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

Masashi Naruse¹, Honami Takahashi¹, Nori Kurata², Yukihiro Ito¹,*

¹Graduate School of Agricultural Science, Tohoku University, 468-1 Aramaki Aza Aoba, Aoba-ku, Sendai 980-8572, Japan;
²National Institute of Genetics, 1111 Yata, Mishima, Shizuoka 411-8540, Japan

*E-mail: yukito@tohoku.ac.jp Tel: +81-22-757-4233 Fax: +81-22-757-4232

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
expression induced by cytokinin

OSH1 of 2 mg/l, and cells were harvested after the indicated time.

fresh medium, BA was added to the medium at a concentration

continuous shaking. Suspension cells were transferred to the

mediated transformation was carried out as

Agrobacterium

harboured the cDNA sequence between a cauliflower mosaic

(Ito et al. 2001; Miyoshi et al. 2003), and resultant plasmids

(AB246780) were used in this study

signalling genes are positive regulators of the

shoot regeneration raised a possibility that the induction

expression and mediate the

signalling of cytokinin is well studied, downstream

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,

Rice (L.) cultivar Nipponbare was used for all

Materials and methods

Table 1. Primers used in this study.

| Gene | Primer Sequence |
|------|----------------|
| OK3  | C3-F10 CTTGAAGTGTACTAACAGG |
|      | C3-R1 CATCGGCAACTGAAAC |
| OsR9 | R7-F1 GGTTGTGATCATGTCAGGA |
|      | R7-R1 AACGATGTCCTGTTAAG |
| OSH1 | H24-1 GCTCTTCTGAGGAGGACCAA |
|      | H-1 AACCAGTTGATCTTGCT |
| Actin| RAc-1 AACTGGGATGATATGGAAG |
|      | RAc-2 CCTCAGATGCCAGACCTTG |

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 clororan, and

incubated at 28°C with continuous shaking. Suspension cells

were maintained and treated with cytokinin as described above.

Expression analysis

RNA isolation and RT-PCR were carried out as described

previously (Ito et al. 2001). Briefly, poly(A)’RNAs were

purified from 5µg 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 OK3, R7-F1 and R7-R1 for OsR9, 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

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

Copyright © 2018 The Japanese Society for Plant Cell and Molecular Biology

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.

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
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.

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
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 (1h after treatment) with that of a type-A RR gene OsRR9 (within 30min) 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.

Acknowledgements

Full-length cDNAs were developed by the Rice Genome Project of the National Institute of Agrobiological Sciences, Japan, and were provided by the Rice Genome Resource Center. We thank Tomomi Makino, Satomi Sakai, Akemi Ishii and Yoko Shiroto for their technical assistance. This work was supported in part by Kuriyayashi Education and Science foundation.

References

Bauer P, Lubkowitz M, Tyers R, Nemoto K, Meeley RB, Goff SA, Freland M (2004) Regulation and a conserved intron sequence of liguleless3/4 knox class-1 homeobox genes in grasses. Planta 219: 359–368
Brenner WG, Ramireddy E, Heyl A, Schmülling T (2012) Gene regulation by cytokinin in Arabidopsis. Front Plant Sci 3: 8
El-Showk S, Ruonala R, Harlariutta Y (2013) Crossing paths: Cytokinin signalling and crosstalk. Development 140: 1373–1383
Foster T, Yamaguchi J, Wong BC, Veit B, Hake S (1999) Gnr1 is a dominant mutation in the knox4 homeobox gene affecting cell shape and identity. Plant Cell 11: 1239–1252
Frugis G, Giannino D, Mele G, Nicolodi C, Chiappetta A, Bitonti MB, Innocenti AM, Dewitte W, Van Onckelen H, Mariotti D (2001) Overexpression of KNOTTED1 in lettuce shifts leaf cell determinate growth to a shoot-like indeterminate growth associated with an accumulation of isopentenyl-type cytokinins. Plant Physiol 126: 1370–1380
Hay A, Craft J, Tsiantis M (2004) Plant hormones and homeoboxes: Bridging the gap. BioEssays 26: 395–404
Hiei Y, Ohta S, Komari T, Kumashiro T (1994) Efficient transformation of rice (Oryza sativa L.) mediated by Agrobacterium and sequence analysis of the boundaries of the T-DNA. Plant J 6: 271–282
Hirose N, Makita N, Kojima M, Kamada-Nobusada T, Sakakibara H (2007) Overexpression of a type-A response regulator alters rice morphology and cytokinin metabolism. Plant Cell Physiol 48: 523–539
Hwang I, Sheen J (2001) Two-component circuitry in Arabidopsis cytokinin signal transduction. Nature 413: 383–389
Inoue T, Higuchi M, Hashimoto Y, Seki M, Kobayashi M, Kato T, Tabata S, Shinozaki K, Kakimoto T (2001) Identification of CRE1 as a cytokinin receptor from Arabidopsis. Nature 409: 1060–1063
Ito Y, Eiguchi M, Kurata N (2001) KNOX homeobox genes are bridging the gap. BioEssays 26: 395–404
sufficient in maintaining cultured cells in an undifferentiated state in rice. *Genesis* 30: 231–238

Ito Y, Kurata N (2006) Identification and characterization of cytokinin-signalling gene families in rice. *Gene* 382: 57–65

Jasinski S, Piazza P, Craft J, Hay A, Woolley L, Rieu I, Phillips A, Hedden P, Tsiantis M (2005) KNOX action in *Arabidopsis* is mediated by coordinate regulation of cytokinin and gibberellin activities. *Curr Biol* 15: 1560–1565

Kerstetter RA, Laudencia-Chingcuanco D, Smith LG, Hake S (1997) Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. *Development* 124: 3045–3054

Kurakawa T, Ueda N, Maekawa M, Kobayashi K, Kojima M, Nagato Y, Sakakibara H, Kyoizuka J (2007) Direct control of shoot meristem activity by a cytokinin-activating enzyme. *Nature* 445: 652–655

Kusaba S, Kano-Murakami Y, Matsuoka M, Tamaoki M, Sakamoto T, Yamaguchi I, Fukumoto M (1998) Alteration of hormone levels in transgenic tobacco plants overexpressing the rice homeobox gene *OSH1*. *Plant Physiol* 116: 471–476

Long JA, Moan EI, Medford JI, Barton MK (1996) A member of the KNOTTED class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. *Nature* 379: 66–69

Miyoshi K, Ito Y, Serizawa A, Kurata N (2003) *OsHAP3* genes regulate chloroplast biogenesis in rice. *Plant J* 36: 532–540

Muehlbauer GJ, Fowler JE, Girard L, Tyers R, Harper L, Freeling M (1999) Ectopic expression of the maize homeobox gene *Liguleless3* alters cell fates in the leaf. *Plant Physiol* 119: 651–662

Ohira K, Ojima K, Fujitaka A (1973) Studies on the nutrition of rice cell culture I. A simple, defined medium for rapid growth in suspension culture. *Plant Cell Physiol* 14: 1113–1121

Ori N, Juarez MT, Jackson D, Yamaguchi I, Banowetz GM, Hake S (1999) Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene *knotted1* under the control of a senescence-activated promoter. *Plant Cell* 11: 1073–1080

Reiser L, Sanchez-Baracaldo P, Hake S (2000) Knots in the family tree: Evolutionary relationships and functions of *knox* homeobox genes. *Plant Mol Biol* 42: 151–166

Schneeberger RG, Beccraft PW, Hake S, Freeling M (1995) Ectopic expression of the *knox* homebox gene *rough sheath1* alters cell fate in the maize leaf. *Genes Dev* 9: 2292–2304

Sentoku N, Sato Y, Kurata N, Ito Y, Kitano H, Matsuoka M (1999) Regional expression of the rice KN1-type homeobox gene family during embryo, shoot, and flower development. *Plant Cell* 11: 1651–1663

Tsuda K, Ito Y, Sato Y, Kurata N (2011) Positive autoregulation of a KNOX gene is essential for shoot apical meristem maintenance in rice. *Plant Cell* 23: 4368–4381

Ueguchi C, Sato S, Kato T, Tabata S (2001) The *AHK4* gene involved in the cytokinin-signaling pathway as a direct receptor molecule in *Arabidopsis thaliana*. *Plant Cell Physiol* 42: 751–755

Vollbrecht E, Reiser L, Hake S (2000) Shoot meristem size is dependent on inbred background and presence of the maize homeobox gene, *knotted1*. *Development* 127: 3161–3172

Yamada H, Suzuki T, Terada K, Takei K, Ishikawa K, Miwa K, Yamashino T, Mizuno T (2001) The *Arabidopsis* AHK4 histidine kinase is a cytokinin-binding receptor that transduces cytokinin signals across the membrane. *Plant Cell Physiol* 42: 1017–1023

Yanai O, Shani E, Dolezal K, Tarkowski P, Sablowski R, Sandberg G, Samach A, Ori N (2005) *Arabidopsis* KNOXI proteins activate cytokinin biosynthesis. *Curr Biol* 15: 1566–1571