PCP
PLANT & CELL PHYSIOLOGY

Strigolactone and Cytokinin Act Antagonistically in Regulating Rice Mesocotyl Elongation in Darkness

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(Received June 21, 2013; Accepted October 15, 2013)

Regular Paper

Strigolactones (SLs) are a group of phytohormones that control plant growth and development including shoot branching. Previous studies of the phenotypes of SL-related rice (*Oryza sativa*) *dwarf* (*d*) mutants demonstrated that SLs inhibit mesocotyl elongation by controlling cell division. Here, we found that the expression of cytokinin (CK)-responsive type-A *RESPONSE REGULATOR* (*RR*) genes was higher in *d10-1* and *d14-1* mutants than in the wild type. However, CK levels in mesocotyls of the *d* mutants were not very different from those in the wild type. On the other hand, application of a synthetic CK (kinetin) enhanced mesocotyl elongation in the *d* mutants and the wild type. *d10-1* and *d14-1* mesocotyls were more sensitive to CK than wild-type mesocotyls, suggesting that the up-regulation of the CK-responsive type-A *RR* genes and the higher elongation of mesocotyls in the *d* mutants are mainly due to the increased sensitivity of the *d* mutants to CK. Co-treatment with kinetin and a synthetic SL (GR24) confirmed the antagonistic functions of SL and CK on mesocotyl elongation. *OsTCP5*, which encodes a transcription factor belonging to the cell division-regulating TCP family, was also regulated by SL and CK and its expression was negatively correlated with mesocotyl length. These findings suggest that OsTCP5 contributes to the SL- and CK-controlled mesocotyl elongation in darkness.

Keywords: Cytokinin • Mesocotyl • Rice • Strigolactone.

Abbreviations: CK, cytokinin; CPPU, 1-(2-chloro-4-pyridyl)-3-phenylurea; cZ, *cis*-zeatin; iP, N^6-(Δ^2-isopentenyl)-adenine; qRT–PCR, quantitative reverse transcription–PCR; RR, response regulator; SL, strigolactone; TCP, TEOSINTE BRANCHED1/CYCLOIDEA/PCFs; tZ, *trans*-zeatin.

Introduction

Strigolactones (SLs) are a group of terpenoid lactones that have been found in root exudates of various plant species. They were first identified as compounds that stimulate the germination of seeds of weeds such as *Striga* and *Orobanche* that parasitize the roots of other plants (Cook et al. 1966). SLs or their metabolites also act as phytohormones that suppress shoot branching (Gomez-Roldan et al. 2008, Umehara et al. 2008). In addition, SLs reduce lateral roots, but enhance plant height, secondary growth, senescence and root hairs (reviewed by Seto et al. 2012, Brewer et al. 2013, Ruyter-Spira et al. 2013). Both SL biosynthetic mutants and SL signaling mutants have been identified in several plant species. They include *more axillary growth* (*max*) in Arabidopsis (*Arabidopsis thaliana*), *ramosus* (*rms*) in pea (*Pisum sativum*), *decreased apical dominance* (*dad*) in petunia (*Petunia hybrida*) and *dwarf* (*d*) or *high-tillering dwarf* (*htd*) in rice (*Oryza sativa*) (reviewed by Leyser 2009, Beveridge and Kyozuka 2010). Each of these mutants has increased branching and reduced plant height. Genetic analyses of these mutants have revealed that biosynthesis of SLs is mediated by two carotenoid cleavage dioxygenases, CCD7 (e.g. rice D17/HTD1) and CCD8 (e.g. rice D10), one Cyt P450 monooxygenase (e.g. Arabidopsis MAX1) and one novel iron-containing protein (e.g. rice D27) (Beveridge and Kyozuka 2010, Leyser 2009). Alder et al. (2012) and Waters et al. (2012a) suggest that D27 is a β-carotene isomerase, which works upstream of CCD7 and CCD8 to produce an SL precursor (carlactone) prior to the MAX1-mediated step. On the other hand, one F-box leucine-rich repeat protein (e.g. rice D3) is involved in SL signaling, and rice D14/D88/HTD2, which is homologous to a hydrolase/esterase, is involved in SL signaling or

Plant Cell Physiol. 55(1): 30–41 (2014) doi:10.1093/pcp/pct150, available online at www.pcp.oxfordjournals.org

downstream metabolism (Leyser 2009, Beveridge and Kyozuka 2010). In Arabidopsis, AtD14 (a D14 ortholog) and KARRIKIN INSENSITIVE 2 (KAI2; an AtD14 paralog) appear to act with MAX2 (a D3 ortholog) (Waters et al. 2012b). AtD14 regulates SL signaling, and KAI2 and MAX2 regulate both karrikin signaling and SL signaling (Nelson et al. 2011, Waters et al. 2012b). In petunia, DAD2 (a D14 ortholog) was found to hydrolyze GR24 (a synthetic SL analog) and to interact with PhMAX2A (a D3 ortholog) in the presence of GR24 to initiate the SL signaling pathway (Hamiaux et al. 2012). In addition, an ATP-binding cassette (ABC) transporter (PDR1) was shown to be involved in SL transport (Kretzschmar et al. 2012). The PDR1 transporter is responsible for controlling SL-dependent symbiotic signaling and branching.

Auxin and cytokinin (CK) control tillering and shoot branching (Müller and Leyser 2011). Auxin, which originates from the shoot apex, represses shoot branching. CK is a class of plant hormones that were first identified as cell division-promoting factors (Miller et al. 1955, Miller et al. 1956) and were subsequently identified as factors that control various processes in plant growth and development (Sakakibara 2006). Root-derived CK has traditionally been considered to promote axillary bud outgrowth (Thimann and Skoog 1933, Sachs and Thimann 1967). However, CK synthesized in shoots appears to have a stronger influence on axillary bud outgrowth than root-derived CK (i.e. xylem sap CK) (Faiss et al. 1997, Tanaka et al. 2006).

SLs are a third class of branching factors that may interact with the two classical hormones, auxin and CK. How SL interacts with auxin for the control of shoot branching has been extensively studied (reviewed by Domagalska and Leyser 2011, Brewer et al. 2013). Other studies have examined the interactions between SL and CK in the control of shoot branching. In pea and Arabidopsis, export of CK from the roots (i.e. xylem sap CK) is dramatically reduced in SL-related increased-branching mutants (Beveridge et al. 1997, Morris et al. 2001, Foo et al. 2007). The export of CK is reduced through a feedback loop in the SL signaling pathway (Foo et al. 2005). However, no clear differences can be found in the CK levels of shoot apices between the SL-related mutant and the wild type in pea, Arabidopsis (Foo et al. 2007) or rice (Arite et al. 2007). Interestingly, expression of a gene involved in CK biosynthesis in pea, *Adenosine phosphate-isopentenyltransferase-1* (*PsIPT1*), is enhanced in SL-related mutants, implying that the increased CK biosynthesis by the enhanced *PsIPT1* gene expression in the shoots compensates for the reduced export of CK from the roots (Dun et al. 2012). Furthermore, SL and CK were found to act antagonistically in controlling bud outgrowth by regulating the expression of the pea TCP (TEOSINTE BRANCHED1/CYCLOIDEA/PCFs) family transcription factor *PsBRC1* in axillary buds (Dun et al. 2012). Indeed, CK down-regulates and SL up-regulates the expression of *PsBRC1* in buds (Braun et al. 2012, Dun et al. 2012).

The plant-specific TCP family of non-canonical basic helix–loop–helix (bHLH) transcription factors is involved in the regulation of cell proliferation (Cubas et al. 1999). TCP proteins can be classified into two classes (I and II). Class I proteins (also known as PCF class or TCP-P class proteins) have a four amino acid deletion in a basic domain of the protein, and class II proteins (also known as TCP-C class proteins) do not have this deletion (Martín-Trillo and Cubas 2010). The phenotypes observed in single and multiple mutants suggest that the class II TCP proteins prevent growth and proliferation. The axillary bud-specific regulators with proven functional importance in bud activity include the class II TCP protein-encoding genes, *TEOSINTE BRANCHED 1* (*TB1*) in maize (Doebley et al. 1997) and its homologs in other plant species including *AtBRC1* and *AtBRC2* (Arabidopsis; Aguilar-Martínez et al. 2007, Finlayson 2007), *PsBRC1* (pea; Braun et al. 2012) and *FC1/OsTB1* (rice; Takeda et al. 2003, Minakuchi et al. 2010). Defects in the *TCP* genes result in an increased branching phenotype (Takeda et al. 2003, Aguilar-Martínez et al. 2007, Finlayson 2007, Minakuchi et al. 2010, Braun et al. 2012) , indicating a negative correlation between the *TCP* gene expression and axillary bud outgrowth activity.

In rice, the mesocotyl is a tissue located between the coleoptilar node and the basal part of the seminal root in a young seedling (Hoshikawa 1989). The mesocotyl is highly elongated when seedlings are grown in darkness, whereas mesocotyl elongation is dramatically inhibited under light conditions. We previously found that SL negatively regulates mesocotyl growth by inhibiting cell division in rice in darkness (Hu et al. 2010). In this study, we attempted to better understand the relationship between SL and CK. One objective was to examine how SL and CK regulate mesocotyl elongation in rice. For this, we compared two mutants of cv. Shiokari, SL-deficient *d10-1* (Ishikawa et al. 2005, Arite et al. 2007) and SL-insensitive *d14-1* (Ishikawa et al. 2005, Arite et al. 2009), with the wild type (cv. Shiokari). A second objective was to examine whether an SL-responsive transcription factor *TCP* gene (*OsTCP5*) is involved in the control of mesocotyl elongation by SL and CK. For this we also used an SL-deficient mutant of cv. Nipponbare (*d10-2*) and its wild type (cv. Nipponbare) as plant materials.

Results

Up-regulation of CK-responsive *OsRR* gene expression in mesocotyls of SL-related *d* mutants

We previously showed that enhancement of mesocotyl elongation in the SL-related *d* mutants was due to enhanced cell division in the mesocotyls (Hu et al. 2010), raising the possibility that CK (a plant hormone that has a role in promotion of cell division) is involved in the enhancement of cell division in the mesocotyls of the *d* mutants. To examine this possibility, we investigated the expression of certain rice *RESPONSE REGULATOR* (*RR*) genes that are responsive to CK in the mesocotyls. Such genes are often used as CK-inducible marker genes (e.g. López-Bucio et al. 2007). In this study, rice tillering dwarf

mutants of cv. Shiokari, *d10-1* and *d14-1*, and their wild type (cv. Shiokari) were used as plant materials. To identify which rice *RR* genes are CK responsive, we checked the effects of a synthetic CK (kinetin) and a cytokinin oxidase/dehydrogenase inhibitor [1-(2-chloro-4-pyridyl)-3-phenylurea (CPPU), which is known to induce the accumulation of active endogenous CKs in plant tissues (Laloue and Fox 1989, Bilyeu et al. 2001)] on the expression of each of 13 rice *RR* genes (*OsRR1*–*OsRR13*) in the wild-type mesocotyl. These treatments significantly induced the expression of several of the *OsRR* genes (*OsRR4*, *OsRR5*, *OsRR7*, *OsRR9/10* and *OsRR11*; *OsRR9* and *OsRR10* are difficult to distinguish because their nucleotide sequences are highly homologous to each other) (**Supplementary Fig. S1**). The expression of these genes in 2- to 4-day-old mesocotyls was higher in *d10-1* and *d14-1* than in the wild type (**Fig. 1**), implying that CK levels and/or a sensitivity to CK are increased in the *d10-1* and *d14-1* mesocotyls.

Comparison of CK levels in mesocotyls of SL-related *d* mutants and the wild type

To check whether the CK levels are increased in the *d10-1* and *d14-1* mesocotyls, we measured endogenous concentrations of several common natural isoprenoid CKs, namely *trans*-zeatin (tZ), *cis*-zeatin (cZ) and N^6-(Δ^2-isopentenyl)-adenine (iP), all of which generally exhibit high CK activities in rice roots, and their riboside derivatives (tZR, cZR and iPR) (Sakakibara 2006, Kudo et al. 2012) in 4-day-old mesocotyls of the wild type, *d10-1* and *d14-1* (**Fig. 2**). The tZ level in the *d10-1* mesocotyl was 1.4-fold higher than the level in the wild-type mesocotyl (**Fig. 2A**), whereas the difference in the tZ levels in the mesocotyls between the *d14-1* mutant and the wild type was not significant (**Fig. 2A**). Moreover, the levels of cZR in the *d10-1* and *d14-1* mesocotyls were 2- to 2.5-fold lower and the levels of iPR in the *d10-1* mesocotyl was 1.4-fold lower than the level in the wild-type mesocotyl (**Fig. 2D, F**). On the other hand, the levels of

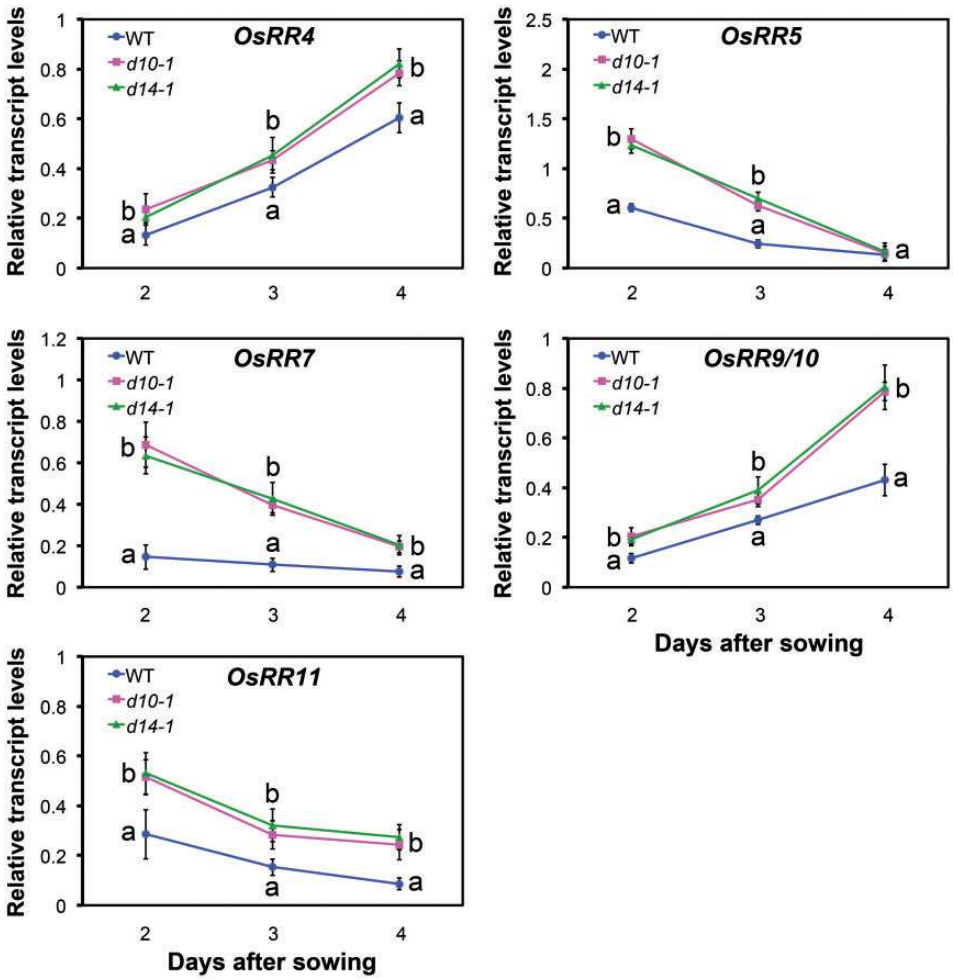

Fig. 1 Type-A *OsRR* gene expression in mesocotyls. qRT–PCR analyses of type-A *OsRR* genes (*OsRR4*, *OsRR5*, *OsRR7*, *OsRR9/10* and *OsRR11*) were performed using RNAs extracted from the mesocotyls of the wild type (WT) and *d* mutants (*d10-1* and *d14-1*) grown in darkness for 2–4 d. The *Ubiquitin* gene was used as a control. Different lower case letters denote significant differences among relative transcript levels of the WT and *d* mutants on each day ($P < 0.05$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ($n = 6$) ± SD.

tZR, cZ and iP were comparable with those of the wild type (Fig. 2B, C, E). A synthetic SL analog, GR24 (1 μM), decreased the tZ levels in the *d10-1* mesocotyl to the tZ levels in the wild type, whereas GR24 did not affect the levels of any CKs or their riboside derivatives in the *d14-1* mesocotyl (Fig. 2). These results showed that the CK levels in the 4-day-old mesocotyls were not very different between the *d* mutants and the wild type (Fig. 2), and the pattern of changes of some CK levels (i.e. tZ, cZR and iPR levels) in *d10-1* and *d14-1* in response to the GR24 treatment (Fig. 2) did not seem to correspond to the pattern of changes of mesocotyl lengths (Hu et al. 2010).

Increased sensitivity of mesocotyl elongation in *d10-1* and *d14-1* mutants to CK

To examine the role of CKs in mesocotyl elongation, seedlings were grown in darkness on agar plates containing 0 or 100 μM

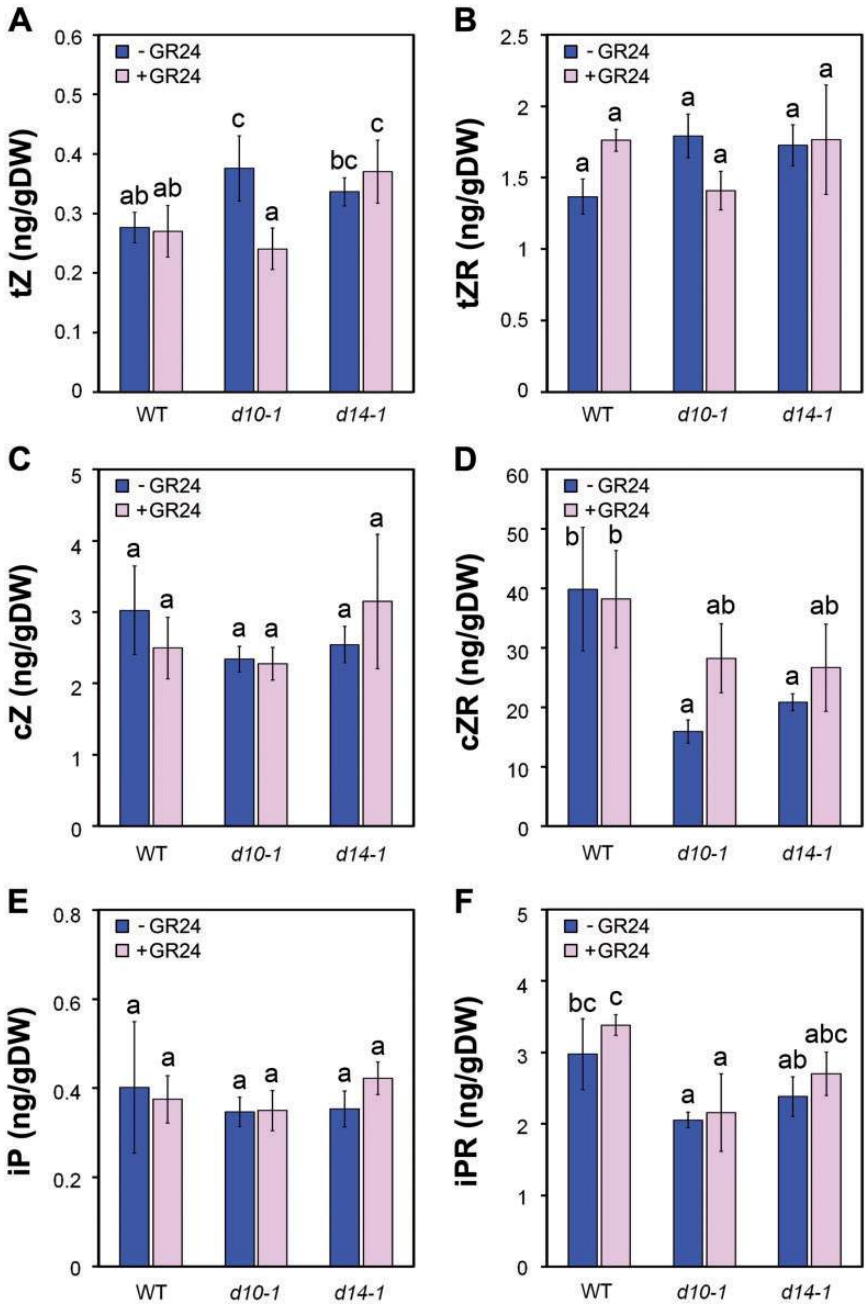

Fig. 2 Cytokinin levels in mesocotyls under dark conditions. Endogenous cytokinin levels, (A) tZ, (B) tZR, (C) cZ, (D) cZR, (E) iP and (F) iPR, in mesocotyls of the wild type (WT) and *d* mutants (*d10-1* and *d14-1*) were quantified by LC-MS/MS after germination and grown in darkness for 4 d. Blue and pink columns indicate untreated samples (−GR24) and GR24-treated samples (1 μM GR24; +GR24), respectively. Different lower case letters denote significant differences among hormone levels ($P < 0.05$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ($n = 4$) ± SD.

of a synthetic CK, kinetin. Kinetin at 100 μM enhanced the lengths of mesocotyls of the wild type (**Supplementary Fig. S2A**), making the lengths similar to those of the *d10-1* and *d14-1* mesocotyls without kinetin treatment (**Supplementary Fig. S2B, C**). Kinetin also accelerated the mesocotyl elongation in *d10-1* and *d14-1*, causing the mesocotyls to plateau (i.e. reach their maximal length) earlier than untreated mesocotyls (**Supplementary Fig. S2B, C**). After day 4, the increases were not as great in the mutants as they were in the wild type because the mutants were near their maximal length.

To examine whether *d10-1* and *d14-1* mesocotyls are more sensitive to CK than wild-type mesocotyls, seedlings were grown in darkness on agar plates containing 0, 0.5, 1 or 5 μM kinetin for 4 d. In wild-type seedlings, 5 μM kinetin slightly but significantly increased mesocotyl length, while lower concentrations had no effect (**Fig. 3**). However, in *d10-1* and *d14-1* seedlings, 0.5 μM kinetin stimulated mesocotyl elongation, indicating that *d10-1* and *d14-1* mesocotyls are more sensitive to CK than wild-type mesocotyl (**Fig. 3**). Together, these findings suggest that the increased sensitivity of the *d* mutants to CK (**Fig. 3**) mainly contributes to the up-regulation of the CK-responsive type-A *RR* genes and the higher elongation of mesocotyls in the *d* mutants.

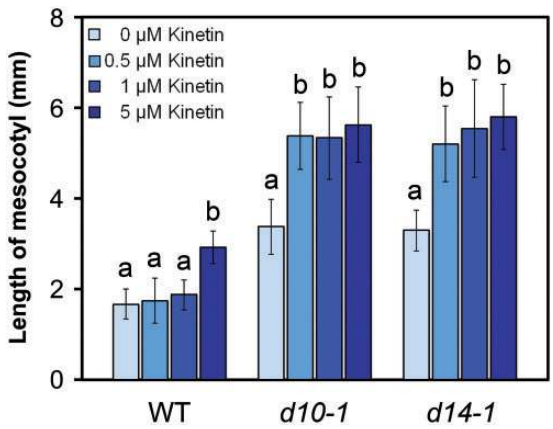

Fig. 3 Dose–response of mesocotyl elongation of dark-grown wild-type (WT), *d10-1* and *d14-1* seedlings to CK. Seedlings of the WT, *d10-1* and *d14-1* were germinated and grown on an agar plate without any treatments or on agar plates containing 0.5, 1 or 5 μM kinetin for 4 d. Different lower case letters denote significant differences among mesocotyl lengths ($P < 0.01$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ($n = 12$) ± SD.

Interaction between SL and CK in mesocotyl elongation

To examine interaction between SL and CK in the rice mesocotyl, we treated the wild-type, *d10-1* and *d14-1* seedlings with GR24, kinetin and both GR24 and kinetin. In seedlings grown in the dark on agar plates for 4 d, GR24 and kinetin partly antagonized each other's effect on mesocotyl elongation in the wild-type and *d10-1* seedlings (**Fig. 4**). In contrast, GR24 did not affect the kinetin-induced mesocotyl growth in *d14-1* (**Fig. 4**). These results suggest that SL and CK have an antagonistic relationship in the control of mesocotyl elongation in rice under dark conditions.

Roles of SL and CK in controlling *OsTCP5* expression in mesocotyls

To identify genes regulated by SL during mesocotyl elongation in rice, we carried out a microarray analysis using RNA extracted from mesocotyls of *d10-1*, *d14-1* and the wild type grown under dark conditions for 4 d, with or without treatment with 1 μM GR24 (data not shown). One of the SL-up-regulated genes encoded a TCP family transcription factor, *OsTCP5* (Os01g0763200; LOC_Os01g55750; Navaud et al. 2007; Martín-Trillo and Cubas 2010). We selected *OsTCP5* for further examination because some TCPs have roles in SL-controlled axillary bud outgrowths (Minakuchi et al. 2010, Braun et al. 2012). OsTCP5 is a CIN-type protein of the class II TCP subfamily (Martín-Trillo and Cubas 2010). CIN-type proteins limit cell proliferation at the margins of the developing leaf primordia in

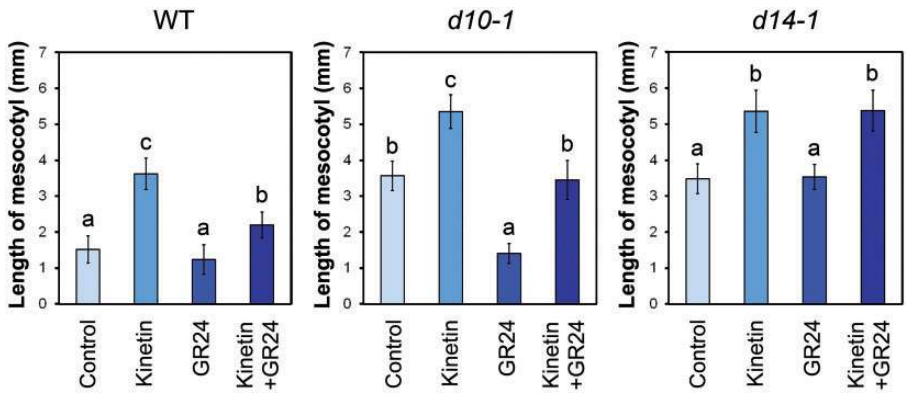

Fig. 4 Action of kinetin and GR24 in controlling mesocotyl elongation under dark conditions in the wild type (WT), *d10-1* and *d14-1*. Seedlings of the WT, *d10-1* and *d14-1* were germinated and grown on agar plates with or without 100 μM kinetin and with or without 1 μM GR24 for 4 d. Different lower case letters denote significant differences among mesocotyl lengths ($P < 0.01$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ($n = 10$) ± SD.

snapdragon, Arabidopsis and tomato. In snapdragon and Arabidopsis *cin* mutants, the leaf cells divide for a longer time than they do in the wild-type plants (Nath et al. 2003, Palatnik et al. 2003).

Quantitative reverse transcription–PCR (qRT–PCR) analysis revealed that *OsTCP5* was expressed at lower levels in *d10-1* and *d14-1* than in the wild type, and GR24 induced its expression in the *d10-1* mesocotyl, but not in the *d14-1* mesocotyl (**Fig. 5A**). Kinetin repressed *OsTCP5* expression more strongly in the wild type than in *d10-1* and *d14-1* (**Fig. 5B**). Together, these observations suggest that OsTCP5 is involved in the control of cell division by SL and CK in the mesocotyl.

As shown in **Fig. 6**, GR24 and kinetin antagonize each other's effect on *OsTCP5* expression in the wild-type and *d10-1* seedlings. CK (kinetin) stimulated mesocotyl elongation (**Fig. 4**) when *OsTCP5* was down-regulated by CK (**Fig. 6**), whereas SL (GR24) repressed mesocotyl elongation (**Fig. 4**) when *OsTCP5* was up-regulated by SL (**Fig. 6**). In other words, the response of *OsTCP5* expression in the wild type and *d10-1* to the CK and SL treatments was negatively correlated with the response of the mesocotyl elongation to the CK and SL treatments.

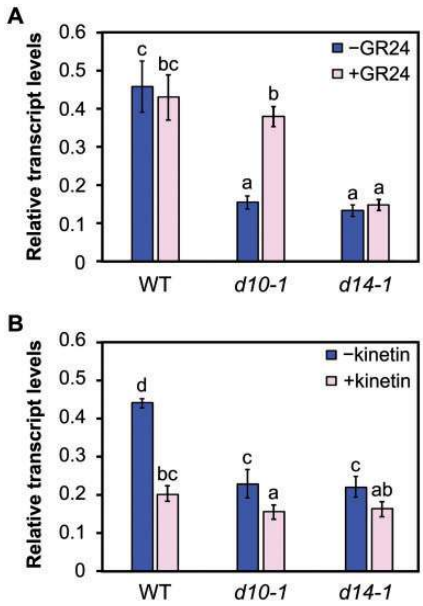

Fig. 5 Effects of SL and CK on *OsTCP5* expression in mesocotyls. (A) Expression of the *OsTCP5* gene in 4-day-old mesocotyls of the wild type (WT), *d10-1* and *d14-1* germinated in darkness with or without GR24 treatment was examined by qRT–PCR analysis. Blue and pink columns indicate untreated samples (−GR24) and GR24-treated samples (1 μM GR24; +GR24), respectively. (B) Expression of the *OsTCP5* gene in 4-day-old mesocotyls of the WT, *d10-1* and *d14-1* germinated in darkness with or without kinetin treatment, was examined by qRT–PCR analysis. Blue and pink columns indicate untreated samples (−Kinetin) and kinetin-treated samples (100 μM kinetin; +Kinetin), respectively. The *Ubiquitin* gene was used as a control. Values are means ($n = 6$) ± SD. Different lower case letters denote significant differences among relative transcript levels ($P < 0.05$, one-way ANOVA and then Tukey's test for multiple comparisons).

Role of *OsTCP5* in mesocotyl elongation

To investigate the function of *OsTCP5* in mesocotyl elongation, we examined the phenotypes of two independent transgenic lines (*pACT1::OsTCP5-1* and *pACT1::OsTCP5-2*), whose backgrounds were cv. Nipponbare (**Fig. 7**). We used cv. Nipponbare instead of cv. Shiokari for these experiments because the transformation efficiency of cv. Shiokari is very low compared with that of cv. Nipponbare and because we also had a *d10* mutant with a cv. Nipponbare background (*d10-2*). In seedlings grown in the dark on agar plates for 4 d, the *OsTCP5* gene was expressed at lower levels in *d10-2* than in the wild type (cv. Nipponbare) (**Fig. 7A**). Kinetin (100 μM) significantly repressed *OsTCP5* expression in the wild-type mesocotyl. The *OsTCP5* mRNA levels in *d10-2* tend to be reduced by the kinetin treatment, but this reduction was not significant (**Fig. 7A**), unlike *OsTCP5* expression in *d10-1* mesocotyls (**Fig. 6**). The reason for the difference in the repression levels of *OsTCP5* expression in *d10-1* and *d10-2* is unclear, but one possibility is that the difference is due to the different backgrounds of the two mutants (cv. Shiokari and cv. Nipponbare, respectively). In the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines, the *OsTCP5* expression was slightly, but significantly, higher than in the wild type in the absence of kinetin (**Fig. 7A**). The expression levels in the *pACT1::OsTCP5* lines were still high even after the treatment with kinetin (**Fig. 7A**), whereas *OsTCP5* expression was significantly reduced by kinetin in the wild type. It should be noted that we were not able to find *OsTCP5*-overexpressor lines, in which the *OsTCP5* expression levels were very high, among the transgenic *pACT1::OsTCP5* lines. However, the *pACT1::OsTCP5* lines were still useful for investigating the role of OsTCP5 in mesocotyl elongation, because the *OsTCP5* expression in the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines was not affected by the kinetin treatment (**Fig. 7A**).

After germination in darkness for 4 d, the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines had almost the same mesocotyl

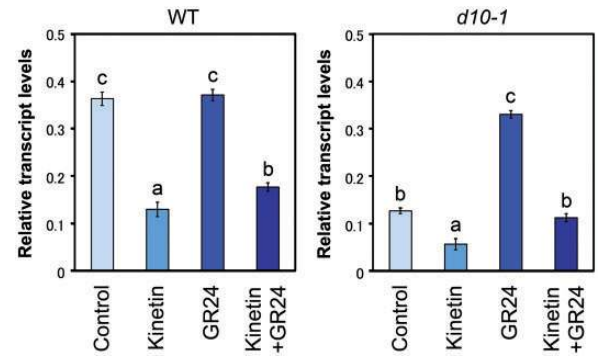

Fig. 6 Effects of kinetin and GR24 on *OsTCP5* expression in wild-type (WT) and *d10-1* mesocotyls. Mesocotyls were germinated and grown on agar plates with or without 100 μM kinetin and with or without 1 μM GR24 for 4 d. *OsTCP5* gene expression was measured by qRT–PCR. The *Ubiquitin* gene was used as a control. Different lower case letters denote significant differences among relative transcript levels ($P < 0.05$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ($n = 6$) ± SD.

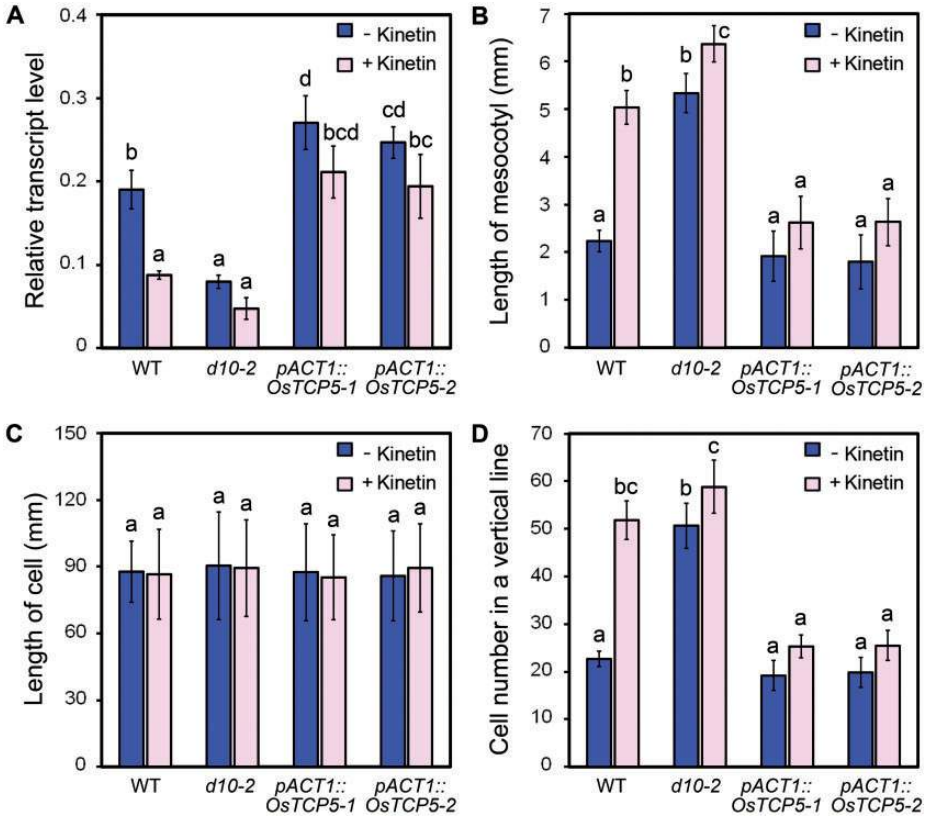

Fig. 7 Function of *OsTCP5* in CK-regulated mesocotyl elongation. *OsTCP5* expression (A), mesocotyl elongation (B), cell length (C) and cell number (D) in mesocotyls of the wild type (WT), the *d10-2* mutant and the transgenic *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines with or without kinetin treatment were examined. All samples had a cv. Nipponbare background. Blue and pink columns indicate untreated samples (−Kinetin) and kinetin-treated samples (100 μM kinetin; +Kinetin), respectively. Different lower case letters denote significant differences among the values ($P < 0.01$, one-way ANOVA and then Tukey's test for multiple comparisons). Values are means ± SD [$n = 6$ (A, B, D) and $n = 300$ (C)].

lengths as the wild type, whereas the *d10-2* mutant had a longer mesocotyl (**Fig. 7B**). The kinetin treatment greatly enhanced mesocotyl elongation in the wild type, and kinetin had a slight positive effect on mesocotyl elongation in *d10-2*. However, this response was dramatically impaired in the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines (**Fig. 7B**).

To examine whether cell division and/or cell elongation in the mesocotyls are affected by kinetin, we measured cell length in vertical sections of the mesocotyls in the wild type, *d10-2* and the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines. Cell lengths in all the mesocotyls were comparable and the treatment with kinetin had no visible effect on cell length (**Fig. 7C**). The average number of cells from the bottom to the top of the mesocotyl was ∼23 in the wild-type, ∼51 in *d10-2*, ∼19 in the *pACT1::OsTCP5-1* and ∼20 in *pACT1::OsTCP5-2* lines (**Fig. 7D**). When the seedlings were grown in the presence of kinetin, the cell number in the wild-type mesocotyl was increased to almost the cell number of the *d10-2* mesocotyl (without the kinetin treatment). Moreover, the cell number in the *d10-2* mesocotyl was also slightly increased by the kinetin treatment, whereas kinetin did not affect the cell numbers in the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* mesocotyls (**Fig. 7D**), suggesting that the change in lengths of mesocotyls in the wild type, *d10-2* and the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines is due to changes in their cell numbers, but not in cell lengths. Taken together, these results suggest that (i) *OsTCP5* negatively controls cell division during mesocotyl elongation and (ii) the promotion of mesocotyl elongation in *d10-1* and *d14-1* is at least partly caused by down-regulation of *OsTCP5* gene expression.

Discussion

SL may regulate sensitivity to CK in the mesocotyl

There is increasing evidence that SL and CK interact in plants (Beveridge et al. 1997, Morris et al. 2001, Foo et al. 2007, Braun et al. 2012, Dun et al. 2012). One of the roles of CK is to promote cell division (Sakakibara 2006). In this study, all of the CK-responsive type-A *RR* genes (*OsRR4*, *OsRR5*, *OsRR7*, *OsRR9/10* and *OsRR11*) in 2- to 4-day-old mesocotyls were more highly expressed in the *d10-1* and *d14-1* mutants than in the wild type (**Fig. 1**), suggesting that CK is involved in the enhancement of cell division in the *d10-1* and *d14-1* mesocotyls.

Exogenously applied kinetin accelerated the mesocotyl elongations in *d10-1* and *d14-1* as well as in the wild type,

and the growth of kinetin-treated *d10-1* and *d14-1* mesocotyls plateaued earlier than that of untreated mesocotyls (**Supplementary Fig. S2**). Interestingly, the growth curve of the wild-type mesocotyls treated with 100 μM kinetin was relatively similar to the growth curves of untreated *d10-1* and *d14-1* mesocotyls (**Supplementary Fig. S2**). Moreover, the *d10-1* and *d14-1* mesocotyls are more sensitive to supplied CK than the wild-type mesocotyls (**Fig. 3**). These results suggest that the enhanced elongation in the mesocotyls of the *d10-1* and *d14-1* mutants results from the increased sensitivity to CK.

The tZ levels in 4-day-old mesocotyls were slightly (1.4-fold) higher in the *d10-1* mutant than in the wild type, and GR24 decreased the tZ levels in the *d10-1* mesocotyl (**Fig. 2A**). However, the difference in the tZ levels in the mesocotyls without the GR24 treatment between the *d14-1* mutant and the wild type was not significant (**Fig. 2A**). In Arabidopsis, AtD14 (a D14 ortholog) is involved in SL signaling, whereas KAI2 (an AtD14 paralog) is involved in both karrikin signaling and SL signaling (Waters et al. 2012b). A rice KAI2 ortholog might also mediate some SL responses in rice. If this is the case, it might affect the accumulation of tZ in the *d14-1* mutant. On the other hand, the levels of other active CKs (i.e. cZ and iP) in the mesocotyls of the *d10-1* and *d14-1* mutants were roughly similar to those in the wild-type (**Fig. 2C, E**). Among riboside derivatives (tZR, cZR and iPR) of the active CKs, the levels of cZR in the *d10-1* and *d14-1* mesocotyls were 2- to 2.5-fold lower and the levels of iPR in the *d10-1* mesocotyl was 1.4-fold lower than the level in the wild-type mesocotyl (**Fig. 2D, F**). Together, these results showed that the CK levels in the 4-day-old mesocotyls of the *d* mutants were not very different from those in the wild type (**Fig. 2**) and the pattern of changes of some CK levels in *d10-1* and *d14-1* in response to the GR24 treatment (**Fig. 2**) did not seem to correspond to the pattern of changes of mesocotyl lengths (Hu et al. 2010; **Fig. 4**). Thus, the increased sensitivity of the *d* mutants to CK (**Fig. 3**) may contribute more greatly to the up-regulation of the CK-responsive type-A *RR* genes and the higher elongation of mesocotyls in the *d* mutants. In pea, axillary buds are more sensitive to CK in the SL-deficient *rms1* mutant than in the wild type (Dun et al. 2012) although CK levels in the shoots of the wild type and SL-related mutants (including *rms1*) are comparable (Foo et al. 2007), implying that the SL–CK interaction is similar in pea axillary buds and rice mesocotyls.

SL and CK antagonistically affect mesocotyl elongation in SL-deficient mutants

The effects of SL, CK and both SL and CK on mesocotyl length indicate that SL and CK have an antagonistic relationship in the wild type and the SL-deficient *d10-1* mutant under dark conditions (**Fig. 4**). In contrast, such an antagonistic relationship between SL and CK was not observed in the SL-insensitive *d14-1* mutant (**Fig. 4**). The expression levels of SL biosynthetic genes such as *D10*, *D17* and *D27* in the mesocotyls were not altered by the treatments with CK, SL or both CK and SL (**Supplementary Fig. S3**), which suggests that the antagonistic relationship between SL and CK is not caused by the changed efficiency of SL biosynthesis by the CK treatment.

Dun et al. (2012) reported that SL treatment was effective at reducing CK-induced bud outgrowth in a pea SL-deficient mutant, but not in an SL-insensitive mutant, and that SL and CK acted antagonistically in bud outgrowth control. These results suggest that the antagonistic effects of SL and CK on growth are conserved between pea axillary buds and rice mesocotyls.

SL- and CK-regulated OsTCP5 inhibits cell division in the mesocotyl

We found that the expression level of *OsTCP5*, which belongs to a subfamily of the class II TCP proteins that prevent growth and proliferation, was negatively correlated with mesocotyl elongation. GR24 enhanced the expression levels of *OsTCP5* in *d10-1* (**Fig. 5A**), suggesting that up-regulation of the expression of *OsTCP5* is SL dependent. In contrast, GR24 (1 μM) did not affect the expression of *OsTCP5* in *d14-1* (**Fig. 5A**) because the sensitivity to SL is much reduced in *d14-1*. Moreover, *OsTCP5* expression was not affected in the wild type by the GR24 treatment (**Fig. 5A**). It must be noted that 1 μM GR24 also did not affect the mesocotyl length of the wild type (Hu et al. 2010; **Fig. 4**), whereas treatment with higher concentrations of GR24 (10 μM) significantly reduced the length of the wild-type mesocotyl (data not shown). Similarly, application of 1 μM GR24 to the wild-type plants of rice did not affect the outgrowth of tiller buds (Umehara et al. 2008, Minakuchi et al. 2010), but 10 μM GR24 suppressed the bud outgrowth (Umehara et al. 2008). Moreover, it is worth noting that branching of the wild type in Arabidopsis is also poorly responsive to application of exogenous GR24 (5 μM) (Gomez-Roldan et al. 2008). These results suggest that a higher dose of GR24 is necessary to inhibit mesocotyl elongation significantly in the wild-type rice seedlings and bud outgrowth in the wild-type rice and Arabidopsis seedlings. These observations explain why the application of 1 μM GR24 to the wild type did not affect mesocotyl elongation (**Fig. 4**) or the expression of *OsTCP5* (**Fig. 5A**).

Expression of *OsTCP5* in the wild type was repressed by kinetin (**Fig. 5B**), suggesting that it can be down-regulated by CK. The effects of SL, CK and both SL and CK on *OsTCP5* expression indicate that SL and CK antagonize each other's effect on *OsTCP5* expression in the wild type and *d10-1* (**Fig. 6**). The *OsTCP5* expression, which is up-regulated by SL or down-regulated by CK (**Figs. 5, 6**), can nicely explain the antagonistic relationship of SL and CK in the control of mesocotyl elongation in rice (**Fig. 4**). To determine whether *OsTCP5* directly regulates the mesocotyl elongation, we attempted to produce *OsTCP5*-overexpressor and -repressor lines. However, for unknown reasons, the expression levels of *OsTCP5* in the *pACT1::OsTCP5* lines (for overexpressor) were only modestly increased (**Fig. 7A**). On the other hand, we failed to produce *OsTCP5* repressor lines by transgenic techniques. The failure to

produce *OsTCP5*-overexpressor or *OsTCP5*-repressor lines may be because overexpression or repression of *OsTCP5* negatively affected the growth and viability of rice plants. Nevertheless, constitutive *OsTCP5* expression in the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines impaired the function of CK in cell division in the mesocotyl, thereby repressing mesocotyl elongation (**Fig. 7**). This shows that OsTCP5 directly regulates mesocotyl elongation. These findings suggest that mesocotyl elongation is at least partly controlled by OsTCP5. However, the treatments of the *pACT1::OsTCP5* lines and the wild type with 1 μM GR24 did not affect the mesocotyl lengths (**Supplementary Fig. S4**). This may be because *OsTCP5* expression was not affected in the wild type by the treatment with 1 μM GR24 (**Fig. 5A**) and thus a higher dose of GR24 is necessary to inhibit mesocotyl elongation significantly.

In pea, SL up-regulates expression of the class II *TCP* gene, *PsBRC1*, at axillary buds of the wild type and the SL-deficient *rms1* mutant, but not at axillary buds of the SL-insensitive *rms4* mutant. CK down-regulates the expression of *PsBRC1* in the wild type and SL-related mutants (*rms1* and *rms4*) (Braun et al. 2012). These results suggest that *PsBRC1* works as a common point in the SL–CK branching regulatory pathway (Braun et al. 2012, Dun et al. 2012). SLs did not suppress the increased branching phenotype in the pea *Psbrc1* mutant (Braun et al. 2012) or rice *fc1* mutant (Minakuchi et al. 2010), whereas overexpression of *FC1/OsTB1* partially rescued the defects (in plant height, tiller number and stem thickness) observed in the rice SL-insensitive *d3* mutant (Minakuchi et al. 2010). The expression of *FC1/OsTB1* in wild-type rice is negatively regulated by CK (Minakuchi et al. 2010). Unlike the expression of *PsBRC1*, however, the *FC1/OsTB1* expression is not altered by SL treatment (Minakuchi et al. 2010), and thus further studies are needed to elucidate the molecular basis for the interaction between SL and *FC1/OsTB1* function. Taken together, these results suggest that SL and CK control bud outgrowth in many plant species by regulating the expression of *TB1*-related *TCP* genes (e.g. *PsBRC1* and *FC1/OsTB1*) and control mesocotyl elongation in rice by regulating the expression of *TCP* genes (e.g. *OsTCP5*).

In conclusion, we found that endogenous SLs in wild-type rice reduce the sensitivity of the mesocotyl to CK, thereby derepressing the expression of *OsTCP5*. The increased level of OsTCP5 protein, which negatively regulates cell division in the mesocotyl, may contribute to a shorter mesocotyl in darkness. Although OsTCP5 has a role in SL- and CK-controlled mesocotyl elongation in darkness, further studies are needed to determine whether it is the main factor.

Materials and Methods

Plant materials and growth conditions

The rice (*Oryza sativa*) tillering dwarf mutants, *d10-1* and *d14-1*, and their wild type (cv. Shiokari) were used as plant materials (Kinoshita and Takahashi 1991, Ishikawa et al. 2005, Arite et al. 2007, Arite et al. 2009). The transgenic *pACT1::OsTCP5* lines (see Construct for transgenic *pACT1::OsTCP5* lines), the tillering dwarf mutant *d10-2* and their background cultivar 'Nipponbare' also were used in this study.

Dehusked rice caryopses were sterilized in a 0.5% (v/v) sodium hypochlorite solution for 1 h. After washing with deionized water, seeds were kept in the water for 10 h at 4°C in darkness, and then seeds were sown on separate 0.7% (w/v) agar plates. The agar plates were placed in dark boxes made of black cardboard (Haga and Iino 2004) and then covered with black cloths. The boxes were put in the growth chamber, and the seeds were germinated and grown at 28°C under conditions of complete darkness.

RNA extraction

Dark-grown seedlings were fixed in 100% acetone (on ice) for 1 h in a dark room and subjected to a vacuum on ice for 5 min. This fixation was repeated twice, with replacement of the fixative solution with fresh solution in darkness. The mesocotyls were then cut off under a stereo-microscope and frozen in liquid nitrogen. Total RNA was extracted from the frozen fixed tissues by using an RNeasy Plant Mini Kit (Qiagen).

qRT–PCR

For qRT–PCR, 0.1 μg of total RNA extracted from the mesocotyls was used as a template. Transcript levels were measured by qRT–PCR using a LightCycler (Roche Diagnostics) and QuantiTect SYBR Green RT-PCR kit (Qiagen) in accordance with the manufacturers' protocols. A cDNA clone of each gene was used to draw standard curves for quantification. Primer sequences used to amplify the transcripts are shown in **Supplementary Table S1**. Note that *OsRR9* and *OsRR10* possess >99% identity within the coding region and flanking sequences (Jain et al. 2006) so that primer sets specific to *OsRR9* and *OsRR10* could not be designed. All transcript levels were normalized to transcript levels of the *Ubiquitin* gene as a control. qRT–PCR was performed using total RNA from three biological replicates. Data are expressed as the means ± SD of six separate experiments.

Measurement of cytokinin levels

Rice seeds were germinated and grown on separate 0.7% (w/v) agar plates at 28°C under dark conditions. After 4 d, the mesocotyls were harvested under a green safety light immediately after opening the dark box. CK in the mesocotyls was extracted and analyzed by using liquid chromatography–electrospray–tandem mass spectrometry (LC-MS/MS) as described by Katsumata et al. (2011). Endogenous concentrations of CK were calculated as described by Kojima et al. (2009).

Hormone and chemical treatments

GR24 was synthesized as described previously (Umehara et al. 2008). For SL treatment, 0.7% (w/v) agar was melted by autoclaving, and then GR24 (100 mM; dissolved in 100% acetone)

was added to the melted agar (~50°C) to prepare agar plates with 1 μM GR24 (final concentration) [0.001% (v/v) acetone was contained in each plate]. Sterilized seeds were placed on the agar plates with 1 μM GR24 and were germinated and grown at 28°C under complete darkness. For negative controls, agar plates with 0.001% (v/v) acetone were used.

For CK and CPPU treatment, 10 mM kinetin and 100 mM CPPU (dissolved in 100% acetone) were added to the melted agar as described above. Agar plates with 0.5, 1, 5 or 100 μM kinetin (final concentration) or 10 μM CPPU [final concentration; 0.001% (v/v) acetone was contained in each plate] were used in this experiment. For double treatment, kinetin was added to melted agar together with GR24.

Construct for transgenic *pACT1::OsTCP5* lines

The DNA sequence of the reading frame of a full-length *OsTCP5* cDNA (accession No. AK071964) was amplified using primers AK071964-Fw (5′-ggggacaagtttgtacaaaaaagcaggctccTCGAGGAGGCGTTGATAAGGATGAT-3′) and AK071964-Rv (5′-ggggaccactttgtacaagaaagctgggtccGCTTCCTGTCTGATCATGGAAAGCT-3′), in which the lower case sequences were necessary for the Gateway BP reaction (Hartley et al. 2000), by PCR, and the resulting PCR product was cloned by BP reaction into a Gateway entry vector, pDONR207 (Invitrogen). Subsequently, *OsTCP5* cDNA was transferred by LR reaction into Gateway destination vectors, which are derivatives of the pSMAHdN636L-GateA binary vector (Hakata et al. 2010) pSMAHdN638GW, for overexpression analysis. In the resulting expression construct, a rice *ACTIN1* promoter directed *OsTCP5* cDNA expression. The recombinant plasmids were transformed into a rice cultivar, Nipponbare, via *Agrobacterium tumefaciens* strain EHA105, according to Toki et al. (2006) to obtain *pACT1::OsTCP5* lines.

Measurement of cell length and cell numbers in vertical sections of mesocotyls

The mesocotyls of six dark-grown 4-day-old seedlings from the wild type, *d10-2* mutant and the *pACT1::OsTCP5-1* and *pACT1::OsTCP5-2* lines were embedded in 5% (w/v) agar and cut in 20–25 μm thick vertical sections with a vibratome (Leica VT1200S, Leica Microsystems). The sections were observed with a fluorescence microscope (BX60, Olympus Corporation) and photographed with a CCD camera (Dp70, Olympus Corporation). The lengths of 50 cells in the mesocotyl (a total of 300 cells for each mesocotyl) were measured.

The cells in the outer layers (including the epidermis) of the mesocotyls from the coleoptilar node to the basal part of the seminal root in vertical sections were counted under a microscope ($n = 6$ for each line of rice).

Statistical analysis

For statistical analyses, one-way analysis of variance (ANOVA) and the post-hoc Tukey test were performed using SPSS Statistics Version 19 (IBM software, Inc.; www-01.ibm.com/software/).

Supplementary data

Supplementary data are available at PCP online.

Funding

This work was supported by the Ministry of Education, Culture, Sports, Science, and Technology of Japan [grants-in-aid to M.N.]; the Ministry of Agriculture, Forestry, and Fisheries of Japan [Genomics for Agricultural Innovation (grant No. AMR0001) to H.I.].

Disclosures

The authors have no conflicts of interest to declare.

Acknowledgments

We thank Drs. Moritoshi Iino and Ken Haga for instructions on how to grow rice seedlings under completely dark conditions. We thank Drs. Mingfang Zhang, Masahiko Maekawa, Yasuo Nagato, Hitoshi Sakakibara, Shin-ichi Arimura, Hirokazu Takahashi, Shunsaku Nishiuchi, Sae Shimizu-Sato, Yoshiaki Inukai and Yutaka Sato for stimulating discussions. We thank Ms. Ritsuko Motoyama for help in the microarray analysis, Ms. Yumiko Takebayashi for help in the CK measurement, and Ms. Keiko Iida-Okada and Mr. Akihiko Horikawa for help in the generation of transgenic rice.

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