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Functional Analysis of *OsKANADI1*, A Florigen *Hd3a* Interacting Protein in Rice (*Oryza sativa* L.)

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Abstract

OsKANADI1 is considered as a florigen Hd3a interacting protein. To study the function of *OsKANADI1*, the expression pattern of *OsKANADI1* was performed by semiquantitative RT-PCR with various wild-type tissues in the floral transition stage. The results demonstrated that *OsKANADI1* was expressed in all organs of wild-type plants, but was highest in roots and leaves. We hypothesize that *OsKANADI1* is a transcription factor in rice because it contains a GARP domain and posses a nuclear localization signal. To determine whether *OsKANADI1* encodes a nuclear protein, full-length *OsKANADI1* fused to GFP was introduced into onion epidermis cells by particle bombardment. The result revealed that *OsKANADI1* was localized in the nucleus, suggesting that *OsKANADI1* may be a transcription factor. Functional analysis was carried out using a reverse genetics approach to generate gain of function mutant (overexpression) and knockdown mutant (RNAi). The results showed that suppression of *OsKANADI1* by RNAi displayed branching and increasing tiller number in several lines. This phenotype resembles to the Hd3a overexpressed plants indicating they possibly function in similar pathway.

Key words : *OsKANADI1*, Transcription factor, Hd3a interacting protein, Rice

Introduction

The shoot apical meristem (SAM) plays a central role in the formation of lateral organs such as leaves and floral organ primordia. The adaxial-abaxial axis is fundamental for the subsequent asymmetric growth of the leaf and lamina expansion (McConnell and Barton, 1998).

Analysis of Hd3a interacting proteins in yeast two-hybrid screening identified a novel putative transcription factor belonging to the KANADI domain protein family, namely *OsKANADI1*. In *Arabidopsis*, *KANADI* genes function in lateral polarity in organs including roots, leaves and flowers. *KANADI* is required for abaxial identity in both leaves and carpels. It encodes a nuclear-localized protein in the GARP family of putative transcription factors (Riechmann *et al.* 2000). GARP homologs constitute a large family of DNA-binding proteins in plants that may be needed for a variety of key cellular functions including regulation of transcription, phosphor transfer signaling and differentiation. A GARP motif was also found in the identified *KANADI* product (Kerstetter *et al.* 2001).

KANADI acts antagonistically to the *class III HZ-Zip* genes. *KANADI* genes are expressed in a pattern complementary to that of the *class III HD-Zip* genes in the shoot; *KANADI* expression occurs in the phloem and abaxial regions of lateral organs early in development (Kerstetter *et al.* 2001, Emery *et al.* 2003, Eshed *et al.* 2004). While *KANADI* genes do not appear to be required for proper meristem function, they are needed for pattern formation of organs produced by the shoot apical and vascular meristems.

Class III HD-Zip gene family members (*PHABULOSA* [*PHB*], *PHAVULOSA* [*PHV*],

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REVOLUTA [*REV*], *ATHB8* and *ATHB15*) are putatative transcription factors that have been shown to be required for the establishment of the apical meristem, and for proper pattern formation in lateral organs and vascular tissue in the aerial portion of the plant body. Their expression in the shoot is confined to the apical meristem, the adaxial portions of lateral organs, and procambium and xylem tissue (McConnell and Barton, 1998).

In *Arabidopsis*, adaxial identity is specified by class III homeodomain leucine zipper (HD-ZIPIII) transcription factors, the transcription factor ASYMMETRIC LEAVES2 (AS2), and the transacting siRNA tasiARF, whereas abaxial identity is specified by KANADI (KAN), YABBY (YAB), AUXIN RESPONSE FACTOR (ARF), and LITTLE ZIPPER (ZPR) transcription factors and by the miRNAs miR165/miR166. Genetic analysis indicates that many of these genes interact antagonistically: loss-of-function mutations in adaxial genes typically produce an abaxialized phenotype that is accompanied by the expanded expression of abaxial genes, whereas loss of function mutations in abaxial genes produce an adaxialized phenotype that is associated with the expanded expression of adaxial genes (Wu *et al.* 2008).

KANADI and *class III HD-Zip* genes families are expressed in complementary patterns in developing lateral roots, and play functional roles in lateral root formation (Hawker and Bowman 2004). Recently, Wu *et al.* (2008) reported that *Arabidopsis* KANADI1 (AtKAN1) promotes abaxial identity by directly repressing the transcription of *AS2* in abaxial tissue. AtKAN1 binds to a site in the promoter of *AS2*, and that a single nucleotide mutation in this site interferes with AtKAN1 binding and produces an adaxialized phenotype that is associated with the inappropriate expression of *AS2* in abaxial tissue.

Few reports have been published about the function of KANADI family members in rice (Luo *et al.* 2007, Yan *et al.* 2008) and maize (Candela *et al.* 2008). *RL9* (*Rolled leaf 9*) was identified by map-based cloning in two rice allelic rolled-leaf mutants which displayed very similar phenotypes, with completely adaxialized leaves and malformed spikelets. *RL9* encodes a GARP protein, and is an ortholog of *Arabidopsis KANADIs*. Therefore, functional studies of *OsKANADI1* in rice plant will contribute to assessment of the possible function of OsKANADIs in Hd3a signaling. Further functional studies using reverse genetics to obtain mutants (either gain or loss of function mutants), or knock-down mutant using RNAi of Hd3a partners, should yield insights into the function of Hd3a in plant growth and development, particularly during the floral transition.

Materials and Methods

Expression pattern of OsKANADI1

Total RNA from seeds, seedlings, leaf blades, leaf sheath, stems, shoot apices, roots and rice suspension culture cells were extracted using TRIZOL reagent (Invitrogen) and treated with DNaseI (Invitrogen). The cDNA was synthesized from 1 µg of total RNA using SuperScript II reverse transcriptase (Invitrogen). The cDNA was subjected to semi-quantitative analysis of gene expression using PCR with primers specific for *OsKANADI1*, *Hd3a* and ubiquitin. The primers used in this research were : *OsKANADI1*-F (CACCATGCGGTGGACGACGTCGCT), *OsKANADI1*-R(TCTCTCGTGGCCTCCGAGAA), Hd3a-F (GCTCACTATCCATCCAGCATG), Hd3a-R (CCTTGCTCAGCTATTTAATTGCATAA),UBQ-F (CACAAGAAGGTGAAGCTCGC) and UBQ-R (GCCTTCTGGTTGTAGACGTAGG)

Subcelluler localization of OsKANADI1

To gain insights into the molecular function of *OsKANADI1*, we made a fusion construct to express GFP-linked *OsKANADI1* driven by the CaMV 35S promoter, to identify its intracellular localization. Five micrograms of DNA containing GFP-*OsKANADI1* construct was coated with 1.0 µg diameter gold particle Au Powder (Bio-Rad, Hercules, Ca, USA). Resuspended coated particles were

spread and dried on Microcarrier (Millipore, Bedford, MA, USA) and delivered into onion (*Allium cepa*) epidermal cells using a PDS-100/He Particle Delivery System (Bio-Rad). After overnight incubation (12-16 h) at 30°C, expression and localization of the GFP fusion protein were observed with a confocal microscope (LSM 10-META, Carl Zeiss) using an excitation filter of 488 nm.

Transgenic plant (overexpression and RNAi) construction

Mutant plants overexpressing *OsKANADI1* were generated under the control of 35S CaMV promoter and suppressing plants by RNAi. Full-length *OsKANADI1* was subcloned into the pENTR/D-TOPO cloning vector (Invitrogen). These constructs were introduced by LR clonase into pGWB17 vector to obtain *OsKANADI1*-tagged 4 x myc under the CaMV 35S promoter. This construct was introduced into rice calli by *Agrobacterium*-mediated transformation.

To generate RNAi transgenic plants, the gene sequence of *OsKANADI1* (including 383 and 309-bp fragments, respectively, as 3' untranslated /UTR regions), for which inverted repeats were made, were amplified using specific primers *OsKANADI1*-F (CAC CAG GAT CCA GCA TGT GT) and *OsKANADI1*-R (CCC TCT TCA CCC AAT CTC CG) and subcloned into the pENTR/D-TOPO cloning vector to yield entry vectors pENTR-*OsKANADI1* RNAi.

The final RNA silencing vector was produced by an LR clonase reaction between entry clone RNAi and pANDA (Miki and Shimamoto, 2005). Transgenic rice plants were generated by *Agrobacterium*-mediated transformation of rice calli in N8 background, performed according to a published protocol (Hiei *et al.* 1994).

Growth and screening of OsKANADI1 transgenic plants

To determine *OsKANADI1* transgenic plants phenotypes, all plants were grown in climate chambers with 24-h temperature cycles (10 h, 30°C during subjective day; 14 h, 25°C during subjective night). The humidity was 70%. The fluence of light was ~300 $\mu mol^{-2}s^{-1}$ (400-750 nm) under SD conditions. Transgenic plants were screened to investigate transgene insertion by PCR using specifics primers as follow : *OsKANADI1*-F (TGTACCGGACCATCAAGACC), *OsKANADI1*-R (GAGCTGGTGATGTT GGTGGT), GUS-F (GTATCAGTGTGCA TGGCTGG), GUS-R (CATCAGCACGTTA TCGAATCC), OsKAN-F (5UTR) CCAAGCA ACAAGTTACACCCCC, OsKAN-R (5UTR) CTCTTCTTGTCCTTGCGATGAC, HPT-F (GAGCCTGACCTATTGCATCTCC), HPT-R (GGCCTCCAGAAGAAGATGTTGG)

Results and Discussion

In response to floral stimulus, the apical meristem stop producing leaves to initiate floral development; this switch in morphogenesis involves a change in the identity of the primordial initiated and in phyllotaxis (Corbesier and Coupland, 2006). In the previous study, we identified a novel putative transcription factor, *OsKANADI1*, as an Hd3a interacting protein in rice (Purwestri *et al.* 2008, Taoka *et al.* 2011).

To further study the function of *OsKANADI1*, the expression pattern of *OsKANADI1* have been performed by semiquantitative RT-PCR with various wild-type tissues in the floral transition stage. The results demonstrated that *OsKANADI1* was expressed in all organs of wild-type plants, but was highest in roots and leaves (Figure 1A)

Previous studies indicated that the Arabidopsis KAN1 protein and GARP domain-containing proteins are localized in the nucleus (Kerstetter *et al.* 2001, Hosoda *et al.* 2002). We hypothesize that *OsKANADI1* is a transcription factor in rice because it contains a GARP domain and posses a nuclear localization signal. To determine whether *OsKANADI1* encodes a nuclear protein, full-length *OsKANADI1* fused to GFP was introduced into a vector. Vector isolated from

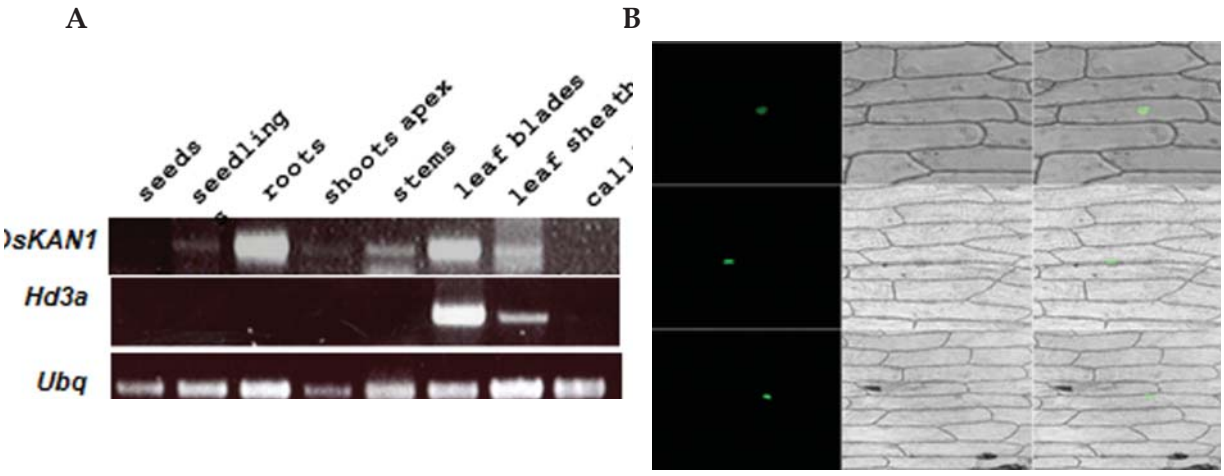

Figure 1. *OsKANADI1* expression and subcellular localization. (A) Semi-quantitative RT-PCR of *OsKANADI1* in various organs of wild-type N8 rice. (B) Subcellular localization of OsKAN1-GFP fusion protein in the nucleus

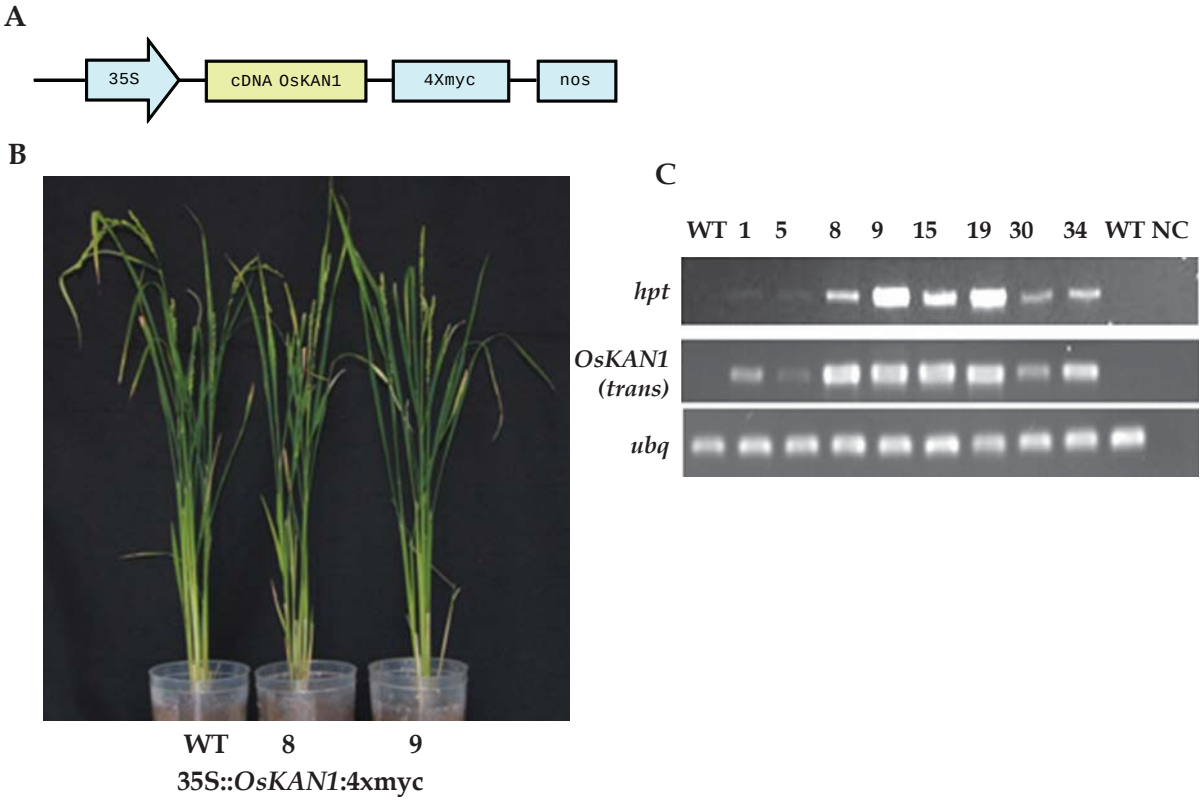

Figure 2. Characterization of *OsKANADI1*-overexpressing plants. (A*) OsKANADI1*-overexpression construct was controlled by CaMV 35S promoter and tagged with 4Xmyc. (B) Growth of N8 wild-type and *OsKANADI1*-overexpressing plants under SD conditions. (C) Semi quantitative RT-PCR in transgenic plants. The *OsKANADI1* transgene was detected in transgenic plants using specific primers. Several lines showed overexpression of *OsKANADI1* (lines 8, 9,15,19). Ubiquitin was used as loading control.

cells expressing the *OSKANADI1*-GFP fusion protein was introduced into onion epidermis cells by particle bombardment. This revealed that *OsKANADI1* is localized in the nucleus, suggesting that *OsKANADI1* may be a transcription factor (Figure 1B). Our previous results showed that Hd3a was localized in nucleus and interact with 14-3-3 protein and FD, a bZIP transcription factor (Purwestri *et al*. 2009; Taoka *et al*. 2011). The expression pattern and subcellular localization of *OsKANADI1* provided important insights into its function.

We generated mutant plants overexpressing *OsKANADI1* (OsKAN1-ox) fusion with 4xmyc tag under the control of

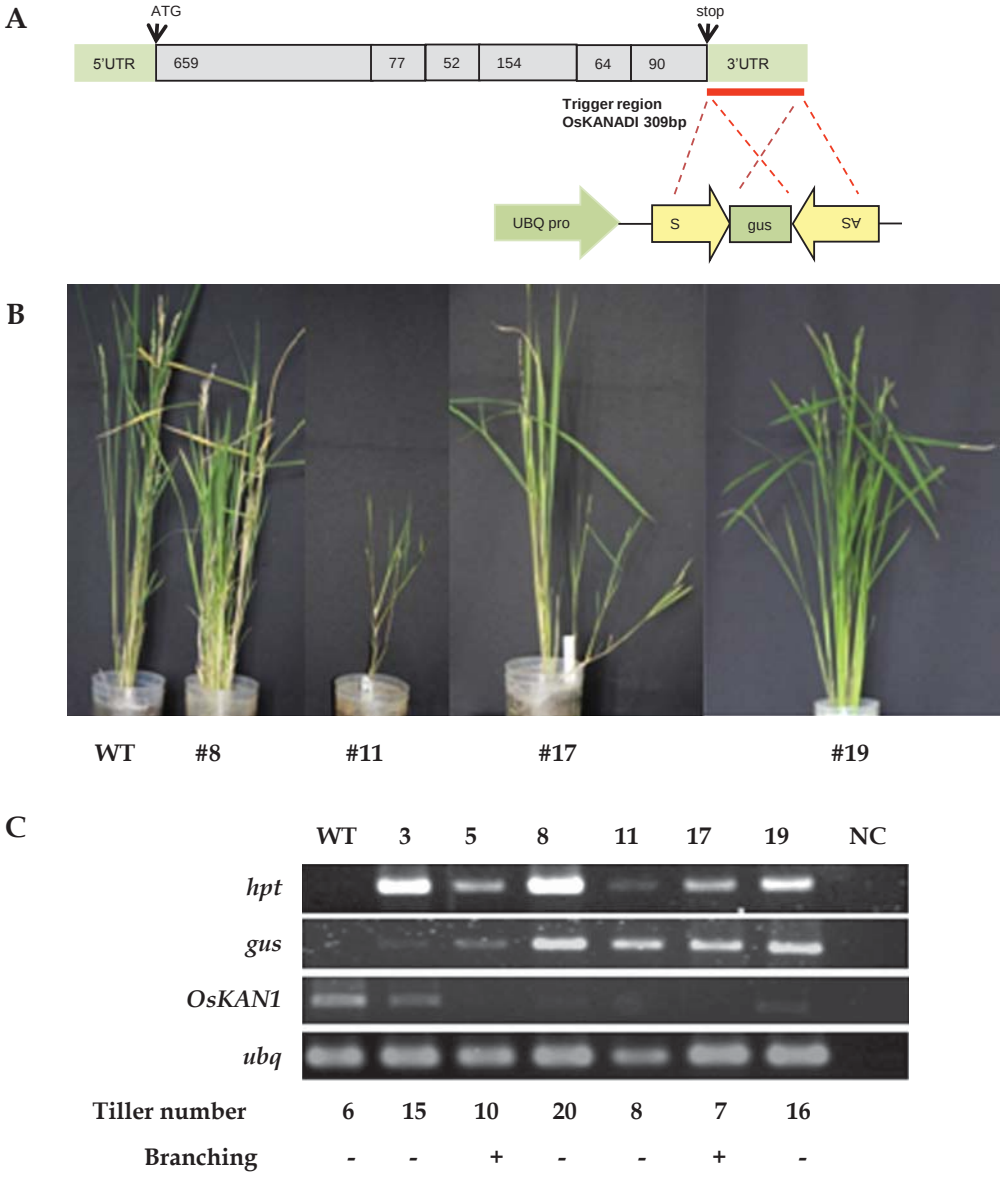

Figure 3. Characterization of *OsKANADI1*-RNAi plants. (A*) OsKANADI1-RNAi* construct. Gene specific region (3UTR) was used for *OsKANADI1 RNAi* construct. (B) Growth of N8 wild-type and *OsKANADI1* RNAi plants under SD conditions. (C) Semi quantitative RT-PCR in transgenic plants. The *OsKANADI1* transgene was detected in transgenic plants using *gus* primers. Using specific primers, several lines showed supression of *OsKANADI1.* Ubiquitin was used as loading control. NC : negative control (without DNA template)

the 35S CaMV promoter (Figure 2A), and *OsKANADI1*-suppressing plants by RNAi (Figure 3A). At present, only T0 generation plants are available, and therefore we have not yet analyzed the flowering time. However, we found interesting phenotypes of *OsKANADI1*-RNAi plants. The knocked-down OsKANADI plants displayed two stem folks and increased tiller number (Figure 2B). This phenotype resembles to Hd3a overexpressing plants, indicating that they may be involved in similar pathways. *OsKANADI1* expression was confirmed in both T0 generation *OsKANADI1*-ox (Figure 2C) and *OsKANADI1*-RNAi mutants plants (Figure 3C). Analysis of these mutants in the next generation is important for understanding the possible role of *OsKANADI1* in association with Hd3a during plant development.

OsKANADI1 has at least seven homologs in rice. *OsKANADI1* has a potential phosphorylation site for Ca^{2+} dependent protein kinase and might function as a target binding site of 14-3-3 proteins (RSXpSXP- pS motif in C-terminal region). This feature also presence in FD, a FT interacting protein in *Arabidopsis* (Abe *et al.* 2005). The possibility that *OsKANADI1*, 14-3-3 proteins and Hd3a may form a complex to regulate their signaling should be interesting to address in future.

In *Arabidopsis*, members of the KANADI family of GARP transcriptional regulators have been found to play essential roles in the specification of abaxial fate in leaf development. Loss of function mutations in individual genes have relatively weak effects on organ polarity (Kerstetter *et al.* 2001, Eshed *et al*. 1999), but *kan1 kan2* (Eshed *et al.* 2001) and *kan1 kan2 kan3* (Eshed *et al.* 2004) mutants are strongly adaxialized. It is interesting to investigate possible target genes of the *OsKANADI1*-Hd3a complex, as well as the tissue specificity of *OsKANADI1* expression to determine whether expression is also localized in the phloem, similar to *Hd3a*. It has been reported that *Arabidopsis KANADI* and *class III HD-ZIP* genes exhibit complementary expression patterns in the vasculature as well as in the leaves. *KANAD*I expression is restricted to the developing phloem, positioned abaxially, and *class III HD-ZIP* expression is limited to developing xylem, positioned adaxially. While *KAN2* and *KAN3* are expressed in developing phloem throughout the plant (leaf and stem), *KAN1* expression in the phloem is largely limited to the root (Emery *et al.* 2003).

In *Arabidopsis* asymmetric leaf development, KANADI proteins have antagonistic function with HD-ZIP III proteins (Emery *et al.* 2003), and *KANADI* genes are necessary for *YABBY* expression (Eshed *et al.* 2004). However, in rice no *HD-ZP III* genes have yet been characterized, but four *YABBY* genes were characterized, i.e. *DROPPING LEAF*, *YAB1*, *YAB3*, and *OsYAB4* (Jang *et al.* 2004, Yamaguchi *et al.* 2004, Dai *et al.* 2007, Liu *et al.* 2007). These YABBY genes in rice have been proved not to determine the abaxial cell fate, unlike their function in *Arabidopsis*.

Recent study in rice identified *RL9* as a member of OsKANADI family. The loss of function of this gene results in the rolled-leaf phenotype and malformed spikelets, suggesting that RL9 function similar to *Arabidopsis* KANADIs (Yan *et al.* 2008). In this study, we generated *OsKANADI1* mutants, either overexpressed or RNAi plants. Suppression of *OsKANADI1* by RNAi displayed branching and increasing tiller number in several lines. This phenotype resembles to the *Hd3a* overexpressed plants (unpublished data) indicating they possibly function in similar pathway. Further characterization using the multiple OsKANADI mutants plants in the next generation remain to be investigated.

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