Overexpression of a rice long-chain base kinase gene OsLCBK1 in tobacco improves oxidative stress tolerance

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Abstract Sphingolipids and their metabolites including long-chain bases (LCBs) and long-chain base 1-phosphates (LCBPs) have been shown to be involved in regulation of various aspects of biological processes in plants. However, little is known about the biological function of LCB kinases (LCBKs), which catalyze the phosphorylation of LCBs to form LCBPs in plant abiotic stress tolerance. In the present study, we performed a functional analysis in transgenic tobacco to explore the possible involvement of a rice LCBK gene OsLCBK1 in abiotic stress tolerance. Root elongation of the transgenic tobacco seedlings with constitutive overexpression of OsLCBK1 was less sensitive to exogenous abscisic acid as compared with the vector-transformed seedlings. The OsLCBK1-overexpressing transgenic tobacco plants showed increased tolerance against oxidative stress after treatment with methyl viologen or H2O2, and up-regulated expression of oxidative stress-related genes. However, the OsLCBK1-overexpressing transgenic tobacco plants showed similar phenotype as vector-transformed plants in response to salt stress and had no change in expression of salt stress-related genes. Our results suggest that OsLCBK1, an enzyme involved in synthesis of LCBPs, may be involved in ABA response and has functions in regulation of oxidative stress tolerance in plants.

Key words: Abscisic acid (ABA), long-chain base kinase (LCBK), long-chain base 1-phosphates (LCBPs), oxidative stress tolerance, transgenic tobacco

Plants suffer adverse conditions such as salt and oxidative stresses during their lives and have developed a plenty of complex and sophisticated signaling pathways to regulate their response to abiotic stresses (Mittler et al. 2004; Seki et al. 2007; Shimozaki and Yamaguchi-Shinozaki 2007; Zhu 2002). Extensive biochemical, molecular, genetic and genomics studies have identified a lot of stress-responsive genes that are activated during abiotic stress responses in a number of plant species, especially in Arabidopsis and some of such stress-responsive genes have been explored for their potential in improving stress tolerance in plants (Ashraf 2010; Bhatnagar-Mathur et al. 2008; Umezawa et al. 2006; Wang et al. 2003; Yamaguchi and Blumwald 2005).

Recent studies have demonstrated that lipid signaling is an integral part of the complex regulatory network in plant response to biotic and abiotic stresses (Munnik and Testerink 2009; Wang et al. 2006). Long-chain base (LCB) 1-phosphates (LCBPs) are widely conserved bioactive lipid molecules in eukaryotic cells and have been shown to be signaling and regulatory molecules in multiple pathways involved in cell proliferation, cell death and stress responses (Alden et al. 2011; Markham et al. 2011; Zäuner et al. 2010). LCB kinase (LCBK) catalyzes the phosphorylation of LCBs to form LCBPs (Funato et al. 2003). The first plant LCBK gene AtLCBK1 was identified from Arabidopsis (Imai and Nishiura 2005). Bioinformatics analysis identified three LCBKs, AtLCBK1 (At5g23450), AtLCBK2/SPHK1 (At4g21540), and At2g46090 in Arabidopsis (Imai and Nishiura 2005; Worrall et al. 2008). AtLCBK1 can phosphorylate D-erythro-dihydrosphingosine and phytosphingosine (Coursol et al. 2005; Imai and Nishiura 2005), whereas AtLCBK2 (AtSPHK1) is a functional sphingosine kinase (SphK)(Worrall et al. 2008). The majority of leaf SphK activity in Arabidopsis plants is associated with membrane fractions (Coursol et al. 2005). Recently, it was found that SphK was involved in ABA-induced stomatal closure (Coursol et al. 2003; Guo et al. 2012; Worrall et al. 2008). On the other hand, degradation of LCBPs by LCBP phosphatase or LCBP lyase has also been shown to play roles in abiotic stress response. For
examples, mutations in Arabidopsis AtSPP1 (encoding a functional LCBP phosphatase) or AtDPL1 (encoding a functional LCBP lyase) affect the ABA responsiveness and drought tolerance (Nishikawa et al. 2008; Nakagawa et al. 2011).

During our studies on the molecular biology of rice disease resistance responses, we identified a rice LCBK gene OsLCBK1, encoding a putative LCBK in rice, and found that overexpression of OsLCBK1 in transgenic tobacco plants affected disease resistance response. The objective of the present study was to explore the biological functions of OsLCBK1 in plant abiotic stress response. Results from comparison of the phenotypes of the transgenic plants with vector-transformed plants in multiple abiotic stresses revealed that overexpression of the OsLCBK1 gene in tobacco plants led to less abscisic acid (ABA) sensitivity, enhanced tolerance to oxidative stress and increased responsiveness of the stress-related gene expression. Our data suggest that OsLCBK1 plays an important role in plant abiotic stress response.

Materials and methods

Plant growth

The coding sequence of the OsLCBK1 gene was cloned into plant binary vector CHF3pp2p212 under the control of the califlower mosaic virus (CaMV) 35S promoter and transformation of tobacco was performed using Agrobacterium-mediated leaf disc transformation as described previously (Luo et al. 2005). Two independent transgenic homozygous lines with single-copy of the OsLCBK1 transgene and a CHF3pp2p212 vector-transformed line were used in the present study. All tobacco plants used were grown in a growth room under a 16 h/8 h day/night regime at 20–25°C and 8-week-old plants were used for all experiments.

ABA sensitivity assay

Seeds of the transgenic lines and vector-transformed plants were surface-sterilized and then placed on 1/2 MS medium containing indicated concentrations of ABA under 16 h/8 h day/night regime at 22–25°C for germination. Root lengths were recorded 2 weeks after germination and at least 40 individual seedlings were measured. The experiments were repeated independently for three times.

Abiotic stress tolerance assay

Fully expanded leaves from 8-week-old plants of the vector-transformed and OsLCBK1-overexpressing lines were rinsed with sterile distilled water and leaf discs (13 mm in diameter) were made by a hole puncher. Salt tolerance assay was done by floating the leaf discs in 10 ml NaCl solution (0, 0.4, and 0.8 M) for 3 days. Oxidative stress assay was performed by floating the leaf discs in methyl viologen (MV) or H$_2$O$_2$ solutions at different concentrations. In MV experiment, leaf discs were incubated in the dark for 1 h and then placed under illumination condition at moderate light intensity (200 µmol m$^{-2}$ s$^{-1}$) for 19 h at 25°C. In H$_2$O$_2$ experiment, leaf discs were incubated for 1 day under illumination condition at moderate light intensity (200 µmol m$^{-2}$ s$^{-1}$). At least 30 leaf discs from fully expanded leaves of 5 tobacco plants were included in each experiment and all experiments were repeated independently for three times.

Measurement of chlorophyll contents

Chlorophyll content was measured as described before (Veronese et al. 2003). The content of chlorophyll was tested by spectrophotometer, quantified according to the formula Chl (A+B) = 5.24A$_{664}$+22.24A$_{648}$, where Chl is the chlorophyll concentration in µg per ml and A is the absorption (Lichtenthaler 1987). At least 30 leaf discs from fully expanded leaves of 5 tobacco plants were included in each experiment and the experiments were repeated independently for three times.

Gene expression analysis by RT-PCR

Leaf discs were harvested after treatments with salt or oxidative stresses and total RNA was extracted by TRIZOL reagent (Invitrogen, Shanghai, China) according to the manufacturer’s instructions. The first strand cDNAs were synthesized using the SuperScript III Kit (Invitrogen, Shanghai, China) and 1 µg of the synthesized cDNAs were used for semi-quantitative RT-PCR in a total volume of 25 µl. PCR conditions were set as 94°C 30 s, 48–65°C 30 s and 72°C 30 s for 25–30 cycles based on the abundance of transcript for each gene, followed by 5 min of final extension at 72°C. PCR products were electrophoresed on a 1.2% agarose gel. Stress-related and internal reference genes and their gene-specific primers used are listed in Table 1. At least 30 leaf discs from fully expanded leaves of 5 tobacco plants were included in each experiment and the experiments were repeated independently for three times.

Results and discussion

In our previous studies aiming at elucidating the molecular basis of rice disease resistance responses, we identified a rice LCBK gene OsLCBK1 that encodes a putative LCBK in rice. Bioinformatics analysis revealed that there are two putative LCBKs in rice genome, Os10g37280 (OsLCBK1) and Os04g45800. OsLCBK1 encodes a 757 aa protein, which contains a conserved diacylglycerol kinase catalytic domain and conserved C1–C5 domains, similar to the characteristic structures in AtLCBK1. OsLCBK1 and Os04g45800 show an identity of 61.4% to each other and shows identity of 58% to AtLCBK1, indicating that OsLCBK1 is likely to be a LCBK in rice. To explore the biological function of OsLCBK1 in disease resistance response, we generated transgenic tobacco lines that overexpress the OsLCBK1 gene. The transgenic lines were allowed to grow for 3 generations and homozygous and single-copy lines were screened and confirmed by 3:1 segregation ratio.
of the antibiotic resistance marker on 1/2 MS medium containing 200 µg ml⁻¹ kanamycin. Two independent T3 generation homozygous lines (K1–5 and K1–44) that contain single copy of the OsLCBK1 gene were chosen for this study. In the present study, we examined whether OsLCBK1 has functions in plant abiotic stress response by comparison of the phenotypes of the OsLCBK1-overexpressing transgenic plants with the vector-transformed plants.

**Decreased ABA sensitivity in the OsLCBK1-overexpressing plants**

Previous studies have shown that AtLCBK1 was slightly induced by low humidity or ABA (Imai and Nishiura 2005; Nishiura et al. 2000). Therefore, we first compared the sensitivity of the OsLCBK1-overexpressing transgenic and the vector-transformed plants to exogenous ABA. Without the treatment of ABA, the root growth showed no difference between the OsLCBK1-overexpressing seedlings and the vector-transformed seedlings (Figure 1A and B). After the treatment of ABA, the root growth of the vector-transformed seedlings was inhibited and this inhibition was more obvious when the concentration increased. Compared with the vector-transformed seedlings, root growth of the OsLCBK1-overexpressing seedlings was less inhibited by exogenous ABA and the roots were much longer than the vector-transformed seedlings at high concentrations of ABA (2 µM and 4 µM) (Figure 1A). Grown on 1/2 MS containing 4 µM ABA, the root length was only ca. 15% of those grown on 1/2 MS without ABA in the vector-transformed seedlings, while the root length was ca. 30% of those grown on 1/2 MS without ABA in seedlings of the transgenic lines (Figure 1B). These results suggest that overexpression of OsLCBK1 in transgenic tobacco resulted in a decreased ABA sensitivity. Our observation differs from a previous finding that the stomata of the AtLCBK2/AtSPHK2-overexpressing Arabidopsis plants were more sensitive,
than wild type to exogenous ABA (Worrall et al. 2008). The difference in ABA responsiveness between the OsLCBK1-overexpressing tobacco seedlings and the AtLCBK2/AtSPHK2-overexpressing Arabidopsis plants may be due to the facts that OsLCBK1 encodes a putative LCBK while AtSPHK1 is a functional SphK (Worrall et al. 2008), and that the root elongation assays were used for examining ABA sensitivity of the OsLCBK1-overexpressing tobacco plants in our experiments while stomatal response assays were carried out for assessing ABA sensitivity of the AtSPHK1-overexpressing Arabidopsis plants (Worrall et al. 2008). It was found that sphingolipids including S1P can function as intracellular messengers in response to ABA and that AtSPHK1 and phyto-S1P play important roles in mediating the ABA response (Coursol et al. 2003; Guo et al. 2012; Ng et al. 2001). For example, LCBPs were shown to be involved in ABA inhibition of stomatal opening and promotion of stomatal closure in Arabidopsis (Coursol et al. 2003, 2005). Therefore, it is likely that overexpression of OsLCBK1 in transgenic tobacco plants may promote synthesis of LCBPs and thus affect the balance of LCBPs, which in turn interfere with the ABA signaling leading to changes in ABA sensitivity. However, mutations in Arabidopsis LCBP phosphatase gene AtSPP1, which is likely to suppress the degradation of LCBPs, did not affect the ABA-mediated inhibition of root elongation (Nakagawa et al. 2011). Further investigations with combined biochemical and genetic approaches are required to elucidate the details of the functions for OsLCBK1 in ABA signaling and possible involvement of LCBPs in ABA-mediated root elongation.

Enhanced oxidative stress tolerance in the OsLCBK1-overexpressing plants

Possible involvement of OsLCBK1 in oxidative stress tolerance was studied using H2O2 and MV as artificial stress conditions. During our experiment time, no significant phenotype appeared on the leaf discs from the transgenic lines and the vector-transformed plants without H2O2 or MV treatment (Figures 2A, 3A). With the treatment of H2O2 or MV, bleaching or chlorosis were observed in leaf discs from OsLCBK1-overexpressing and the vector-transformed plants (Figures 2A, 3A). Bleaching or chlorosis symptom in leaf discs from the OsLCBK1-overexpressing plants was less severe than those of the vector-transformed plants (Figures 2A, 3A). These observations were further confirmed by measuring chlorophyll contents in leaf discs from the transgenic and the vector-transformed plants after MV or H2O2 treatments (Figures 2A, 3A). After treatments with MV or H2O2, relative chlorophyll contents in leaf discs of the OsLCBK1-overexpressing plants were markedly higher than those from the vector-transformed plants (Figures 2A, 3A). Notably, the difference in relative chlorophyll contents in leaf discs of the OsLCBK1-overexpressing and the vector-transformed plants was increased along with the increase of the concentrations of MV or H2O2 (Figures 2A, 3A). These results indicate that overexpression of OsLCBK1 in transgenic tobacco plants can improve oxidative stress tolerance. It was recently found that mutations in Arabidopsis FBR11 (encoding a subunit of serine palmitoyltransferase that catalyzes de novo synthesis of LCBS) led to attenuated formation of LCBS in response to Fumonisin B1 and that exogenous LCBS efficiently induced generation of reactive oxygen species (ROS) while exogenous LCBPs specifically blocked LCB-induced ROS generation in fbr1 mutant plants (Shi et al. 2007). These observations demonstrated...
that the homeostatic balance between LCBs and LCBPs plays important roles in regulating ROS level in cells. In our study, when overexpressed, the OsLCBK1 may accelerate the rate of LCBP synthesis, resulting in reduced levels of LCBs and increased levels of LCBPs, and thus affect the homeostatic balance between LCBs and LCBPs, which favors, probably through regulating cellular ROS level, to improve oxidative stress tolerance in the OsLCBK1-overexpressing transgenic tobacco plants. However, whether overexpression of OsLCBK1 in transgenic tobacco plants affects ROS generation and the physiological mechanisms of enhanced oxidative stress tolerance regulated by OsLCBK1 in transgenic plants need further examined.

Unaltered salt tolerance in OsLCBK1-overexpressing plants

Possible role of OsLCBK1 in salt stress tolerance was also studied by testing the tolerance of leaf discs from 8-week-old transgenic and the vector-transformed plants to different concentrations of NaCl solution. Without NaCl treatment, leaf discs remained green both in the OsLCBK1-overexpressing and vector-transformed plants. With the increasing of NaCl, the leaf discs showed slight bleaching symptom in the OsLCBK1-overexpressing and vector-transformed plants (Figure 4A). At the concentration of 0.4 M and 0.8 M, the relative chlorophyll contents were 81% and 52% of those without NaCl in the vector-transformed plants, respectively (Figure 4B). The
relative chlorophyll contents were 85% and 55% of those without NaCl treatment in the OsLCBK1-overexpressing plants (Figure 4B). Based on the bleaching symptom and reduction of chlorophyll contents, no significant difference was observed in salt tolerance between the OsLCBK1-overexpressing and vector-transformed plants. These results suggested that overexpression of OsLCBK1 in transgenic tobacco does not affect the salt stress tolerance and thus OsLCBK1 has limited function in salt stress tolerance. This is contrary to the observations that, in Arabidopsis, AtLCBK2 (AtSPHK1) plays important roles in ABA signaling and defense response against drought and chilling stresses (Coursol et al. 2003; Dutilleul et al. 2012; Guo et al. 2011, 2012; Worrall et al. 2008). However, it is not clear whether or not AtLCBK2 has a function in salt stress response. Signaling pathways require for salt stress response are not identical to those required from drought stress response, although crosstalking between signaling pathways involved in salt and drought stress responses exist (Zhu 2002).

**Differential expression of stress-related genes in OsLCBK1-overexpressing plants**

Expression of some selected stress-related genes was analyzed to get insights into the possible mechanisms of the enhanced oxidative stress tolerance in the OsLCBK1-overexpressing transgenic tobacco plants. To this purpose, expression of oxidative stress-related genes, including genes encoding ascorbate peroxidase (APX), catalase (CAT) and glutathione S-transferases (GST), was first analyzed. In water-treated controls, expression of GST was not detected in leaf discs from the OsLCBK1-overexpressing and the vector-transformed plants, while expression of APX and CAT with different patterns was detected in leaf discs from the OsLCBK1-overexpressing and the vector-transformed plants (Figure 5A). When compared with those in the vector-transformed plants, increased expression of APX in leaf discs of the OsLCBK1-overexpressing plants was observed (Figure 5A). After incubation with H2O2 or MV, expression of APX, GST and CAT was up-regulated in leaf discs of the vector-transformed plants, indicating an oxidative stress was applied to leaf discs (Figure 5A). However, the expression levels of APX, GST and CAT in leaf discs of the OsLCBK1-overexpressing plants were higher than those in leaf discs from the vector-transformed plants (Figure 5A). Increased expression levels for GST and CAT were much evident in leaf discs from the OsLCBK1-overexpressing plants, as compared with those of the vector-transformed plants (Figure 5A). It is well established that enhanced oxidative stress tolerance is often associated with high levels of expression of genes that are involved in oxidative stress responses in plants (Mittler et al. 2004, 2006). Therefore, it is likely that the up-regulated expression of the oxidative stress-responsive genes after treatment with MV or H2O2 may be responsible for the increased oxidative stress tolerance observed in the OsLCBK1-overexpressing tobacco plants.

It is previously shown that NtSAM1 (encoding S-adenosyl-l-Met synthetase), NtTOBLT (encoding a lipid transfer protein) and NtLEA5 (encoding one of late embryogenesis abundant proteins) were induced during salt stress response (Espartero et al. 1994; Masuta et al. 1992; Torres-Schumann et al. 1992; Xu et al. 1996). When treated in water without NaCl, no significant expression of NtSAM1 and NtTOBLT was detected in leaf discs from the vector-transformed plants, while no change in expression of NtLEA5 was observed in leaf discs between the OsLCBK1-overexpressing and the vector-transformed plants (Figure 5B). After treatment with NaCl, expression levels of the NtSAM1, NtTOBLT and NtLEA5 genes were increased in leaf discs from the vector-transformed plants and the OsLCBK1-overexpressing plants; however, expression levels of the NtSAM1, NtTOBLT and NtLEA5 genes in leaf discs from the OsLCBK1-overexpressing plants were comparable to those in the vector-transformed plants (Figure 5B). The unchanged expression patterns of these salt stress-related genes in the OsLCBK1-overexpressing plants further support our observations that overexpression of OsLCBK1 in transgenic tobacco plants does not affect the salt stress tolerance.

![Figure 5](image_url)
tolerance.

In some previous studies, enhanced oxidative stress tolerance and increased disease resistance were simultaneously observed in transgenic tobacco overexpressing pepper peroxidase and ascorbate peroxidase genes (Choi et al. 2012; Sarowar et al. 2005). Similar results were also observed for the OsLCBK1-overexpressing transgenic tobacco as overexpression of OsLCBK1 in transgenic plants resulted in enhanced oxidative stress tolerance and disease resistance (Zhang and Song unpublished data). These findings indicate a link between oxidative stress tolerance and disease resistance. It is well known that ROS homeostasis play important roles in oxidative stress and disease resistance responses (Miller et al. 2008; Mittler et al. 2011; Suzuki et al. 2012). Excessive ROS is generally cytotoxic and thus leads to oxidative stress; however, lower concentrations of ROS have been demonstrated to be important signal transduction molecules (Miller et al. 2008; Mittler et al. 2011; Suzuki et al. 2012). Up-regulated expression of the oxidative stress-responsive genes in the OsLCBK1-overexpressing transgenic plants may increase the ability to scavenger excessive ROS and thus maintain ROS to a level that can act as signaling molecules in regulating oxidative stress and disease resistance responses rather than act as cytotoxic factors to cause cellular damage.

Sphingolipids and their metabolites such as LCBPs are important regulators in animal cells (Hannun et al. 1986; Merrill et al. 1986; Wilson et al. 1986). Recently, it was demonstrated that LCBPs play important roles in regulating biotic and abiotic stress responses in plants (Alden et al. 2011; Chen et al. 2012; Dunn et al. 2004; Dutilleul et al. 2012; Lynch et al. 2009; Lynch and Dunn 2004; Michaelson et al. 2009; Ng and Hetherington 2001; Worrall et al. 2003). The present study explored the function of a rice LCB kinase gene OsLCBK1 in abiotic stress and showed that overexpression of OsLCBK1 in transgenic tobacco resulted in a decreased ABA sensitivity and improved oxidative stress tolerance but not salt stress tolerance. However, the involvement of OsLCBK1 in other abiotic stresses (e.g. cold, heat and drought stresses) needs to be examined further by comparing the different phenotype of the OsLCBK1-overexpressing transgenic and the vector-transformed plants under different abiotic stress conditions. Most importantly, the physiological and biochemical mechanisms and the signaling pathway involved in OsLCBK1-regulated abiotic stress responses also need further investigations.

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