

OsHox4 regulates GA signaling by interacting with DELLA-like genes and GA oxidase genes in rice

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Abstract Gibberellic acid (GA) plays an essential role in many plant growth and developmental processes. Overexpression of rice (*Oryza sativa* L.) HOMEBOX4 (*OsHox4*) gene in rice variety IR64 under the control of CaMV 35s promoter caused varying degrees of dwarfism and bushy tillers. Further investigation showed that over-expression of *OsHox4* in indica rice induced semi-dwarf by repressing stems cell elongation. This repression could be eliminated by exogenously applied Gibberellin-4 (GA₄) suggested that *OsHox4* was involved in GA metabolism. Investigations of the expressions of rice DELLA-like subfamily genes, GA 3-oxidase family genes and GA 2-oxidase family genes by qRT-PCR showed that *OsHox4* played an important role in GA deactivation and signaling by controlling the expression of rice DELLA-like subfamily genes, rice GA 2-oxidase family genes and rice GA 3-oxidase

family genes. Our experiment provided an evidence that over-expression of *OsHox4* in transgenic rice resulted in a semi-dwarf phenotype that could be fully rescued by application of exogenous GA₄. The *OsHox4* plays an important role in GA deactivation by controlling the expression of rice DELLA subfamily genes, rice GA 2-oxidase family genes and rice GA 3-oxidase family genes.

Keywords *OsHox4* · GA signaling · Morphogenesis · Rice (*Oryza sativa* L.)

Introduction

Gibberellic acid (GA) plays an essential role in many plant growth and developmental processes, including seed germination, hypocotyl and stem elongation, leaf expansion, reproductive development, and circadian and light regulation (Olszewski et al. 2002; Sun and Gubler 2004; Fleet and Sun 2005). Over the past decade, biochemical and genetic studies have identified most of the genes in GA-biosynthetic pathway and GA-deactivation enzymes (Sakamoto et al. 2004; Thomas et al. 2005). Nevertheless, the mechanisms of GA signaling are still poorly understood.

Derepression of the repressed state is currently considered to be the key step of GA action in the GA signaling pathway. In this model, DELLA subfamily proteins of the GRAS superfamily play an important role in the negative control of GA signaling (Gomi and

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Matsuoka 2003; Hartweck 2008). GRAS proteins are a recently discovered family of plant-specific proteins named after GAI, RGA and SCR, the first three of its members isolated. They are important regulatory components in a number of different cellular processes ranging from meristem maintenance, signal transduction to hormone signaling (Bolle 2004). It has been suggested that GA-dependent degradation of DELLA proteins is a key event for GA-signaling (Ueguchi-Tanaka et al. 2007). DELLA protein is rapidly degraded by GA through ubiquitination is reported in several organism, such as rice, Arabidopsis and barley (Sasaki et al. 2003; Silverstone et al. 2001; Itoh et al. 2002). The disappearance of SmDELLA1 protein after GA₄ treatment is reported in *selaginella moellendorffii* also (Hirano et al. 2007). DELLA protein degradation causes derepression of the repression state of GA action. The processing of the GA signal in the nucleus depends directly on the presence or absence of DELLA proteins, which are therefore presently considered to be a ‘molecular switch’ for GA signaling (Gomi and Matsuoka 2003). The rice genome contains one DELLA protein ortholog, *Os-GAI/SLR1*. The *slr1* mutant is a recessive mutation and phenocopies a constitutive GA response (Ogawa et al. 2000; Ikeda et al. 2001). It has been postulated that DELLAs function as transcriptional repressors of genes involved in GA signaling (Olszewski et al. 2002). Taken together, bioactive GA promotes growth by turning on de-DELLA-repressing system. Conversely, if the bioactive GA is reduced, the degradation of DELLA protein will slow down or not at all, and the plant growth will be inhibited. This might be the reason why so many semi-dwarfs appear in the mutants where bioactive GA is reduced.

HD-Zip proteins constitute a large family of transcription factors and several lines of evidence have also shown that HD-Zip proteins play roles in regulating plant responses to hormones (Sawa et al. 2002; Johannesson et al. 2003; Rosin et al. 2003; Hay et al. 2004). Rice *OsHox4* is a drought-repressed HD-Zip family I gene act as activators and express predominantly in vascular (Agalou et al. 2008, Meijer et al. 2000). Loss-of-function analysis of *OsHox4* reveals no specific phenotype, but over-expression of *OsHox4* displayed a prolonged vegetative phase and reduced internodes elongation; the flowering time of *OsHox4* overexpression lines is variant from as normal as wild type to no flowering (Agalou et al. 2008).

The objectives of this research are: (1) to figure out the mechanism of semi-dwarf caused by *OsHox4* overexpression; (2) confirm the role of *OsHox4* in GA metabolism pathway; and (3) to further explore the GA signaling pathway in rice if it is possible. Our results showed that *OsHox4* involves in GA metabolism because the semi-dwarf phenotype in *OsHox4* transgenic rice could be fully eliminated by applied GA. A further research provided evidence that *OsHox4* regulate GA signaling and plant morphogenesis by interacting with DELLA-like subfamily genes and GA 2, 3-oxidasze family genes.

Materials and methods

Crop husbandry and sampling

Seeds of rice (*Oryza sativa* L. cv. IR64, IRTP A004) were obtained from the International Network for the Genetic Evaluation of Rice, International Rice Research Institute (IRRI), Philippines. Plants were grown individually in pots containing clay loam soil under glasshouse conditions with natural sun light and photoperiod. The tissue samples were frozen immediately in liquid nitrogen and stored at -80°C for future RNA extraction.

Rice transformation

The binary vector for over-expression was constructed based on pCAMIA-1301 (CAMBIA, Genbank accession number: AF234297) and pRTL2 (Mason et al. 1992). A *Hind*III fragment with a double CaMV 35S enhancer/promoter and the nopaline synthase gene terminator from pRTL2 was inserted into pCAMBIA-1301. The resulting vector was named as p1301DS. For the over-expression of *OsHox4*, the full-length cDNA was inserted into p1301DS digested with *Bam*HI and *Kpn*I. The PCR fragments were sequenced and inserted into pDS1301. The constructs were introduced into rice plants by *Agrobacterium tumefaciens* (strain EHA105)-mediated transformation following modified protocol of Hiei and Komari (2006).

Growth regulators treatments

An amount of 10 and 30 μM gibberellin acid (GA₄, Sigma) was sprayed to transgenic rice at heading stage

on 35 s::*OsHox4* transgenic plants. The treatments were repeated every 2 days for 3 times. The length of the internodes was measured 7 days after the beginning of the treatments. For RNA extraction and quantitative RT-PCR analysis, the flag leaves were collected 24 h after GA₄ spraying.

Light microscopy

2nd internodes (which are beneath peduncle) are used to compare the stem cell elongation. Stems were harvested from wild type, *OsHox4* transgenic plant and 7 days after GA₄ treatment *OsHox4* transgenic plants. The microtome sections (10 μm) were mounted on glass slides for imaging.

Statistical analysis

Analyses of variances (ANOVA) were made with the General Linear Model Procedure of SPSS statistical software version 16.0 (IBM SPSS Statistics 2007). ANOVA was used to determine the treatment effects on each variable and confidence intervals derived from these analysis used to establish significant effects. Correlation analysis and regression analysis was performed by SPSS statistical software version 16.0 (IBM SPSS Statistics 2007) also.

RNA isolation and real-time quantitative PCR analysis

Total RNA was extracted from flag leaves of Null (wild type IR64) and transgenic plants (IR64-*OsHox4*-014, IR64-*OsHox4*-027, IR64-*OsHox4*-022) 24 h after different concentration GA₄ applied using RNA extracting kit (TRIzol reagent, Invitrogen). 4 μg total RNA was pre-treated with RNase-free DNase I (Invitrogen), according to the manufacturer's instruction. RT reaction was performed with SuperScriptTM II reverse transcriptase (Invitrogen) following the manufacturer's instructions. Primers used for real-time PCR are listed in Supplementary 1. Real-time PCR was performed in an optical 96-well plate with an ABI PRISM[®] 7500 Real-time PCR System (Applied Biosystems) by using SYBR[®] Green to monitor dsDNA synthesis. All reactions contained 10 μl 2×SYBR[®] Green Master Mix Reagent (Applied Biosystems), 2.0 ng cDNA and 200nM of each gene-specific primer in a final volume of 20 μl. Thermal

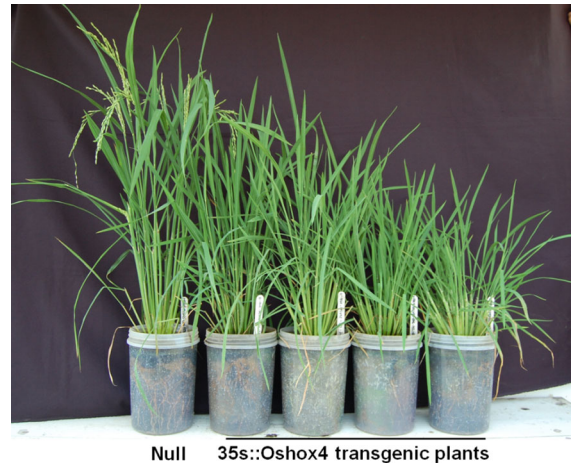


Fig. 1 Phenotype of Null (wild type) and transgenic lines. Overexpression of *OsHox4* under CaMV 35s promoter caused a reduced plant height and increased tiller number at different level compare to Null (wild type)

cycling was as follows: 50 °C for 2 min; 95 °C for 10 min; 50 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min. Relative expression level of reporter and target genes was determined based on the 2(-Delta Delta C(T)) method (Livak and Schmittgen 2001) by using rice GAPDH as internal control. Transcript levels from Null (wild type) were set at 1.

Result

Overexpression of *OsHox4* induced semi-dwarf plants along with increased tiller number

Rice HD-ZIP I family gene *OsHox4* was overexpressed in IR64 under CaMV 35s promoter, which induced shorter plant height than Null (wild type of IR64, as a control) with ranging from a very strong dwarf phenotype (with essentially no stem) to semi-dwarf or wild type phenotype (Figs. 1, 2). The average plant height of Null was 100.96 cm; the average height of three *OsHox4* overexpression lines, IR64-*OsHox4*-014, IR64-*OsHox4*-027 and IR64-*OsHox4*-022 was 48.667, 52.786 and 62.643 respectively (Table 1). ANOVA (Analyses of variances) analysis results showed that the plant height of three *OsHox4* overexpression transgenic lines, IR64-*OsHox4*-027, IR64-*OsHox4*-022 and IR64-*OsHox4*-014 were significantly shorter than Null (Table 1). The difference of plant

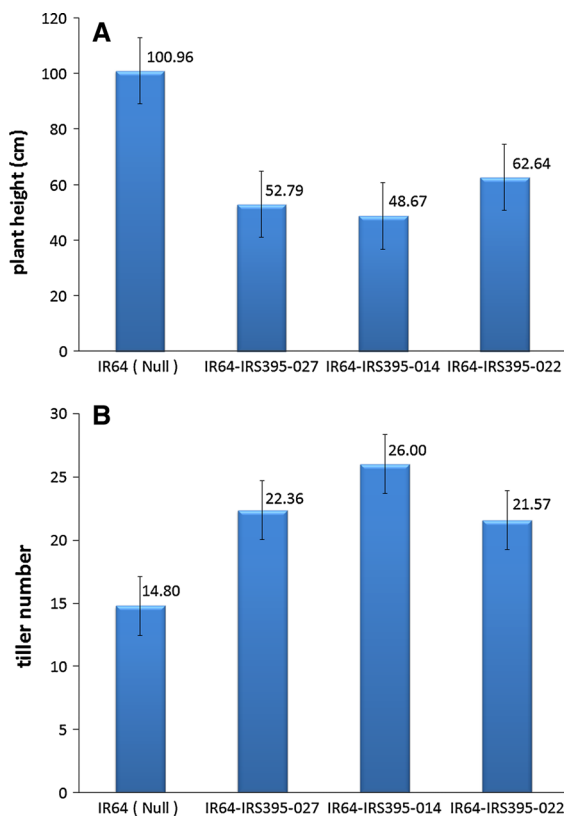


Fig. 2 Comparison of plant height and tiller number among Null (wild type) and *OsHox4* transgenic plants. Comparison of plant height between *OsHox4* transgenic plant and Null showed a repression of plant height in *OsHox4* overexpression plants (a); comparison of tiller number between *OsHox4* transgenic plant and Null (wild type) showed a increasing of tiller number in *OsHox4* overexpression plants (b). Data are mean \pm SD (standard error) ($n \geq 5$ biological repeats)

height between transgenic line IR64-*OsHox4*-027 and IR64-*OsHox4*-014 was not significant (Table 1; Fig. 2). The difference of plant height between transgenic line IR64-*OsHox4*-027 and IR64-*OsHox4*-022 was not significant also (Table 1; Fig. 2). The plant height of transgenic line IR64-*OsHox4*-014 was

significant shorter than transgenic line IR64-*OsHox4*-022, the P value < 0.0001 (Table 1; Fig. 2).

Compare to wild type, over expression of rice *OsHox4* gene induced more tillers also. The average tiller number of Null was 14.80; the average tiller number of three *OsHox4* overexpression lines, IR64-*OsHox4*-014, IR64-*OsHox4*-027 and IR64-*OsHox4*-022 was 26.00, 22.357 and 21.571 respectively. ANOVA analysis results showed that the tiller numbers in 3 single copy *OsHox4* overexpression transgenic lines, IR64-*OsHox4*-027, IR64-*OsHox4*-014 and IR64-*OsHox4*-022, were significantly higher than in wild type plants (Table 1; Fig. 2). The tiller numbers among the *OsHox4* overexpression transgenic lines were not significant (Table 1).

Exogenous GA₄ eliminates the semi-dwarf stature of *OsHox4* transgenic plant

In order to investigate the causing of *OsHox4* induced semi-dwarf and the role of *OsHox4* in plant development, 10 and 30 μ M exogenous GA₄ was applied on *OsHox4* transgenic rice at heading stage, the semi-dwarf phenotype which induced by the overexpression of *OsHox4* was reversed in different extent (Fig. 3).

Further investigations showed that the internodes of *OsHox4* transgenic rice plant increased differently after exogenous GA₄ applied. Compare to *OsHox4* overexpression transgenic plants without GA₄ treatment, 7 days after 30 μ M exogenous treatment at heading stage, the 1st internodes (peduncle) was 2 times the length as those without GA₄ treatment plants, which was the same as average peduncle length of Null; the average length of 2nd internodes (the internodes beneath peduncle) increased by 5 times long as those without GA₄ treated *OsHox4* overexpression transgenic plants, which was 2 times higher than Null. But the elongation of 3rd, 4th and 5th internodes was not significant faster than those in

Table 1 Statistics analysis of plant height and tiller numbers ($\alpha = 0.05$)

Source of Variation	Plant height (cm)				Tiller number			
	Mean	Std. Deviation	Std. Error	Duncan group	Mean	Std. Deviation	Std. Error	Duncan group
IR64- <i>OsHox4</i> -014	48.667	2.513	0.837	a	26.000	3.500	1.167	a
IR64- <i>OsHox4</i> -027	52.786	8.833	2.361	a	22.357	4.069	1.087	ab
IR64- <i>OsHox4</i> -022	62.643	6.083	2.298	b	21.571	3.645	1.378	b
IR64 (Null Control)	100.96	5.823	2.603	c	14.800	1.789	0.800	c



Fig. 3 Exogenous GA₄ eliminate the plant height repression of *OsHox4* overexpression plant. Comparison of *OsHox4*-overexpressing plants treated with GA₄ at different concentrations showed GA₄ could eliminate the plant height repression of *OsHox4* transgenic rice. *OsHox4* overexpressing plants were treated with water (control) (a), 10 µM exogenous GA₄ (b) and 30 µM exogenous GA₄ (c) showed different levels increase in plant height. This confirmed *OsHox4* involved in GA deactivation in *OsHox4* transgenic plant

OsHox4 overexpression transgenic plants without GA₄ treatment (Fig. 4). It showed GA₄ could increase internodes elongation only if they were at elongation stage. These results confirmed exogenous GA₄ could reverse the depression of stem elongation induced by *OsHox4* overexpression and the elongation degree of internodes depends on their development stage while exogenous GA₄ were applied.

A comparison of longitudinal section of 2nd internodes between Null and *OsHox4* overexpressing plant without GA₄ treatment showed overexpression of *OsHox4* reduced plant height by inhibiting stem cell elongation (Fig. 5); and comparison between *OsHox4* overexpression transgenic plants with 30 µM GA₄ treatment and without GA₄ treatment showed the exogenous GA₄ eliminated the semi-dwarf stature of *OsHox4* transgenic plant by increasing the stem cell elongation (Fig. 5). Therefore, over-expression of *OsHox4* induced semi-dwarf by repressing stems cell elongation and application of exogenous GA₄ could reverse dwarf phenomena by also eliminating this repression.

Expression analysis of rice GA 3-oxidase and GA 2-oxidase family genes

To investigate the molecular mechanism of *OsHox4* on GA metabolism and characterize the role of *OsHox4* in GA signaling, we checked the expression of 2 rice GA 3-oxidase family genes (*OsGA3ox1* and *OsGA3ox2*), and 9 rice GA 2-oxidase family genes (*OsGA2ox1*, 2, 3, 4, 5, 6, 7, 8 and 9) in flag leaves of Null and *OsHox4* overexpression transgenic plants which were treated by different concentration levels exogenous GA₄ after 24 h by qRT-PCR. Our qRT-PCR results showed, compare to Null, the expressions of two rice GA 3-oxidase family genes, *OsGA3ox1* and *OsGA3ox2*, were depressed in *OsHox4* overexpression transgenic plants (Fig. 6). The average relative expression of *OsGA3ox1* and *OsGA3ox2* were lower to 16 and 29 % of their expression in Null respectively (Fig. 6). 24 h after different 50 ml 10 µM GA₄ treatments, the expression of *OsGA3ox1* upregulated to almost the same level as in null and the expression of *OsGA3ox2* upregulated to 25 % higher than in Null (Fig. 6). After 30 µM GA₄ treatment, the relative expression of *OsGA3ox1* did not do change too much but the expression of *OsGA3ox2* in 30 µM GA₄ applied plant was lower to 45 % of its expression in 10 µM GA₄ applied plants (Fig. 6). It showed the *OsGA3ox2* was subjected to GA feedback.

In rice GA 2-oxidase family, the expression of *OsGA2ox4*, 5, 6, 8, 9 in *OsHox4* transgenic plant was differently lower than in Null, from 22 to 62 %. After 10 µM GA₄ treatment, their expressions were highly up-regulated in *OsHox4* transgenic plants. In 30 µM GA₄ treated plant, the expressions of *OsGA2ox3*, 4, 5, 8 and 9 were decreased (Fig. 7).

From our qRT-PCR analysis, the expressions of *OsGA3ox1*, *OsGA3ox2*, *OsGA2ox4*, 5, 6, 8 and 9 were down-regulated in *OsHox4* transgenic rice; 10 µM GA₄ treatment could induce their expression to a level higher than in Null, but 30 µM GA₄ treatment could inhibit their expression in some extent (except *OsGA2ox6*, its expression still up-regulated after 30 µM GA treatment, that showed 30 µM GA₄ treatment could not reach its responding threshold) (Figs. 6, 7). All of these showed the expressions of these genes were mainly controlled by active GA in vivo. Rice *OsHox4* could regulate their expression indirectly by active GA level in plant.

The expression of *OsGA2ox2* and *OsGA2ox3* was 2.7 times and 1.6 times higher in *OsHox4* overexpression

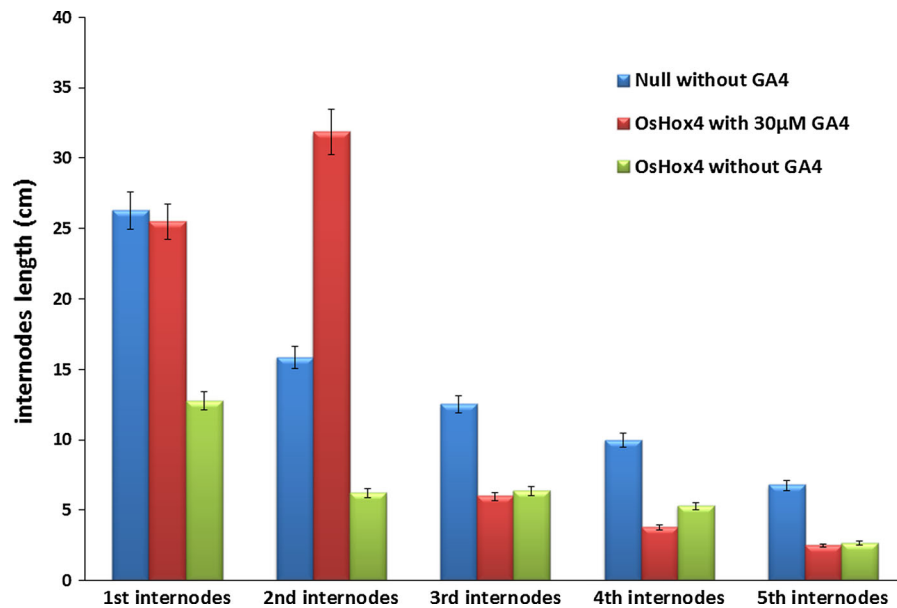


Fig. 4 Comparison of different internodes in Null (wild type) and *OsHox4* transgenic plant. Comparison of internodes length among Null without GA₄ treatment (blue column), *OsHox4* overexpression plants without GA₄ treatment (green column) and *OsHox4* overexpression plant 7 days after 30 µM GA₄ treatment (red column) showed the internodes elongation

increasing by exogenous GA₄ depending on plant growth stage. 1st internodes is peduncle, 2nd is the one beneath peduncle, so forth for 3rd, 4th, and 5th internodes. The average values were calculated from measures of at least ten plants ± SD (SE). (Color figure online)

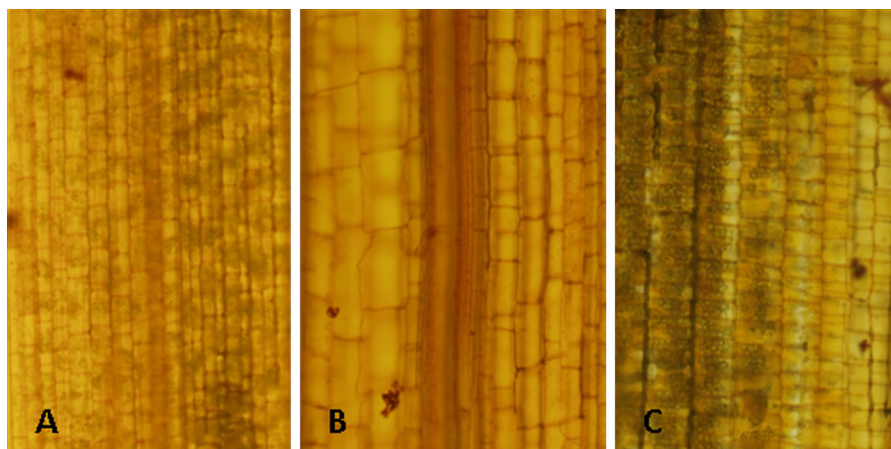


Fig. 5 Longitudinal section comparison of Null (wild type), *OsHox4* transgenic plant and GA₄ applied *OsHox4* transgenic plant. A comparison of longitudinal section of 2nd internodes beneath peduncle among Null (a), *OsHox4* overexpressing plant treated with 50 ml 30 µM exogenous GA₄ (b) and *OsHox4*

overexpressing plant without GA₄ treatment (c) showed overexpression of *OsHox4* reduced plant height by inhibiting stem cell elongation (c); application of exogenous GA₄ could eliminate this repression (b). Null without GA₄ treatment was used as control (a)

transgenic plants than in Null respectively and was highly upregulated in 10 µM GA₄ treated plants, 11 times and 42 times higher than in Null respectively. After 30 µM GA₄ treatment, the expression of *OsGA2ox3*

was down-regulated a little bit, but the expression *OsGA2ox2* was still up-regulated (Fig. 7). The expression analysis showed *OsGA2ox2* and *OsGA2ox3* were regulated by both of *OsHox4* and active GA.

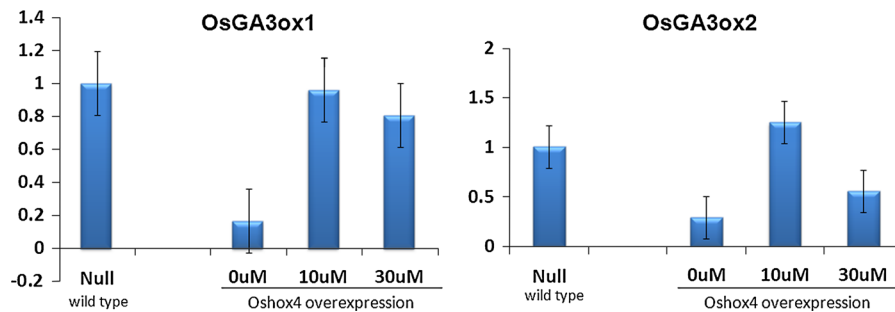


Fig. 6 Real-time PCR analysis of 2 rice GA 3-oxidase family genes. Transcripts of 2 rice GA 3-oxidase family genes, *OsGA3ox1* and *OsGA3ox2*, in Null (wild type) and three *OsHox4*-over-expressing lines (IR64-*OsHox4*-027, IR64-

OsHox4-022, IR64-*OsHox4*-017) were quantified by real time RT-PCR. cDNA samples were derived from flag leaves of Null and *OsHox4* transgenic rice plants in different GA treatments. Data are mean \pm SD (SE) ($n = 3$ biological repeats)

There was no expression of *OsGA2ox1* and *OsGA2ox7* in both of Null plants and *OsHox4* transgenic plants.

Expression analysis of rice DELLA protein subfamily genes

The expressions of 5 rice DELLA protein subfamily genes *OsGAI*, *OsSCR*, *OsSCRL*, *OsSCL14* and *OsRGA1* were investigated in Null and GA₄ applied transgenic plants. qRT-PCR results showed that the expression of rice DELLA protein gene *OsGAI* in *OsHox4* transgenic plants was as low as 51 % in Null but up-regulated to 1.8 times as its in Null after 50 ml 10 μ M GA₄ applied. In 30 μ M treated plants, the expression of *OsGAI* was down-regulated to lower than in 10 μ M GA₄ treated plants and Null (Fig. 8). It showed the expression of *OsGAI* was affected by active GA in plant. Compare to Null, the expression of *OsSCR* up-regulated in *OsHox4* overexpression transgenic plants and was further increased in 10 μ M GA₄ treated transgenic plant (Fig. 8). It showed both of *OsHox4* and exogenous GA₄ had a positive role in its expression. Comparing to wild type, the expression of *OsRGA1* and *OsSCRL* were down-regulated in *OsHox4* transgenic plants but up-regulated in *OsHox4* transgenic plants which were treated by 10 μ M GA₄ after 24 h. The expressions of all of these 4 genes (*OsGAI*, *OsSCR*, *OsSCRL* and *OsRGA1*) in 30 μ M GA₄ treated transgenic plant were lower than their expressions in 10 μ M GA₄ treated plants. It showed a GA feedback mechanism involved in their expression. Compare to Null, the expression of *OsSCL14* was down-regulated in *OsHox4* transgenic plant, application of GA₄ intensified this down-regulation (Fig. 8). It showed

Oshxo4 was a negative factor in expression of *OsSCL14*; GA₄ could not eliminate the depression of its expression. The molecular mechanism needs a further investigation.

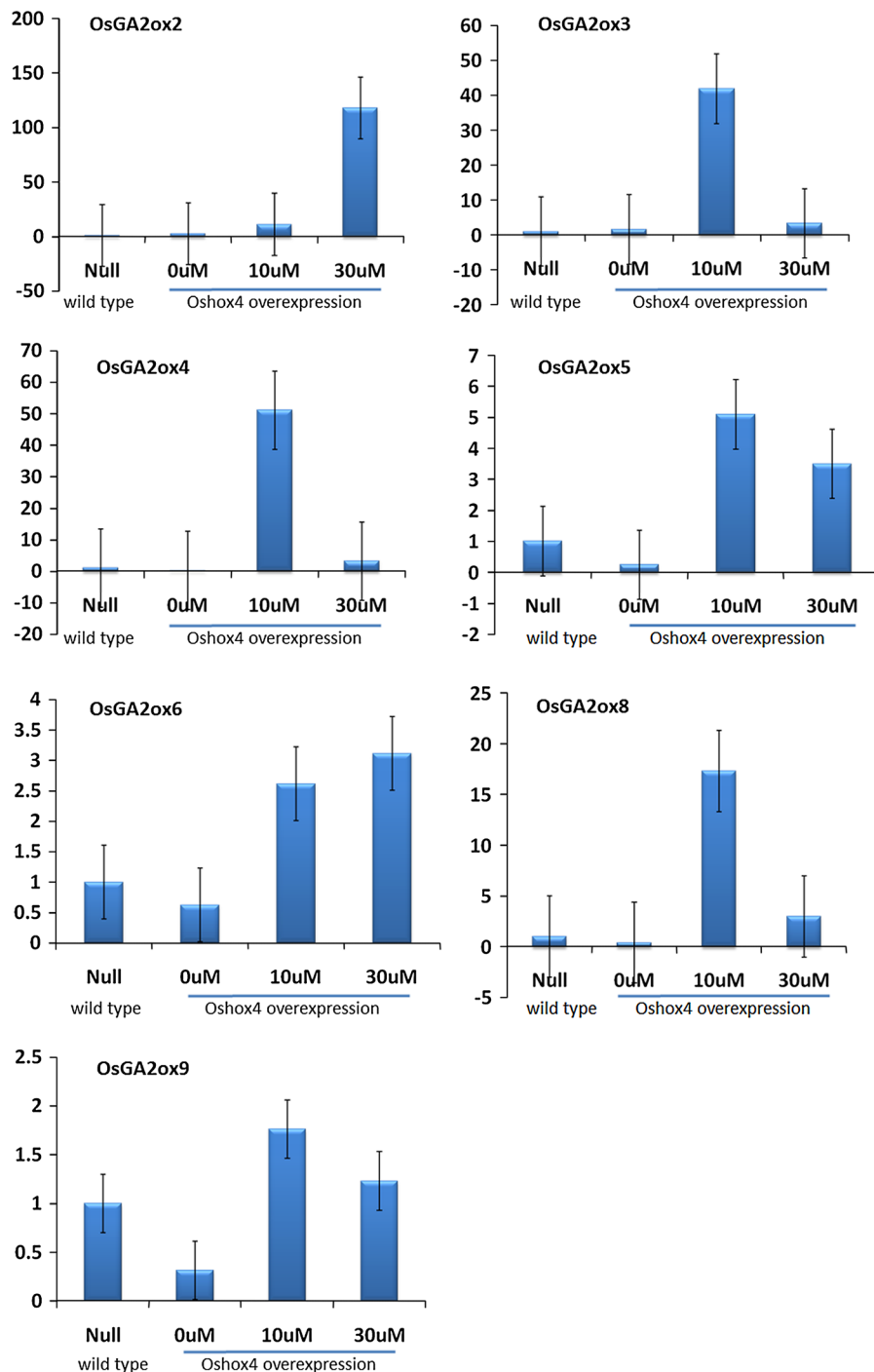
Discussion

Rice *OsHox4* gene regulate the GA metabolism and signaling

The semi-dwarf which is produced by *OsHox4* over-expression indicates *OsHox4* has a role of GA deactivation or/and GA signal response (Fig. 3). Expression analysis shows rice *OsHox4* gene involves in repression GA response by rice GRAS family genes and regulates the GA metabolism by GA 2-oxidase family and GA 3-oxidase family genes (Figs. 6, 7, 8). Our results confirmed that GA is deactivated in *OsHox4* transgenic plant because the exogenous GA₄ can eliminate the semidwarf statures which are caused by *OsHox4* over-expression (Fig. 3).

It is well know that rice GA3-oxidases are response for GA activation (Hartweck, 2008). From our expression analysis, the down-regulation of rice GA3-oxidase genes, *OsGA3ox1* and *OsGA3ox2*, in *OsHox4* transgenic plants implies the *OsHox4* deactivate GA by inhibiting the expression of OsGA3-oxidases (Fig. 6). Except for rice GA3-oxidase genes, the expression analysis result shows *OsGA2ox2* and *OsGA2ox3* are regulated by *OsHox4* also. The up-regulation of GA deactivation factors *OsGA2ox2* and *OsGA2ox3*, together with down regulation of GA biosynthesis factors *OsGA3ox1* and *OsGA3ox2*,

Fig. 7 Real-time PCR analysis of 7 rice GA 2-oxidase family genes. Transcripts of 7 GA 2-oxidase family genes in Null (wild type) and three *Oshox4*-over-expressing lines (IR64-*Oshox4*-027, IR64-*Oshox4*-022, IR64-*Oshox4*-006) were quantified by real time RT-PCR. cDNA samples were derived from flag leaves of Null and *Oshox4* transgenic rice plants in different GA treatments. Data are mean \pm SD (SE) ($n = 3$ biological repeats)

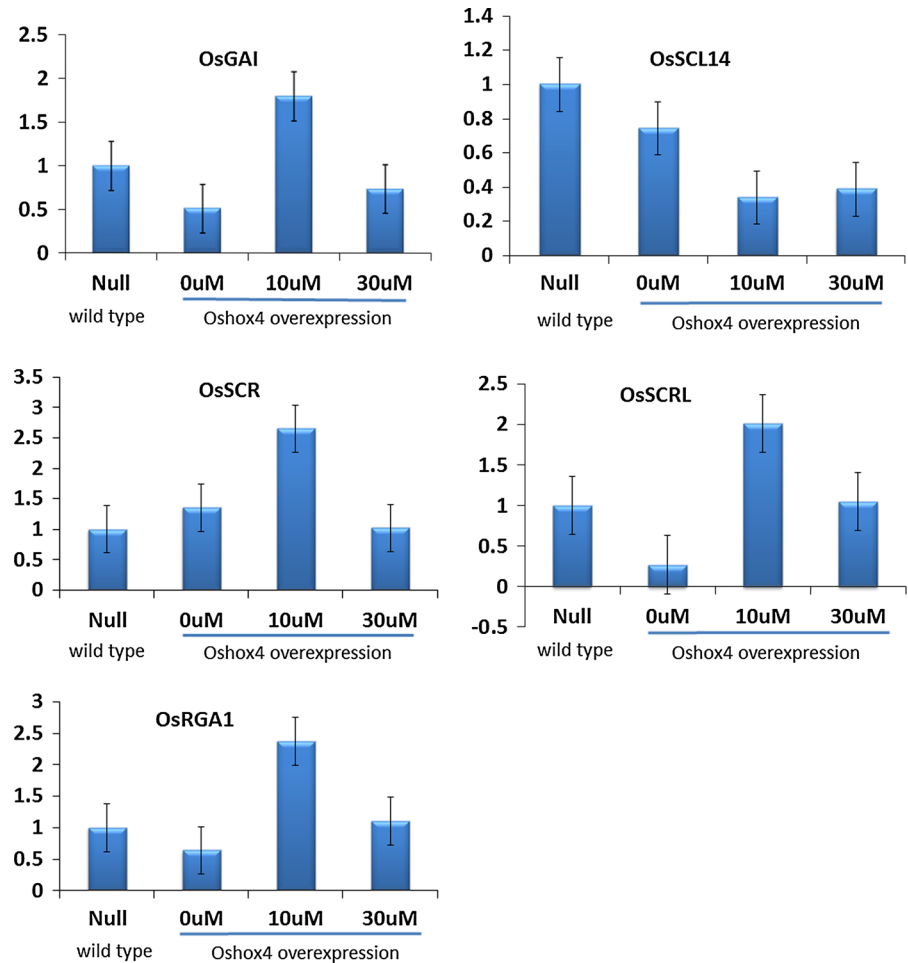


implies *Oshox4* controls GA metabolism at biosynthesis and deactivation two directions.

It has been suggested that GA-dependent degradation of DELLA proteins is a key event for GA-signaling (Ueguchi-Tanaka et al. 2007). The

processing of the GA signal in the nucleus depends directly on the presence or absence of DELLA proteins, which are therefore presently considered to be a ‘molecular switch’ for GA signaling (Gomi and Matsuoka 2003). But the direct targeted upstream and

Fig. 8 Real-time PCR analysis of 5 rice DELLA protein subfamily genes. Transcripts of 5 rice DELLA protein subfamily genes in Null (wild type) and three *OsHox4*-over-expressing lines (IR64-*OsHox4*-027, IR64-*OsHox4*-022, IR64-*OsHox4*-006) were quantified by real time RT-PCR. cDNA samples were derived from flag leaves of Null and *OsHox4* transgenic rice plants in different GA treatments. Data are mean \pm SD (SE) ($n = 3$ biological repeats)



downstream components of DELLA proteins are still unknown. Promoter analysis based on PLACE database which is described by Higo et al. (1999) showed that except the *OsGA3ox2*, all of the genes analyzed in this paper have homeodomain binding sites (Supplementary 2). But only the expressions of *OsGA2ox2* and *OsGA2ox3* are upregulated in *OsHox4* transgenic plant (Figs. 6, 7, 8), it implies *OsHox4* directly/indirectly regulates a complex GA signaling network. Comparing to application of 10 μ M GA₄, the expressions of most genes are lower after 30 μ M GA₄ applied (Figs. 6, 7, 8), which hints a GA feed-back mechanism in controlling these genes at transcription level.

Directly/indirectly regulating the expression of rice GA3 oxidase family, GA2 oxidase family and GRAS family genes implies the complex role of *OsHox4* in rice development. An extended biological and biochemical

analysis will help to find a common mechanism of function, if it exists. Determining the specific roles of these proteins in response to developmental and environmental signals, as well as their cross-talk with other pathways, is an important future task.

Dai et al. (2008) report that the semidwarf induced by over-expression of *OsHox4* could not be complemented by applied GA₃ at seedling stage. Our result showed clearly that exogenous GA₄ could reverse the semidwarf phenotype at different stem elongation stages (Figs. 3, 4, 5). It confirms our conclusion that the exogenous GA₄ only works on the cells which are at elongation stage. All of these hints GA₄ alone is not enough to increase cell elongation, it should work together with other factors which started the internodes cells elongation and reverse the semidwarf.

They also observed that the over-expression plants accumulated elevated levels of bioactive GA, while

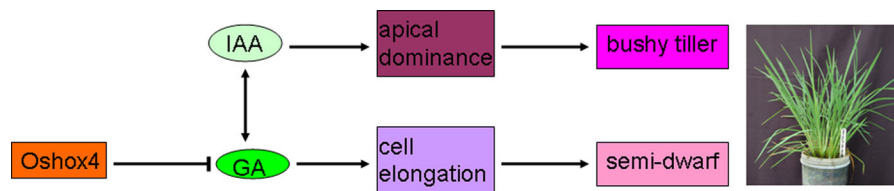


Fig. 9 Illustration of GA and IAA in controlling plant height and tillers. Schematic representation of the interaction of GA and IAA metabolic pathway showed how plant morphogenesis was controlled

the GA catabolic gene *OsGA2ox3* was upregulated in the transgenic plants (Dai et al. 2008). In some extent, the upregulation of *OsGA2ox3* is contradicted with the elevation of bioactive GA in over-expression plants. Our results showed the *OsHox4* control GA signaling in two directions, one was disturbing the bioactive GA metabolism by controlling the expression of GA 2-oxidase and GA 3-oxidase genes; another was disturbing the GA signaling through DELLA subfamily genes. The accumulation of bioactive GA in *OsHox4* transgenic plants implies the *OsHox4* mainly control GA activity by involving in GA signaling pathway. The related mechanism needs to be confirmed and unraveled by a further investigation.

Rice *OsHox4* involves in rice morphogenesis by hormones coordination (372 words)

Dwarfism is one of the most valuable traits in crop breeding because semi-dwarf cultivars are more resistant to damage by wind and rain (lodging resistant) and are associated with stable increased yields (Evans 1993). Overexpression of *OsHox4* can not only reduce the plant height but can increase the tiller number at same time (Fig. 1; Table 1). Further investigation shows exogenous GA₄ can eliminate the semi-dwarf stature of *OsHox4* transgenic plant that means the GA metabolism is deactivated in *OsHox4* transgenic plant.

It has been long known that there is cross-talking between GA action and other hormones signaling to control the plant growth and development, but the mechanism remains unclear. It is reported that auxin can positively affect GA biosynthesis also, and GA signaling through DELLA proteins is mediated by auxin (Fu and Harberd 2003). Over 95 % of the transcriptional changes associated with GA application were included among the changes induced by IAA application in populus and arabidopsis (Covington and

Harmer 2007, Bjorklund et al. 2007). IR64 *OsHox4* transgenic plant has more tillers and short stature probably because the IAA which is repressed together with GA. Since the GA can positively affect auxin biosynthesis (Nemhauser et al. 2006; Desgagne-Penix and Sponsel 2008), the active GA is depressed in *OsHox4* transgenic rice which causes a lower level of IAA in *OsHox4* transgenic plant than in wild type plant. The lower level of active GA is the reason of semi-dwarf in *OsHox4* transgenic plant; and the lower level of IAA is the reason of multiple tillers in *OsHox4* transgenic plant because the elimination of apical dominance when IAA level is low. Fig. 9 is an illustration of discussion in above.

Conclusion

HD-ZIP family gene *OsHox4* is over-expressed in IR64 under the control of CaMV 35 s promoter; 10uM and 30uM GA₄ application result showed that the exogenous GA can rescue the semi-dwarf caused by *OsHox4* over-expression; internodes section result showed that the cell length was increased after GA spraying. The expression of rice DELLA protein subfamily genes, rice GA 3-oxidase family genes and rice GA 2-oxidase family genes were checked by qRT-PCR; our result showed *OsHox4* impaired the biosynthesis of active GA by down-regulating rice GA 3-oxidase family genes and deactivate GA by upregulating rice GA 2-oxidase family genes. *OsHox4* also involves in repression GA response by rice GRAS family genes.

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Conflict of interest Authors declared that they have no conflict of interests.

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