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Antisense inhibition of a BRI1 receptor reveals additional protein kinase signaling components downstream to the perception of brassinosteroids in rice

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Abstract Plants express a variety of proteins at the cell surface responsible for the transduction of regulatory information into the cell via receptors. In the present study, an attempt has been made to identify the components of the brassinosteroids (BRs) signaling transduction cascades in transgenic rice (*Oryza sativa*) expressing the antisense strand of *OsBRI1* transcript. A 60 kDa protein, immunologically characterized as mitogen-activated protein kinase (MAPK), showed reduced phosphorylation activity in the membrane fractions of the *OsBRI1* antisense rice over control, demonstrating the inhibition in the perception of BRs by the BRI1 receptor, when compared with the exogenously applied brassinolide. The phosphorylation activity of the 50 kDa Ca^{2+}-dependent protein kinase was however increased in the cytosolic fractions of *OsBRI1* antisense over control. The data obtained suggest that MAPK and Ca^{2+}-dependent protein kinase in rice are discrete but parallel signaling cascades and might involve receptors other than BRI1 in response to BR stimulus. **© 2001 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Brassinosteroid; Brassinolide-insensitive receptor; Mitogen-activated protein kinase; Ca^{2+}-dependent protein kinase; Rice (*Oryza sativa*)

1. Introduction

Plant hormones are important signaling molecules involved in the regulation of plant growth and development. Brassinosteroids (BRs), a group of naturally occurring growth-promoting plant steroids, are capable of eliciting strong growth responses and physiological changes [1–4]. Significant progress has been made over the last few years in elucidating the BR biosynthetic pathway. The discovery of BR-deficient (e.g. *dwf1-6/cbb1*, dwarf, cabbage; *cbb3/cpd*, constitutive photomorphogenic dwarf; *det2*, de-etiolated; *ikb*) and BR-insensitive (e.g. *BRI1*, brassinolide-insensitive; *cbb2*; *d61-2*, rice dwarf; *ika*) mutants in *Arabidopsis*, pea, tomato and rice [4,5] led to the isolation of *BRI1* gene. Molecular characterization of the product of this gene revealed it to be a receptor-like transmembrane kinase (RLK) that transduces steroid signals across the plasma membrane [4,6–8]. The protein kinase domain of *BRI1* was found to be related to the kinase domain of other RLKs in higher plants such as *ERECTA* [9], *CLV1* [10], *RLK5* [11] and *Xa21* [7,12].

Loss or gain of the function of such receptor-like kinases, either through mutation or an antisense approach, is expected to lead to severe physiological consequences. This was demonstrated by our earlier studies where, for example, a mutant of an *OsBRI1* gene as well as transgenic plants expressing the antisense strand of the *OsBRI1* transcript exhibited severe shortening of internodes in rice plants as compared to the normal plants [4], even though the endogenous level of BRs, especially castasterone, registered a four times increase in these plants as compared to the wild type. In the present study, therefore, anti-*OsBRI1* transgenic plants along with their control were used for dissecting the BRs signaling pathways following with or without an exogenous application of brassinolide (BL). Since plant hormones have been shown to operate through mitogen-activated protein kinase (MAPK)- and Ca^{2+}-dependent protein kinase (CDPK)-based signaling cascades [13–15], activities of both MAPK and CDPK were studied in anti-*OsBRI1* transgenic rice for elucidating the components of the BR signaling cascade downstream to the perception of BRs.

2. Materials and methods

2.1. Plant materials

Two cultivars of transgenic rice, *Oryza sativa* cv. Nipponbare and cv. Dontokoi, overexpressing the antisense strand of the *OsBRI1* transcript under a promoter-terminator cassette (pBIAct1nos) and their controls, were used as described previously [4]. Plants were maintained in the greenhouse at 30°C (day) and 24°C (night).

2.2. Preparation of protein extracts and subcellular fractionations

Leaf-sheath segments from the young tillers of 3-month-old rice plants were used for the isolation of protein extract and subcellular fractions as described previously [16].

2.3. In-gel protein kinase assays

Samples containing an equal amount of protein were subjected to SDS–PAGE in 15% polyacrylamide gels containing 0.5 mg ml^{-1} bovine myelin basic protein (MBP, Sigma, USA) as a substrate. After electrophoresis, SDS was removed by washing the gel in washing buffer containing 25 mM Tris (pH 7.5), 0.5 mM dithiothreitol (DTT), 0.1 mM Na_3VO_4, 0.5 mg ml^{-1} bovine serum albumin and 0.1% Triton X-100 three times, each for 30 min at room temperature. The proteins were renatured in 25 mM Tris, pH 7.5, 1 mM DTT and 0.1 mM Na_3VO_4 at 4°C overnight with three changes of buffer. Kinase reactions were performed in a kinase assay buffer containing 25

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mM Tris (pH 7.5), 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, 0.1 mM Na_3VO_4, 200 nM ATP and 5 μM [γ-^{32}P]ATP (110 TBq mmol^{-1}; Amersham Pharmacia Biotech, UK) for 60 min, and stopped by washing extensively in 5% (w/v) trichloroacetic acid and 1% (w/v) sodium pyrophosphate. Dried gels were analyzed either by an X-ray autoradiography or with a phosphorimager (Molecular Dynamics, USA). The in-gel kinase assay for the CDPK activity was performed using histone III-S (Sigma) as a substrate [17].

2.4. Immunodetection of MAPK

Membrane fractions were fractionated by SDS–PAGE and transferred onto a PVDF membrane as described previously [18]. Membrane was cross-linked with an anti-ERK1 antibody (Sigma) and detection of antigen–antibody complexes was performed with enhanced chemiluminescence using an ECL™-Plus kit (Amersham Pharmacia Biotech).

2.5. Immunoprecipitation and in vitro MAPK activity

Membrane fractions were incubated with shaking for 2 h at 4°C with 5 μg of anti-ERK1 (Sigma) in an immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM Na_3VO_4, 1 mM PMSF and 0.5% Triton X-100). About 30 μl of prewashed Protein A-Sepharose (Sigma) was added and incubated with continuous shaking at 4°C overnight. The Sepharose beads–protein complexes were centrifuged for 5 min at 6000×*g* and the pellet was washed twice with 0.5 ml of buffer followed by two further washes with a kinase assay buffer as described previously (Section 2.3). The MAPK assay was carried out in 15 μl of kinase buffer to which 0.25 mg ml^{-1} MBP, 40 mM ATP and 5 μM [γ-^{32}P]ATP (110 TBq mmol^{-1}) were added. The reaction was allowed to proceed for 30 min at room temperature and was terminated by the addition of an SDS sample buffer. The samples were denatured by boiling for 5 min and electrophoresed on a 15% SDS–PAGE gel. The gel was subsequently stained in Coomassie brilliant blue to confirm equal protein loading, destained, dried and analyzed as described in Section 2.3.

2.6. In vitro phosphorylation of endogenous proteins

In vitro phosphorylation assay was carried out in a 25 μl reaction mixture containing 20 mM Tris, pH 7.5, 10 mM $MgCl_2$, 0.2 mM $CaCl_2$, 39 mM [γ-^{32}P]ATP (0.44 TBq nmol^{-1}) and 5 μl of protein extract [16]. The basal level of phosphorylation was measured in the presence of 4 mM EGTA instead of $CaCl_2$. The reaction mixture was incubated for 10 min at 30°C and terminated by the addition of a lysis buffer containing 8 M urea, 2% Triton X-100, 2% ampholines (pH 4–11) and 10% polyvinylpyrrolidone-40. Denatured proteins were subjected to isoelectric focusing in tube gels and were analyzed on two-dimensional (2D) PAGE [19].

3. Results

3.1. MBP phosphorylating activity is reduced in BRI1 antisense transgenic plants

In an in-gel kinase assay with MBP as a substrate, it was found that phosphorylation of MBP was associated with a protein of 60 kDa in the membrane fractions, which was significantly reduced in anti-*OsBRI1* transgenic rice as compared to the control plants (Fig. 1A). No significant kinase activation was detected in the cytosolic fractions (Fig. 1A). To distinguish whether the reduced kinase activity in anti-*OsBRI1* was specific to the inhibition of the BRI1 receptor, we monitored the kinase activation in control and transgenic rice (cv. Dontokoi) in response to the application of exogenous BL for different time periods. As shown in Fig. 1B, MBP phosphorylating activity was found associated with a protein of 60 kDa in both control and anti-*OsBRI1* membrane extracts. However, the constitutive level of phosphorylation activity in anti-*OsBRI1* rice was much lower as compared to control fractions, suggesting the inhibition in the perception of BL by a BRI1 receptor in the transgenic rice. Moreover, exogenous BL transiently activated this kinase within 15–30 min of treatment, suggesting the involvement of this kinase during early response in BRs signaling pathway.

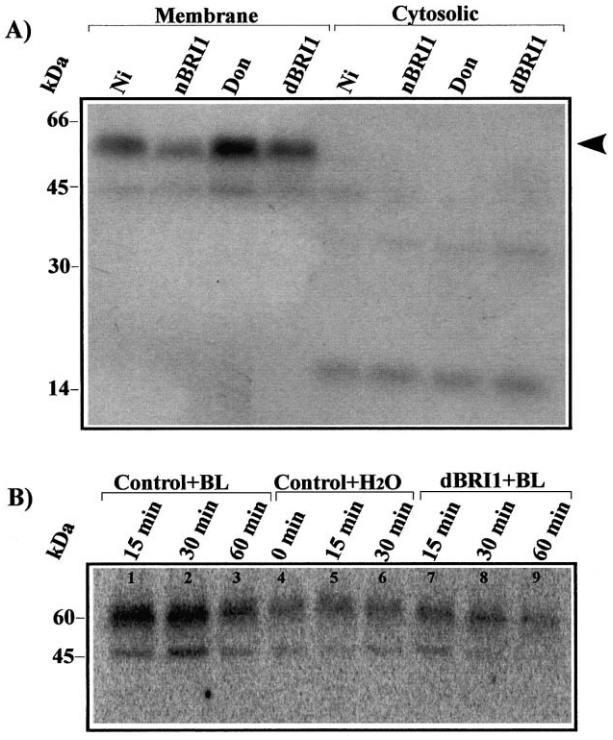

Fig. 1. In-gel protein kinase assay showing MBP phosphorylating activity. A: Membrane and cytosolic fractions of leaf sheath in control (Ni, Nipponbare; Don, Dontokoi) and anti-*OsBRI1* transgenic rice (nBRI1, Nipponbare; dBRI1, Dontokoi). The arrow indicates a protein kinase of 60 kDa in membrane fractions. B: Leaf-sheath segments of control (Dontokoi) and anti-*OsBRI1* (dBRI1) were treated with 1 μM BL for different time periods as indicated. As controls, Milli-Q water was added rather than a solution of BL. Membrane and cytosolic fractions were prepared and subjected to in-gel kinase assay using MBP as a substrate as described in Section 2. Molecular weight markers (in kDa) are indicated on the left.

3.2. Ca^{2+}-dependent kinase activity is increased in BRI1 antisense transgenic plants

To assess the possible involvement of CDPKs in BRs response, both membrane and cytosolic fractions, prepared from leaf sheath from control and transgenic plants, were analyzed by an in-gel kinase assay using histone III-S as a substrate. Interestingly, the increased phosphorylation activity was found associated with a protein of 50 kDa in the cytosolic fractions of anti-*OsBRI1* transgenic rice as compared to control (Fig. 2A). No significant kinase-like activity was observed in the membrane fractions (Fig. 2A).

To demonstrate the BL-induced activation of this CDPK in anti-*OsBRI1* rice, leaf-sheath segments from control and transgenic rice were treated with exogenous BL for different time periods. As shown in Fig. 2B, histone III-S phosphorylation activity was found associated with 50 kDa CDPK in both control and anti-*OsBRI1* cytosolic fractions within 15 min, as compared to water control. The appearance of an additional 52 kDa band might be induces due to the water stress, which could not be observed in Fig. 4A. The above results suggest the involvement of CDPK during early response in the BRs signaling pathway. Furthermore, kinase activity was not detected in gels without added protein sub-

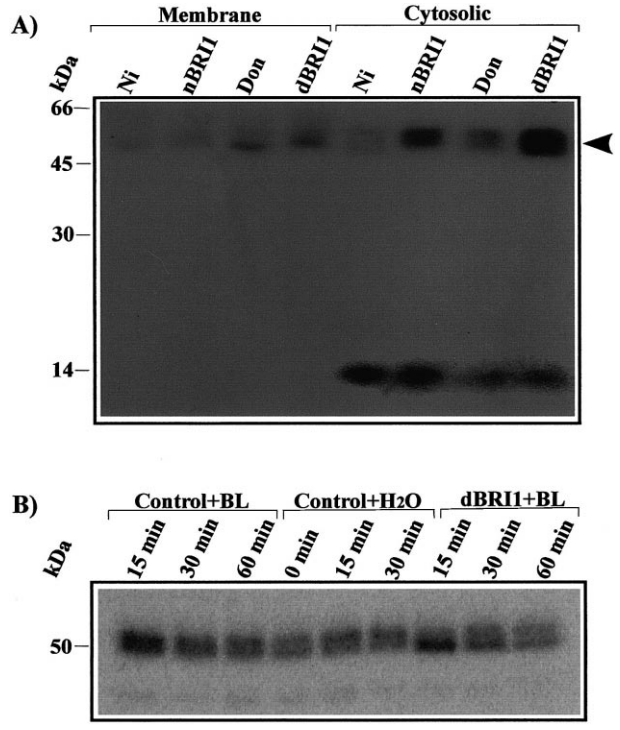

Fig. 2. In-gel protein kinase assay showing the phosphorylation of histone III-S by CDPK. A: Membrane and cytosolic fractions of leaf sheath in control (Ni, Nipponbare; Don, Dontokoi) and anti-*OsBRI1* transgenic rice (nBRI1, Nipponbare; dBRI1, Dontokoi). The arrow indicates a protein kinase of 50 kDa in cytosolic fractions. B: Leaf-sheath segments of control (Dontokoi) and anti-*OsBRI1* (dBRI1) were treated with 1 μM BL for different time periods as indicated. As controls, Milli-Q water was added rather than a solution of BL. Membrane and cytosolic fractions were prepared and subjected to in-gel kinase assay using histone III-S as a substrate as described in Section 2. Molecular weight markers (in kDa) are indicated on the left.

strate, indicating that the phosphorylation observed was not due to autophosphorylation of the 50 kDa protein (data not shown).

3.3. BL-induced kinase activities have characteristics of MAPKs and CDPKs

3.3.1. Membrane fractions contain proteins immunologically related to MAPKs. To examine whether the BRs-responsive MBP phosphorylation activity could be attributed to a MAPK, the membrane fractions used in Fig. 1B were subjected to Western analysis (Fig. 3A), using an anti-ERK1 antibody. Previous results on immunodetection with anti-ERK1 have shown that it reacts with at least five human ERK isoforms and MAPK of *Arabidopsis* [13]. Fig. 3A shows the presence of a band at 60 kDa that was in agreement with the results obtained by an in-gel kinase assay, suggesting the activation of ERK-like MAPKs in the BRs signaling pathway.

To further assess whether this 60 kDa kinase had the characteristics of MAPK, protein extracts of membrane fractions from BL-treated control (Fig. 1B, lane 1) and anti-*OsBRI1* plants (Fig. 1B, lane 7) were subjected to immunoprecipitation with anti-ERK1 antibodies followed by in vitro MBP kinase assay (Fig. 3B). The kinase activity at 22 kDa shows the capacity to phosphorylate MBP, and was up-regulated in anti-ERK immunoprecipitates of extracts prepared from BL-treated control rice as compared with the anti-*OsBRI1* plants.

3.3.2. Characterization of 50 kDa kinase as a CDPK in cytosolic fractions. To confirm that the BL-induced activation of kinase in anti-*OsBRI1* rice is a CDPK, in vitro analysis with Ca^{2+} chelator (EGTA), protein kinase inhibitor and calmodulin antagonist was carried out. The activity of 50 kDa kinase was completely inhibited by EGTA (Fig. 4A). H-7, an inhibitor of protein kinase C, and W-7, a potent calmodulin antagonist, strongly inhibited the kinase activity of 50 kDa kinase, suggesting it to be a CDPK (Fig. 4A). No obvious influence on the 17 kDa kinase activity was detected, which has been attributed to the nucleoside diphosphate kinase as previously reported [17].

We further investigated whether the increase in BRs-induced CDPK activity would be reflected in changes in the in vitro phosphorylation pattern. Fig. 4B shows that when total protein extract from control and anti-*OsBRI1* rice was incubated in the presence of [γ-^{32}P]ATP and Ca^{2+} and analyzed by 2D PAGE, several endogenous proteins became phosphorylated. It is interesting that anti-*OsBRI1* plants showed a specific increase in the degree of phosphorylation of several proteins with molecular weight ranging from 40 kDa to 70 kDa, when compared with the control plants. Protein phosphorylation was Ca^{2+}-dependent because no phosphorylation was observed in the presence of EGTA (Fig. 4B).

4. Discussion

BRs, a group of unconventional hormones, have been shown to play a crucial role in plant development and growth [1–4]. Information on their mode of action is just beginning to emerge. The availability of BR-deficient and BR-insensitive mutants in several plants has been of a great help in the isolation and characterization of the *BRI1* gene [4,5]. The *BRI1* gene from rice and other plants encodes a RLK that transduces steroid signals across the plasma membrane [4,9–12]. Our earlier studies on mutants of *OsBRI1* and anti-*OsBRI1* transgenic plants exhibited severe shortening of intern-

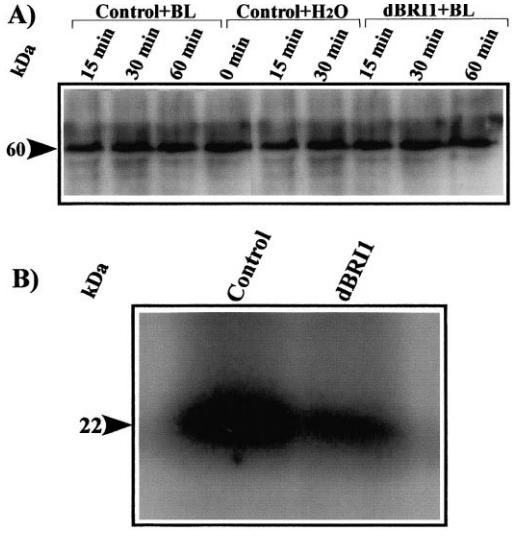

Fig. 3. BL-induced protein kinase has characteristics of ERK-like MAPKs. A: Membrane fractions used in in-gel kinase assay (Fig. 1B) were immunoblotted with an anti-ERK1 antibody. B: Membrane fractions used in in-gel kinase assay (Fig. 1B; lanes 1 and 7) were immunoprecipitated with an anti-ERK1 antibody and than analyzed using an in vitro MBP kinase assay.

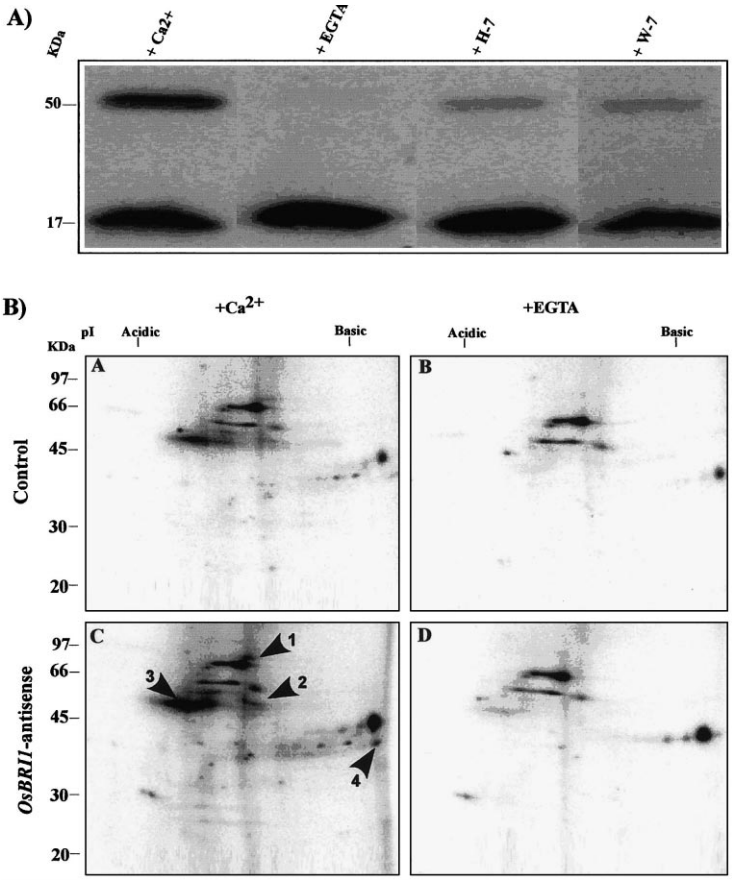

Fig. 4. Characterization of plant CDPKs. A: In vitro effects of Ca^{2+}, EGTA, H-7 (inhibitor of protein kinase C) and W-7 (calmodulin antagonist) on the 50 kDa kinase in the cytosolic fractions of anti-*OsBRI1* transgenic plants. To each reaction mixture was added 4 mM EGTA, 100 μM H-7 and 100 μM W-7. All reaction mixtures contained 0.2 mM $CaCl_2$ except those for EGTA. Histone III-S was used as substrate for the in-gel kinase assay. B: 2D PAGE pattern showing in vitro phosphorylation in the rice leaf extract of control and anti-*OsBRI1* transgenic plants. A,C: Phosphorylation in the presence of 0.2 mM $CaCl_2$; B,D: phosphorylation in the presence of 4 mM EGTA. Right to left: isoelectric focusing for first dimension, top to bottom: SDS–PAGE for second dimension. Numbers in the figures show positions of proteins having a relative molecular mass of 70 kDa (1), 50 kDa (2), acidic 50 kDa (3) and basic 40 kDa (4).

odes in rice plants [4], suggesting thereby the role of BRs in cell division and cell elongation. Interestingly, the dwarf phenotype in these mutants could not be rescued in spite of a four-fold increase in the endogenous levels of BRs, internally modulated in the transgenic plants. These observations suggest that (i) the elevation in the levels of BRs in transgenics is an attempt by the plants to compensate for their diminished sensitivity to BRs [4] and (ii) it is the state of the BR receptor and not the levels of BRs per se which play a crucial role in the BR signaling pathway. The present study was therefore planned to study the impact of BRI1 receptor function in elucidating the signal cascades mediated by BRs. Since plant hormones [13,14] and elicitors [20] as well as different types of abiotic and biotic stresses [21,22] are known to operate through MAPK- and CDPK-based signaling cascades, the activity of these kinases was studied in the control as well as in anti-*OsBRI1* transgenic plants.

The results obtained in the present study revealed that the transgenic rice, impaired in the BRI1 receptor, showed a decreased activity of 60 kDa MBP-like kinase in the membrane fractions (Fig. 1A) that was restored in BL-treated control plants but not in anti-*OsBRI1* transgenics (Fig. 1B). Additionally, MAPK antibodies (ERK1) immunoprecipitated a 60 kDa protein, which showed increased phosphorylation activity in BL-treated control tissues (Fig. 3B). These results demonstrated that 60 kDa kinase is in fact a MAPK. The signal transduction following the perception of BRs by MAPK in plants is not known but studies by Treisman et al [23] have shown that in animals the activated MAPK is translocated to the nucleus where it phosphorylates target transcription factors. In plants, there has been evidence that MAPKs are involved in the G_1 phase of the cell cycle to induce G_1 cyclins, which are key regulators in cell division [24,25]. Since MAPK is shown to be involved in cell division and growth in plants, it is reasonable to expect that the anti-*OsBRI1* transgenic plants, not expressing the BRI1 receptor, will not respond to exogenous and/or elevated endogenous levels of BRs in restoring the normal phenotype. These results therefore further support the fact that BRs activate the membrane-bound MAPK and trigger a signal cascade eventually switching on the processes involved in cell division and cell elongation. Hence in the absence of BRs-mediated signal cascade due to reduced or inactivation of MAPK, there is a severe reduction in the elongation of the internodes in anti-*OsBRI1* transgenic rice.

An unexpected but interesting observation made in the present study was the increased activity of CPDK following the exogenous application of BL in control as well as in anti-

OsBRI1 transgenic rice plants (Fig. 2A,B). This activity was associated with a 50 kDa protein and was restricted only to the cytosolic fractions. These results suggest that BRs, in addition to activating the membrane-bound MAPK, also activate cytosolic CDPK-mediated signal cascades, influencing in turn the pathways known to be involved in transcription, membrane transport and cell structure [15]. In fact, in animals hormone receptors are released from the cytoplasmic complexes upon hormonal stimuli and are translocated to the nucleus [26]. These receptors in the nucleus act independently as transcription factors. The CDPK activity observed in the present study might involve a class of receptors similar to that reported in animals [26]. However, no evidence of nuclear receptors has hitherto been found in plants, including the complete sequencing of the *Arabidopsis* genome [27].

Taken together the results obtained in the present study reveal that exogenous application of BL or indigenously naturally occurring biologically active BRs activate membrane-bound MAPK and cytosolic CDPK. In transgenic plants expressing the antisense strand of the *OsBRI1* gene, MAPK could not be activated and therefore, the transgenic plants depicted BRs unrescuable phenotype of stunted internodal segments. Furthermore, the increased induction of CDPK activity in the cytosolic fraction of anti-*OsBRI1* as well as control plants suggests that signaling pathways following BRs activation of MAPK and CDPK in rice are discrete but parallel and therefore it is reasonable to speculate that there is an involvement of additional receptor(s) other than BRI1 for BRs response in plants.

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