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Constitutive expression and silencing of a novel seed specific calcium dependent protein kinase gene in rice reveals its role in grain filling

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ABSTRACT

Ca^{2+} sensor protein kinases are prevalent in most plant species including rice. They play diverse roles in plant signaling mechanism. Thirty one CDPK genes have been identified in rice and some are functionally characterized. In the present study, the newly identified rice CDPK gene *OsCPK31* was functionally validated by overexpression and silencing in Taipei 309 rice cultivar. Spikelets of overexpressing plants showed hard dough stage within 15 d after pollination (DAP) with rapid grain filling and early maturation. Scanning electron microscopy of endosperm during starch granule formation confirmed early grain filling. Further, seeds of overexpressing transgenic lines matured early (20–22 DAP) and the average number of maturity days reduced significantly. On the other hand, silencing lines showed more number of unfilled spikelet without any difference in maturity duration. It will be interesting to further decipher the role of *OsCPK31* in biological pathways associated with distribution of photosynthetic assimilates during grain filling stage.

Introduction

The calcium ion (Ca^{2+}) plays a significant central role as second messenger in various signal transduction pathways to control their metabolism and to adapt to their environment (Sanders et al., 2002; Hetherington and Brownlee, 2004; Dodd et al., 2010; Asano et al., 2012). In plants, changes in cytoplasmic Ca^{2+} concentration occurs in response to various signals, including phytohormones, abiotic stress such as drought, high and low temperature or light, biotic stress stimuli such as pathogen elicitors, symbiotic nodulation factors, altered protein phosphorylation and gene expression pattern (Knight and Knight, 2001; Dodd et al., 2010; Asano et al., 2012; Romeis and Herde, 2014). In plants, an array of Ca^{2+} sensors: calcium-dependent protein kinases (CDPKs), calmodulins and calmodulin-like proteins and calcineurin B-like proteins are reported to be involved in cell signaling and response to specific stimuli (Asano et al., 2012). CDPKs sense the changes in Ca^{2+} concentration in plant cells in response to environmental stimuli and translate these perceived signals into subsequent downstream signaling events to trigger response mechanisms/pathways (Boudsocq and Sheen, 2013). CDPKs comprise a large family of serine/threonine kinases in plants and protozoans but not in animals (Ludwig et al., 2004; Harper and Harmon, 2005; Nagamune and Sibley, 2006). CDPK proteins have four-domain structure consisting of a variable N-terminal domain, catalytic kinase domain, autoregulatory domain, and a calmodulin-like domain with four EF hand motifs. Ca^{2+} binding to the CDPK regulatory domain triggers an intra-molecular conformational change, in which an inactive protein kinase is converted to an active one and as a consequence subsequent signal transduction processes are initiated (Hrabak et al., 2003; Asano et al., 2012; Liese and Romeis, 2013).

Genome wide characterization of *Arabidopsis thaliana* predicted 34 CDPK encoding genes and many of them have been characterized to show distinct expression patterns during plant development (Cheng et al., 2002; Hrabak et al., 2003). Similarly, in wheat (*Triticum aestivum*), 20 CDPKs have been identified and most of these are involved in multiple signal transduction pathways (Li et al., 2008). Recently, a genome wide analyses of maize (*Zea mays*) and cotton (*Gossypium raimondii*) predicted about 40 and 41 CDPKs encoding genes exhibiting differential expression in different tissues and developmental or physiological stages (Kong et al., 2013; Liu et al., 2014). Expression studies of CDPK genes in wild growing grapevine (*Vitis amurensis*) showed 13 CDPK genes were actively expressed under osmotic and temperature stress treatment (Dubrovina et al., 2013). Similarly, in *Vitis vinifera*, 17 CDPK genes have been identified. Among them, a few VvCDPK genes expressed prevalently in pollen/stamen suggesting new functions with an important role in pollen development (Chen et al., 2013a). Some CDPKs display an isoform-specific expression pattern which

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is not only organ- or tissue-specific but also dependent on growth conditions (Hrabak, 2000). Overexpression of *AtCPK6* in *Arabidopsis* provided tolerance to salt/drought stress (Xu et al., 2010). Among the 34 CPK genes in *Arabidopsis*, 12 show evidence of significant expression in pollen (Pina et al., 2005). *AtCPK17* and *AtCPK34* act as a Ca^{2+} sensor-responder that functions as a key regulator of pollen tube tip growth (Myers et al., 2009). Similarly, the other studies showed that *AtCPK2* and *AtCPK20* were Ca^{2+} dependent which regulates pollen tube growth (Gutermuth et al., 2013). Expression of novel tomato CDPK gene (*LeCPK2*) was reported to be involved in plant growth, development, abiotic and biotic stresses (Chang et al., 2009). A stress-responsive CDPK gene, *ZoCDPK1* was isolated from a stress cDNA generated from ginger (*Zingiber officinale*). The gene was abundantly expressed in rhizome and also induced by salt, drought and Jasmonic acid (JA) treatment. Overexpression of *ZoCDPK1* in tobacco conferred tolerance to salinity and drought stresses without any yield penalty and up-regulation of stress-related genes *RD21A* and *ERD1* (Vivek et al., 2013). A novel CDPK gene (*PeCPK10*) was cloned from *Populus euphratica* and the gene was induced by abiotic stresses: salt, cold and drought stress. Overexpression of *PeCPK10* in *Arabidopsis* showed better drought and freezing tolerance than control plants. Further, several abscisic acid-responsive genes and stress-responsive genes were enhanced during stress treatment (Chen et al., 2013b). Though, various CDPKs from different plant species were identified and reported, their biological functions in plant growth and development has not yet been completely understood.

In rice, 31 CDPK genes have been identified and their putative functions have been characterized through expression profiling under various stresses and phytohormone treatments (Ray et al., 2007; Ye et al., 2009). Majority of rice CDPK genes expressed in a tissue specific manner and induced under defined growth conditions (Wan et al., 2007). A few of the rice CDPK genes have been functionally validated by reverse genetics approach. Ectopic expression of OsCDPK7 (*OsCPK13*) gene in rice plants showed its involvement in response to cold and salt/drought stresses (Saijo et al., 2000). Functional characterization of OsCDPK13 (*OsCPK7*) protein revealed its up-regulation during cold and gibberellic acid treatment suggesting a possible role in cell elongation process and involvement in cold signal transduction pathways (Abbasi et al., 2004). Spatial and temporal expression analysis of the rice CDPK proteins*viz*., OsCDPK2 (*OsCPK19*), OsCDPK11, and SPK (*OsCPK23*) indicated increased level of mRNA and protein during seed development (Kawasaki et al., 1993; Frattini et al., 1999). The *OsCPK23* (*spk*) gene was reported to be involved in modulation of biosynthetic pathways of storage products in the seed. Functional studies of this gene by antisense *spk* transformants showed spikelets with watery seeds and large amount of sucrose instead of starch. *OsCPK23* was implicated in the activation of sucrose synthase enzyme which supplies the substrates for the biosynthesis of storage products (Asano et al., 2002). Overexpression of *OsCPK19* gene severely impaired seed development in rice (Morello et al., 2000). Constitutive expression of rice *OsCPK10* in *Arabidopsis* and rice showed increased the resistance to infection with *Pseudomonas syringae* pv *tomato* and to blast fungus *Magnaporthe grisea* respectively. The result indicated that the *OsCPK10* plays a crucial role in plant defense and it may regulate both salicylic acid and JA defense pathways (Fu et al., 2013). Recently, *OsCPK9* was demonstrated as a positive regulator of abiotic stress tolerance, spikelet fertility, and ABA sensitivity (Wei et al., 2014).

Genome wide expression analysis of rice CDPK gene using Gene chip revealed a number of genes that express specifically or are common to the developmental stages. The gene *OsCPK31* showed characteristic domains found in CDPK proteins along with four EF hands and was included in CDPK gene family (Ray et al., 2007). *OsCPK31* belongs to an independent clade of CDPK family (Ray et al., 2007; Ye et al., 2009; Campos-Soriano et al., 2011). The expression profiling of rice *CPKs* with 27 different rice tissues suggested that the *OsCPK31* preferentially expressed in endosperm and could have an important role in seed development (Ye et al., 2009). The present work was undertaken to characterize the *OsCPK31* gene of rice through overexpression and silencing approach.

Materials and methods

Plasmid constructs and seed material

Seeds of japonica rice, *Oryza sativa* (L.) cv. Taipei 309, available at the Directorate of Rice Research (DRR), Hyderabad, India were used for transformation. The binary vectors and details of constructs were kindly provided by Prof. A.K. Tyagi, NIPGR, India. The overexpression construct (pB4NU-CK31-Ox) was made by isolating a rice cDNA clone of 3373 bp (*OsCPK31* gene) from indica rice IR64 and then cloned into the vector pCAMBIA1301. The gene was driven by maize *ubiquitin* promoter and *nos* terminator. It also contains *gusA* reporter gene and *hpt* as plant selection marker. The pANDA-CK31-Si (RNAi) vector contains the hairpin of *OsCPK31* gene for its silencing. The PCR-derived 334 bp (Forward: 5′-TTCTACAACCTGCTGCGT-3; Reverse: 5′-ATATGGATTGAGCGGCTG-3′) fragment was inserted into two regions flanked by two recombination sites (*attB1* and *attB2*) in opposite directions and the gus linker sequence was flanked by the two inverted repeats. The gene is driven by maize *ubiquitin* promoter with first intron and splicing acceptor site and *nos* terminator gene. The detailed construction of pANDA-RNAi binary vector was described (Miki and Shimamoto, 2004). The RNAi vector harbored kanamycin as bacterial selection marker and hygromycin as plant selection marker. The recombinant vector was maintained in *Escherichia coli* strain *DH5α* and mobilized into *Agrobacterium* strain EHA105 by triparental mating method (Lichtenstein and Draper, 1985).

Rice transformation and molecular characterization

Rice (*O. sativa* (L.) cv. Taipei 309) transformation was carried out through *Agrobacterium* using scutellum-derived calli with two different constructs namely pB4NU-CK31-Ox (overexpression gene cassette) and pANDA-CK31-Si (silencing gene cassette) as per the protocol developed in our laboratory. Mature seeds were dehulled manually and sterilized with 70% ethanol for 2 min followed by 0.1% (w/v) $HgCl_2$ for 4 min followed by three washing with autoclaved distilled water. After blot drying the seeds were inoculated on autoclaved callus induction medium containing MS basal salts (Murashige and Skoog, 1962) supplemented with 2 mg/L 2,4-D, 0.5 mg/L kinetin, 500 mg/L L-proline, 500 mg/L casein hydrolysate, 30 g/L maltose, solidified with 0.3% phytagel and maintained the culture under dark at 26 ± 2 °C for 21 d. Twenty-one-day old embryogenic calli were infected for 15 min with 0.5 OD_{600} of *Agrobacterium* strain (EHA105) culture which was previously suspended in MS suspension medium (MS basal salt + 2 mg/L 2.4-D + 68.5 g/L sucrose + 36 g/L glucose + 200 μM acetosyringone (AS) + pH 5.2) and co-cultivated on MS-AS solid medium (MS suspension medium + 200 μM AS + pH 5.2 + 0.3% phytagel) for 3 d in dark at 26 ± 2 °C. The washed calli were maintained in selection medium (callus induction medium) supplemented with 50 mg/L hygromycin B (Sigma–Aldrich, USA) for 15 d at dark. After the third selection, resistant calli were transferred to regeneration medium (MS basal salt, 2 mg/L kinetin, 0.3 mg/L NAA, 30 g/L sucrose, 30 g/L D-sorbitol and 4% phytagel) supplemented with 25 mg/L of hygromcyin. The regenerated plantlets were maintained in rooting medium (1/2 MS basal salt + 15 g/L sucrose + 0.4% phytagel) and then