Cytokinin Activity of cis-Zeatin and Phenotypic Alterations Induced by Overexpression of Putative cis-Zeatin-O-glucosyltransferase in Rice

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cis-Zeatin (cZ) is generally regarded as a cytokinin with little or no activity, compared with the highly active trans-zeatin (tZ). Although recent studies suggested possible roles for cZ, its physiological significance remains unclear. In our studies with rice (Oryza sativa), cZ inhibited seminal root elongation and up-regulated cytokinin-inducible genes, and its activities were comparable to those of tZ. Tracer experiments showed that exogenously supplied cZ-riboside was mainly converted into cZ derivatives but scarcely into tZ derivatives, indicating that isomerizations of cZ derivatives into tZ derivatives are a minor pathway in rice cytokinin metabolism. We identified three putative cZ-O-glucosyltransferases (cZOGT1, cZOGT2, and cZOGT3) in rice. The cZOGTs preferentially catalyzed O-glucosylation of cZ and cZ-riboside rather than tZ and tZ-riboside in vitro. Transgenic rice lines ectopically overexpressing the cZOGT1 and cZOGT2 genes exhibited short-shoot phenotypes, delay of leaf senescence, and decrease in crown root number, while cZOGT3 overexpression lines did not show shortened shoots. These results propose that cZ activity has a physiological impact on the growth and development of rice.

Cytokinins, a class of phytohormones, are involved in the regulation of various biological processes, including organogenesis (Kurakawa et al., 2007; Pernisová et al., 2009; Marhavý et al., 2011), senescence (Gan and Amasino, 1995; Kim et al., 2006), and nutrient responses (Takei et al., 2004a; Sakakibara et al., 2006; Hirose et al., 2008; Ruffel et al., 2011). Natural cytokinins detected in plants are N5-substituted adenine derivatives and are structurally classified into isoprenoid cytokinins or aromatic cytokinins carrying an isoprene-derived or aromatic N6 side chain, respectively (Mok and Mok, 2001; Sakakibara et al., 2006). Both groups of cytokinins include members with minor modifications of the N6 side chain, such as hydroxylations. Isoprenoid cytokinins include N6-(5'-isopentenyl)adenine (iP), trans-zeatin (tZ), dihydrozeatin, and cis-zeatin (cZ), whereas aromatic cytokinins include 6-benzyladenine, ortho-topolin (oT), and meta-topolin. Isoprenoid cytokinins have been detected in all plant species examined so far, while aromatic cytokinins have been found only in a subset of these (Strnad, 1997; Sakakibara, 2006).

The initial products of isoprenoid cytokinin biosynthesis in plants are iP-ribotides formed by adenosine phosphate-isopentenylationtransferase (Kakimoto, 2001; Takei et al., 2001). After transhydroxylation of the prenyl side chain (Takei et al., 2004b), the tZ- and iP-ribotides are converted into the active free-base forms tZ and iP, respectively (Kurakawa et al., 2007; Kuroha et al., 2009; Tokunaga et al., 2012). The cZ biosynthesis pathway has not been fully elucidated, but a major step in Arabidopsis (Arabidopsis thaliana) is the release of precursors in the course of tRNA degradation (Miyawaki et al., 2006). For inactivation, the free-base cytokinins may be degraded by cytokinin oxidase (Houbé-Hérin et al., 1999; Werner et al., 2001; Bartrina et al., 2011) or conjugated by glucosyltransferases (Dixon et al., 1989; Martin et al., 1999, 2001; Mok et al., 2000; Mok and Mok, 2001; Haberer and Kieber, 2002; Veach et al., 2003; Hou et al., 2004; Wang et al., 2011) and converted to cytokinin-ribotides in the purine salvage pathway (Moffatt et al., 1991; Allen et al., 2002). A pathway to convert cZ or cZ-riboside (cZR) into tZ or tZ-riboside (tZR) was also proposed following partial purification of a putative cis-trans-isomerase from endosperm of Phaseolus vulgaris (Bassil et al., 1993).

The relationship between side chain variation and activity has been investigated by classical bioassays and the characterization of cytokinin signaling components. For instance, in callus growth assays using tobacco (Nicotiana tabacum) and Phaseolus lunatus, tZ showed the highest activity followed by iP and cZ (Leonard et al., 1971; Schmitz et al., 1972; Mok et al., 1978). In Arabidopsis, the promoter activity of the
cytokinin-inducible ARABIDOPSIS RESPONSE REGULATOR5 gene was efficiently up-regulated by lower concentrations of tZ than of cZ (Gajdošová et al., 2011). The first identified cytokinin receptor, CRE1/AHK4, complemented a yeast snl1 mutant lacking an endogenous osmosensing histidine kinase (HK) in the presence of iP and tZ but not cZ (Inoue et al., 2001). Thus, while tZ is considered a highly active cytokinin and has been the main focus of cytokinin research, cZ is regarded as a derivative of low activity.

However, there are several lines of evidence suggesting that cZ could be an active cytokinin in maize (Zea mays), which contains abundant cZ derivatives (Veach et al., 2003). For instance, two maize cZ-O-glucosyltransferases (cZOGTs), cisZOG1 and cis-ZOG2, preferentially catalyzed O-glucosylation in the N6 side chain of cZ rather than tZ using UDP-Glc as a Glc donor in vitro (Martin et al., 2001; Veach et al., 2003), although P. lunatus ZOG1 specifically catalyzed tZ-O-glucosylation (Dixon et al., 1989; Martin et al., 1999). Cytokinin O-glucosides have been assumed to represent reversibly inactivated storage forms (Pineda Rodó et al., 2008). Thus, the discovery of cisZOGs raises the possibility that cZ derivatives may play a more important role in cytokinin homeostasis than previously recognized (Martin et al., 2001). Furthermore, in a bacterial system, the maize cytokinin receptor ZmHK1 responded to cZ and tZ with comparable affinities (Yonekura-Sakakibara et al., 2004; Lomin et al., 2011). cZ and its derivatives are abundant not only in maize but also in other species, including chickpea (Cicer arietinum; Emery et al., 1998) and rice (Oryza sativa; Takagi et al., 1985, 1989; Kojima et al., 2009). In addition, a recent comprehensive screen showed that higher levels of cZ than tZ derivatives can be found in species across the complete evolutionary tree of land plants (Gajdošová et al., 2011). However, the physiological significance of cZ in plants is still poorly understood.

In this study, we demonstrate that cZ itself can act as an active cytokinin in rice in the physiological concentration range. We identified rice genes encoding cisZOGs (cZOGTs) and characterized transgenic rice plants overexpressing the cZOGT genes to gain further insights into the role of cZ. The transgenic plants exhibited developmental phenotypes with modified cytokinin profiles. In summary, we suggest that cZ is an active cytokinin playing a role in normal growth and development in rice.

RESULTS

Cytokinin Activity of Exogenously Supplied cZ in Rice

Exogenously supplied cytokinins inhibit root elongation via cytokinin receptors in a dose-dependent manner (Inoue et al., 2001; Higuchi et al., 2004). To test whether cZ is an active cytokinin in rice, bioactivities of cZ and tZ were compared in root growth assays using Arabidopsis and rice seedlings. In Arabidopsis, the inhibitory effect of cZ was significantly weaker than that of tZ above 10 nM (Fig. 1A). On the other hand, the effects of the two inhibitors were comparable in rice (Fig. 1B). This result suggested that exogenously supplied cZ has cytokinin activity, at least in long-term treatments.

To further examine the cytokinin activity of cZ in rice, mRNA accumulation of type A response regulator genes (OsRRs; Ito and Kurata, 2006; Jain et al., 2006; Pareek et al., 2006) was analyzed in seedling roots. Quantitative reverse transcription (qRT)-PCR analysis showed that the levels of OsRR1, OsRR2, OsRR6, and OsRR9/10 mRNA were significantly increased within 15 min after exposure to 100 nM cZ or 100 nM tZ (Fig. 2). This immediate response of OsRRs indicated that cZ also exhibits cytokinin activity in the short term.

Isomerization of cZ and tZ Derivatives in Rice Seedlings

Since isomerization from cZ or cZR to tZ or tZR was proposed to occur (Bassil et al., 1993), the possible conversion of cZ to tZ should be considered when evaluating cytokinin activity. Therefore, we carried out tracer experiments using [1013C,515N]cZR and [1013C,515N]tZR (denoted cZR(+15) and tZR(+15), respectively, hereafter). In roots fed with cZR(+15), cZ-O-glucoside (cZOG) was the major derivative containing the isotope labels, followed by cZ-ribotides-O-glucoside (cZRPsoG), cZ, cZ-O-glucoside (cZOG), cZ, and cZ-ribotides (cZRPs) at 1 h after feeding (Fig. 3). Although isotope labels were detected also in tZ-ribotides (tZRPts), tZR, tZ, tZ-O-glucoside (tZOG), and tZ-O-glucoside (tZOG), cZ derivatives represented 99.1% of all cytokinins derived from cZR(+15). On the other hand, in roots fed with tZR(+15), tZRPts were the major derivatives containing isotope labels, followed by tZ, tZ-9-N-glucoside, tZR, and tZRPts-O-glucoside.
Label was also detected in cZRPsOG and cZROG, but more than 99.9% of all cytokinins derived from tZR(+15) were tZ derivatives. Similar results were obtained from shoots fed with cZR(+15) and tZR(+15): cZ derivatives and tZ derivatives amounted to 98.8% and 99.1% of all cytokinins derived from cZR(+15) and tZR(+15), respectively. When we monitored the isotope labels until 24 h after feeding, the proportions remained essentially unchanged (Supplemental Tables S1 and S2). These results suggested that isomerization, if any occurs, is negligible compared with other reactions of cytokinin metabolism such as glucosylation and phosphorylation. Furthermore, isomerization can be neglected when rice seedlings are treated with zeatin derivatives at concentrations in the micromolar range for short periods. In combination with the results of the expression analysis of OsRR genes (Fig. 2), these findings led us to conclude that cZ functions as an active cytokinin in rice roots.

**In Silico Exploration of Candidate Rice Genes Encoding Putative cZOGTs**

BLAST searches in rice genome databases (the Rice Annotation Project Database [RAP-DB; http://rapdb.dna.affrc.go.jp/] and the Rice Genome Annotation Project [http://rice.plantbiology.msu.edu/]) using amino acid sequences of maize cisZOGs as queries revealed six nuclear genes (RAP-DB identifiers Os04g0556400, Os04g0556500, Os04g0556600, Os04g0556200, Os04g0565400, and Os07g0660500) potentially encoding cZOGTs. All of the putative cZOGT genes seemed to lack introns. The complete predicted amino acid sequences for the cZOGT genes were highly similar to maize cisZOGs (Supplemental Fig. S1). A C-terminal...
conserved glycosyltransferase domain was predicted by National Center for Biotechnology Information Conserved Domain Search analysis (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi; Supplemental Fig. S1). However, the protein encoded by Os04g0556400 seemed to lack glycosyltransferase activity due to a stop codon in the glycosyltransferase domain (Supplemental Fig. S1). All of the putative cZOGTs have been classified into the UDP-glucuronosyltransferase (UGT) family (GT1) in the Rice GT Database (http://ricephylogenomics.ucdavis.edu/cellwalls/gt/; Cao et al., 2008). We analyzed sequence similarities using the entire amino acid sequences of the rice putative cZOGTs, maize cisZOGs, and P. lunatus ZOG1 (Martin et al., 1999). The maize cisZOGs were nested within the rice proteins, but no cZOGT was closely related to P. lunatus ZOG1 (Fig. 4A). Os04g0556500 and Os04g0556600 were particularly closely related to the maize cisZOGs. When P. lunatus ZOG1 was used as the query in BLAST searches of rice databases, no counterparts were found.

In Vitro Activity of Rice cZOGTs

To examine O-glucosylation activity of the putative cZOGTs, poly-His tagged proteins were expressed in Escherichia coli cells, purified by metal-chelate affinity chromatography (Fig. 4B), and used for in vitro assays. Preliminary tests to determine activities were carried out using tZ, tZR, tZR 5'-monophosphate (tZRMP), cZ, cZR, cZR 5'-monophosphate (cZRMP), and oT as Glc acceptors (Fig. 4C). The recombinant proteins originating from Os04g0556500, Os04g0556600, and Os04g0565400, which were later named cZOGT1, cZOGT2, and cZOGT3, respectively, showed significant activities for cZ (Fig. 4C). Interestingly, cZOGT1 and cZOGT2 showed comparable activities for cZR and cZ, while cZOGT3 showed weaker activity for cZR than for cZ (Fig. 4C). cZOGT1 and cZOGT2 also exhibited notable activity for oT, but cZOGT3 did not (Fig. 4C). While cZOGT1 and cZOGT2 were weakly active for tZ but not tZR, cZOGT3 showed activities for both tZ and tZR (Fig. 4C). In other tested combinations of a recombinant protein and a Glc acceptor, no significant O-glycosylation was detected. Substrate specificity for sugar donors, requirements of Mg2+ and ATP, and optimum pH were also examined. The results suggested that cZOGTs catalyze O-glycosylation in a Mg2+-dependent and ATP-independent manner, specifically using UDP-Glc as a Glc donor, at optimum pH values of 6.0 for cZOGT1 and cZOGT3 and of 7.0 for cZOGT2 (Supplemental Fig. S2).

The kinetic parameters of the recombinant cZOGTs were determined for the substrates cZ and cZR (Table I; Supplemental Fig. S3). The Km values of rice cZOGTs for cZ were a little higher than the previously reported Km of maize cisZOGs (cisZOG1, 46 μM; cisZOG2, 96 μM; Veach et al., 2003). The affinity and specificity constant (Km/Ks) for cZR were similar to those for cZ in cZOGT1 and cZOGT2. The kinetic parameters of cZOGT3 for cZR and tZ could not be determined because of insufficient affinities.

Expression Profiles of cZOGT Genes in Rice

The accumulation of mRNAs of cZOGT genes was analyzed by qRT-PCR in various rice organs: total shoots and roots at the seedling stage, flowers before anthesis, panicle branches, top and basal parts of internode I, node I, blade and sheath of the flag leaf, and blades of leaves 2 and 4 counted down from the flag
leaf (LB-2 and LB-4, respectively) at the reproductive stage. The cZOGT1 mRNA was detected in all tested organs, with the maximum level found in LB-4 (Fig. 5A). cZOGT2 and cZOGT3 mRNAs were detected in almost all organs examined, with the highest levels in blades of flag leaves as well as LB-2 and LB-4 (Fig. 5, B and C).

To reveal tissue specificities of cZOGT expression, promoter-reporter analysis was carried out. DNA fragments of 3,052, 3,952, and 3,089 bp upstream of the starting Met codon of cZOGT1, cZOGT2, and cZOGT3 (designated cZOGT1pro, cZOGT2pro, and cZOGT3pro, respectively) were fused with the GUS coding sequence. The cZOGT1pro-GUS, cZOGT2pro-GUS, and cZOGT3pro-GUS constructs were transformed into rice, and the T1 seedlings were stained for GUS activity. Unfortunately, no GUS activity was detected in cZOGT3pro-GUS seedlings. In cZOGT1pro-GUS and cZOGT2pro-GUS seedlings, significant GUS activity was reproducibly detected in leaf blades (Fig. 6, A and D) but not in roots. In cZOGT1pro-GUS seedlings, more or less uniform GUS activity was observed in leaf blades, with a somewhat stronger signal at the laminar joints (Fig. 6, A and B). In cZOGT2pro-GUS seedlings, GUS activity was stronger in the leaf tips and disappeared toward the laminar joints (Fig. 6, D and E). In cZOGT2pro-GUS but not in cZOGT1pro-GUS seedlings, GUS activity was detected also in vascular bundles of coleoptiles (Fig. 6, C and F). To analyze the tissue distribution of the promoter activities, cross-sections of fully expanded leaf blades were prepared from mature plants and stained for GUS activity. Tissues in which cZOGT1pro-GUS was active could not be specified because the signals were too weak. In cZOGT2pro-GUS plants, however, GUS activity appeared localized to phloem parenchyma cells of large and small vascular bundles (Fig. 6, G and H).

### Visible Phenotypes of Transgenic Rice Overexpressing cZOGT Genes

We had demonstrated that cZOGT1, cZOGT2, and cZOGT3 preferentially catalyzed O-glucosylation of cZ and cZR rather than tZ in vitro and that cZOGT1 and cZOGT2 also catalyzed O-glucosylation of tT (Fig. 4C). However, we had not detected tT, tT-riboside, and their O-glucosides in any sample (data not shown). In order to better understand the physiological significance of these findings, we perturbed cZ metabolism by generating transgenic rice in which cZOGT genes were ectopically overexpressed under the control of a rice actin promoter (McElroy et al., Table I. Enzymatic properties of recombinant cZOGTs with various cytokinin substrates

| Symbol | Substrate | $K_m$ ($\mu$M) | $k_{cat}$ ($s^{-1}$) | $k_{cat}/K_m$ ($10^4$ s$^{-1}$) |
|--------|-----------|---------------|---------------------|-------------------------------|
| cZOGT1 | cZ        | 330           | 31.8               | 9.6 $\times$ 10$^4$          |
|        | cZR       | 95            | 14.5               | 1.5 $\times$ 10$^4$          |
| cZOGT2 | cZ        | 124           | 9.47               | 7.6 $\times$ 10$^4$          |
|        | cZR       | 177           | 14.5               | 8.2 $\times$ 10$^4$          |
| cZOGT3 | cZ        | 196           | 53.9               | 2.8 $\times$ 10$^5$          |

Figure 5. Accumulation of cZOGT1 (A), cZOGT2 (B), cZOGT3 (C), and Gpc1 (D) mRNAs in various organs of rice. Total RNAs were extracted from shoots (Sht) and roots (Rt) of 2-week-old seedlings and from flowers (Flw), panicle branches (PBr), top and basal parts of internode I (InNt and InNb, respectively), node I (Nd), leaf blades of flag leaves (FLB), and blades of leaves 2 and 4 below the flag leaf (LB-2 and LB-4, respectively) of older plants. Total RNAs were subjected to qRT-PCR using gene-specific primers. The mRNA levels are indicated as amounts per total RNA without normalization by an internal control gene. The Gpc1 gene encoding glyceraldehyde-3-phosphate dehydrogenase is presented as an unrelated gene used to control for expression in the tissues. Mean values ± SD of three plants are shown. ND, Not detected.

Visible Phenotypes of Transgenic Rice Overexpressing cZOGT Genes

We had demonstrated that cZOGT1, cZOGT2, and cZOGT3 preferentially catalyzed O-glucosylation of cZ and cZR rather than tZ in vitro and that cZOGT1 and cZOGT2 also catalyzed O-glucosylation of tT (Fig. 4C). However, we had not detected tT, tT-riboside, and their O-glucosides in any sample (data not shown). In order to better understand the physiological significance of these findings, we perturbed cZ metabolism by generating transgenic rice in which cZOGT genes were ectopically overexpressed under the control of a rice actin promoter (McElroy et al., Table I. Enzymatic properties of recombinant cZOGTs with various cytokinin substrates

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Because the transgenic lines for overexpression of cZOGT3 (cZOGT3-ox) were generated later than those for cZOGT1 and cZOGT2 (ZOGT1-ox and cZOGT2-ox, respectively), the cZOGT1-ox and cZOGT2-ox lines in the T2 or T3 generation and cZOGT3-ox lines in the T1 generation were compared with vector control (VC) lines in the corresponding generations.

Significantly reduced shoot growth was observed in cZOGT1-ox and cZOGT2-ox but not in cZOGT3-ox, although the transgenes were overexpressed in all lines (Fig. 7). Growth analysis in plants of the T2 generation showed that cZOGT1-ox and cZOGT2-ox plants remained smaller than the VCs throughout their lifetime while the plastochron was not altered (Fig. 8, A and B). This suggested that the phenotype was due to reduced elongation growth rather than a disturbed sequence of developmental events. For instance, the third leaf sheaths were approximately 30% and approximately 50% shorter in cZOGT1-ox and cZOGT2-ox, respectively, than in the VCs (Fig. 8, C and D). Epidermal cell lengths were similar in all plants (Fig. 8E), suggesting that decreased cell production was involved in causing the short-shoot phenotype. The short-shoot phenotype in cZOGT1-ox and cZOGT2-ox lines occurred independently of the offspring generation in T1, T2, and T3 plants (data not shown).

Mature cZOGT2-ox plants looked conspicuously dark green (Fig. 7A). Chlorophyll contents in leaf blades at various nodal positions remained high in aging cZOGT2-ox leaves at times when the corresponding VC began to die (Fig. 9). A similar trend was
observed in cZOGT1-ox (Fig. 9). These results suggested that leaf senescence was delayed in cZOGT1-ox and cZOGT2-ox. The delay of leaf senescence in cZOGT2-ox was further confirmed by an analysis of the levels of the large subunit of Rubisco in leaf crude extracts (Supplemental Fig. S5).

Seminal roots of cZOGT1-ox were longer than those of VC, and fewer crown roots developed in cZOGT1-ox and cZOGT2-ox than in VC (Supplemental Fig. S6).

Endogenous Cytokinins in Transgenic Rice Overexpressing cZOGT Genes

To investigate the effects of ectopic overexpression of cZOGT genes on the rice cytokinin profile, endogenous cytokinins were quantified in shoots of transgenic lines. For this analysis, the same plants were used from which the data presented as Figure 7, B and C, had been produced. Significant differences between cZOGT-ox and VC lines were found regarding the contents of cZRPsOG, cZROG, cZOG, tZROG, and tZOG but not with respect to the other cytokinins analyzed (Supplemental Table S3).

In all cZOGT1-ox and cZOGT2-ox lines examined, the contents of cZOG, cZRPsOG, tZOG, and tZROG were increased compared with those in the VC lines, although the differences were not always statistically significant (Fig. 10, A and C–E; Supplemental Table S3). On the other hand, in cZOGT3-ox lines, cZOG and cZRPsOG contents were unaffected, whereas tZOG and tZROG levels were dramatically increased (Fig. 10, A and C–E; Supplemental Table S3). No clear trends were observed in the tZROG contents of transgenic lines, even though a statistically significant difference was detected between a cZOGT3-ox line (3ox-9) and a VC line (VC-3; Fig. 10B; Supplemental Table S3).

DISCUSSION

Based on studies with Arabidopsis, cZ has generally been thought to be a cytokinin derivative with no or low activity (Inoue et al., 2001; Spíchal et al., 2004; Romanov et al., 2006; Stolz et al., 2011). Although recent studies with nonmodel plants suggested possible roles for cZ (Vyroubalová et al., 2009; Goggin et al., 2010; Lomin et al., 2011), its physiological significance was yet to be clarified. Our root growth assay clearly demonstrated comparable activities of cZ and tZ in the inhibition of rice seminal root elongation (Fig. 1). The capacity of cZ to induce ZmRR1 gene expression in maize cultured cells has been reported to resemble that of tZ (Yonekura-Sakakibara et al., 2004). Our results further indicated that cZ has tZ-like cytokinin activity not only in the induction of cytokinin-responsive genes but also in the control of root growth at physiological
concentration ranges. Thus, contrary to widespread assumptions, cZ plays a role as an active cytokinin in several aspects of rice growth and development. Previous biochemical studies on HK cytokinin receptors showed that the affinity to cZ was similar to that to tZ in maize HK (ZmHK1; Lomin et al., 2011), while cZ affinities were much lower than tZ affinities in Arabidopsis HKs (AHK2, AHK3, and CRE1/AHK4; Romanov et al., 2006; Stolz et al., 2011). Four cytokinin receptors have been identified in rice (OsHK3, OsHK4, OsHK5, and OsHK6; Ito and Kurata, 2006; Pareek et al., 2006; Du et al., 2007; Schaller et al., 2007). A recent study of ligand specificities of the OsHKs using a heterologous protoplast system showed that OsHK3 and OsHK4 have similar sensitivities to cZ and other cytokinins (Choi et al., 2012). These receptors may be involved in the cZ response in roots.

Our tracer experiments showed that cZR mostly was converted into O-glucosides, whereas tZR was initially converted into tZRPs and then into tZ-9-N-glucoside (Fig. 3; Supplemental Tables S1 and S2). However, rapid accumulation of cZRPs was also observed following the application of cZR, even though the cZRPs levels reached were lower than the tZRPs levels established following exposure to tZR (Supplemental Tables S1 and S2). Thus, tZR as well as cZR phosphorylation may occur, but the more rapid O-glucosylation may result in a lower accumulation of cZRPs. Interestingly, the accumulation of tZOG and tZROG after cZR application was comparable to that following tZR application, while the accumulation of tZ and tZMR after the application of cZR was less pronounced than after tZR application (Fig. 3). These results suggest that isomerization may occur in the O-glucoside forms. However, our data indicate that isomerizations between cZ derivatives and tZ derivatives represent a minor pathway in rice cytokinin metabolism. No cis-trans-isomerization was observed in tobacco BY-2 cells and oat (Avena sativa) leaf segments exposed to radioisotope-labeled tZ or cZ (Gajdošová et al., 2011). The isomerization might occur predominantly in specific organs or tissues such as the endosperm of immature seeds (Bassil et al., 1993).

We identified three cZOGTs as putative cZOGTs in rice. Enzymatic characterization showed that the cZOGTs catalyzed not only cZ-O-glucosylation but also cZR-O-glucosylation. Although maize cisZOG1 did not recognize cZR as a substrate in a previous study (Martin et al., 2001), recombinant cisZOG1 catalyzed cZR-O-glucosylation in our tests (data not shown). The activities of rice cZOGT1 and cZOGT2 as well as maize cisZOG1 directed at the riboside form separate these enzymes from P. lunatus ZOG1, which does not accept tZR as a substrate (Dixon et al., 1989); the biological relevance of this difference, however, remains unclear. Maize cisZOG1 has been reported to catalyze tO-glucosylation (Mok et al., 2005); similarly, cZOGT1 and cZOGT2, but not cZOGT3, catalyzed tO-glucosylation in vitro (Fig. 4C). However, tO and its derivatives including tO-glucoside were not detected in cZOGT1-ox and cZOGT2-ox plants (Supplemental Table S3).

The enzymatic properties and phylogenetic analysis (Fig. 4A) indicated that rice cZOGT1 and cZOGT2 are cognates of maize cisZOGs. However, expression patterns suggested that cZOGT2 might have a different physiological function from that of maize cisZOGs at the seedling stage: cZOGT2 was predominantly expressed in shoots (Figs. 5 and 6), whereas cisZOG genes were active mostly in roots (Veatch et al., 2003; Výroubalová et al., 2009).

The observed alterations of O-glucoside contents in cZOGT1-ox and cZOGT2-ox lines were consistent with the in vitro activities targeting cZ but not with those targeting cZR and cZRMP (Figs. 4 and 10); this suggested that phosphorylation of cZROG might occur in rice. Although adenosine kinases have been reported to be involved in the phosphorylation of iP-ribotides and tZR (Chen and Eckert, 1977; von Schwartzenberg et al., 1998; Kwade et al., 2005; Schoor et al., 2011), no activity directed at O-glucosides has been described. Despite the higher activity of cZOGT3 for cZ than for tZ and tZR in vitro (Fig. 4C), cZOG contents remained unchanged in cZOGT3-ox, whereas tZOG and tZROG levels dramatically increased (Fig. 10). There is an example of a plant UGT whose actual substrate in vivo is determined by the substrate specificity of interacting enzymes that catalyze upstream reactions (SORghum bicolor UGT85B1; Kahn et al., 1999; Nielsen et al., 2008). We can hypothesize that the cZOGTs interact with other proteins affecting the in vivo activity of cZOGTs.

The cZOGT1-ox and cZOGT2-ox plants exhibited conspicuous short-shoot phenotypes apparently caused by decreased cell numbers in affected organs (Fig. 8) and also a delay of leaf senescence (Fig. 9; Supplemental Fig. S5). These phenotypes are consistent with previous observations in cytokinin mutants (Riefler et al., 2006;
Bartrina et al., 2011) and transgenic maize over-expressing *P. lunatus* ZOG1 (Pineda Rodó et al., 2008), suggesting that the action of cytokinins is perturbed in cZOGT1-ox and cZOGT2-ox. It should be noted that increased tZ and tZR O-glucosylation does not seem sufficient to cause the short-shoot phenotype because this phenotype was not found in cZOGT3-ox, which showed significantly increased tZOG and tZROG contents (Fig. 10). On the other hand, alteration of cZOG and cZRPsOG contents appeared to parallel the occurrence of the short-shoot phenotype (Figs. 7 and 10), suggesting that increased cZ O-glucosylation might be involved in the causation of the phenotype.

However, several issues need to be considered carefully. First, the *K*\(_m\) values of rice cZOGTs for cZ and cZR (95–330 \(\mu M\); Table I) seemed high compared with the endogenous cZ and cZR contents (around 1–3 nM; calculated from Supplemental Table S3). Second, the cZ content was not significantly decreased in cZOGT1-ox and cZOGT2-ox (Supplemental Table S3), and the increase of cZOG contents was rather moderate (Fig. 10). These results raise the question of whether there is another, unidentified substrate of cZOGTs controlling the phenotype. On the other hand, *K*\(_m\) values in the micromolar range are not unusual for cytokinin metabolic enzymes, especially for glucosyltransferases. Examples are 50 \(\mu M\) in *P. lunatus* ZOG1 for tZ (Mok et al., 2005) and 70 to 240 \(\mu M\) in Arabidopsis N-glucosyltransferases UGT76C1 and UGT76C2 for iP and tZ (Hou et al., 2004). Moreover, substrate contents of cytokinin glucosyltransferases did not decrease in previously studied transgenic plants overexpressing *P. lunatus* ZOG1 (Pineda Rodó et al., 2008) and UGT76C2 (Wang et al., 2011), while glucosides increased. The moderate increase of cZOG in cZOGT1-ox and cZOGT2-ox may be explained by the natural abundance of cZOG in rice and/or the involvement of the O-glucoside in multiple metabolic pathways. However, we cannot exclude the possibility that cZOGTs are involved in additional mechanisms of plant growth and development. To fully clarify this issue, integrative metabolome and hormone analyses of the cZOGT-ox and cZOGT loss-of-function mutants will be required.

In conclusion, our evidence shown here suggests that cZ functions as an active cytokinin and that its metabolism including O-glucosylation possibly contributes to the control of cytokinin activity in rice.

![Figure 10](image-url)

**Figure 10.** Contents of cZOG and tZOG and their precursors in cZOGT-overexpressing rice. Cytokinin derivatives were extracted from shoots of transgenic plants overexpressing cZOGT1 (1ox-7), cZOGT2 (2ox-2 and 2ox-3), and cZOGT3 (3ox-7 and 3ox-9) and from VC plants (VC-1, VC-3, and VC-4) grown in soil for 21 d after sowing (the same samples as in Fig. 7, B and C, were used) and were analyzed by UPLC-MS/MS. The contents of O-glucosides of cZ (cZOG; A), cZR (cZR OG; B), cZR (cZRPsOG; C), tZ (tZOG; D), and tZR (tZROG; E) are presented as means ± SD of three plants. The generations of transgenic plants are indicated underneath the names of the lines. Different lowercase letters indicate statistically significant differences in the same generation as detected by the Tukey-Kramer test (*P* < 0.05) following one-way ANOVA. FW, Fresh weight; n.s., not significant.
Further investigation into cZ metabolism, including the elucidation of O-glucoside metabolism and the identification of novel genes involved in its biosynthesis, will promote a better understanding of the physiological significance of cZ.

**MATERIALS AND METHODS**

**Plant Materials**

Rice (*Oryza sativa* ‘Nipponbare’) and Arabidopsis (*Arabidopsis thaliana*) ecotype Columbia were used as wild-type rice and Arabidopsis, respectively. Rice plants were grown under three conditions: sterile culture on agar plates, hydroponic culture, and soil culture. For sterile cultures, sterilized rice seeds were sown and germinated on one-half-strength Murashige and Skoog medium containing 1% (w/v) Suc and 1% (w/v) agar at 30°C for 1 to 2 d in the dark. The seedlings were further grown for 5 d in a growth chamber with a 13-/11-h day/night regime at 28°C/24°C. For hydroponic culture, rice seeds were germinated in distilled water at 30°C for 2 d in the dark. The germinated rice seeds were sown in tap water adjusted to pH 5.5 using HCl and grown for 1 wk in a greenhouse with supplemental artificial light at 26°C/23°C. The seedlings were further grown for 1 wk in one-quarter-strength nutrient solution (Makino et al., 1983). For soil culture, germinated rice seeds were sown on a synthetic soil (Mitsui-Toatsu No.3) and grown for 3 to 4 wk in a greenhouse with irrigation and supplemental artificial light at 26°C/23°C. Then, the plants were transplanted with approximately 1 g of slow-release fertilizer per plant and grown in a greenhouse with irrigation and supplemental artificial light at 26°C/23°C until harvest of samples or the late grain-filling stage. Arabidopsis seedlings were aseptically cultured on one-half-strength Murashige and Skoog agar medium in a growth room with a 16-/8-h day/night regime at 22°C for 13 d.

**Oligonucleotide Primers**

Oligonucleotide primers used in this study are shown in Supplemental Table S4.

**Preparation of Total RNA and First-Strand cDNA and Quantitative PCR Analysis**

Total RNA was extracted from plant samples using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions. Single-stranded complementary DNA (cDNA) was prepared from total RNA using the SuperScript III First-Strand Synthesis System (Invitrogen) with oligo(dT)12-18 primers. Quantitative real-time PCR analysis was carried out with the StepOne Plus Real-Time PCR System (Applied Biosystems Japan) using gene-specific primers (Supplemental Table S1) and SYBR Premix Ex Taq II (Perfect Real Time; TaKaRa BIO). Purified plasmids containing the target sequences of the primers were used to obtain linear standard curves (r² > 0.9985), and the mRNA contents were determined quantitatively within the linear range.

**Assays for Cytokinin Activities**

To prepare stock solutions, cytokinins were dissolved in dimethyl sulfoxide (DMSO) and added to agar medium or nutrient solution. In all conditions, the final concentration of DMSO was controlled to 0.02% (v/v). Root growth assays were carried out using plants in sterile culture as described above. The primary root length of Arabidopsis or seminal root length of rice was manually measured in seedlings that were grown in the presence of tZ or cZ at 0, 1, 10, 100, 1,000, or 10,000 μM. Expression analysis of OsRR genes was carried out in plants grown in hydroponic culture. The 2-wk-old rice seedlings were pretreated with one-tenth-strength nutrient solution without nitrogen nutrient (1/10-N) for 1 d. Then, the seedlings were transferred to 1/10-N solution containing no cytokinin, 100 nM tZ, or 100 nM cZ. Whole roots were harvested at 15, 30, and 60 min after transfer. Roots harvested before transfer served as time-zero samples. Harvested roots were immediately frozen in liquid nitrogen and stored at −80°C until total RNA extraction. The expression levels of OsRR1, OsRR2, OsRR3, OsRR4, and OsRR10 (RAP-DB identifiers Os04g0442300, Os02g0575900, Os02g0803200, Os01g0952500, Os04g0673000, and Os11g0143300/Os12g01139400) were analyzed by qRT-PCR.

**Synthesis of Stable Isotope-Labeled tZR and cZR**

Stable isotope-labeled tZRMP was synthesized from [1013C,515N]AMP and 1-hydroxy-2-methyl-2-[(1S)-1,2-dimethyl-1,2-ethanediyl]-4-diphosphate with recombinant IZ secretion (Tsz) protein (Sugawara et al., 2008). Thus, cytokinins derived from this isotope-labeled tZRMP labeled in the uracil ring and ribosyl group; +15 have a molecular mass increased by 15 D. The tZRMP(+15) was dephosphorylated by alkaline phosphatase to form tZR(+15). To purify the tZR(+15), proteins were removed using MicroPrep-EZ columns (Amicon), and the resulting solution was loaded onto an octadecylsilyl reverse-phase column (Symmetry C18, 3.5 μm, 2.1 × 150 mm; Waters) in an HPLC system (Alliance 2695 system/2996 photodiode array detector; Waters). The products were separated at a flow rate of 1.0 mL min⁻¹ with linear gradients of solvent A (2% acetic acid) and solvent B (acetonitrile) set as follows: 0 min, 99% A + 1% B; 1 min, 99% A + 1% B; 3 min, 93% A + 7% B; 11 min, 90% A + 10% B; 12 min, 60% A + 40% B; 13 min, 40% A + 60% B; 22 min, 40% A + 60% B; 23 min, 99% A + 1% B. Fractions containing tZR (+15) were collected and dried. Isomerization from tZR(+15) to cZR(+15) was carried out by nonenzymatic conversion (Bassil et al., 1993). Seventy microliters of 3 μM tZR(15) in DMSO was mixed with 100 μL of 2-mercaptoethanol in a glass tube and incubated under artificial light (D400 [Toshiba], MLBIOC400-U [Mitsubishi/Osam]) at 28°C for 30 min. The resulting solution was dried and dissolved in 2% acetic acid. The cZR(+15) was purified as described above for tZR(+15). The quantity and purity of the synthesized tZR(15) and cZR(+15) were checked by ultraperformance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS analysis) (Supplemental Fig. S7) as described previously (Kojima et al., 2009).

**Tracer Experiments**

Hydroponically grown rice seedlings (2 wk old) were pretreated with 1/10-N solution for 1 d and then transferred into the 1/10-N solution containing 1 μM tZR(+15) or tZR(+15). Whole roots and shoots were separately harvested 1 h after transfer. The harvested roots and shoots were immediately frozen in liquid nitrogen and stored at −80°C until cytokinin analysis (Kojima et al., 2009). Monitored ion transitions for the quantification of labeled cytokinin species were as follows: 367.2 > 230.2 for cZR(+15), 362.2 > 230.2 for cZR(+10), 230.2 > 146.1 for tZR(+10), 529.2 > 230.2 for tZR(+15), 524.2 > 230.2 for tZR(+10), 529.2 > 230.2 for cZR(+15), 522.2 > 230.2 for tZR(15), and 392.2 > 230.2 for tZR(10), and 392.2 > 230.2 for cZR(10). Other conditions for MS/MS analysis were as described previously (Kojima et al., 2009; Tokunaga et al., 2012).

**Cloning of cDNAs, DNA Sequencing, and Phylogenetic Relationships**

BLAST searches in the RAP-DB (http://rapdb.dna.afrc.go.jp/) and the Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) Web sites were carried out using the deduced amino acid sequences of maize (Zea mays) cis-ZOG1 (AF318075) and cisZOG2 (AY082660) as queries. Predicted entire coding regions of Os04g0565200, Os04g0565400 (cZOGT3), and Os07g0660500 were amplified from first-strand cDNA by PCR and put into the pCR-Blunt II-TOPO vector (Invitrogen). DNA sequencing was carried out with a 3100 Genetic Analyzer (Applied Biosystems Japan). The phylogenetic tree was constructed by the neighbor-joining method with a bootstrap test using MEGA 4.0.2 software (http://www.megasoftware.net/).

**Preparation of Recombinant Proteins**

In vitro assays of cytokinin-O-glucosylation activities were performed with purified recombinant proteins expressed in *Escherichia coli*. The DNA fragments containing entire reading frames were inserted into the multicloning site of the pCold-I vector (TaKaRa BIO) to generate translational fusions with translation-enhanced elements and poly-His tags. The resulting expression plasmids were transformed into the E. coli strain BL21. Transfected BL21 cells were cultured and treated to express recombinant proteins according to the pCold-I manufacturer’s instructions. Crude soluble proteins were extracted from the BL21 cells by sonication in extraction buffer (50 mM NaH2PO4-NaOH, pH 8.0, 300 mM NaCl, 10 mM imidazole, and 1 mM dithiothreitol) containing a protease inhibitor cocktail (P8849; Sigma). The recombinant proteins were purified by nickel affinity chromatography using Ni-NTA Superflow (Qiagen).
according to the manufacturer’s instructions. Purified recombinant proteins were stored at −80°C in the presence of 1 mM diethanolamine and 15% (w/v) glycerol. Concentrations of recombinant proteins were determined with a Protein Assay kit (Bio-Rad) and by densitometry of SDS-polyacrylamide gels stained with Bio-safe Coomassie Brilliant Blue G-250 stain (Bio-Rad) using bovine serum albumin as a standard.

Enzyme Assay

The enzyme assay for cytokinin-O-glucosylation was based on a previous study of maize cisZOGs (Veach et al., 2003). One microgram of the purified recombinant protein was incubated in 198 μL of reaction premixure (50 mM Tris-acetate buffer, pH 5.0–7.0, or Tris-HCl, pH 7.0–10.0, 1.5 or 3.0 mM UDP-Glc, and 50 or 100 mM MgCl2) for at least 5 min at 30°C, and then reactions were started by the addition of 2 μL of cytokinin in DMSO as a substrate. To determine kinetic parameters, at least four different concentrations of a cytokinin substrate in the range of 10 to 500 μM were applied. Five minutes later, the reactions were stopped by the addition of 1 mL of cold ethanol, and the mixtures were incubated for at least 20 min at −30°C. After removal of the precipitates by centrifugation (20,400 × g, 10 min at 4°C), the supernatant was vacuum dried, dissolved in 60 μL of 2% (w/v) acetic acid, and loaded onto an octadecylsilan column (Merck, Supersphere RP-select B, 4 mm × 250 mm) in an HPLC system. Compounds were separated at a flow rate of 1.0 mL min−1 with linear gradients of solvent A (2% acetic acid) and solvent B (acetonitrile) as follows: 0 min, 99% A + 1% B; 1 min, 93% A + 7% B; 6 min, 93% A + 7% B; 14 min, 90% A + 10% B; 38 min, 60% A + 40% B; 40 min, 50% A + 50% B; 55 min, 99% A + 1% B.

Promoter-GUS Analysis

A Gateway cassette (Invitrogen) was inserted into a unique HindIII site upstream of the GUS coding sequence of the pCAMBIA1390-GUS vector (Hirose et al., 2005) using the In-Fusion Advantage PCR Cloning Kit (Clontech). The resulting vector was designated as pCAMBIA1390-GW-GUS. Genetic sequences containing putative promoter regions of cZOGT1, cZOGT2, and cZOGT3 (1,305, 2,152, and 2,117 bp from the translational initiation codon, respectively) were designated as cZOGT1pro, cZOGT2pro, and cZOGT3pro and were put into the entry vector pDONR221 (Invitrogen) using the Gateway BP Clonase II Enzyme Mix (Invitrogen). Subsequently, to construct cZOGT1pro-GUS, cZOGT2pro-GUS, and cZOGT3pro-GUS, the putative promoter sequences were put into the pCAMBIA1390-GW-GUS vector using the Gateway LR Clonase II Enzyme Mix (Invitrogen). Transgenic rice plants were generated by the Agrobacterium tumefaciens-mediated method (Hirose et al., 2005) using the A. tumefaciens strain EHA105. Detection of GUS activity and histochemical analysis were carried out as described previously (Hirose et al., 2005).

Generation and Characterization of Transgenic Plants Overexpressing cZOGT Genes in Rice

The binary vector pActnos/Hmz (Sentoku et al., 2000; Hirose et al., 2007) was used to prepare the constructs for overexpression of rice cZOGT genes. The entire coding regions of the cZOGT cDNAs were inserted into an Act1/Smal site located between an Act1 promoter (Zhang et al., 1991) and a NOS terminator. Transformation of rice was carried out using A. tumefaciens (Zheng et al., 2001) and a NOS terminator. Transformation of rice was carried out using A. tumefaciens as described above. The empty vector containing pActnos/Hmz served as a control. We generated four VC lines (VC-1, VC-3, VC-4, and VC-7), four cZOGT1-ox lines (1ox-1, 1ox-3, 1ox-4, and 1ox-7), four cZOGT2-ox lines (2ox-2, 2ox-3, 2ox-7, and 2ox-10), and four cZOGT3-ox lines (3ox-3, 3ox-7, 3ox-9, and 3ox-10); all of these were independent transgenic lines derived from different transformation events. The lengths of shoots and leaves were measured manually. To measure cell lengths of epidermal cells, seedlings were grown hydroponically, and 5 mm of the leaf sheaths was cut at their laminar joints. The leaf sheaths were stained with propidium iodide after fixation in 5% (v/v) formaldehyde solution containing 37% (v/v) formaldehyde, 5% (v/v) acetic acid, and 63% (v/v) ethanol. Propidium iodide fluorescence micrographs of epidermal cells were taken using a confocal laser-scanning microscope (FV1000; Olympus). Cell lengths were determined with ImageJ (http://rsweb.nih.gov/ij/). Relative chlorophyll contents were analyzed in a non-invasive manner using a portable chlorophyll meter (SPAD-502Plus; Konica Minolta). The relative contents of each leaf were determined by three measurements at the tip, middle, and basal parts of the leaf. Crude proteins were extracted from leaf blades in extraction buffer (50 mM NaH2PO4-NaOH, pH 8.0, 1 mM EDTA, 0.1% [v/v] 2-mercaptoethanol, 0.1% [v/v] Tween 20, 5% [v/v] glycerol, and 1% [v/v] proteinase inhibitor cocktail III from Calbiochem). The homogenate was centrifuged at 20,000g for 20 min at 4°C, and the resulting supernatant was used as the crude protein extract. The protein content was determined by the Protein Assay kit (Bio-Rad) using bovine serum albumin as a standard. Cytokinin was quantified with a Xeno TQS (Waters) tandem quadrupole mass spectrometer; conditions for MS/MS analysis were as described previously (Kojima et al., 2009).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Multiple alignment of deduced amino acid sequences of maize cisZOGs and putative rice cZOGTs.

Supplemental Figure S2. Preliminary tests to determine conditions for an enzymatic activity assay for rice cZOGTs.

Supplemental Figure S3. Plots of velocities of O-glucosylation catalyzed by cZOGT recombinant proteins against substrate concentrations.

Supplemental Figure S4. Overexpression of cZOGT genes in transgenic lines.

Supplemental Figure S5. Delay of leaf senescence in cZOGT2-overexpressing rice.

Supplemental Figure S6. Visible phenotypes in roots of cZOGT1- and cZOGT2-overexpressing rice at the seedling stage.

Supplemental Figure S7. UPLC/MS/MS analysis of synthesized [1013C,515N]tZR and [1013C,515N]cZ.

Supplemental Table S1. Time courses of cytokinin derivative contents of rice seedlings after application of [1013C,515N]tZR.

Supplemental Table S2. Time courses of cytokinin derivative contents of rice seedlings after application of [1013C,515N]cZ.

Supplemental Table S3. Cytokinin contents in shoots of cZOGT-overexpressing lines and VC lines.

Supplemental Table S4. Primers used in this study.

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