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Overexpression of a Type-A Response Regulator Alters Rice Morphology and Cytokinin Metabolism

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Genome-wide analyses of rice (*Oryza sativa* L.) cytokinin (CK)-responsive genes using the Affymetrix GeneChip® rice genome array were conducted to define the spectrum of genes subject to regulation by CK in monocotyledonous plants. Application of *trans*-zeatin modulated the expression of a wide variety of genes including those involved in hormone signaling and metabolism, transcriptional regulation, macronutrient transport and protein synthesis. To understand further the function of CK in rice plants, we examined the effects of in planta manipulation of a putative CK signaling factor on morphology, CK metabolism and expression of CK-responsive genes. Overexpression of the CK-inducible type-A response regulator *OsRR6* abolished shoot regeneration, suggesting that OsRR6 acts as a negative regulator of CK signaling. Transgenic lines overexpressing *OsRR6* (OsRR6-ox) had dwarf phenotypes with poorly developed root systems and panicles. Increased content of *trans*-zeatin-type CKs in OsRR6-ox lines indicates that homeostatic control of CK levels is regulated by OsRR6 signaling. Expression of genes encoding CK oxidase/dehydrogenase decreased in OsRR6-ox plants, possibly accounting for elevated CK levels in transgenic lines. Expression of a number of stress response genes was also altered in OsRR6-ox plants.

Keywords: Cytokinin — Gene chip — Microarray — *Oryza sativa* — Response regulator.

Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; AMT, ammonium transporter; AP2/EREBP, APETALA2/ethylene-responsive element binding protein; ARF, auxin response factor; AUX/IAA, auxin/indole-3-acetic acid; bHLH, basic helix–loop–helix; CaMV, cauliflower mosaic virus; CK, cytokinin; CKX, CK oxidase/dehydrogenase; DMSO, dimethylsulfoxide; GA, gibberellin; GFP, green fluorescent protein; HK, His protein kinase; HP, His-containing phosphotransfer protein; IPT, adenosine phosphate-isopentenyltransferase; PHT, phosphate transporter; PUP, purine permease; RR, response regulator; RT–PCR, reverse transcription–PCR; SULTR, sulfate transporter; tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5′-monophosphate.

Nucleotide sequence data from this article have been deposited in the DDBJ/EMBL/GenBank database under accession numbers AB249653 (*OsRR6*), AB249654 (*OsRR5*), AB249655 (*OsRR3*), AB249656 (*OsRR11*), AB249657 (*OsRR7*), AB249658 (*OsRR4*), AB249659 (*OsRR9*), AB249660 (*OsRR10*), AB249661 (*OsRR1*), AB249662 (*OsRR2*), AB249663 (*OsRR8*), AB249664 (*OsRR12*) and AB249665 (*OsRR13*). Microarray data have been deposited in The National Center for Biotechnology Information Gene Expression Omnibus (NCBI GEO) database under accession number GSE6737.

Introduction

Cytokinins (CKs) are a class of plant hormones that regulate many aspects of growth and development, including cell division, apical dominance, leaf senescence, nutrient signaling and shoot differentiation (Mok 1994, Sakakibara 2006). In the past decade, substantial progress has been made in understanding CK biosynthesis, metabolism and signal transduction. Much of this knowledge is based on research in *Arabidopsis*, a dicotyledonous model plant. This has been achieved by identifying a series of genes encoding key enzymes, including CK-synthesizing adenosine phosphate-isopentenyltransferase (*IPT*), CK-degrading CK oxidase/dehydrogenase (*CKX*) and CK-inactivating CK glycosyltransferase (for reviews, see Mok and Mok 2001, Sakakibara 2006, Werner et al. 2006).

The current model of the CK signaling pathway is a multistep His–Asp phosphorelay system comprised of a sensor His protein kinase (HK), His-containing phosphotransfer protein (HP) and response regulator (RR; for recent reviews, see Aoyama and Oka 2003, Heyl and Schmülling 2003, Kakimoto 2003, Grefen and Harter 2004, Mizuno 2004, Ferreira and Kieber 2005, Hwang and Sakakibara 2006, and references therein). Early CK signaling events have been characterized primarily in *Arabidopsis*, in which three genes encoding HKs that function as CK receptors (*AHK2*, *AHK3* and *AHK4*/*CRE1*/*WOL*; Inoue et al. 2001, Suzuki et al. 2001, Ueguchi et al. 2001, Yamada et al. 2001), five encoding HPs (*AHP1–AHP5*; Miyata et al. 1998, Suzuki et al. 1998) and an analog (*AHP6*; Mähönen et al. 2006), and 23 encoding RRs (*ARR1–ARR22* and *ARR24*; Brandstatter and Kieber 1998, Imamura et al. 1998, Sakai et al. 1998, Imamura et al. 1999, D'Agostino et al. 2000, Mason et al. 2004, Mizuno 2004) have been identified. The ARRs have been generally classified into type-A or type-B based on structural features (Imamura et al. 1999, Mizuno 2004). Type-A ARRs contain a receiver domain with a short C-terminal extension, while type-B ARRs have a receiver domain and a long C-terminal extension that contains transcription-regulating domains

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serving as transcriptional regulators for certain target genes, including the type-A *ARR* genes (Sakai et al. 2000, Sakai et al. 2001, Mason et al. 2005, Yokoyama et al. 2007). Expression is rapidly induced in response to exogenous CK in the type-A *ARR* genes but not in the type-B genes (Brandstatter and Kieber 1998, Taniguchi et al. 1998, Imamura et al. 1999, Kiba et al. 1999, D'Agostino et al. 2000). Overexpression of type-A *ARR* genes (*ARR4*, *ARR5*, *ARR6* and *ARR7*) in *Arabidopsis* protoplasts represses the ability of CK to induce *ARR6* promoter activity, indicating that type-A ARRs are transcriptional repressors (Hwang and Sheen 2001). Overexpression of *ARR8* represses shoot regeneration and greening of calli, suggesting that ARR8 plays a role as a negative regulator in CK signaling (Osakabe et al. 2002). Overexpression of *ARR15* in *Arabidopsis* plants resulted in reduced sensitivity to CK (Kiba et al. 2003). Analyses of multiple insertional mutants of type-A *ARR* genes suggested that type-A ARRs perform partially redundant functions, such as negative regulation of CK signaling (To et al. 2004). Recent genome-wide expression profiling indicates that ARR7 is a negative regulator of early CK-regulated genes and acts as a transcriptional repressor (Lee et al. 2007). In contrast, overexpression of *ARR4* in the cultured stems of transgenic plants promotes shoot regeneration in the presence of CKs, suggesting that ARR4 is a positive regulator in CK signaling (Osakabe et al. 2002). However, this positive effect remains uncertain, as a loss-of-function analysis did not support it (To et al. 2004).

Type-A *RR* genes have also been isolated and characterized from the monocots maize (Sakakibara et al. 1998, Sakakibara et al. 1999, Asakura et al. 2003, Giulini et al. 2004) and rice (Ito and Kurata. 2006, Jain et al. 2006). Treating detached maize tissues with CK induces the accumulation of transcripts of type-A *RR* genes (*ZmRR1*–*ZmRR7*; Sakakibara et al. 1998, Sakakibara et al. 1999, Asakura et al. 2003, Giulini et al. 2004). A single loss-of-function mutation in a type-A *RR* (*ZmRR3*/*ABPH1*) in maize alters leaf phyllotaxy, which is probably the result of enlargement of the shoot apical meristem (Giulini et al. 2004). Thirteen putative type-A *RR* genes in rice (*OsRR*) were recently reported to be expressed in an organ-specific manner, and also in response to light conditions (Ito and Kurata 2006, Jain et al. 2006). Application of CK to rice seedlings induced the expression of most of the *OsRRs*, and *OsRR6* expression was also enhanced in response to salinity, dehydration and low temperature stress (Jain et al. 2006). Although the isolation of *RR* genes involved in CK signaling has been reported in the Gramineae, knowledge of the in planta role of type-A RRs in monocotyledonous species is very limited compared with *Arabidopsis*.

The search for genes whose expression is modified by CK has yielded a number of valuable tools that have been used to understand CK signaling and the complex developmental processes under control of this hormone. Genome-wide analyses have identified a variety of CK-responsive genes in *Arabidopsis* (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003, Kiba et al. 2004, Brenner et al. 2005, Kiba et al. 2005, Lee et al. 2007). Completion of the rice genome sequence has made the comprehensive identification of CK-responsive genes in this model monocot plant possible. In this study, we identified rice genes regulated by CK using an Affymetrix rice genome array and examined transgenic rice plants that overexpress *OsRR6* (referred to as OsRR6-ox). Our study showed that OsRR6-ox plants displayed altered morphologies and changes in CK metabolism, probably due to changes in the gene regulatory network.

Results and Discussion

Identification of CK-regulated genes in roots and leaves

One aspect of understanding the action of a regulatory signal depends on knowing which genes are induced or repressed in response to it. To gain insight into the mode of action of CK in rice seedlings, excised whole roots and detached leaf blades were treated with 5 μM *trans*-zeatin (tZ) for 30 or 120 min. Total RNA from these organs was subjected to microarray analysis using an Affymetrix GeneChip® rice genome array, which contains probes to query approximately 48,564 *japonica* and 1,269 *indica* transcripts. Three independent replicate treatments were performed for each organ per time point. To increase the chance of detecting differentially expressed genes, transcripts with a ratio of change >2.0 and an Affymetrix flag call with a 'present' classification for the higher expression value were classified as regulated.

A total of 677 root and 641 leaf genes met the criteria for up-regulation, and 591 root and 962 leaf genes met the criteria for down-regulation at both the 30 and 120 min time points after CK treatment (Fig. 1A and Supplementary Table S1). The up-regulated genes included three type-A *OsRR* genes (Table 1). The up-regulation of *OsRR4* and *OsRR6* was confirmed by semi-quantitative reverse transcription–PCR (RT–PCR) using the same RNA samples used in the microarray analyses (Fig. 1B). Induction of these genes confirms a previous report (Jain et al. 2006) and validates the experimental system used in this study. Jain et al. (2006) showed that rice has at least seven *RR* genes that are induced by CK, yet this study found only three. This discrepancy might be due to the expression of *RR* genes below detectable levels in the tissues used in this study. Although probes for all *RR* genes are present in the gene chip used, the fact that flag calls for seven *RR* genes other than *OsRR2*, *OsRR4* and *OsRR6* are 'absent' supports this possibility (Supplementary Table S2).

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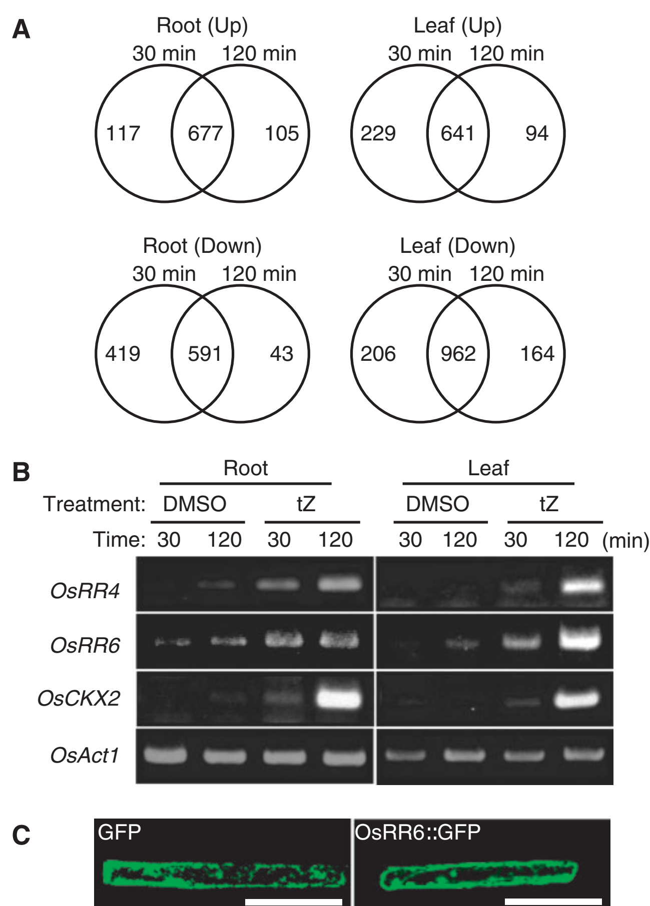

Fig. 1 Identification of cytokinin-responsive genes in rice. (A) Venn diagrams of CK-regulated genes at 30 and 120 min in roots and leaves. The number in each area denotes the number of genes in the set that showed more than a 2-fold change (>2-fold increase [Up] or <0.5-fold decrease [Down]). (B) Semi-quantitative RT–PCR analysis of the type-A response regulator genes *OsRR4* and *OsRR6*. The same RNA samples used for micro-array analysis were subjected to PCR. 'DMSO' indicates solvent-only control. *OsAct1*, encoding rice actin1 (McElroy et al. 1990), is used as an extraction and loading control. (C) Intracellular localization of OsRR6. Constructs with either GFP alone or OsRR6::GFP were introduced into epidermal cells of rice leaf sheaths by particle bombardment. After overnight incubation, the cells were observed using a confocal laser-scanning microscope. Bar = 50 μm.

A large proportion of the genes identified in this study as CK regulated were also reported as CK responsive in *Arabidopsis* (Che et al. 2002, Hoth et al. 2003, Rashotte et al. 2003, Kiba et al. 2004, Brenner et al. 2005, Kiba et al. 2005).

Hormone metabolism and signaling

Most of the identified CK-responsive genes associated with hormone metabolism and signaling were related to CK itself, ethylene, gibberellin or auxin (Table 1 and Supplementary Table S1), as is the case with *Arabidopsis* (Brenner et al. 2005). Seven of the eight genes for the precursor enzyme IPT (*OsIPT1–OsIPT8*) are present on the gene chip used; *OsIPT2* is missing (Supplementary Table S2). These seven *IPT* genes were weakly expressed and flag calls for only two or three of them were 'present' (Supplementary Table S2). No significant CK response from *IPT* genes was observed under our experimental conditions, whereas a longer period of CK application repressed the accumulation of transcripts of some *OsIPTs* (Sakamoto et al. 2006). On first inspection, *OsIPT4* appears to be induced by tZ (Supplementary Table S2). However, RT–PCR assays could not confirm its induction. This discrepancy may be due to low expression of this gene (see Supplementary Table S2, raw values). Moreover, expression levels of two genes encoding putative CK *trans*-hydroxylases were also low, making it difficult to evaluate their CK responsiveness (Supplementary Table S2). Up-regulation of *OsCKX11* expression was observed with CK application (Table 1 and Supplementary Table S1), which is consistent with a subset of *Arabidopsis CKX* genes (*AtCKX3*, *AtCKX4*, *AtCKX5* and *AtCKX6*; Hoth et al. 2003, Rashotte et al. 2003, Zimmermann et al. 2004, Brenner et al. 2005, Kiba et al. 2005, Werner et al. 2006) and a maize *CKX* (*Ckx1*; Brugière et al. 2003). The expression of *OsCKX2*, which is relevant to the regulation of rice grain production (Ashikari et al. 2005), was markedly induced by tZ, although expression of this gene did not meet the criteria for microarray analyses (see Materials and Methods, Fig. 1B and Supplementary Table S2). Transcript abundance of a putative gene encoding CK *O*-glucosyltransferase (accession No. AK068176) also increased in response to tZ application. Cytokinin *O*-glucosyltransferase catalyzes the inactivation of zeatin-type CKs by *O*-glucosylation. Up-regulation of these putative CK-inactivating genes could contribute to homeostatic control of CK activity. Two (accession Nos. AK072660 and AK071680) and three (AK119483, AK060241 and AK071680) *purine permease* (*PUP*) genes were up-regulated by tZ in root and leaf tissues, respectively (Supplementary Table S1). On the other hand, accumulation of transcripts of one *PUP* gene (accession No. AK059161) was down-regulated in response to tZ in both organs (Supplementary Table S1). In *Arabidopsis*, some members of the PUP family may be involved in CK transport (Bürkle et al. 2003). Transcript abundance of *PUP4* was down-regulated by CK (Brenner et al. 2005). In this context, CK-responsive rice PUP might play a defined role in CK transport. Genes encoding putative *HK*, *HP* and type-B *RR* were not CK responsive (Supplementary Table S2), while *AHK4*/*CRE1*/*WOL* was up-regulated by CK (Rashotte et al. 2003, Kiba et al. 2004, Kiba et al. 2005).

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Table 1 Hormone-related genes regulated by cytokinin in rice

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change (30 min, 120 min)[a]		Fold change (OsRR6-ox/control)
				Root	Leaf	
			Cytokinin signaling			
Os.23942.1.A1_at	AK070645	LOC_Os02g35180	Type-A response regulator (OsRR2)	–	U (1.15, 3.52)	0.645
Os.15908.1.S1_s_at	AK101721	LOC_Os01g72330	Type-A response regulator (OsRR4)	U (3.63, 2.65)	U (1.13, 5.22)	0.929
Os.24952.1.S1_at	AK059734	LOC_Os04g57720	Type-A response regulator (OsRR6)	U (2.03, 1.01)	U (1.72, 2.36)	0.744
			Cytokinin metabolism			
Os.27224.2.A1_at	AK103510	LOC_Os08g35860	Cytokinin oxidase/dehydrogenase (OsCKX11)	U (4.79, 3.85)	U (5.77, 6.09)	1.26
Os.51127.1.S1_a_at	AK068176	LOC_Os04g37820	Cytokinin-*O*-glucosyltransferase-like protein	–	U (5.96, 6.74)	0.664
			Auxin signaling			
Os.7203.1.S1_at	AK100337	LOC_Os01g63230	Axi1 (auxin-independent growth promoter)-like protein	D (0.345, 0.404)	D (0.383, 0.329)	1.04
Os.11585.1.S1_at	AK100287	LOC_Os09g27080	Axi1 (auxin-independent growth promoter)-like protein	D (0.232, 0.306)	D (0.349, 0.357)	0.651
Os.39652.1.S1_at	CB667155	LOC_Os09g37330	Auxin-induced protein-related-like protein	U (6.27, 3.28)	U (7.75, 3.17)	4.53
OsAffx.17454.1.S1_s_at	9636.m04191	LOC_Os08g41290	Auxin-induced protein-related-like protein	–	U (12.6, 23.3)	1.23
Os.45885.1.S1_s_at	AU029622	LOC_Os01g43090	Auxin-induced protein-related-like protein	–	D (0.384, 0.36)	0.834
Os.49711.1.S1_at	AK106714	LOC_Os08g35110	Auxin-induced protein-related-like protein	–	D (0.195, 0.195)	2.18
Os.8977.1.S1_at	AK107043	LOC_Os06g50040	Auxin-responsive family protein Auxin transport	–	U (2.46, 1.65)	1.6
Os.6258.1.S1_a_at	AK103981	LOC_Os01g63770	Aux1-like permease	–	D (0.506, 0.492)	0.72
Os.19608.1.S1_at	AK070682	LOC_Os09g38130	Auxin efflux carrier family protein	–	U (2.7, 1.26)	2.21
Os.37330.1.S1_at	AK059229	LOC_Os01g58860	Auxin efflux carrier family protein	U (5.05, 4.72)	–	0.731[b]
Os.23143.1.S1_at	AK073345	LOC_Os09g31478	Auxin efflux carrier family protein	U (2.68, 2.38)	–	1.38
Os.38376.1.S1_s_at	AK069848	LOC_Os01g60230	Auxin efflux carrier family protein Auxin metabolism	D (0.395, 0.658)	–	0.807
Os.11353.1.S1_at	AY187620	LOC_Os01g37960	IAA-amino acid hydrolase homolog	U (4.25, 2.95)	U (2.28, 1.67)	1.2
Os.17898.1.S1_at	AK069855	LOC_Os02g42330	Bitrilase	–	D (0.422, 0.375)	1.22
			Ethylene signaling			
Os.8653.1.S1_at	CB656998	LOC_Os02g32610	CTR1-like protein kinase	U (2.69, 2.77)	U (7.36, 6.35)	1.34
Os.14934.1.S1_at	AY136816	LOC_Os04g08740	Ethylene receptor-like protein (ETR2)	U (3.78, 3.79)	U (4.02, 2.96)	0.896
Os.8423.1.S1_a_at	AY434735	LOC_Os02g57530	Ethylene receptor-like protein (ETR3)	D (0.322, 0.429)	–	0.737
Os.6117.1.S1_at	AK070793	LOC_Os11g37520	ETO1-like protein 1 (ethylene overproducer 1-like protein 1)	D (0.379, 0.571)	D (0.406, 0.369)	0.804
Os.10423.1.S1_s_at	AB074972	LOC_Os07g48630	Ethylene-insensitive-3-like protein (OsEIL2)	–	D (0.493, 0.64)	0.485

(continued)

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Table 1 Continued

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change (30 min, 120 min)[a]		Fold change (OsRR6-ox/ control)
				Root	Leaf	
			Ethylene metabolism			
Os.12268.1.S1_at	AF049889	LOC_Os02g53180	1-Aminocyclopropane-1-carboxylate oxidase (ACO2)	U (1.17, 2.1)	–	0.789
Os.37617.1.S1_at	AK102472	LOC_Os01g39860	1-Aminocyclopropane-1-carboxylate oxidase	–	U (15.1, 35.2)	1.95
Os.6034.1.S1_at	AU173534	LOC_Os01g09700	1-Aminocyclopropane-1-carboxylate synthase (OS-ACS5)	U (11.2, 9.41)	U (33.7, 98.6)	3.94[b]
OsAffx.14625.1.S1_x_at	9633.m00941	LOC_Os05g10780	1-Aminocyclopropane-1-carboxylate synthase	U (3.15, 1.99)	–	1.62[b]
			Gibberellin signaling			
Os.4149.2.S1_x_at	AK062249	LOC_Os03g50810	Gibberellin-induced leucine-rich repeat receptor-like protein kinase (OsTMK)	D (0.382, 0.393)	D (0.095, 0.103)	1.22
Os.48105.1.A1_at	CR287495	LOC_Os06g15620	Gibberellin-stimulated protein	D (0.498, 0.293)	–	1.23
OsAffx.24166.1.S1_at	AY676927	LOC_Os02g08440	WRKY family transcription factor (OsWRKY71)	D (0.444, 0.738)	D (0.375, 0.292)	0.412
			Gibberellin metabolism			
OsAffx.13147.1.S1_s_at	AK101758	LOC_Os05g48700	GA2-oxidase	U (35.1, 36.5)	–	4.1[b]
Os.27439.2.S1_at	CB660961	LOC_Os05g34854	GA20-oxidase (OsGA20ox4)	–	U (4.86, 3.55)	1.53
Os.21634.1.S1_at	AK101713	LOC_Os01g55240	GA2-oxidase (OsGA2ox3)	–	D (0.171, 0.383)	0.344

[a] Transcripts with a ratio of induction (plus or minus cytokinin) of >2.0 at 30 or 120 min after cytokinin application are listed as 'up-regulated (U)' and <0.5 are listed as 'down-regulated (D)'. Genes which do not meet the criteria are marked as –.
[b] Genes whose expression levels are below 150 and flag calls are 'absent'. Annotation was derived from the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/).

Inhibition of hypocotyl elongation by CK in etiolated *Arabidopsis* seedlings is due to CK-induced ethylene production (Cary et al. 1995), suggesting a connection between CK and ethylene action. Post-transcriptional stabilization of at least two 1-aminocyclopropane-1-carboxylate (ACC) synthase enzymes (ACS5 and ACS9) contributes to CK-induced ethylene biosynthesis (Vogel et al. 1998, Chae et al. 2003). The *ACS5* gene is up-regulated at the transcriptional level by application of CK (Vogel et al. 1998). Although the mode of interaction between CK and ethylene in rice remains unknown, the abundance of transcripts for the ethylene synthesis pathway enzymes ACC oxidase and ACC synthase increased in response to CK in both roots and leaves (Table 1 and Supplementary S1). Moreover, several genes involved in ethylene signaling were also modulated, including two putative ethylene receptors, CTR-like protein kinase, ETO1-like protein 1 and ethylene-insensitive-3-like protein (Table 1 and Supplementary S1). The regulatory interactions between CK and ethylene in rice may be at least in part mediated by the regulation of these genes.

Several genes involved in gibberellin signaling and metabolism were modulated by CK (Table 1 and Supplementary Table S1). The expression of gibberellin-inducible *OsTMK*, which encodes a leucine-rich repeat receptor protein kinase in deepwater rice, is down-regulated by CK. The expression of *OsTMK* is particularly high in regions undergoing cell division and elongation, and low in the non-growing region of the internode (van der Knaap et al. 1999), implying that CK affects rice growth regulation mediated by OsTMK. In addition, transcript abundance of the *OsWRKY71* gene, encoding a transcriptional repressor of gibberellin signaling in aleurone cells (Zhang et al. 2004), was also decreased by CK (Table 1 and Supplementary Table S3), suggesting antagonism between CK and gibberellin signaling.

Positive regulation of the metabolic pathway for active gibberellin species might occur (i.e. up-regulation of

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GA20ox4 and down-regulation of *GA2ox3*) in CK-treated leaves (Table 1). This type of regulation apparently does not suit the proposed model of CK and gibberellin interactions in shoot apical meristems for maintenance of the meristem function in *Arabidopsis* (Jasinski et al. 2005, Yanai et al. 2005) or rice (Sakamoto et al. 2006). Although there is no robust explanation at present, there could be different modes of interaction between these hormones in different organs.

Several genes encoding auxin-regulated auxin/IAA (*AUX/IAA*) and auxin response factor (*ARF*) family transcription factors, auxin-responsive proteins and putative auxin transporters were modulated (Table 1, and Supplementary Tables S1, S3). Also, a putative IAA-amino acid hydrolase homolog was up-regulated and an auxin biosynthesizing nitrilase gene was down-regulated. Down-regulation of a nitrilase gene by CK was also reported in *Arabidopsis* (Brenner et al. 2005). Regulation of these genes could contribute to synergy and antagonism between CK and auxin (Coenen and Lomax 1997, Rashotte et al. 2005), but there is as yet too little known about these interactions and the genes that regulate them to suggest a model.

Transcriptional regulators

The rice genome probably encodes >2,000 transcription factors or transcriptional regulators (Gao et al. 2006; http://drtf.cbi.pku.edu.cn./). Application of CK altered the expression of a large number of transcriptional regulators, including APETALA2/ethylene-responsive element-binding protein (AP2/EREBP), basic helix–loop–helix (bHLH), WRKY, bZIP, MYB, NAC and MADS family transcription factors (Supplementary Table S3). In *Arabidopsis*, some CK-inducible AP2 genes, designated *CYTOKININ RESPONSE FACTORS* (*CRFs*), mediate CK responses in concert with the His–Asp phosphorelay system (Rashotte et al. 2006). Phylogenetic analyses found several CK-up-regulated rice AP2 genes that fall into a clade near the *CRF* genes (Supplementary Figs. S1A, S1B), indicating that the rice AP2 and CRF gene products may play a similar role. The expression of most of the affected WRKY genes was lowered in both roots and leaves (17 of 18 in roots, 14 of 17 in leaves) including *OsWRKY71*. This trend was also observed in *Arabidopsis* (Brenner et al. 2005). It is noteworthy that overexpression of *OsMADS18*, which showed CK inductiveness in roots (Supplementary Table S3), induced early flowering in rice, and that the formation of axillary shoot meristems was accelerated (Fornara et al. 2004).

Macronutrient acquisition and distribution

Subsets of genes for transporters of macronutrients such as nitrate, ammonium, phosphate, potassium and sulfate were repressed by the application of CK (Table 2 and Supplementary Table S1). Similar regulation was reported in *Arabidopsis* (Brenner et al. 2005, Kiba et al. 2005, Sakakibara et al. 2006), suggesting a common mechanism in monocots and dicots for controlling macronutrient acquisition and distribution. Regulation of these genes has been well studied in *Arabidopsis*. Some nitrate transport genes are induced by nitrate (Orsel et al. 2002), whereas others, such as ammonium transporter (*AMT*; Gazzarrini et al. 1999), as well as transporters of phosphate (*PHT*; Muchhal et al. 1996), potassium (*HAK*; Rodríguez-Navarro and Rubio 2006) and sulfate (*SULTR*; Grossman and Takahashi 2001), are induced by substrate starvation. In addition, CK-dependent repression of *SULTR1;1* and *SULTR1;2* is mediated by AHK4/CRE1/WOL (Maruyama-Nakashita et al. 2004), and induction of some phosphate starvation-responsive genes, including *PHT1;1*, is mediated by AHK4/CRE1/WOL and AHK3 (Franco-Zorrilla et al. 2002, Franco-Zorrilla et al. 2005). To our knowledge, there is little information about the correlation between macronutrient uptake and distribution and CK content in rice at present. Rice grown in paddy fields predominantly utilizes ammonium rather than nitrate during most of the growing period, since ammonium is the major form of inorganic nitrogen in hypoxic and anaerobic soils (Sasakawa and Yamamoto 1978). Thus, the form of available inorganic nitrogen is different between rice and most other agricultural plants. *IPT3* is selectively and rapidly regulated by nitrate in *Arabidopsis* (Miyawaki et al. 2004, Takei et al. 2004). It would be interesting to know how rice *IPT* genes are regulated by nitrogen and by other macronutrient responses to understand the adaptive mechanisms to differing nutritional habitats and the role of CK as a regulator of macronutrient acquisition and distribution.

Protein synthesis

Previous studies have pointed out the up-regulation of genes coding for ribosomal proteins by CK in soybean (Crowell et al. 1990) and *Arabidopsis* (Brenner et al. 2005, Kiba et al. 2005). In this study, the expression of 120 ribosomal protein genes was up-regulated in leaves, while only one ribosomal protein gene was up-regulated in roots (Supplementary Table S4). This contrasting result is a strong indicator of organ-specific CK regulation of genes encoding ribosomal proteins.

Overexpression of OsRR6 alters plant morphology and CK metabolism

A list of rice CK-responsive genes is now available. It became possible to examine the effects of in planta manipulation of type-A RRs on CK-responsive genes by overexpression of an *RR* gene. Thirteen putative type-A *RR* genes in rice (*OsRR*) were identified (Ito and Kurata 2006, Jain et al. 2006, Supplementary Table S5). We selected

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Table 2 Rice macro-nutrient transport genes regulated by cytokinin

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change (30 min, 120 min)[a] Root	Fold change (30 min, 120 min)[a] Leaf	Fold change (OsRR6-ox/control)
			Nitrate			
Os.49093.2.S1_x_at	AK109733	LOC_Os02g02190	High-affinity nitrate transporter	D (0.287, 0.211)	–	0.68[b]
Os.49093.1.S1_a_at	AB008519	LOC_Os02g02170	High-affinity nitrate transporter	D (0.277, 0.219)	–	0.728[b]
Os.52974.1.S1_at	AK070216	LOC_Os04g50940	Nitrate transporter	D (0.145, 0.270)	–	2.48[b]
Os.22928.1.S1_x_at	AK072036	LOC_Os02g37040	Nitrate transporter	–	D (0.422, 0.410)	1.38
Os.56367.1.A1_at	AK109931	LOC_Os02g47090	Nitrate transporter	U (4.17, 4.62)	–	0.6[b]
			Ammonia			
Os.9328.1.S1_at	AK107204	LOC_Os02g40730	Ammonium transporter (OsAMT1;3)	D (0.409, 0.143)	–	0.519
Os.49050.1.S1_at	AK108711	LOC_Os02g34580	Ammonium transporter (OsAMT2;2)	–	U (2.28, 2.39)	1.3
			Phosphate			
Os.6567.1.S1_at	AK100323	LOC_Os02g56510	Pho1-like phosphate transporter (OsJ023)	D (0.451, 0.373)	–	0.654
Os.9506.1.S1_a_at	AK067498	LOC_Os02g38020	Phosphate transporter 2-1	–	D (0.195, 0.245)	0.5
Os.13467.1.S1_x_at	AF416722	LOC_Os04g10750	Phosphate transporter (OsPT4)	–	D (0.392, 0.518)	0.613
OsAffx.17942.1.S1_at	9637.m02486	LOC_Os09g28160	Mitochondrial phosphate transporter	–	D (0.441, 0.548)	0.528
			Potassium			
Os.27454.1.S1_x_at	AK099800	LOC_Os07g01214	High-affinity potassium transporter	–	D (0.419, 0.369)	0.728
Os.57530.1.S1_x_at	AJ491855	LOC_Os06g48800	High-affinity potassium transporter	–	D (0.501, 0.496)	0.215[b]
Os.19896.2.S1_x_at	CB623166	LOC_Os01g34850	High-affinity potassium transporter	–	D (0.491, 0.458)	0.228[b]
Os.27139.1.A1_at	CB669136	LOC_Os02g39910	Potassium channel tetramerization domain-containing protein	–	D (0.128, 0.152)	1.074
Os.18658.1.S1_at	AK120422	LOC_Os06g36590	Potassium efflux antiporter	–	U (1.87, 2.85)	0.573
Os.17941.1.S1_s_at	AK062977	LOC_Os03g03590	Potassium efflux antiporter	D (0.493, 0.654)	D (0.338, 0.408)	0.399
Os.20541.1.S1_at	AK066919	LOC_Os03g37840	Potassium transporter	–	D (0.47, 0.378)	0.291
Os.15701.2.S1_at	AK059797	LOC_Os07g47350	Potassium transporter	–	D (0.483, 0.379)	0.983
Os.53894.1.S1_x_at	AK100672	LOC_Os04g52120	Potassium transporter	U (3.31, 3.93)	–	1.06
Os.13483.1.S1_at	AK070738	LOC_Os07g48130	Potassium transporter	U (1.71, 2.30)	–	0.477
Os.53102.1.S1_s_at	AK070831	LOC_Os09g21000	Potassium transporter	D (0.489, 0.696)	–	0.85
Os.11262.2.S1_x_at	AK105723	LOC_Os04g32920	Potassium transporter 1 (OsHAK1)	D (0.404, 0.391)	–	0.486
			Sulfate			
Os.5588.1.S1_s_at	AU031675	LOC_Os03g09980	High-affinity sulfate transporter	D (0.36, 0.48)	–	0.375[b]
Os.27076.1.S1_at	AK067270	LOC_Os06g05160	Sulfate transporter	D (0.392, 0.668)	D (0.232, 0.402)	0.608
Os.10281.1.S1_at	AK069796	LOC_Os09g06499	Sulfate transporter	–	D (0.481, 0.402)	0.737
OsAffx.3180.1.S1_at	9631.m00896	none	Sulfate transporter	–	D (0.444, 0.448)	0.865
Os.10754.1.S1_at	AK066932	LOC_Os03g06520	Sulfate transporter	–	D (0.176, 0.176)	0.518

[a] Transcripts with a ratio of induction (plus or minus cytokinin) of >2.0 at 30 or 120 min after cytokinin application are listed as 'up-regulated (U)' and <0.5 are listed as 'down-regulated (D)'. Genes which do not meet the criteria are marked as –.
[b] Genes whose expression levels are below 150 and flag calls are 'absent'. Annotation was derived from the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/).

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OsRR6 for examining the role of type-A RRs in detail, because its pattern of expression is well studied (Jain et al. 2006; Fig. 1B; Table 1 and Supplementary Table S1). To determine the intracellular localization of OsRR6, we employed an *OsRR6::GFP* (green fluorescent protein) fusion. The results of a bombardment expression assay with epidermal cells of rice leaf sheaths showed that OsRR6::GFP displayed a localization profile very similar to that of GFP, suggesting that OsRR6::GFP was localized mainly in the cytoplasmic space (Fig. 1C).

We generated transgenic rice plants that overexpress *OsRR6* (OsRR6-ox) by fusing its coding sequence to the rice *actin1* (*Act1*) promoter, because this promoter generally produces much higher levels of constitutive expression in rice than the cauliflower mosaic virus (CaMV) 35S promoter (Zhang et al. 1991, Sentoku et al. 2000). Callus transformed with *Act1*::*OsRR6* showed severe retardation of shoot regeneration compared with callus transformed with a control vector (Fig. 2). Addition of N^6-benzyladenine, a CK, to the regeneration medium increased the number of shoots that differentiated from wild-type rice calli (Scarpella et al. 2003). Moreover, rice calli transformed with the ipt-type MAT vector regenerated in the absence of exogenous CK (Endo et al. 2002). These observations suggest that CK promotes shoot regeneration in rice. Thus, OsRR6 probably acts as a negative regulator of CK signaling.

Hygromycin B-resistant plants (T_0 generation) derived from transformed callus were individually transferred onto MS-agar plates containing hygromycin B for 7 d and then hydroponically grown for 75 d. Eight of 11 independent transgenic lines carrying *Act1*::*OsRR6* (OsRR6-ox) had a dwarf phenotype with poorly developed root systems, and small panicles with a reduced branching pattern and a reduction in the number of spikelets (Fig. 3A, B). We confirmed that these transgenic plants accumulated large amounts of *OsRR6* transcripts by RT–PCR (Fig. 3C). Two additional experiments using independently transformed *Act1*::*OsRR6* plants gave essentially the same results (data not shown), and confirmed that overexpression of *OsRR6* results in aberrant development.

Using an alternative genetic approach to determine *OsRR6* function, a missense mutant of *OsRR6*, *OsRR6D103E*, was made in which the putative phosphoryl-accepting aspartate residue (Asp103) was replaced with glutamate. Because the aspartate residue is a putative phosphorylation site, the predicted gene product should lose its phosphor-accepting ability. In some experiments, aspartate to glutamate substitutions in RR proteins mimic the active and phosphorylated state (Hass et al. 2004, Leibfried et al. 2005). Thus, the D103E mutation should activate OsRR6. However, callus transformed with *Act1*::*OsRR6D103E* showed no retardation of shoot regeneration (Fig. 2). Moreover, each of 20 OsRR6D103E-ox independent lines was indistinguishable from plants transformed with a control vector (control plants; Fig. 3A, B). These results support the hypothesis that growth defects associated with OsRR6 overexpression are due to a requirement for phosphorylation of OsRR6. The change to glutamate could have failed to mimic the activated form of the RR, or this substituted protein simply was not stable. The latter possibility is not likely because protein gel blot analyses using anti-cMyc antibody confirmed that sufficient amounts of 5× cMyc::OsRR6D103E were accumulated (data not shown). It should be noted that overexpression of a cytosolic RR, *ARR22*, which is quite different from authentic type-A ARRs, caused severe growth defects, particularly in the development of roots (Kiba et al. 2004). Overexpression of a mutated RR, which is changed at a putative phosphoryl-accepting aspartate to glutamate in ARR22 (Asp74), conferred no severe phenotype. Mutations of the conserved aspartate

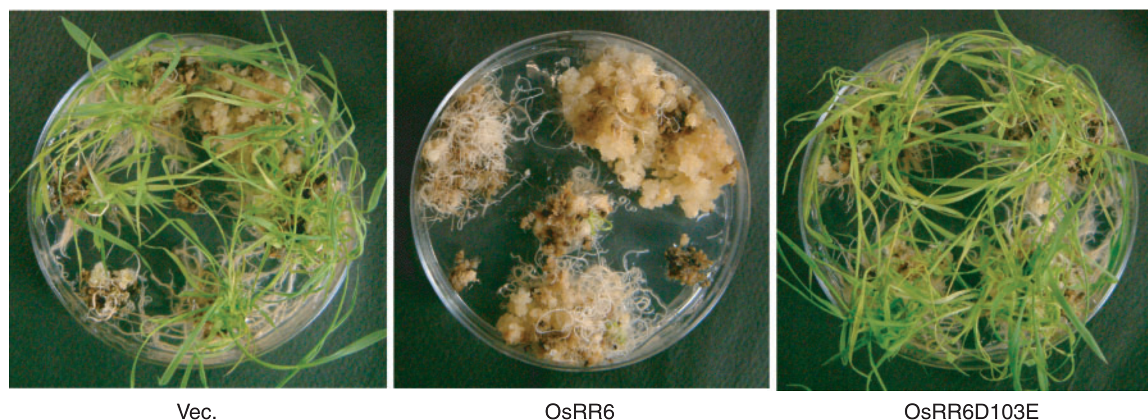

Fig. 2 OsRR6 represses shoot regeneration in rice callus. Callus was transformed with *Agrobacterium* carrying a binary vector pActnos/Hmz (Vec.), *Act1::OsRR6* (OsRR6) or *Act1::OsRR6D103E* (OsRR6D103E). Calli were selected with hygromycin B and grown on regeneration agar plates for 6 weeks.

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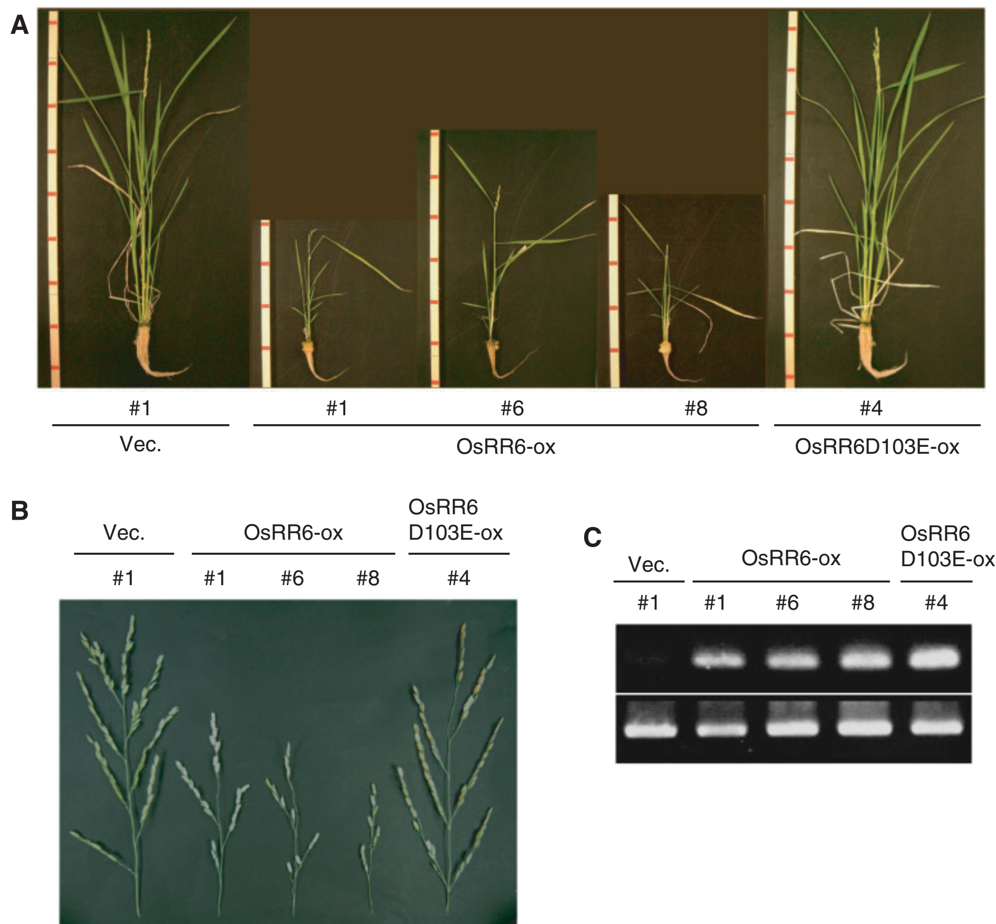

Fig. 3 Morphologies of transgenic plants overexpressing OsRR6. OsRR6-ox transgenic plants (T_0 generation) were grown on MS-agar plates containing hygromycin B for 7 d, and then hydroponically grown for 75 d. Transgenic plants, transformed with vector pActnos/Hmz (Vec.) or *Act1::OsRR6D103E* (OsRR6D103E-ox), were also grown under the same conditions. The typical phenotype of each transgenic line and their panicles are shown in (A) and (B). Total RNA samples were prepared from the shoots of each transgenic plant. (C) Semi-quantitative RT–PCR analysis of the *OsRR6* gene in the transgenic lines. *OsAct1* is an extraction and loading control. The scale bar between red lines = 10 cm for A.

residues to asparagine in ARR4 (Asp95) and ARR6 (Asp86) did not alter their ability to reduce *ARR6* promoter activity (Hwang and Sheen 2001). Moreover, overexpression of a mutated, nuclear-localized ARR7, which had been changed from Asp85 to glutamate, severely affected the function of shoot apical meristems (Leibfried et al. 2005). These lines of evidence imply that the biological function of type-A RRs is not simply to act as a negative regulator of CK signaling in all conditions, but that their phosphorylation state, as dictated by primary protein structure and/or subcellular location, affects the regulation of downstream developmental events.

Neither *Arabidopsis* hextuple type-A *arr* mutants (*arr3*, *4*, *5*, *6*, 8 and *9*) nor plants overexpressing *ARR5*, *ARR6*, *ARR7* or *ARR15* have severe phenotypes when grown under normal conditions, indicating that there are highly overlapping functions among members of this large gene family (Kiba et al. 2003, To et al. 2004, Leibfried et al. 2005). In contrast, maize mutants defective for ABPH1 (*abph 1*) have defects in phyllotaxis and meristem size regulation (Giulini et al. 2004). Considering what is known from *Arabidopsis* and maize, along with our present results, which show that simple overexpression of *OsRR6* causes growth defects, type-A RR genetic redundancy may be lower in grass species.

OsRR6-ox did not set normal flowers, resulting in sterility. Very recently, it has been reported that the rice *LONELY GUY* (*LOG*) gene, which encodes a CK-activating enzyme, is required to maintain meristem activity (Kurakawa et al. 2007). The shoot meristem of *log* mutants flattens and prematurely stops floral organ differentiation, suggesting that CK plays a key role in proper maintenance of shoot meristem activity in rice. In this context, the sterility of OsRR6-ox might be caused

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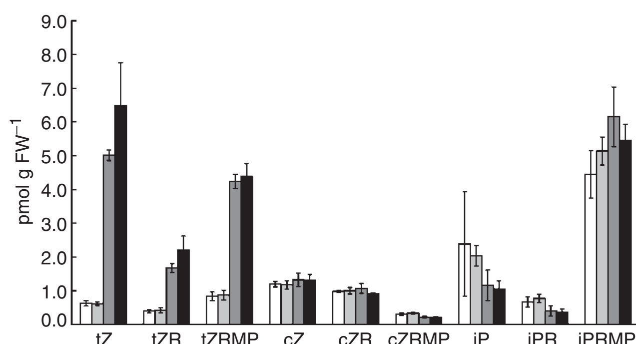

Fig. 4 Endogenous cytokinins in rice transformants. Cytokinin concentrations in whole shoots of two independently grown OsRR6-ox and control plants were determined. Data are means ± SE ($n = 3$). Empty bar; control vector line 1 (corresponding to #1 of Vec. in Fig. 3), light gray bar, control vector line 8; dark gray bar, OsRR6-ox line 1; filled bar, OsRR6-ox line 6 (corresponding to #1 and #6 of OsRR6-ox in Fig. 3, respectively). tZ, *trans*-zeatin; tZR, tZ riboside; tZRMP, tZR 5′-monophosphate; cZ, *cis*-zeatin; cZR, cZ riboside; cZRMP, cZR 5′-monophosphate; iP, isopentenyladenine; iPR, iP riboside; iPRMP, iPR 5′-monophosphate. gFW, g fresh weight. The complete data set is presented in Supplementary Table S6.

by negative regulation of CK signaling in the meristem. To explore whether overexpression of *OsRR6* influences endogenous CK content, we measured CK concentrations in shoots of two independently grown 75-day-old OsRR6-ox T_0 generation and control plants. OsRR6-ox plants had an increased content of tZ-type CKs (Fig. 4 and Supplementary Table S6). The concentrations of tZ, the biologically most active CK, was increased by >8-fold, and the tZ derivatives tZ riboside (tZR) and tZR 5′-monophosphate (tZRMP) were increased by >4-fold. The concentrations of *cis*-zeatin-type, isopentenyladenine-type and dihydrozeatin-type CKs were not significantly affected. Although the increase in CK content may not be sufficient to compensate for the growth defects of OsRR6-ox, the evidence of an increase is an indicator of a homeostatic control mechanism. Interestingly, in *Arabidopsis*, reduced CK signaling also led to an increase in CK content, in particular when *AHK3* was mutated (Riefler et al. 2006).

Mutual regulation between CK signaling and CK metabolism

The balance between CK biosynthesis and degradation determines endogenous CK levels. To gain some insight into the mechanism of increased CK content in OsRR6-ox, we carried out a genome-wide expression profile of OsRR6-ox. Total RNA from the same extractions as those used for quantification of CK was subjected to microarray analysis. There were changes of at least 3-fold in 441 genes in OsRR6-ox plants as compared with control plants (Table 3 and Supplementary Table S7). Interestingly, the expression of *OsCKX4* and *OsCKX5* was substantially reduced in OsRR6-ox transformants (Table 3 and Supplementary Table S7). In our study, none of the six detected rice *IPT* genes had altered transcript levels even if the threshold was set at a low stringency of 2.0 (data not shown). Down-regulation of *OsCKX4* and *OsCKX5* may thus partly contribute to the increased CK content of OsRR6-ox plants. A putative gene for CK-*O*-glucosyltransferase (accession No. AK104985) is up-regulated (Table 3 and Supplementary Table S7), which is in agreement with the observed increase of tZ-*O*-glucosides in OsRR6-ox plants (Supplementary Table S6). The microarray data were verified for a few selected genes by semi-quantitative RT–PCR (Fig. 5A).

Overexpression of *OsRR6* also affected the expression of CK-responsive genes. Of the 441 genes modulated in OsRR6-ox, 165 were CK responsive (Fig. 5B and Supplementary Table S8). Most of the up- or down-regulated genes in OsRR6-ox were up-regulated (17 of 21; 81%) or down-regulated (126 of 144; 88%) by CK, suggesting that the increased cellular CK primarily modulates CK-responsive genes, especially CK-repressive ones, in OsRR6-ox plants. It is likely then that a mutual control mechanism links CK signaling and metabolism, which contributes to a fine-tuning of the CK response. Eighteen CK-inducible genes were down-regulated in OsRR6-ox (Fig. 5B and Supplementary Table S8). The expression of several CK-inducible genes was reduced by the over-expression of a negative regulator of CK signaling in *Arabidopsis*, ARR8, suggesting that CK-inducible genes could be the targets of ARR8 (Osakabe et al. 2002). Considering the fact that OsRR6 acts as a negative regulator of CK signaling (Fig. 2), these 18 genes could be the targets of OsRR6 signaling. Although there are a few exceptions [e.g. an auxin-induced protein-related-like protein (CB667155) and a potassium transporter (AK066919), Tables 2, 3], expression of CK-responsive genes associated with hormone metabolism and signaling, and macronutrient transport was not significantly altered in OsRR6-ox (Tables 1, 2), suggesting that these categories of genes are not under the control of OsRR6 signaling. It may seem counterintuitive that type-A RRs are not included in the list of genes modulated in OsRR6-ox transformants. One possible explanation is that OsRR6 participates in the repression of transcription of some type-A RRs, as is the case with *Arabidopsis*, in which activation of the type-A *RRs* provides a negative feedback mechanism (Hwang and Sheen 2001).

Genes involved in stress responses are altered in OsRR6-ox plants

A major fraction of the genes modulated in OsRR6-ox play a particular role in the plant's response to hormones or

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Table 3 Differentially regulated genes in OsRR6-ox plants compared with control plants (selected)

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change[a]
			Cytokinin-related	
Os.53614.1.S1_at	AK104985	LOC_Os02g51930	Cytokinin-*O*-glucosyltransferase-like protien	3.23
Os.33309.1.S1_at	AK101022	LOC_Os01g56810	Cytokinin oxidase (OsCKX5)	0.251
Os.50470.1.S1_at	AK121317	LOC_Os01g71310	Cytokinin oxidase (OsCKX4)	0.102
			Auxin-related	
Os.39652.1.S1_at	CB667155	LOC_Os09g37330	Auxin-induced protein-related-like protein	4.53
Os.15250.1.S1_at	AK106063	LOC_Os08g24790	Auxin-induced protein-related-like protein	3.31
Os.17449.1.A1_at	AK066552	LOC_Os01g69070	Auxin efflux carrier family protein	0.332
Os.54966.1.S1_at	AK107208	LOC_Os06g47620	IAA-amino acid hydrolase	0.295
Os.22271.2.S1_x_at	CB621018	LOC_Os06g48950	ARF (auxin response factor) family transcription factor (OsARF7a)	0.273
Os.53828.1.S1_at	AK100297	LOC_Os08g41720	PIN1-like auxin transport protein-like	0.171
			Ethylene-related	
Os.27299.1.A1_at	AK065008	LOC_Os01g04800	AP2/EREBP family transcription factor	7.83
OsAffx.27278.1.S1_at	9633.m03916	LOC_Os05g41760	AP2/EREBP family transcription factor	0.319
Os.4893.1.S1_at	AK100184	LOC_Os05g29810	AP2/EREBP family transcription factor	0.284
OsAffx.12799.1.S1_s_at	9631.m00866	LOC_Os03g09170	AP2/EREBP family transcription factor	0.0714
			Gibberellin-related	
Os.17900.1.S1_s_at	BI803020	LOC_Os05g35690	Gibberellin-regulated protein	0.274
			ABA-related	
Os.12986.1.S1_at	AK061581	LOC_Os04g44500	ABA-responsive protein-like	0.213
			Defense-related	
Os.49466.1.S1_at	AK099946	LOC_Os12g43430	Thaumatin-like protein (response to pathogen)	17.8
Os.54304.1.S1_at	M24504	LOC_Os04g09390	Lectin	10.7
Os.6620.1.S1_at	CR292655	LOC_Os03g52390	Type II proteinase inhibitor family protein	8.18
Os.27755.1.S1_at	AK071889	LOC_Os07g35560	β-1,3-Glucanase	7.94
Os.52833.1.S1_at	AK069503	LOC_Os08g02110	Peroxidase	7.04
Os.12761.1.S1_at	AK102970	LOC_Os03g46060	Antifungal thaumatin-like protein	6.44
Os.2210.1.S1_at	AF032972	LOC_Os08g08970	Germin-like protein	6.11
Os.7947.1.S1_x_at	AF323610	LOC_Os05g31140	β-1,3-Glucanase	5.9
Os.27483.1.S1_at	AK059812	LOC_Os08g13440	Germin-like protein	5.54
Os.459.1.S1_at	U77656	LOC_Os03g46070	Thaumatin-like protein (response to pathogen)	5.39

(continued)

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Table 3 Continued

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change[a]
Os.32115.1.S1_at	AK068247	LOC_Os01g71350	β-1,3-Glucanase	5.07
Os.5441.1.S1_at	AK108376	LOC_Os10g38470	Glutathione *S*-transferase (toxin catabolism)	5.02
Os.20230.1.S1_at	AK121059	LOC_Os11g37950	Barwin family protein (response to virus)	4.97
Os.27864.1.S1_at	CB659199	LOC_Os04g59200	Peroxidase (response to pathogen)	4.84
Os.20289.1.S1_at	AY435041	LOC_Os11g37960	Barwin family protein (response to virus)	4.39
Os.158.1.S1_at	D14481	LOC_Os10g02070	Peroxidase (respone to pathogen)	4.17
Os.323.1.S1_at	AF251277	LOC_Os07g03710	PR-1 type pathogenesis-related protein PR-1a	3.92
Os.28139.1.S1_at	AK060655	LOC_Os01g62260	Osmotin-like protein (response to pathogen)	3.45
Os.6867.1.S1_x_at	AT003452	LOC_Os12g43380	Thaumatin-like protein (response to pathogen)	3.45
Os.418.1.S1_at	AK107926	LOC_Os01g28450	Pathogenesis-related protein class 1	3.44
Os.2416.1.S1_a_at	AB027428	LOC_Os01g71340	β 1,3-Glucanase (response to pathogen)	3.34
Os.19861.1.S1_at	AF306651	LOC_Os07g03730	Pathogenesis-related protein 1	3.3
Os.5031.1.S1_at	AB127580	LOC_Os12g36830	Pathogenesis-related protein Bet v I family protein	3.28
Os.2423.1.S1_at	AK070762	LOC_Os12g36850	Pathogenesis-related protein Bet v I family protein	3.15
Os.28124.1.S1_at	AK059767	LOC_Os10g28080	Class III chitinase	3.01
Os.5404.3.S1_a_at	AK066013	LOC_Os01g61880	Respiratory burst oxidase homolog	0.331
Os.49413.1.A1_x_at	AK100778	LOC_Os09g34150	NB-ARC domain-containing resistance protein	0.328
Os.5986.1.S1_at	AK069761	LOC_Os08g44400	Glutathione *S*-transferase	0.322
Os.37621.3.S1_at	AU166305	LOC_Os01g02340	Thaumatin-like protein (response to pathogenic fungi)	0.313
OsAffx.9584.1.S1_at	9629.m05956	LOC_Os01g60600	WRKY family transcription factor	0.279
OsAffx.9584.1.S1_x_at	9629.m05956	LOC_Os01g60600	WRKY family transcription factor	0.262
Os.45516.1.S1_at	AK120089	LOC_Os01g02780	Rust resistance kinase Lr10	0.257
Os.56922.1.S1_at	AK110587	LOC_Os02g26430	WRKY family transcription factor	0.256
Os.11770.1.S1_at	AF014469	LOC_Os07g48040	Peroxidase	0.251
Os.5045.1.S1_at	AK102138	LOC_Os01g03680	Bowman–Birk serine protease inhibitor family protein	0.198
Os.11549.1.S1_at	BU673475	LOC_Os12g02080	Peroxidase	0.182
Os.4976.1.S1_at	AB012855	LOC_Os05g33150	Chitinase	0.151
Os.170.1.S1_at	AB003195	LOC_Os01g64110	Chitinase	0.147
Os.11894.1.S1_at	AK064754	LOC_Os02g39330	Class IV chitinase	0.132
			Abiotic stress-related	
Os.6812.1.S1_at	AK058583	LOC_Os10g21670	Dehydration stress-induced protein	6.03
OsAffx.26649.1.S1_x_at	9632.m05653	LOC_Os04g57880	DnaJ domain-containing heat shock protein	4.23
Os.12633.1.S1_s_at	U60097	LOC_Os11g26790	Dehydrin	3.49
Os.12244.1.S1_at	AB110191	LOC_Os03g15960	Class I low molecular weight heat shock protein	3.43
Os.6863.1.S1_s_at	AK066682	LOC_Os12g14440	Jacalin-related lectin	3.41

(continued)

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Table 3 Continued

Affymetrix ID	Accession No.	TIGR code	Putative function	Fold change[a]
Os.10251.1.S1_at	AK120294	LOC_Os10g33370	Fatty acid elongase 3-ketoacyl-CoA synthase	3.27
Os.57456.1.S1_x_at	D10956	LOC_Os01g24710	Salt stress-induced protein	3.02
OsAffx.13360.1.S1_at	9631.m04381	LOC_Os03g45280	Dehydrin family protein	0.302
Os.12167.1.S1_at	AK070197	LOC_Os02g44870	Dehydrin family protein	0.301
Os.2881.1.S1_at	AK121010	LOC_Os10g40360	Proline oxidase/dehydrogenase	0.295
Os.11323.1.S1_at	AK071812	LOC_Os03g20120	Galactinol synthase (WSI76 protein induced by water stress)	0.29
Os.5194.2.S1_at	AK100713	LOC_Os01g66110	Dehydration-responsive protein	0.285
Os.52767.1.A1_at	AK069153	LOC_Os08g35160	DnaJ domain-containing heat shock protein	0.226
Os.32498.1.S1_at	AK069480	LOC_Os08g06170	Reticuline oxidase (response to oxidative stress)	0.185
Os.7985.1.S1_at	AK058242	LOC_Os03g18070	Fatty acid desaturase	0.119

Transcripts with a ratio of change of >3.0 compared with control plants are listed. Annotation was derived from the TIGR database (http://www.tigr.org/tdb/e2k1/osa1/).
[a] Normalized value ratio of OsRR6-ox plants over control plants.

are related to pathogen defense or responses to abiotic stress with parallel up- and down-regulation within the same category (Table 3 and Supplementary Table S7). These results imply a certain, defined role for OsRR6 in the integration of plant signal transduction. The response of *OsRR6* to both CK and abiotic stresses such as salinity, dehydration and cold supports this idea (Jain et al. 2006). The sterility of transgenic rice plants that overexpress *OsRR6* prevented us from experimentally examining their phenotypes under various stresses. Future analyses of knockout or RNA interference mutants of *OsRR6* will enable us to define further its possible participation in stress responses. The expression of some type-A *ARR* genes is induced by different environmental stresses such as drought, salinity and low temperature (Urao et al. 1998). Moreover, it has been reported that the type-A ARR family participates in light signaling in a complex manner (Sweere et al. 2001, To et al. 2004, Salomé et al. 2006). Taken together, type-A RRs may not only play a role in CK signaling, but may also form a complex network that is predominantly responsible for integration, fine-tuning and cross-talk of many plant signaling pathways.

Materials and Methods

Plant materials and growth conditions

For the identification of CK-responsive genes by microarray analysis, rice (*Oryza sativa* L. cv Nipponbare) seeds were sterilized with 70% ethanol for 1 min and then with 2% (w/v) sodium hypochlorite for 15 min, soaked in distilled water at 30°C for 1 d, and 30 germinated seeds were transferred to a nylon net floating on tap water that had been adjusted to pH 5.5. Seedlings were grown for 2 weeks in an environment-controlled greenhouse with a photoperiod of 12 h light (30°C)/12 h dark (25°C). Whole roots and third leaves, which were cut at the lamina joint with a razor blade in water, were excised from the 2-week-old seedlings and immediately dipped in distilled water containing either 5 μM tZ in dimethylsulfoxide [DMSO; 0.1% (v/v)] or an equal volume of DMSO as a control. Each excised organ was incubated at 30°C for 30 or 120 min, harvested and stored at −80°C until the RNA was extracted. Three independent biological replicates were prepared for each experiment.

Identification of OsRR genes

OsRR genes were identified by BLAST queries in GenBank (www.ncbi.nlm.nih.gov/BLAST/), The Institute for Genomic Research (TIGR rice genome project; http://www.tigr.org/tdb/e2k1/osa1/index.shtml), the DNA Data Bank of Japan (DDBJ; http://www.ddbj.nig.ac.jp/search/blast-j.html) and the rice cDNA database at KOME (http://cdna01.dna.affrc.go.jp/cDNA/) using the *OsRR6* amino acid sequence. *OsRR11* cDNAs were amplified using RT–PCR with the primers 5′-GAGAGGTTGAGA AGTTGAGATGTC-3′ and 5′-AAAAGTCTCCGAAACAAAC AATGC-3′. Total RNA was prepared from 3-week-old seedlings with the RNeasy plant Mini Kit (Qiagen, Hilden, Germany) with RNase-free DNase I (Qiagen). cDNA was synthesized using SuperScript II RT (Invitrogen, Carlsbad, CA, USA) with oligo(dT)$_{12–18}$ primers. The amplified fragment was cloned into pCR-Blunt-TOPO (Invitrogen) and sequenced to confirm the amplification fidelity. Analyses of cDNA and amino acid sequences were carried out using GENETYX-MAC version 11 (Software Development Co., Tokyo, Japan).

Construction and growth of OsRR6-ox transgenic plants

The entire coding sequence of *OsRR6* was amplified from a full-length cDNA clone (accession No. AK059734) as template using the *Bam*HI and *Sal*I integral primers 5′-AGGATCCATG GCGGCAGCGGCGCAGGCT-3′ and 5′-TGTCGACTCATCTG ATACGGCTGCAGAG-3′. The resulting PCR product was

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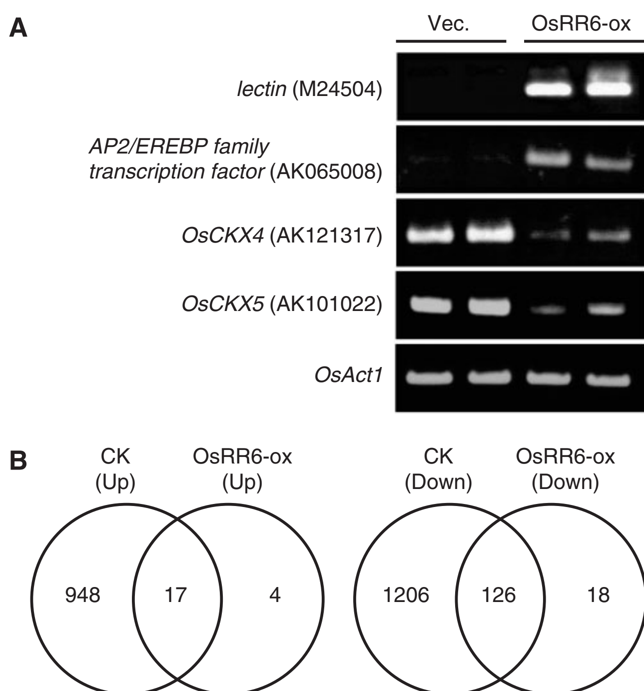

Fig. 5 Genes modulated in OsRR6-ox transformants. (A) Semi-quantitative RT–PCR of selected genes showing differences in the microarray studies. Total RNA was from the same extractions as the cytokinin quantification shown in Fig. 4, and subjected to PCR. *OsAct1* is an extraction and loading control. The accession number of the tested gene is shown in parentheses. (B) Venn diagrams of genes regulated by CK and modulated in OsRR6-ox plants with more than 2-fold changes (>2-fold increase [Up] or <0.5-fold decrease [Down]) and 3-fold changes (>3-fold increase [Up] or <0.33-fold decrease [Down], respectively).

cloned into the *Bam*HI/*Sal*I site of pGBKT7 (Clontech, Palo Alto, CA, USA), and designated pGBKT7-OsRR6. A site-directed mutant of OsRR6, OsRR6D103E, was made by amplifying pGBKT7-OsRR6 with the substitution-introducing primers 5′-CATCACCGAGTACTGGATGCCCG-3′ and 5′-CGGGCAT CCAGTACTCGGTGATG-3′. An *Nde*I/*Bam*HI cassette containing four copies of the c-Myc epitope (*4× cMyc*) was introduced upstream of the *OsRR6* and *OsRR6D103E* genes to give an N-terminal fusion. The resulting constructs, *5× cMyc-OsRR6*, *5× cMyc-OsRR6D103E* were amplified by PCR as *Xba*I/*Eco*RV fragments and introduced into the pActnos/Hmz binary vector (Sentoku et al. 2000) at an *Xba*I/*Sma*I site downstream of the rice actin1 gene promoter. An *Xba*I–*Eco*RV 5× cMyc cassette was also introduced into the pActnos/Hmz binary vector and used as a control. *Agrobacterium tumefaciens* (EHA101) carrying the above constructs was used to transform rice following the method of Hiei et al. (1994). Hygromycin B-resistant plants (defined as transgenic plants of the T_0 generation) from callus grown on regeneration agar plates for 6 weeks were transferred to agar plates containing 1× Murashige and Skoog salts, 3% (w/v) sucrose and 50 μg ml^{-1} hygromycin-B (MS-agar plate) for 7 d, and then hydroponically grown until heading in an environment-controlled greenhouse with a photoperiod of 12 h light (30°C)/12 h dark (25°C) as described by Kamachi et al. (1991).

Particle bombardment

The coding sequence of *OsRR6* was fused to the 5′ terminus of the GFP gene, which was under the control of the CaMV 35S promoter (35S-sGFP [S65T]). This construct was introduced into rice leaf sheath cells by bombardment with 1 μm gold particles as described in the supplier's protocol (PDS-1000/He, Biorad, Hercules, CA, USA). After overnight incubation, transient expression was observed by confocal laser-scanning fluorescence microscopy (Fluoview IX5, Olympus, Melville, NY, USA).

Semi-quantitative reverse transcription–PCR

Total RNA was prepared using an RNeasy plant Mini Kit (Qiagen) with RNase-free DNase I (Qiagen). Approximately 1 μg of RNA was used as template for first-strand cDNA synthesis, which was performed by SuperScript II RT (Invitrogen, Carlsbad, CA, USA) with oligo$(dT)_{12-18}$ primers in a reaction volume of 20 μl. A 1 μl aliquot of the reaction mixture was amplified by PCR using ExTaq DNA polymerase (TAKARA SHUZO CO. LTD, Kyoto, Japan) in a reaction volume of 50 μl with the primers 5′-ATGGCTGACGCCGAGGATATCCAG-3′ and 5′-TTAGA AGCATTTCCTGTGCACAATG-3′ for *OsAct1* (*actin1*; McElroy et al. 1990), 5′-TCAGAGGACGAAGCCAAGATT GTC-3′ and 5′-TTCATAGTAGCAGTAGCACTTAGC-3′ for *OsRR4*, 5′-ATGACCGGCTACGAGCTCCTCAAG-3′ and 5′AT TCTAACTTGCTAAGGCAGTAAG-3′ for *OsRR6*, 5′-AACAA CATGTGCTGCAGCCAGTGG-3′ and 5′-GATCCCAAAGCTA GCTAGCTCACC-3′ for *lectin* (accession No. M24504), 5′-TCATCGACTGCAAGAAGAACAACG-3′ and 5′-ATCATT TGCAATCTCTGACCTGAC-3′ for *AP2/EREBP family transcription factor* (accession No. AK065008), 5′-GTCCACGACGG CGAGCTCAA-3′ and 5′-TCATGCGAGTGGTGACGTGA-3′ for *OsCKX2*, 5′-ACGATGAACTTCAACCACGATGAG-3′ and 5′-CATTGAGGTAGCACAGCTCTTCTC-3′ for *OsCKX4*, and 5′-CTCATCTACCCCATGAACAG-3′ and 5′-CAGAATTGTCT ACACATCAG-3′ for *OsCKX5*. The number of cycles varied depending on the gene to avoid saturation of the amplification as follows: *OsAct1*, 22 cycles; *OsRR4* and *OsRR6*, 25 cycles; and *lectin*, *AP2/EREBP family transcription factor*, *OsCKX2*, *OsCKX4* and *OsCKX5*, 28 cycles. A 5 μl aliquot of the reaction was separated on 0.8% (w/v) agarose gels and stained with ethidium bromide.

DNA microarray analysis

Microarray analysis was performed using a GeneChip® rice genome array (Affymetrix, Santa Clara, CA, USA). Preparation of labeled target cRNA, subsequent purification and fragmentation were carried out using One-cycle target labeling and control reagents (Affymetrix). Double-stranded cDNA was prepared from 10 μg of total RNA. Hybridization, washing, staining and scanning were performed as described in the supplier's protocol. A 10 μg aliquot of fragmented cRNA was subjected to hybridization. It should be noted that all the above manipulations were carried out independently for each RNA sample.

Data analysis was performed using GeneChip® Operating Software (GCOS; Affymetrix) and GeneSpring 7 (Agilent Technologies, Palo Alto, CA, USA). Normalization per chip and per gene of the log_2 values was performed as recommended by the GeneSpring manual for Affymetrix gene chips. Briefly, per chip normalization with the 50th median of all measurements on the chip was performed. In addition, for CK treatment experiments, each treatment chip was specifically normalized to a control chip treated with DMSO for a similar time to the actual treatment.

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Three independent replicates were used for CK treatment experiments. The Affymetrix flag calls (present, P; marginal, M; or absent, A) were computed to give expression classes as follows: a gene was classified as present (P) when the expression call was P for all six gene chips; a gene was classified as 'turning from present to absent' (P → A) when the expression calls were P for three gene chips of the DMSO time point controls and either A or M for three gene chips of the experimental samples; and a gene was classified as 'turning from absent to present' (A → P) when the expression calls were either A or M for three gene chips of the DMSO controls and P for three replicates of the experimental samples. Genes that did not meet these criteria were classified as 'absent' and eliminated from further analysis, and any gene whose mean from all six experiments for each time point was lower than 500 was discarded as being unreliable data. Furthermore, one-way analysis of variance (ANOVA) Welch's *t*-tests were performed using the Statistical Group Comparison tool in GeneSpring 7 software. Comparisons were made between treatments of tZ and DMSO for individual time points. A *P*-value cut-off of 0.05 was selected for all tests. Finally, transcripts with a ratio of induction of >2.0 and either a P or an A → P classification were regarded as up-regulated. Transcripts with a ratio of induction of <0.5 and either a P or a P → A classification were regarded as down-regulated.

Two independent transgenic plants were used for each micro-array experiment with OsRR6-ox and control transgenic plants carrying empty vector. Only those transcripts whose flag calls were declared 'present' in all four chips were taken into account. Moreover, a raw cut-off level of 150 was assigned to all four-gene chip experiments. Finally, genes with a 3.0-fold change in expression were chosen. Changes in signal intensity were calculated from the mean value of the normalized values obtained from independent gene chip experiments.

Quantification of CK

Extraction and determination of CK contents from ~100 mg of rice tissue was performed using a liquid chromatography–tandem mass chromatography system (model 2695/Quattro Ultima Pt, Waters, Milford, MA, USA) as described previously (Nakagawa et al. 2005).

Supplementary material

Supplementary material mentioned in the article is available to online subscribers at the journal website www.pcp.oxfordjournals.org.

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