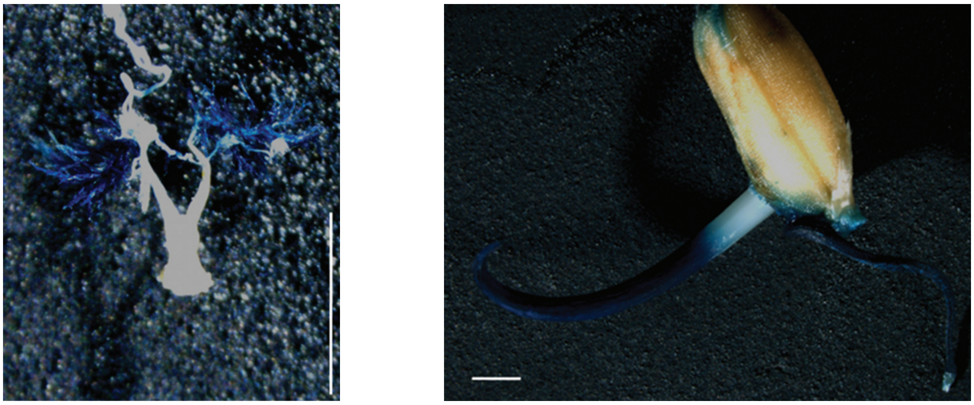
Figure 2 Spatio-temporal transcription pattern and dark treatment

3. *OMTN4* spatial investigation via GUS activity

To determine the *OMTN4* spatial expression, assessment of *OMTN4*$_{pro}$*::β-glucuronidase (GUS)* activity in transgenic lines were performed. The result elucidated GUS activity was strongly detected in leaf (Figure 3(a) and (b)) and in consistent with previous study, which elucidated that *OMTN4* expression was detectable in leaf [5]. Other than a role in leaf, *OMTN4*$_{pro}$::GUS activity also appeared in root (Figure 3(c)), seed husk (Figure 3(d), stigma (Figure 3(e), as well as seedling shoot and root (Figure 3(f). GUS activity in husk and stigma implied that *OMTN4* might be related to seed development.



(a) Leaf (b) Closed up leaf (c) Root (d) Seed



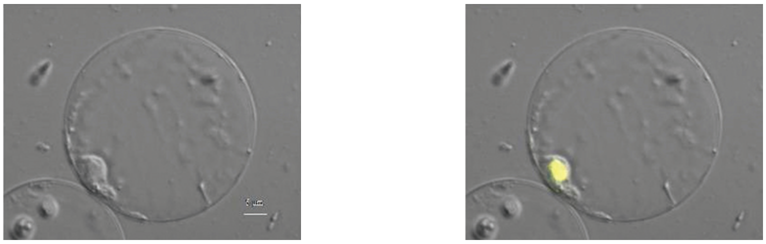
(e) Stigma (f) Seedling. Scale bars; 1 mm

Figure 3 GUS histochemical staining of *OMTN4*$_{pro}$::GUS transgenic line

4. Subcellular localization of *OMTN4*

Previous reports indicated that most of transcription factors were in the nucleus, although, some NAC family members appeared in plasma membrane or endoplasmic reticulum [5]. To bioinformatical forecast this issue, we performed subcellular localization prediction by SLP-Local (http://sunflower.kuicr.kyoto-u.ac.jp/~smatsuda/slplocal.html), which indicated that OMTN4 proteins were probably located in nucleus or cytosol (data not shown). In order to experimentally determine whether the *OMTN4* localized in nucleus, subcellular location of *OMTN4*-GFP were analyzed using full length cDNA without stop codon merging in-frame with GFP coding protein in rice protoplast. Previous study has clarified that *OsMADS3* is in nucleus [12]; hence, *OsMADS3*-mCherry was employed as a nuclear positive control. The construct was transiently expressed in rice protoplast. Laser scanning confocal image of green florescent signal resulted from In-frame fusion of *OMTN4*-GFP, which were co-expressed with mCherry signal from those of *OsMADS3*-mCherry (Figure 4(a) - (h)). The co-localization confirmed that *OMTN4*-GFP were localized in nucleus, suggesting that OMTN4 was nuclear protein. These evidences are in consistent with *OMTN4* role as a transcription factor.

(a) *OMTN4*-GFP (b) *OsMADS3*-mCherry



(c) Bright field (d) Merged field

Figure 4 Subcellular localization of the OMTN4 protein in rice protoplasts under confocal laser scanning microscope

Discussion

Senescence is regulated by complex gene network and many NAC family genes play a crucial role in senescence regulatory network [1], other than the control of leaf senescence, transcription factors might possess multi-functional roles in various development mechanisms. *ANAC092/ORE1*, a NAC gene member targeted by *miR164*, was first discovered in *Arabidopsis* [2]. In rice, six *miR164*-targeted NAC genes, designated as *OMTN1-6*, among them, *OMTN2*-, *OMTN3*-, *OMTN4*-, and *OMTN6*-ox contain negative effect on drought stress resistance. *OMTN4*-ox caused 76, and 371 genes up- and down-regulation, respectively [5]. It should be noted that high number of down-regulated genes possibly due to *OMTN4* induced signal cascade. As a result, a lot of genes have shut or reduced their functions, in order to relocate the nutrients and energy to sink organs [5].

In this study, we elucidated that *OMTN4*-ox expressed in root, culm, sheath, blade and panicle at varying degrees (Figure 2(a)). Interestingly, its expression pattern in FL, L2, L3, L4 was in line with the role to negatively regulate drought resistance in rice (Figure 2(b)). Further, *OMTN4-ox* might not only affect increasing susceptibility to drought stress but also grain development (Figure 1(b) and (c)) suggesting that *OMTN4* possesses a negative effect on grain development process. GUS histochemical result elucidated that *OMTN4* participates in shoot and root development in rice seedling (Figure 4(f)). According to previous study, *OMTN4* expression profile was highly detectable in stamen [5], suggesting that *OMTN4* plays a crucial role in pollen development. Moreover, GUS activity results implied that *OMTN4* expressed in stigma (Figure 3(e)). It is possible that higher expression of *OMTN4* may interfere with stigma development process, directly or indirectly via recruiting other transcription factors. A previous report identified that MYB transcription factor members participate in stigma development [16]. *OMTN4* may act as negative control to MYB to regulate stigma development. Stigma development malfunction or abortion owing to *OMTN4*-ox may be necessary to save the plant life in special conditions, in response to higher *OMTN4* expression. Present knowledge elucidates that *OMTN4* function responds to drought stress signal. NAC overexpression study in *Arabidopsis* elucidated that *ANAC046*-ox lines germinate later than in wild type, and at 40 days after sowing *ANAC046*-ox lines exhibited wavy rosette leaves and smaller size than in WT plants while knock-out lines and SRDX lines were slightly larger. When senescence is considered, *ANAC046*-ox lines showed yellowing leaves at 35 and 50 days after sowing. Moreover, in the case of incubation in the dark for 13 days, *ANAC046*-ox exhibited pale green and smaller-sized leaves than WT [17]. In line with *ANAC046* where negative regulation to plant growth rate and positive regulation to senescence was reported, therefore, *OMTN4* negative effect may not only restrict negative drought stress and sterile seed regulation but whenever plants encountered stresses, many signal cascades occurred, including reproductive organ abortion. These evidences together lead to the conclusion that *OMTN4* probably acts as negative regulator to various processes in rice development, in order to fine-tune downstream process in response to the environmental changes.

Previous report conducted by Vialette-Guiraud, A., Chauvet, A., Gutierrez-Mazariegos, J., Eschstruth, A., Ratet, P., and Scutt, C. [7] elucidated that NAM gene targeted by *miR164* expression was reduced during carpel margin fusion. Moreover, *miR164* resistant form led to failure in carpel margin fusion in *MtNAM* with different defects such as unfused or absent stamens, unfused carpel margins although some flowers' development were normal. Prior study showed that reduced NAM expression via *miR164* strategy is essential for flower development, especially carpel margin fusion, in *Arabidopsis* and *Medicago truncatula* [7]. In consistent to our study, *OMTN4* overexpression may cause incomplete flower development in some flowers, as a result of failure of seed development.

To date, a lot of NAC transcription factors have been reported to play an important role in plant development. Our study has provided evidence on how *OMTN4* may be required for balancing seed amount and input factor. Stringent conditions lead to abortion of seed development. Dealing with environmental challenges is essential for plant survival to extend its longevity. Further study about the mechanism involved in seed abortion influenced by *OMTN4* will establish a valuable knowledge for basic rice molecular genetics which will be useful for understanding gene function.

Conclusion

Plants' largest and specific transcription factor family, the NAC (NAM, ATAF, and CUC2) plays an important role in vital diverse processes and responses to stress. Belonging to one of the NAC TFs, *OMTN4* was investigated in this study. *OMTN4* overexpression, qRT-PCR, GUS, and subcellular localization result were elucidated. Ectopic inflorescence morphology and *OMTN4* spatial expression were revealed. As NAC transcription factor, *OMTN4* was in rice cell nuclear region. GUS staining and qRT-PCR elucidated that *OMTN4* expression was detected in various developmental organs. Future study including CRISPR gene knockout will elucidate *OMTN4* loss-of-function characteristic. Finally, this study contributed to *OMTN4* gain-of-function and negative inflorescence development as key results.

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