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Original Paper

Cytokinin-induced expression of *OSH1* in a shoot-regenerating rice callus

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Abstract The expression of a *KNOX* class 1 gene *OSH1* is induced by cytokinin during regeneration of shoots from callus in *Oryza sativa* L. (rice). This cytokinin-induced expression was enhanced by overexpression of homologues of cytokinin-signalling phosphorelay genes such as a histidine kinase gene *OHK3*, a phosphotransmitter gene *OHP2* and a response regulator gene *ORR1* in cultured cells. Regionally overlapped expression of these genes and *OSH1* was observed in shoot apex. These results suggest that these cytokinin-signalling genes are positive regulators of the expression of *OSH1*, and mediate the *OSH* expression upon shoot regeneration from callus in rice.

Key words: cytokinin, *KNOX* gene, rice, shoot regeneration.

Introduction

In higher plants the SAM is generated during embryogenesis and maintains its meristematic activity throughout development. The SAM also successively generates lateral organs such as leaf and flower. These two antagonistic functions of the SAM are controlled by the expression of *KNOX* class 1 genes, which encode homeodomain proteins. *KNOX* genes are expressed in the meristematic tissues including SAM and play an important role in SAM formation and maintenance. For example, loss-of-function mutations of *Arabidopsis SHOOTMERISTEMLESS* (*STM*) and maize *KNOTTED1* (*KN1*) resulted in defects in the formation and maintenance of the SAM, respectively (Kerstetter et al. 1997; Long et al. 1996; Vollbrecht et al. 2000). Rice *osh1* mutant showed failure of the maintenance of the SAM, and *osh1 osh15* double mutant showed no regeneration of shoots from callus (Tsuda et al. 2011). Conversely, gain-of-function mutations of *KN1* or other *KNOX* genes such as *ROUGH SHEATH1* (*RS1*), *GNARLEY1* (*GN1*), *LIGULELESS3* (*LG3*) or *LG4a*, whose corresponding genes were ectopically expressed in the leaf, resulted in abnormal leaf morphologies (Bauer et al. 2004; Foster et al. 1999; Muehlbauer et al. 1999; Schneeberger et al. 1995). Constitutive overexpression of *KNOX* genes in various plant species also brought about abnormal leaf development, and in an extreme case, regeneration of shoot from callus was inhibited by maintaining the callus cells in an undifferentiated state in rice (*Oryza sativa* L.) (Ito et al. 2001; Reiser et al. 2000). These functional and expression analyses of *KNOX* genes demonstrate importance of the regulation of their expression.

Detail analyses of the *KNOX* gene expression were carried out in rice, and it was shown that positive autoregulation is involved in maintaining the *OSH1* expression in the SAM (Tsuda et al. 2011). OSH1 was shown to bind to its regulatory sequences, and its binding was essential for the proper function of *OSH1*. Mutations in the OSH1-binding sites resulted in the failure of the expression of *OSH1* and complementation of the *osh1* mutation. These results clearly indicate that positive autoregulation is essential for proper expression of *OSH1* and its functionality.

In a previous study, we found that expression of *OSH1* is induced upon shoot regeneration from callus on a regeneration medium containing phytohormone cytokinin (Ito et al. 2001; Tsuda et al. 2011). Cytokinin perception and signalling have been well studied in *Arabidopsis*. Cytokinin is perceived by three partially redundant receptor HKs CYTOKININ RESPONSE1 (CRE1, also known as AHK4 or WOL), AHK2 and AHK3 (Inoue et al. 2001; Ueguchi et al. 2001; Yamada et al. 2001). The cytokinin signal is then transduced to

Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzylaminopurine; HK, histidine kinase; HPt, histidine-containing phosphotransmitter; NAA, 1-naphthaleneacetic acid; RR, response regulator; SAM, shoot apical meristem.
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HPt proteins AHPs, and further to type-B RR proteins ARRs by a phosphotransfer mechanism (Hwang and Sheen 2001). Type-B ARRs are transcription factors, and phosphorylated and activated type-B ARRs induce or reduce expression of target genes including type-A ARRs (Hwang and Sheen 2001). Transcriptome analyses have also been carried out, and a number of genes that are up-regulated or down-regulated by cytokinin were identified (Brenner et al. 2012). Although this phosphotransfer signalling of cytokinin is well studied, downstream events triggered by activated ARRs still have not been well understood, and genetic networks which connect cytokinin signalling and cytokinin responses such as shoot regeneration remain to be elucidated.

In this paper we examined whether cytokinin-signalling genes such as an HK gene, HPt gene and type-B RR gene are involved in the *OSH1* expression upon shoot regeneration from callus in rice. The fact that the expression of *OSH1* was induced by cytokinin upon shoot regeneration raised a possibility that the induction might be mediated by rice homologues of cytokinin signalling genes. Our results suggested that the cytokinin signalling genes are positive regulators of the *OSH1* expression and mediate the *OSH1* expression upon shoot regeneration from callus in rice.

Materials and methods

Plant materials

Rice (*Oryza sativa* L.) cultivar Nipponbare was used for all experiments. For callus induction, seeds were dehusked, surface-sterilized and incubated on an N6CI medium (N6 salts, N6 vitamins, 2 mg/l 2,4-D, 3% sucrose, pH 5.8) at 25°C. Calli were transferred onto an MS medium (MS salts, MS vitamins, 3% sucrose, pH 5.8) containing 1 mg/l NAA, 2 mg/l BA or both. The calli were harvested after 4-day incubation at 25°C. Suspension cells were generated from seed-derived calli. The calli were put into a liquid R2 medium supplemented with 2 mg/l 2,4-D (Ohira et al. 1973) and incubated at 28°C with continuous shaking. Suspension cells were transferred to the fresh R2 medium every 2 weeks. Four days after transfer to the fresh medium, BA was added to the medium at a concentration of 2 mg/l, and cells were harvested after the indicated time.

Transgenic rice

cDNAs for OHK3b (DDBJ accession no. AB246778), *OHP2* (AK072521) and *ORR1* (AB246780) were used in this study (Ito and Kurata 2006). These cDNAs were inserted into a cloning site of a binary vector plasmid of pBCH1 or pBCH2 (Ito et al. 2001; Miyoshi et al. 2003), and resultant plasmids harboured the cDNA sequence between a cauliflower mosaic virus 35S promoter and a terminator of a nopaline synthase gene in a sense orientation. These plasmids were introduced into *Agrobacterium tumefaciens* EHA101 by electroporation. *Agrobacterium*-mediated transformation was carried out as described (Hiei et al. 1994). The transformed callus was put into a liquid R2 medium (Ohira et al. 1973) supplemented with 2 mg/l 2,4-D, 50 mg/l hygromycin and 200 mg/l claforan, and incubated at 28°C with continuous shaking. Suspension cells were maintained and treated with cytokinin as described above.

Table 1. Primers used in this study.

Gene	Primer	Sequence
OHK3	C3-F10	CTTCGAAGTGTACTAACAGG
	C3-R1	CACTGTGGCCACTAGCAAAC
OsRR9	R7-F1	GTTGTGATCATGTCATCGGA
	R7-R1	AAGCAGTATCCGTTAACGGA
OSH1	H24-1	GCTCTTCTGAGGAGGACCAA
	H-1	AACCAGTTGTTGATCTGCTT
Actin	RAc-1	AACTGGGATGATATGGAGAA
	RAc-2	CCTCCAATCCAGACACTGTA

Expression analysis

RNA isolation and RT-PCR were carried out as described previously (Ito et al. 2001). Briefly, poly(A)$^+$RNAs were purified from 5 μg of total RNAs and half of them was reverse-transcribed with an oligo(dT) primer and Superscript III reverse-transcriptase (Invitrogen). The remaining half was similarly treated without the reverse-transcriptase. PCR was carried out with four primers, two for a gene of interest and two for actin. After electrophoresis, signals were visualized with ethidium bromide staining or Southern blot probed with entire *OSH1* cDNA. Primers used for RT-PCR were C3-F10 and C3-R1 for *OHK3*, R7-F1 and R7-R1 for *OsRR9*, H24-1 and H-1 for *OSH1* and RAc-1 and RAc-2 for actin (Table 1).

In situ hybridization of paraffin sections of rice shoot apex was carried out as previously described (Ito et al. 2001) using entire cDNAs of *OHK3* (AB246778), *OHP2* (AK072521) and *ORR1* (AB246780) and a partial cDNA of *OSH1* (a 1,002 bp fragment from its 3′ end) as probes.

Results

Cytokinin-induced expression of OSH1

We previously showed that expression of *OSH1* was induced upon transfer of rice callus to a regeneration medium containing auxin and cytokinin (Ito et al. 2001; Tsuda et al. 2011). We first confirmed cytokinin-induced expression of *OSH1* in calli cultured on a regeneration medium containing both auxin and cytokinin. Growing calli were transferred onto a medium supplemented with auxin alone, cytokinin alone or both auxin and cytokinin, and expression of *OSH1* was examined by RT-PCR. The result showed that the *OSH1* expression was observed in the calli on the cytokinin-containing medium, and auxin is not required for its expression in the callus (Figure 1A). We next examined a time course of *OSH1* induction by cytokinin in suspension cells. Weak expression of *OSH1* was first detected 1 h after cytokinin treatment, and the high-level expression was observed at 6 h after

the treatment (Figure 1B). We also examined expression of *OsRR9*, a type-A RR gene which is an early response gene of cytokinin and a direct target of a type-B RR (Hwang and Sheen 2001; Ito and Kurata 2006), to determine the relevance of this early induction of the *OSH1* expression. The result showed that the expression of *OsRR9* was detected within 30 min after the cytokinin treatment, and that the cytokinin response of *OsRR9* preceded that of *OSH1* (Figure 1B). These results indicate that the *OSH1* expression is induced by cytokinin, but *OSH1* may not be a direct target of a type-B RR in the cytokinin-signalling phosphorelay pathway.

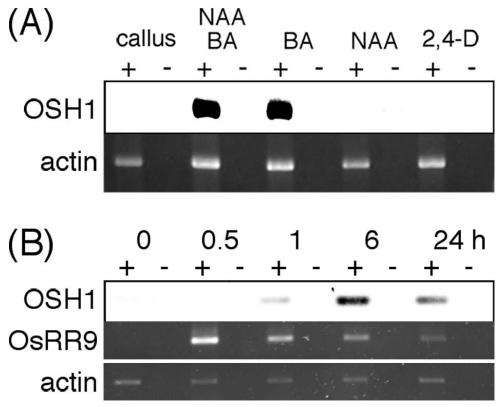

Figure 1. Cytokinin-induced expression of *OSH1* in cultured cells. (A) Expression of *OSH1* in callus on media containing phytohormones. Growing callus on the 2,4-D-containing N6CI medium was transferred onto the MS medium containing NAA, BA or 2,4-D. RNAs were isolated 4 days after the transfer. (B) Expression of *OSH1* in suspension cells by cytokinin treatment. Suspension cells were treated with BA, and RNA was isolated at indicated time points after the treatment. *OsRR9* is a member of the type-A RR family and is an early response gene of cytokinin (Ito and Kurata 2006). + and − indicate whether reverse-transcriptase was added to or omitted from the reverse transcription mixture, respectively. RT-PCR products were visualized by Southern blot probed with an *OSH1* probe (*OSH1*) or ethidium bromide staining (*OsRR9* and actin). Actin was used as an internal control.

Expression of OSH1 in OHK3, OHP2 and ORR1 overexpressing cells

Because cytokinin-signal transduction is known to be mediated by a phosphorelay system (El-Showk et al. 2013), it was speculated that the *OSH1* expression by cytokinin treatment could be mediated by a phosphorelay system. To test this possibility we examined the effect of overexpression of phosphorelay genes such as *OHK3* (an HK gene), *OHP2* (an HPt gene) and *ORR1* (a type-B RR gene) on the *OSH1* expression by cytokinin (Ito and Kurata 2006). These three genes were selected from each gene family based on higher expression levels in cultured cells among members of each family.

We generated transgenic suspension cell lines overexpressing *OHK3* (Figure 2A) and examined *OSH1* expression in these cell lines upon cytokinin treatment. The suspension cells were treated with cytokinin BA for 24 h or not treated, and RNAs were isolated for detection of *OSH1* expression. In these suspension cell lines, *OSH1* expression was detected without the cytokinin treatment, and the cytokinin treatment further induced *OSH1* expression (Figure 2B). The expression level of *OSH1* in the cytokinin-treated *OHK3* overexpressing suspension cell lines was higher than that of a cytokinin-treated control line that was transformed with an empty vector (Figure 2B). The control line showed no expression of *OSH1* without BA-treatment (Figure 2B). These results indicate that *OHK3* has the ability to promote an induction pathway of *OSH1* expression upon cytokinin treatment in cultured cells.

We also generated transgenic suspension cell lines overexpressing *OHP2* or *ORR1*. As was the case with *OHK3*-overexpressing lines, these lines also showed

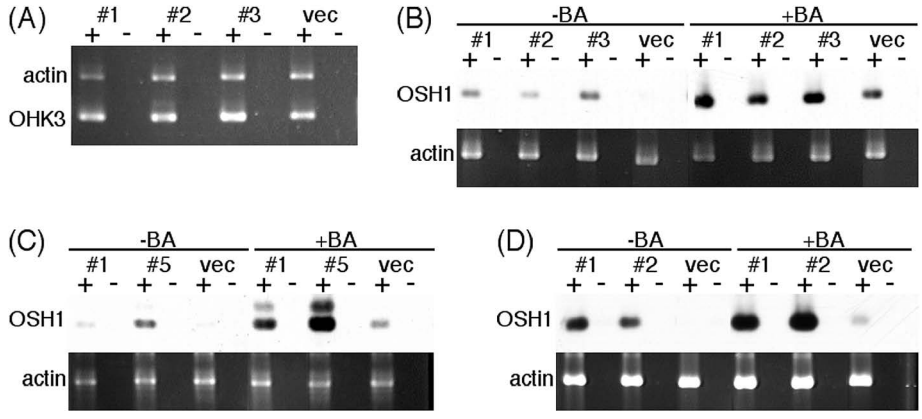

Figure 2. Expression of *OSH1* in the transgenic rice cultured cells. (A) Overexpression of *OHK3* in the 35S-OHK3 transgenic suspension cells. (B) Expression of *OSH1* in the 35S-OHK3 transgenic suspension cells. (C) Expression of *OSH1* in the 35S-OHP2 transgenic suspension cells. (D) Expression of *OSH1* in the 35S-ORR1 transgenic suspension cells. #1 to #5 and vec indicate independent transformed lines and a vector-transformed control line, respectively. + and − indicate whether reverse-transcriptase was added to or omitted from the reverse transcription mixture, respectively. Suspension cells were treated or not treated with BA, and RNAs were isolated after 24 h incubation. RT-PCR products were visualized by Southern blot probed with an *OSH1* probe (*OSH1*) or ethidium bromide staining (*OHK3* and actin). Actin was used as an internal control.

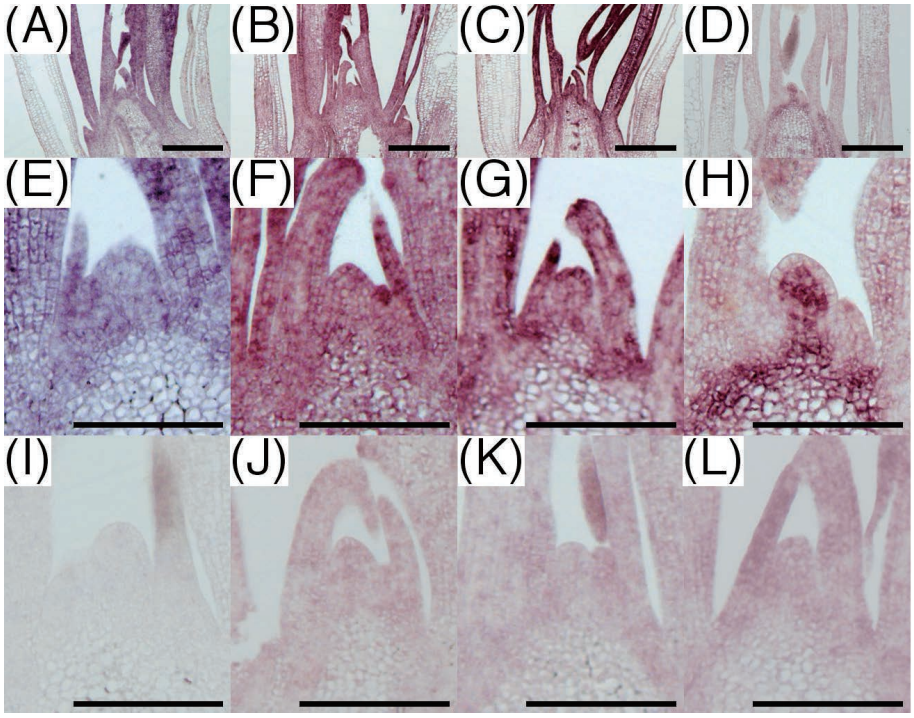

Figure 3. Overlapped expression of the cytokinin-signalling phosphorelay genes in shoot apex. Sections of shoot apex of 1-week seedling were hybridized with antisense RNA probes of *OHK3* (A, E), *OHP2* (B, F), *ORR1* (C, G) and *OSH1* (D, H). Only a faint background staining was observed with control sense RNA probes of *OHK3* (I), *OHP2* (J), *ORR1* (K) and *OSH1* (L). Bars, 100 μm (A to D) and 50 μm (E to L).

OSH1 expression without the cytokinin treatment and further enhancement of *OSH1* expression by cytokinin (Figure 2C, D). These results indicate that *OHP2* and *ORR1* also have the ability to induce *OSH1* expression in cultured cells.

When *OHK3*, *OHP2* or *ORR1*-overexpressing lines were regenerated, they showed no morphological alteration and grew normally.

Overlapped expression of OHK3, OHP2, ORR1 and OSH1

Our analyses of the transgenic cells suggested that the components of the histidine phosphorelay mediate the cytokinin-induced expression of *OSH1* in callus. If this is the case, these genes should show regionally overlapped expression patterns. We previously showed that *OHK3*, *OHP2* and *ORR1* were ubiquitously and overlappingly expressed in various organs including growing and regenerating calli by RT-PCR analysis (Ito and Kurata 2006).

To further examine regionally overlapped expression, we carried out in situ hybridization analyses of these genes in shoot apex of 1-week seedling, where *OSH1* is expressed (Sentoku et al. 1999). Sections of shoot apex were hybridized with antisense probes of *OHK3*, *OHP2*, *ORR1* and also *OSH1*. The results showed that these four genes were expressed in overlapping regions of shoot apex (Figure 3). Signals of the three cytokinin-signalling genes *OHK3*, *OHP2* and *ORR1* were detected in the SAM and developing young leaves, although the signal of *OHK3* in the SAM was rather weak (Figure 3A–C, E–G). The expression of *OSH1* was detected in the SAM, but not in leaves (Figure 3D, H). In control experiments using sense probes of these genes, no clear signals were obtained (Figure 3I–L). These results showed that *OHK3*, *OHP2* and *ORR1* are expressed overlappingly in shoot apex including SAM, where *OSH1* expression was detected, and young leaf.

Discussion

Cytokinin-induced expression of OSH1

In this study, we focused our analysis on the regulation of *OSH1* expression on shoot regeneration from rice callus. Several previous reports showed a link between *KNOX* gene expression and cytokinin (Hay et al. 2004). For example, overexpression of *OSH1* in tobacco or *Arabidopsis KNAT1* in lettuce resulted in the increase of the cytokinin level in the leaf (Frugis et al. 2001; Kusaba et al. 1998). Expression of *KN1* driven by a senescence-inducible promoter also increased the cytokinin level in the tobacco leaf (Ori et al. 1999). In addition, it was reported that *KNOX* expression induced expression of a cytokinin biosynthesis gene in *Arabidopsis* (Jasinski et al. 2005; Yanai et al. 2005). These results suggested that *KNOX* genes control the cytokinin level through expression of a cytokinin biosynthesis gene.

We showed that *OSH1* expression was induced upon

shoot regeneration from callus, and this induction depended on cytokinin in a medium (Figure 1A) (Ito et al. 2001; Tsuda et al. 2011). The cytokinin-induced expression of *OSH1* was also observed in suspension cells (Figure 1B). In addition to *OSH1*, it was reported that other *KNOX* class 1 genes such as *OSH6*, *OSH15* and *OSH71*, but not *OSH45*, are induced by cytokinin in callus (Tsuda et al. 2011). Comparison of the induction of *OSH1* (1 h after treatment) with that of a type-A RR gene *OsRR9* (within 30 min) suggested that *OSH1* may not be a direct target of a type-B RR, and that other transcription factor(s) may intervene between them, although the possibility that *OSH1* is a direct target of type-B RR still cannot be ruled out. Our results indicate that cytokinin induces *KNOX* gene expression in rice cultured cells and raise, together with previous reports, a possibility of a positive feedback control between a *KNOX* gene expression and cytokinin. In this circuit, cytokinin induces *KNOX* gene expression through cytokinin-signalling phosphorelay genes, and the *KNOX* gene in turn increases the cytokinin level through up-regulation of cytokinin biosynthesis genes. Reduced expression of *OSH1* in the SAM of *log* mutants, which have a mutation in the cytokinin biosynthesis gene (Kurakawa et al. 2007), is consistent with this notion. This positive feedback loop may help maintenance of *KNOX* expression in the SAM, in addition to the positive autoregulation of *OSH1* in which OSH1 protein binds to its own regulatory sequence for its expression (Tsuda et al. 2011).

Cytokinin signalling phosphorelay genes mediates the OSH1 expression upon shoot regeneration

Studies in *Arabidopsis* showed that a cytokinin signal is transduced by a phosphorelay mechanism (El-Showk et al. 2013). We previously showed that the *KNOX* class 1 gene *OSH1* was induced upon regeneration on a medium containing cytokinin in rice (Ito et al. 2001; Tsuda et al. 2011). We also identified all the members of cytokinin-signalling phosphorelay genes in rice (Ito and Kurata 2006). In this paper we examined whether histidine phosphorelay genes identified in rice, such as *OHK3*, *OHP2* and *ORR1*, mediate cytokinin-induced expression of *OSH1*. Overexpression of each of these genes brought about the enhanced expression of *OSH1* in cultured cells (Figure 2). We detected the weak *OSH1* expression in the *OHK3*, *OHP2* or *ORR1*-overexpressing cultured cells even without cytokinin treatment (Figure 2). This is probably caused by basal activities of the encoded proteins. The three genes used in the transgenic analyses showed expression in growing and regenerating calli (Ito and Kurata 2006), and they also showed the overlapped expression in the SAM (Figure 3). These results suggest that OHK3–OHP2–ORR1 phosphorelay mediates *OSH1* induction upon shoot regeneration, and that *OHK3*, *OHP2* and *ORR1* are positive regulators of the *OSH1* expression in callus. Type-A RRs are known to be negative regulators of cytokinin signalling, and its overexpression was shown to abolish shoot regeneration in rice (Hirose et al. 2007). Since *OSH1* expression is induced by cytokinin and its induction is mediated by cytokinin-signalling genes, *OSH1* expression may be down-regulated by the overexpression of the type-A RR.

In this paper, we showed that cytokinin-induced expression of *OSH1* is mediated by phosphorelay genes in cultured cells. However, since we used a gain-of-function overexpression approach in suspension cells, the relevance of this notion to normal shoot development was not clear. Loss-of-function approaches will clarify this question.

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