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Characterization of a novel cyclase-like gene family involved in controlling stress tolerance in rice

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ABSTRACT

A novel cyclase-like gene family (*CYL*) encodes proteins containing cyclase domain, but their functions are largely unknown. We report the systematic identification and characterization of *CYL* genes in the rice genome. Five putative CYL protein sequences (OsCYL1 to 4b) were identified. These sequences and other *CYL* homologs were classified into four subgroups based on phylogenetic analysis. Distinct diversification of these CYL proteins exists between plants and non-plants. The *CYL* family has conserved exon-intron structures, and the organizations of putative motifs in plants are specifically diverse. All *OsCYL* genes were expressed in a wide range of tissues or organs and were responsive to at least one of the abiotic stresses and hormone treatments applied. Protein OsCYL4a is targeted to the cell membrane. The overexpression of one stress-responsive gene *OsCYL4a* in rice resulted in decreased tolerance to salt, drought, cold, and oxidative stress. The expression levels of some abiotic stress-responsive factors, including H_2O_2-accumulating negative factors *DST* and *OsSKIPa* in *OsCYL4a*-overexpressing plants, were reduced compared with the wild type under normal condition and drought stress. These results suggest that rice *CYL* family may be functionally conserved polyketide cyclase, resulting in the rapid accumulation of reactive oxygen species to decrease tolerance to abiotic stresses.

Introduction

Various external signals including hormones do not enter the targeting cells, but specifically bind to the outer membrane receptor and form hormone-receptor complex (Urao et al., 2001; Guo et al., 2007). The hormone-receptor complex promotes the exchange between the intracellular guanosine triphosphate (GTP) and guanosine diphosphate (GDP) associated with G proteins to form GTP-G protein complex, which can activate cyclase for production of cyclic nucleotides as secondary signals. Well-documented cyclases include adenyl cyclase and guanyl cyclase, which catalyzes adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) and GTP to cyclic guanosine monophosphate (cGMP), respectively (Ma et al., 2009).

Adenyl cyclase, an important signaling enzyme identified originally in bacteria and motile algae, has been reported also in plants (Ruzvidzo et al., 2013). Several different types of adenyl cyclases have been characterized for the synthesis of cAMP. Adenyl cyclases consist of a large gene family. Some adenyl cyclases are soluble in the cytoplasm, located in the plasma membrane, and dependent in Ca^{2+} (Ma et al., 2009). Signals that directly activate or inhibit adenyl cyclase are usually mediated by specific types of G-proteins. The involvement of cAMP has been indicated in many plant-specific processes. For example, cAMP may be involved in stomatal closure because the activity of guard cell channels can be modified by cAMP-dependent phosphorylation, and some plant potassium channel proteins contain a cAMP-binding domain (Martinez-Atienza et al., 2007). Pollen tube growth is regulated by cAMP, and adenyl cyclase has been proposed to mediate

Abbreviations: ABA, abscisic acid; ADP, adenosine diphosphate; ATP, adenosine triphosphate; BR, brassinosteroid; CYL, cyclase-like; cAMP, cyclic adenosine monophosphate; cGMP, cyclic guanosine monophosphate; GA, gibberellin; GFP, green fluorescence protein; GTP, guanosine triphosphate; IAA, indole-3-aceticacid; JA, jasmonic acid; KT, kinetin; ROS, reactive oxygen species; SA, salicylic acid; WT, wild type.

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incompatibility between the stigma and pollen tube (Rato et al., 2004; Wu et al., 2011). Plant cell cycle and rhizoidal interactions with root hairs are accompanied by a change in cAMP concentration (Pislariu and Dickstein, 2007). Adenylate cyclase MAC1 and the catalytic subunit of cAMP-dependent protein kinase A (CPKA) are required for appressorium development and pathogenesis in the rice blast pathogen *Magnaporthe grisea* (Kulkarni and Dean, 2004). More information on the function of this multifunctional secondary messenger in plants can be anticipated in the future.

Cyclic GMP is an important secondary messenger in various biological processes. In plant cells, cGMP may participate in transducing the signals of fungal invasion (Vieira et al., 2009), red light signals mediated by phytochrome (Pislariu and Dickstein, 2007; Ulijasz et al., 2008), and gibberellin (GA) signals that regulate the synthesis of aleurone amylase (Teng et al., 2010). The tomato *aurea* mutant, which lacks phytochrome A, has been used to demonstrate the involvement of cGMP in phytochrome transduction (Szmidt-Jaworska et al., 2008).

Increasing evidence has suggested that other cyclases are also involved in diverse pathways in biosynthesis, development, and stress responses. A tocopherol-deficient mutant (*vte1*) isolated from *Arabidopsis thaliana* lacked all four tocopherol forms and was deficient in tocopherol cyclase activity (Porfirova et al., 2002). Transgenic tobacco plants overexpressing *VTE1* from *Arabidopsis* enhance the tolerance to drought stress (Liu et al., 2008). Delta-cadinene synthase (DCS) is a sesquiterpene cyclase that catalyzes the cyclization of farnesyl diphosphate in the first committed step of the biosynthesis of gossypol, which is a phytoalexin that protects the plant from bacterial and fungal pathogens (Gennadios et al., 2009). A novel lycopene epsilon-cyclase in maize is also involved in the perturbation of carotenoid biosynthesis (Bai et al., 2009). Moreover, lycopene beta-cyclase is responsible for a high level of beta-carotene accumulation in *Dunaliella salina* (Zhu et al., 2008). Some lycopene beta-cyclase, as a key factor in carotenogenesis and carotenoid biosynthesis-related gene expression in *D. salina*, are up-regulated in response to many stress conditions (Ramos et al., 2008). In addition, *LCYE* and *LCYB1* also mediated carrot root development. Allene-oxide cyclase from *Camptotheca acuminata* confers tolerance against low temperature and salt stress in tobacco and bacteria (Pi et al., 2009). Abscisic acid (ABA) activates adenosine diphosphate (ADP)-ribose cyclase, and cADPR induces a subset of ABA-responsive genes in *Arabidopsis* (Sanchez et al., 2004).

In addition to the cyclases with known substrates, some proteins are annotated as putative cyclases in the protein database. A protein family (PF04199) is considered as cyclase enzymes. The sequences of typical cyclases contain a signature motif HXGTHXDXPXH, which is replaced by QXXXQXDXXXH in some putative cyclases including those of the PF04199 family (Camara et al., 2008). The motif QXXXQXDXXXH in the putative cyclase protein 1r61 (accession no. P84132) from *Bacillus stearothermophilum* was elucidated (Maderova et al., unpublished). This motif comprises 45 to 55 residues and partially occupied Zn ions that are coordinated by H45 and H49 with E167, and by D51 with H155 and E167. This motif is even conserved exclusively, and may be a functionally important site. Some cyclase proteins, such as *Streptomyces peucetius* dpsY, are involved in antibiotic synthesis, and *trans*-dienelactone hydrolase from *Pseudomonas reinekei* MT1 is a novel zinc-dependent hydrolase (Camara et al., 2008).

However, no systematic identification and functional insight of the PF04199 family in a given plant species had been reported. In this study, we systematically analyzed the sequences of the cyclase-like (*OsCYL*) genes belonging to the PF04199 family in rice, and investigated their expression profiles in various tissues or organs under stress and hormone treatments. In addition, we investigated the phenotypic changes of transgenic plants overexpressing one member (*OsCYL4a*) in relation to the development or responses to abiotic stresses and hormone treatments. Our results suggested that the *OsCYL* family has an important function in response to environmental stress and hormonal pathways.

Materials and methods

Identification and sequence analysis of CYL *family*

Using the BLAST program, protein sequences of *Streptomyces peucetius* dpsY and *Pseudomonas reinekei trans*-DLH from the protein sequence database ExPASy (http://www.expasy.org/, Gasteiger et al., 2003) were used to search for homologous proteins in the databases of *Japonica* rice (The Institute for Genomic Research, TIGR, http://www.tigr.org, Yuan et al., 2005; Knowledge-Based Oryza Molecular Biological Encyclopedia, KOME, http://cdna01.dna.affrc.go.jp/cDNA, Kikuchi et al., 2003) and *Arabidopsis* (The *Arabidopsis* Information Resource, TAIR, http://www.arabidopsis.org, Huala et al., 2001). The Profile Midden Markov Models (PHMM) was used to identify new CYL sequences in the rice and *Arabidopsis* genomes. The HMM profile of cyclase domain (accession no. PF04199) was downloaded from the Pfam database (http://www.sanger.ac.uk/Software/Pfam; Bateman et al., 2004). All hits with expected values less than 1.0 were collected. We used a BLASTN search to determine the chromosome locations of *OsCYL* genes, the rice BAC/PAC clones to which they were mapped, and the intron-exon structures of *OsCYL* genes by mapping cDNAs to genomic sequence.

The gene structure display server was used to draw the gene structure schematic diagram (Guo et al., 2007). Multiple sequence alignment of CYL proteins was performed with CLUSTALX (Van de Graaff et al., 1982), and refined manually. Phylogenetic tree was reconstructed by the program MEGA version 4.0 (Ronquist and Huelsenbeck, 2003; Tamura et al., 2007) with conserved amino acids. The tree was edited with TreeView 1.5 (http://taxonomy.zoology.gla.ac.uk, Page, 1996).

Isolation of OsCYL4a *and rice transformation*

The primers (GCTCGATCAAAGTTGGGAAG/GGTGGATGCATCTGACTTGA) were designed to amplify the predicted full-length cDNA from the different tissues of Minghui 63 (*Oryza sativa* L. ssp. *indica*) for the *OsCYL4a* gene. An Ex-Taq DNA polymerase (Takara) was used in the PCR under the following cycling profile: 94 °C for 4 min; 30 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 1 min; and extension at 72 °C for 10 min. The amplified products were cloned into a pGEM-T vector (Promega, Madison, WI, USA). The products were sequenced from both ends using a BigDye Terminator sequencing ready kit (version 2.0 or 3.0) in an ABI PRISM 377 or 3730 sequencer (Applied Biosystems, Foster City, CA, USA) by the vector-border primers T7 and SP6 (Promega). The sequence-confirmed fragments of *OsCYL4a* were cut by *Kpn*I and *Bam*HI from pGEM-T clones, and ligated into the transformation vector pCAMBIA1301U under the control of the promoter of maize ubiquitin gene. The *Agrobacterium*-mediated transformation method was used to introduce the constructs into rice Zhonghua 11 (ZH11, *O. sativa* L. ssp. *japonica*, Hiei et al., 1994).

Plant growth, treatments, and measurements

The rice plants of ZH11 were grown under normal growth conditions and 2-weeks-old seedlings were treated with hormone treatments and abiotic stresses to measure the transcript level of the *OsCYL* genes. Hormone treatments were performed by spraying leaves with brassinosteroid (BR, 200 μM/L), kinetin (KT, 200 μM/L), GA (200 μM/L), ABA (200 μM/L), indole-3-aceticacid (IAA, 200 μM/L), jasmonic acid (JA, 200 μM/L), ethylene