

# Expression of a Gibberellin-Induced Leucine-Rich Repeat Receptor-Like Protein Kinase in Deepwater Rice and Its Interaction with Kinase-Associated Protein Phosphatase<sup>1</sup>

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We identified in deepwater rice (*Oryza sativa* L.) a gene encoding a leucine-rich repeat receptor-like transmembrane protein kinase, *OsTMK* (*O. sativa* transmembrane kinase). The transcript levels of *OsTMK* increased in the rice internode in response to gibberellin. Expression of *OsTMK* was especially high in regions undergoing cell division and elongation. The kinase domain of *OsTMK* was enzymatically active, autophosphorylating on serine and threonine residues. A cDNA encoding a rice ortholog of a kinase-associated type 2C protein phosphatase (*OsKAPP*) was cloned. KAPPs are putative downstream components in kinase-mediated signal transduction pathways. The kinase interaction domain of *OsKAPP* was phosphorylated *in vitro* by the kinase domain of *OsTMK*. RNA gel-blot analysis indicated that the expression of *OsTMK* and *OsKAPP* was similar in different tissues of the rice plant. In protein-binding assays, *OsKAPP* interacted with a receptor-like protein kinase, *RLK5* of *Arabidopsis*, but not with the protein kinase domains of the rice and maize receptor-like protein kinases *Xa21* and *ZmPK1*, respectively.

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The successful existence of all plants depends on their ability to coordinate complex developmental changes and to sense and respond to fluctuations in their environment. A stimulus is perceived by the cell, a signal is generated and transduced, and a biochemical response is elicited. Deepwater or floating rices belong to a group of cultivars whose survival is based on their capacity for rapid internodal elongation when they become submerged during flooding in the rainy season. Under field conditions, growth rates of up to 25 cm/d have been reported, result-

ing in plants that are up to 6 m long (Catling, 1992). The signal for accelerated growth is an increase in the internal ethylene concentration (Raskin and Kende, 1984a), which, via a decrease in ABA levels (Hoffmann-Benning and Kende, 1992), enhances the responsiveness of the internode to GA (Raskin and Kende, 1984b). Whereas ethylene and ABA are intermediates in signaling the change in the environment, the growth response is ultimately elicited by GA.

In several plant species, the putative components of the GA signal transduction pathway have been identified by genetic and biochemical approaches (Swain and Olszewski, 1996; Bethke et al., 1997; Jones et al., 1998; Ritchie and Gilroy, 1998; Steber et al., 1998). *GAMYB*, a transcription factor in the cereal aleurone system, has been isolated and appears to mediate GA-induced expression of a high-pI  $\alpha$ -amylase gene (Gubler et al., 1995). In addition to *GAMYB*, two putative transcription factors with high sequence similarity to each other, *GAI* (Peng et al., 1997) and *RGA* (Silverstone et al., 1998), were identified in genetic screens for GA signal transduction mutants. It has been proposed that these putative transcription factors function as negative regulators of GA signal transduction. Another negative regulator of GA signal transduction is encoded by the *SPY* gene. The *SPY* protein is thought to posttranslationally modify target proteins of the GA-signaling pathway (Jacobsen et al., 1996; Peng et al., 1997; Silverstone et al., 1998). To our knowledge, despite this progress in the identification of GA signal transduction components, the other potential elements of GA transduction (e.g. specific protein kinases, phosphatases, channel proteins, or heterotrimeric G-proteins) have not yet been found, nor has the GA receptor been identified. However, circumstantial evidence has pointed to the plasma membrane as the site of GA perception (Hooley et al., 1991; Gilroy and Jones, 1994).

Many signals are initially perceived by transmembrane receptors, a large number of which function by activation of an intrinsic protein kinase domain. In recent years, many plant RLKs have been identified. Whereas the majority of animal RLKs autophosphorylate on Tyr residues, the ma-

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Abbreviations: GST, glutathione S-transferase; KAPP, kinase-associated type 2C protein phosphatase; KID, kinase interaction domain; LRR, Leu-rich repeat; MBP, maltose-binding protein; RLK, receptor-like kinase; TKD, transmembrane kinase domain; TMK, transmembrane kinase.

majority of plant RLKs autophosphorylate on Ser and/or Thr residues (Braun and Walker, 1996). A petunia RLK, PRK1, exhibits dual specificity, phosphorylating on Tyr as well as Ser residues (Mu et al., 1994). Another class of plant RLKs shows sequence similarity to members of the prokaryotic two-component signal transduction systems, which act as His kinases. This group includes the ethylene receptors (Chang et al., 1993; Schaller and Bleecker, 1995; Wilkinson et al., 1995) and *CK1L*, whose protein product is involved in cytokinin signaling (Kakimoto, 1996). RLKs with predicted or demonstrated substrate specificity for Ser and Thr residues can be classified according to the amino acid composition of their putative extracellular domains. The two largest subclasses of RLKs are those whose extracellular domain contains LRRs and those whose extracellular domain shows sequence similarity to the *S*-locus glycoprotein. The extracellular domains of several other plant RLKs are unique.

We are interested in the mechanism by which GA promotes internodal elongation in deepwater rice (*Oryza sativa*; Kende et al., 1998). In a search for GA-regulated transcripts, we identified the LRR-RLK *OsTMK* (*O. sativa* transmembrane kinase). The expression of *OsTMK* increased during GA treatment of rice stem sections, indicating a role for this gene in plant growth. A potential downstream signal transduction component of RLKs is KAPP, originally identified in Arabidopsis by its *in vitro* interaction with RLK5, an LRR-RLK (Stone et al., 1994). *OsKAPP*, a rice ortholog of the Arabidopsis KAPP, was cloned, and the interaction between KID of *OsKAPP* and the kinase domains of the LRR-RLKs *OsTMK*, RLK5, and Xa21 of rice (Song et al., 1995) and the *S*-locus-like RLK, ZmPK1, of maize (Zhang and Walker, 1993) were investigated.

## MATERIALS AND METHODS

### Plant Material

Seeds of deepwater rice (*Oryza sativa* L. cv Pin Gaew 56) were obtained from the International Rice Research Institute (Los Baños, Philippines). Plants were grown as described by Stünzi and Kende (1989). Twenty-centimeter-long stem sections containing the growing internode were excised and treated with 50  $\mu\text{M}$  GA<sub>3</sub> (Raskin and Kende, 1984a, 1984b). Incubation was allowed to proceed for various periods, after which the different regions of the internode were excised, frozen immediately, and stored at  $-80^{\circ}\text{C}$  until use.

### Identification of *OsTMK* and *OsKAPP* Clones

The derived amino acid sequence of a partial 913-bp cDNA (*OsKIN*), which was isolated during a library screen for cyclin genes, turned out to have similarity to protein kinases. A 305-bp fragment from the 5' end of *OsKIN* was used to screen an intercalary-meristem-specific, unamplified cDNA library, and a full-length clone, *OsTMK*, was isolated. The phage insert was inserted into the *NotI* site of the pBluescript SK(-) phagemid (Stratagene), and the DNA sequence was determined as described previously

(Van der Knaap et al., 1996). The sequences were aligned using the Sequencher program (version 3.0, Gene Codes Corp., Ann Arbor, MI).

To isolate a *KAPP* ortholog, a rice cDNA library (Song et al., 1995) was screened under low-stringency conditions (Walker, 1993) using a 0.7-kb *EcoRI* fragment corresponding to the maize KID (Braun et al., 1997) as a probe. To isolate a full-length cDNA of rice *KAPP*, a second rice cDNA library (W.-Y. Song and P.C. Ronald, unpublished data) was screened under high-stringency conditions (Wang et al., 1996) with the partial cDNA clone that had been isolated in the first screen. The two cDNAs were sequenced using a cycle-sequencing kit (Sequitheer, Epicentre Technologies, Madison, WI) and an automated sequencer (model 4000L, LI-COR, Lincoln, NE).

### RNA Gel-Blot Analysis

Twenty micrograms of total RNA, isolated according to the method of Puissant and Houdebine (1990), was separated electrophoretically in a 1.2% (v/v) formaldehyde-agarose gel (Ausubel et al., 1987) and transferred to a Hybond-N membrane (Amersham). DNA fragments containing the inserts of *RL5*, *E37*, and *OsKIN* were isolated from agarose gels by digestion with  $\beta$ -agarase (New England Biolabs). *RL5* encodes the 5S rRNA-binding protein (Kim and Wu, 1993), and *E37* is a truncated cDNA corresponding to a chloroplast inner membrane protein (Van der Knaap and Kende, 1995). *RL5* and *E37* are constitutively expressed transcripts and served as loading controls. Fifty nanograms of template DNA was labeled in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP (3000 Ci mmol<sup>-1</sup>) using a random-primer labeling kit (Boehringer Mannheim). RNA gel blots were hybridized and washed as previously described (Van der Knaap et al., 1997). For gel-blot analysis of *OsKAPP* expression, an RNA probe was prepared from the region encoding KID in the presence of [ $\alpha$ -<sup>32</sup>P]UTP. Blots were prehybridized and hybridized overnight in 3 $\times$  SSPE, 10 $\times$  Denhardt's solution, 0.5% (w/v) SDS, 50  $\mu\text{g mL}^{-1}$  denatured salmon-sperm DNA, and 50% formamide at 65°C. Blots were washed twice for 5 min each time in 2 $\times$  SSC and 0.5% (w/v) SDS at 65°C and then washed twice in 0.1 $\times$  SSC and 0.5% (w/v) SDS at 65°C for 30 min each. The radioactivity on blots was quantified with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

### DNA Gel-Blot Analysis

Rice genomic DNA was isolated from a CsCl gradient according to the method of Ausubel et al. (1987). Four micrograms of genomic DNA was digested with the appropriate restriction enzyme, and the DNA fragments were separated on a 0.8% (w/v) agarose gel for 20 h at 30 V. The gel was treated following standard protocols (Ausubel et al., 1987), and the DNA was transferred to a Hybond-N+ membrane (Amersham). *OsKIN* was used to prepare a probe by random-primer labeling in the presence of [ $\alpha$ -<sup>32</sup>P]dCTP. Blots were prehybridized and hybridized overnight in 6 $\times$  SSC, 5 $\times$  Denhardt's solution, and 1% (w/v) SDS at 65°C. Nonspecific hybridization was re-

moved by stringent washes in  $0.1\times$  SSC and 0.1% (w/v) SDS at 65°C.

### Overexpression and Purification of the Fusion Proteins

To facilitate the cloning of the kinase domain of *OsTMK* in-frame with MBP, a restriction enzyme site was introduced via PCR. The primers used were 5'-ATGGAATTCTC AATTCAAGTCCTC-3' and the reverse primer present in pBluescript SK(-). The product was amplified from a plasmid containing the full-length clone of *OsTMK* with Pwo polymerase (Boehringer Mannheim). The PCR product was digested with *EcoRI* and *HindIII* and inserted directionally in the same sites of pMAL-*cRI* (New England Biolabs). The construct created, pMBP-*OsTKD*, encoded MBP fused to residues 596 to 962 of *OsTMK*. pMBP-*OsTKD* was sequenced over the primer region to verify that the proper fusion construct had been obtained and was introduced into the *Escherichia coli* host ER2508 (New England Biolabs). The cells were grown in rich medium (containing, per liter: 10 g of Bactotryptone, 5 g of yeast extract, 5 g of NaCl, and 2 g of Glc) supplemented with 1 mM  $MnCl_2$  and 50  $\mu g$  mL<sup>-1</sup> carbenicillin at 37°C until an  $A_{600}$  of 0.6 was reached.

The cells were induced by the addition of 50  $\mu M$  isopropyl  $\beta$ -D-thiogalactoside for 2 h at room temperature and harvested by centrifugation. The bacterial pellet was resuspended in lysis buffer (10 mM Tris-HCl, pH 7.3, 150 mM NaCl, 1 mM DTT, 0.1% [v/v] Tween 20, and 1 mM PMSF) and stored at -20°C overnight. The cells were lysed with a cup sonicator at a 30% duty cycle, a probe setting of 4 to 5, or with a French press. MBP and MBP-*OsTKD* fusion protein (83 kD) were allowed to bind to amylose resin (New England Biolabs) for 20 min, and the resin was washed several times with lysis buffer. The final wash was performed in lysis buffer without Tween 20, and the two proteins were eluted with 10 mM maltose and 10 mM Tris-HCl, pH 7.0.

KID of rice KAPP (KID defined according to Stone et al., 1994) was amplified using the primers 5'-GTGATTCTG GCAGGACTTATCCTGC-3' and 5'-AACACCCTGCAGA GGGCACTGGC-3' and inserted into the pGEX-2T-derived expression vector (Pharmacia). The construct created, pGST-*OsKID*, contained the region encoding the rice KID fused in-frame to GST. Both GST and GST-*OsKID* contained a protein kinase A recognition site. Overexpression of GST-*OsKID* (55 kD) and GST was performed essentially as described for MBP-*OsTKD*, except that  $MnCl_2$  was omitted from the rich medium. After the cells were lysed, the proteins were purified by affinity binding to glutathione agarose resin (Sigma). After several washes, the proteins were eluted with 10 mM GSH in 10 mM Tris-HCl, pH 7.0. Protein concentrations were determined by comparison of band intensity against known standards on a Coomassie Blue-stained polyacrylamide gel. The fusion proteins used in the filter binding assay shown in Figure 6 were overexpressed and purified according to the method of Horn and Walker (1994). The GST-Xa21CAT fusion protein contained the catalytic domain of Xa21 fused in-frame to GST (W.-Y. Song and P.C. Ronald, unpublished data). The MBP-RLK5CAT and GST-PK1CAT fusion proteins contained the

catalytic domains of RLK5 and ZmPK1 fused in-frame to MBP or GST, respectively (Stone et al., 1994; Braun et al., 1997).

### Filter Binding Assay

MBP, GST, GST-Xa21CAT, MBP-RLK5CAT, and GST-PK1CAT were separated on a 7.5% SDS-PAGE gel and electrophoretically transferred to PVDF membranes. The GST-*OsKID* fusion protein was labeled with <sup>32</sup>P at the protein kinase A recognition site at the junction of the fusion using bovine heart-muscle kinase (Sigma) and allowed to bind to the proteins on the membrane (Stone et al., 1994). The filter was then subjected to autoradiography.

### Auto- and Transphosphorylation Assays and Phosphoamino Acid Analysis

Purified MBP-*OsTKD*, in the presence or absence of GST-*OsKID*, was incubated in 15  $\mu L$  of 50 mM Tris-HCl, pH 7.3, containing 10  $\mu Ci$  [ $\gamma$ -<sup>32</sup>P]ATP (6000 Ci mmol<sup>-1</sup>), 20  $\mu M$  nonradioactive ATP, 1 mM DTT, and 10 mM  $MnCl_2$ . The reaction was allowed to proceed for the appropriate time at room temperature or at 30°C and then was stopped by the addition of 35  $\mu L$  of ice-cold 10% (v/v) TCA. The radiolabeled proteins were collected by centrifugation, washed with 50  $\mu L$  of ice-cold 10% (v/v) TCA, and resuspended in 4  $\mu L$  of 1 M Tris base and 5  $\mu L$  of 2 $\times$  SDS sample buffer (Bio-Rad). The resuspended proteins were directly loaded onto a 10% or 12% (w/v) polyacrylamide gel and electrophoretically separated with a constant current of 15 mA. The gel was stained with Coomassie Blue to verify equal loading, dried, and radiographic film was exposed to the gel at room temperature. The radioactivity was quantified with a PhosphorImager.

For phosphoamino acid analysis, the autophosphorylated MBP-*OsTKD* was eluted from the polyacrylamide gel overnight in 50 mM  $NH_4HCO_3$ . The sample was precipitated with TCA and hydrolyzed with acid in 6 M HCl at 110°C for 1 h. The HCl was evaporated, and the pellet was resuspended in electrophoresis buffer (2.2% [v/v] formic acid and 7.8% [v/v] acetic acid, pH 1.9) containing phosphoamino acid standards and applied to a cellulose TLC plate (Merck, Rahway, NJ) as described by Boyle et al. (1991). Samples were subjected to electrophoresis at 1.5 kV for 20 min in pH 1.9 buffer in the first dimension and then in pH 3.5 buffer (5% [v/v] acetic acid and 0.5% [v/v] pyridine) at 1.3 kV for 16 min in the second dimension. Phosphoamino acid standards were visualized by spraying the plate with 0.25% (w/v) ninhydrin in acetone and heating it at 65°C for 30 min. Radiographic film was exposed to the TLC plate for 2 d at -80°C.

## RESULTS

### *OsTMK* Encodes an RLK with High Sequence Similarity to Two LRR-RLKs from Arabidopsis

A screen of the rice cDNA library with the partial clone *OsKIN* resulted in the isolation of three independent inserts





**Table I.** Amino acid identity between LRR-RLKs

The numbers indicate overall percentages of identity between the pairs, based on pairwise comparisons using ALIGN. The last column shows the number of LRRs found in the presumed extracellular domain of the kinases.

RLK	OsTMK	TMK1	LRRs
	%		no.
OsTMK			13
TMK1 <sup>a</sup>	59.0		13
AC000103 <sup>b</sup>	49.2	53.3	13
Xa21 <sup>c</sup>	24.9	26.5	23
BR11 <sup>d</sup>	24.4	25.2	25
CLV1 <sup>e</sup>	25.6	27.9	21
ERECTA <sup>f</sup>	25.6	28.2	19
RLK5 <sup>g</sup>	25.8	27.2	21
DcSERK <sup>h</sup>	23.3	22.6	5
PRK1 <sup>i</sup>	20.9	21.2	5

<sup>a</sup> Chang et al. (1992). <sup>b</sup> Accession number for genomic sequence of Arabidopsis BAC F21J9. <sup>c</sup> Song et al. (1995). <sup>d</sup> Li and Chory (1997). <sup>e</sup> Clark et al. (1997). <sup>f</sup> Torii et al. (1996). <sup>g</sup> Walker (1993). <sup>h</sup> Schmidt et al. (1997). <sup>i</sup> Mu et al. (1994).

of approximately 3100 bp, a size similar to that expected from RNA gel-blot analysis, indicating that a full-length clone had been isolated. When the longest insert was sequenced, no in-frame stop codon was observed upstream of the putative start Met. However, an otherwise identical rice expressed sequence tag (D41598) was found to have a five-nucleotide extension with an in-frame stop codon at the 5' end. As shown in Figure 1A, the putative signal sequence is followed by an extracellular LRR region containing nine potential N-glycosylation sites (consensus N-X-S/T). The first 10 LRRs are flanked by two Cys residues spaced 11 and 8 amino acids apart. One Cys pair, spaced 8 amino acids apart, was found at the N-terminal side of the last three LRRs.

LRRs are believed to play a role in protein-protein interaction, and several LRR domains are flanked by Cys clusters (Kobe and Deisenhofer, 1994). The extracellular domain is followed by a putative transmembrane region and the C-terminal intracellular portion of the protein contains the 12 characteristic kinase subdomains (Hanks and Quinn, 1991). Database searches with the BLAST program (Altschul et al., 1990) indicated amino acid sequence similarity to RLKs from plants. Because the highest similarity was to TMK1 from Arabidopsis (Chang et al., 1992), we named the rice gene *OsTMK*. A high level of similarity was also found to an Arabidopsis open reading frame (locus 2213607 on BAC F21J9; accession no. AC000103).

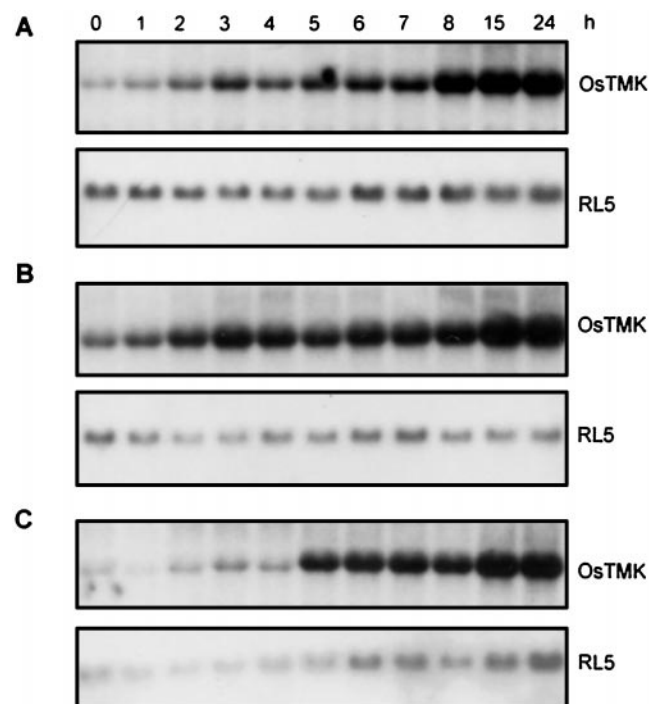
The spacing of the LRRs and the presence of the Cys pairs is conserved in all three proteins. The alignment of the LRRs in the three proteins is shown in Figure 1B. The consensus amino acids shown below the alignment are commonly found in other plant proteins containing LRRs (Li and Chory, 1997), except for the presence of S/T and S at positions 5 and 6, respectively. The alignment of the sequences surrounding the two Cys residues located at either the N-terminal or the C-terminal side of the LRRs in several different LRR-RLKs is shown in Figure 1C. The Cys

residues are often spaced seven amino acids apart; for OsTMK, TMK1, and AC000103, they are usually eight amino acids apart.

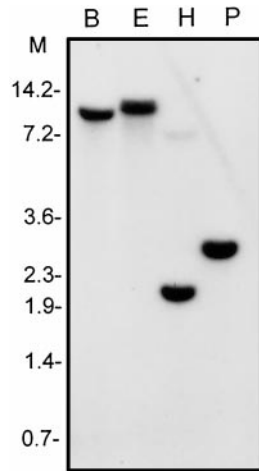
The percentage of amino acid identity, including the putative signal sequences and the transmembrane regions, between several LRR-RLKs is shown in Table I. Whereas the amino acid identity among OsTMK, TMK1, and AC000103 ranges from 49% to 59%, the identity among all other LRR-RLKs is approximately 25%. The kinase domain of OsTMK is 79% identical to that of TMK1 and 63% identical to that of AC000103. The LRR domain of OsTMK is 49% identical to that of TMK1 and 40% to that of AC000103. This indicates that OsTMK, TMK1, and AC000103 belong to a subgroup within the larger LRR-RLK family.

### Changes in Transcript Levels of *OsTMK* in Response to GA and the Gene Copy Number of *OsTMK* in Rice

We were interested in determining whether *OsTMK* plays a role in GA-mediated internodal growth. In rice stem sections, GA enhances the rate of cell production in the intercalary meristem at the base of the growing internode and also results in a 3- to 4-fold increase in the final size of



**Figure 2.** Expression of *OsTMK* in GA-treated stem sections. Stem sections were incubated in 50  $\mu\text{M}$  GA<sub>3</sub> for the times indicated above the lanes. Shown are gel blots containing RNA (20  $\mu\text{g}$  per lane) isolated from the 0- to 5-mm region above the second-highest node, which includes the intercalary meristem and the basal part of the elongation zone (A), 5 to 10 mm above the second highest node, which includes the elongation zone (B), and 10 to 20 mm above the second highest node, which includes the upper part of the elongation zone and the differentiation zone (C). All blots were hybridized to *RL5*, a constitutively expressed gene encoding a 5S rRNA-binding protein.



**Figure 3.** DNA gel-blot analysis of *OsTMK* in rice. A blot containing genomic DNA (5  $\mu$ g per lane), digested with either *Bam*HI (B), *Eco*RI (E), *Hind*III (H), or *Pst*I (P) was probed with random-primer-labeled *OsKIN*, which corresponds to the kinase domain of *OsTMK*. M, Molecular size markers (in kb).

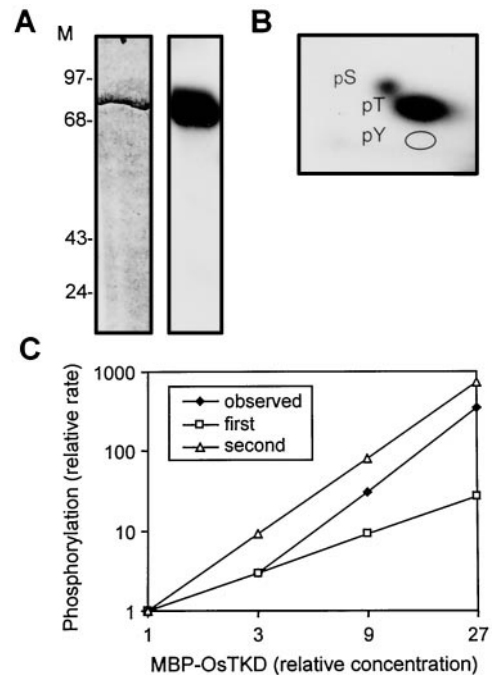
cells in the elongation zone (Raskin and Kende, 1984b; Sauter and Kende, 1992). In deepwater rice internodes, several genes have been identified whose transcript levels increase in response to GA (Kende et al., 1998). Results of gel-blot analyses of RNA isolated from different regions of GA-treated internodes are shown in Figure 2. The top panels in Figure 2, A to C, represent blots hybridized to *OsTMK*; the bottom panels show hybridization of the same blots to *RL5*, a cDNA corresponding to a gene whose expression did not change significantly in the intercalary meristem (Fig. 2A) and in the growing zone (Fig. 2B) of the internode during treatment with GA. The signals were quantified with a PhosphorImager and normalized for equal loading with the signals for *RL5*. *OsTMK* transcript levels increased more than 3-fold in the region of the intercalary meristem and the lower elongation zone after 2 to 3 h of treatment with GA and reached a 9-fold increase after 15 h (Fig. 2A). *OsTMK* transcript levels also increased in the elongation zone (Fig. 2B). In the differentiation zone (Fig. 2C), the increase in *OsTMK* expression was accompanied by an increase in *RL5* mRNA. The expression of *OsTMK* was very low in the oldest part of the internode and did not change during GA treatment (Fig. 9 and data not shown). The transcript levels for *OsTMK* did not increase in control stem sections (data not shown). These results indicate that the mRNA levels of *OsTMK* increase in growing tissues of the internode in response to GA.

High-stringency DNA gel-blot analysis of the region encoding the kinase domain as a probe showed, with the exception of the *Hind*III digest, only one band in each lane (Fig. 3). The additional faint band seen in the lane containing the *Hind*III digest was expected because the insert used as probe had an internal *Hind*III restriction site. The size of the smallest band was 2 kb, which eliminated the possibility that the rice genome contains two linked copies of *OsTMK*. The presence of a single band in each digest on the

DNA gel blot indicates that *OsTMK* is represented by a single gene in the rice genome.

### Phosphorylation Assays with the Kinase Domain of *OsTMK*

To examine whether *OsTMK* constituted an active kinase and to determine which amino acid residues were phosphorylated, the kinase domain, *OsTKD*, was inserted in-frame into a cDNA encoding MBP and was overexpressed in *E. coli*. The resulting fusion protein, MBP-*OsTKD*, was purified to homogeneity and possessed autophosphorylating activity (Fig. 4A). To determine substrate specificity, the autophosphorylated fusion protein was eluted from the polyacrylamide gel and hydrolyzed with HCl; the hydrolysate was analyzed by electrophoresis (Fig. 4B).  $^{32}$ P-labeled spots corresponding to the positions of phospho-Thr and phospho-Ser, but not to that of phospho-Tyr, were detected. This indicates that *OsTMK*, like *TMK1*, is a Ser/Thr protein kinase. Most RLKs undergo intermolecular phosphorylation (second order with respect to enzyme concentration; Horn and Walker, 1994). An intramolecular phosphorylation mechanism, which is a first-order reaction, would result in a linear increase of phosphorylation as a function of enzyme concentration. Because the relationship between the rate of autophosphorylation and the MBP-*OsTKD* concentration was not linear but approached



**Figure 4.** Autophosphorylation of MBP-*OsTKD*. A, Purified MBP-*OsTKD* (500 ng) was allowed to autophosphorylate for 20 min at room temperature. Right, Autoradiogram of the Coomassie Blue-stained gel shown on the left. M, Molecular mass markers (in kD). B, Electrophoretic analysis of phosphoamino acids autophosphorylated by MBP-*OsTKD*. pS, Phospho-Ser; pT, phospho-Thr; pY, phospho-Tyr. C, Semilogarithmic plot of the rate of phosphorylation as a function of MBP-*OsTKD* concentration. The amount of MBP-*OsTKD* used ranged from 30 to 810 nM (38 ng to 1  $\mu$ g per 15- $\mu$ l reaction). The phosphorylation reaction was performed at 30°C for 20 min.



OskAPP	MAAPPLPFVLA AAAIASLVILVLVVFACRRWRRAVVAAPQPPRAAADVVAASPVRSQNE DLNKP LL-EILDDHSSQSN	79
ZmKAPP	. . V . . . . . L . V . V . A . S . . . . . F . C . . . . . RRQRH . LQ . . . . . V . PT . . AV . D . . IDR . . . . . NQS . . . . . R . NI	72
AtKAPP	. . MIGMNIIGL FVMV LML . L . SLIIL . . . . . KP . . YFSRFRSSSRFSSTFKVGD LQR . LI SDDGN . IQGQTS . VTREYDLEGA	80
OskAPP	TFPGNVVGE SSKVHTS-RSDTSPRSHGISDSGRTPAD SCTPQGETHVIDVDTDDTSEEFHLG STLKCTKQTSWSRPDKKH	158
ZmKAPP	S . L . SS . . . . . P . IQ . N . . . . . N . . . . . A . A . T . . I . . EC . VT . . . . . N . EN . . . . . Q . . . . . RR . PPPK . PT . . Q . .	151
AtKAPP	CYQNDGL LH . . LTEGRFYKQRL . S . SPHFSQ . ESFVLEVI SEPSDNALVGQ . LK LPA . . . . . KGS . AEVQTYD . QNNRNEN	157
OskAPP	KRWGSGEDNKNGSISLKDNTYRSNLDVEVIAGPSHGISC SRQSTSPT-IPITLGRVPPSDLV LK DSEVSGKHARINWNAK	237
ZmKAPP	R . RV . . D . . H . . VP . . . . . H . R . . L . . . . . SR . SML . . . . . Q . . . . . G .	231
AtKAPP	LQYNLEK . RLINLSPRLVEDQ . W . FL . . . . . AI . LQHAVN . . . . . SSKL . VK . . . . . S . . . . . A . . . . . Q . T . . ST	237
OskAPP	TLKWEIVDMGSLNGTFVNSRAVHHPNVGSRHWGEP AELADGDIITLGTSSKLSVQISLQNQ-RVPAGIGMASDPMVGRRS	316
ZmKAPP	. . . . . L . . . . . L . Q . . . . . SA . . . . . H . . . . . L . . . . . V . . . . . A . . . . .	310
AtKAPP	KF . . . . . L . . . . . L . . . . . HSIS . DL . . . . . K . N . V . . . . . SD . . . . . TT . VY . R . . . . . EFQI . FK . . . . . AM . G	317
	motif 1 motif 2 motif 3	
OskAPP	<u>GKKLAMEDISFCQCPLQGVGEQGLFGIFDGHGGDGAARAVSKIFPENVA TLSSHETKEKVLSDASDVLRYAFTMTEA</u>	396
ZmKAPP	. . . . . P . . . . . H . . . . . K . . . . . L . . . . . HLGYL . . . . . P . . . . . R . Q . . . . . L . . . . . D	390
AtKAPP	. R . . . . . P . . . . . VCHYKW . . P . ANK . . . . . CVC . . . . . S . . . . . QSAI . . . . . I . . . . . VL . NI . . . . . DSLR . . . . . KR . . . . . DM . AK . . . . .	397
	motif 4 motif 5 motif 5a motif 5b motif 6	
OskAPP	<u>AI-DHEYEGCTATVLLIWFQDKKDCFAQCANLGD SACVMSVNGKMIEMTEDHRVASVTERARIARAGQALKA GEVVRINGL</u>	475
ZmKAPP	T . - . . . . Q . . . . . G . A . . . . . N . . . . . T . D . . . . . T . A . . . . . T . P . RD . . A . LS . .	469
AtKAPP	RLEE . Q . . . . . V . K . NEENF . . . . . IHLG . RY . Q . . . . . V . LS . . K . FQE . . L . . RD . . T . LF . I	477
	motif 7 motif 8 motif 9 motif 10	
OskAPP	<u>NLARMFGDKFLKEQDSRFSSEFPYVSQAVHITKAC-TAF AVIASDGLWDVISTKRAVQLVVEGRE-RNSGDSAS-ADKVAN</u>	552
ZmKAPP	. . . . . P . . . . . P . . . . . R . . . . . K . R . S . . . . . AT . A . AR . . S	548
AtKAPP	. . . . . L . . . . . P . Q . . . . . A . . . . . I . EPLR . DQSSKDV . . . . . L . . . . . V . P . K . . . . . LQM . DKERGRE . . . . . S . E . I . .	555
	motif 11	
OskAPP	<u>RILSEARNLRTKDNTSVIFVDFDILRTDHCI AK</u>	585
ZmKAPP	. V . D . . . . . S . . . . . V . . . . . R . P . . SPVD	583
AtKAPP	GL . N . . . . . AM . . . . . I . YL . . . . . TSL	581

**Figure 5.** Alignment of KAPPs from rice (OsKAPP), maize (ZmKAPP), and Arabidopsis (AtKAPP). Amino acids identical to those in OsKAPP are indicated by periods, and gaps in the alignment with dashes. The 11 structurally conserved motifs of type 2C protein phosphatases are underlined and numbered along the top. Accession numbers are as follows: OsKAPP, AF075603; ZmKAPP, U81960; and AtKAPP, U09505.

the curve expected for a second-order reaction, MBP-OsTKD appears to phosphorylate itself via an intermolecular, higher-order reaction mechanism (Fig. 4C).

### Identification of OsKAPP

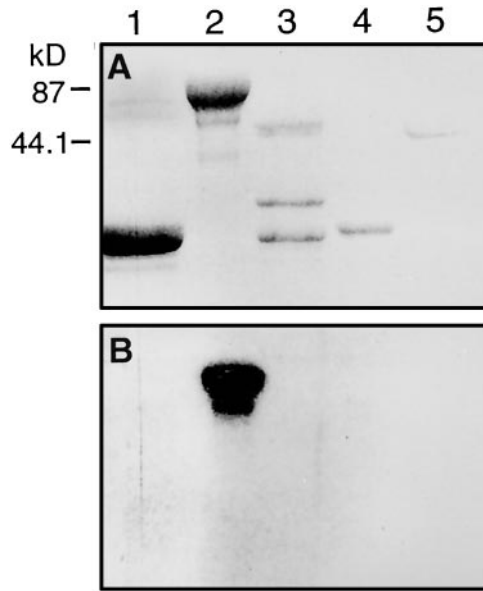
A potential downstream component of several RLKs in plants is KAPP. Arabidopsis and maize KAPP have been shown to interact with TMK1 via KID (Braun et al., 1997). For this reason, we identified the rice ortholog of KAPP and determined its interaction with plant RLKs. *OsKAPP* contains an open reading frame encoding 585 amino acids (Fig. 5). Sequence comparison indicated that OsKAPP has a high level of overall similarity to maize KAPP (81.3% similarity, 77.1% identity, Fig. 5) and Arabidopsis KAPP (54.3% similarity, 45.4% identity, Fig. 5). Like the Arabidopsis and maize KAPP, the predicted rice protein consists of three domains: a potential N-terminal membrane anchor, a centrally located KID, and a carboxy-terminal type 2C protein phosphatase. The N-terminal anchor shares relatively low similarity to the corresponding regions of the Arabidopsis (42.9% similarity, 25.0% identity) and maize KAPP (73.1% similarity, 69.2% identity). In contrast, KID and the protein phosphatase domain of OsKAPP are more related to those of Arabidopsis (KID: 53.5% similarity, 46.9% identity; phosphatase: 68.3% similarity, 57.9% identity) and maize KAPP (KID: 83.3% similarity, 80.0% iden-

tity; phosphatase: 87.5% similarity, 83.2% identity). The protein phosphatase domain of OsKAPP carries the 11 structurally conserved motifs of type 2C protein phosphatases, which is a strong indication for protein phosphatase activity (Bork et al., 1996; Fig. 5).

### Filter Binding Assays of OsKID to RLKs and the Phosphorylation of OsKID by MBP-OsTKD

To determine the protein-binding properties of rice KID, filter binding assays were performed as described for Arabidopsis and maize KID (Stone et al., 1994; Braun et al., 1997). OsKID was expressed in *E. coli* as a GST fusion protein and phosphorylated with  $^{32}\text{P}$  at the protein kinase A site. The probe was allowed to interact with the recombinant kinase domains MBP-RLK5CAT, GST-PK1CAT, and GST-Xa21CAT. The results of the protein-protein interaction assays showed that GST-OsKID interacted with MBP-RLK5CAT but not with GST-PK1CAT (Fig. 6), as has also been found for the Arabidopsis and maize KID (Braun et al., 1997). Furthermore, the labeled GST-OsKID did not bind to GST, MBP, or GST-Xa21CAT. These results indicate that OsKID interacts with RLK5, an LRR-RLK from Arabidopsis, but not with Xa21, an LRR-RLK from rice.

Arabidopsis and maize KID have been shown to interact with TMK1 (Braun et al., 1997). We wanted to determine whether rice KID also interacted with OsTMK and whether

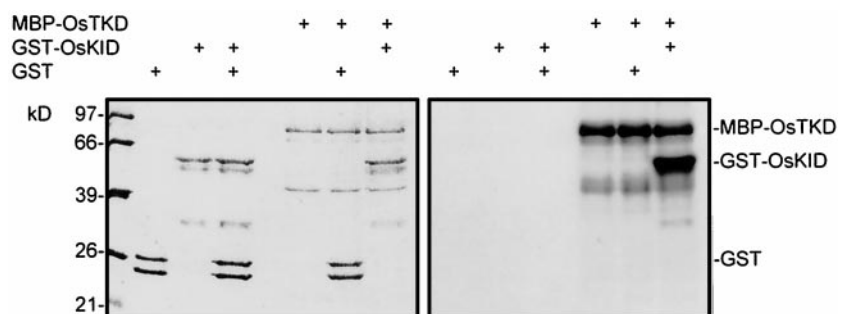


**Figure 6.** KID interacts with the kinase domain of Arabidopsis RLK5 but does not interact with the kinase domains of Xa21 and ZmPK1. A, Coomassie Blue-stained SDS-PAGE gel. Lane 1, GST-Xa21CAT; lane 2, MBP-RLK5CAT; lane 3, GST-PK1CAT; lane 4, GST; and lane 5, MBP. B, Corresponding autoradiogram probed with the <sup>32</sup>P-labeled rice GST-OsKID. The strongly interacting protein (80.5 kD) corresponds to the full-length MBP-RLK5CAT, whereas the weaker, smaller protein may be a degraded form of MBP-RLK5CAT.

it was a substrate for phosphorylation by OsTMK. As shown in Figure 7, MBP-OsTKD phosphorylated GST-OsKID but not GST alone. GST and GST-OsKID did not autophosphorylate or phosphorylate each other. This indicates that GST-OsKID is a substrate for MBP-OsTKD and that these proteins interact in vitro.

Because MBP-OsTKD and GST-OsKID are both substrates for phosphorylation, the level of phosphorylation was determined as a function of protein concentration. Increasing amounts of MBP-OsTKD led to an increase in autophosphorylation of MBP-OsTKD and an increase in phosphorylation of GST-OsKID (Fig. 8A). However, the level of autophosphorylation was greatly increased by decreasing amounts of GST-OsKID. Autophosphorylation is inhibited by increasing amounts of inactive kinase (Horn and Walker, 1994; Williams et al., 1997). To investigate whether inhibition of in vitro autophosphorylation of MBP-OsTKD was specific to its substrate, a phosphoryla-

**Figure 7.** Phosphorylation of GST-OsKID. Left, Coomassie Blue-stained gel. The reaction mixture contained 1.5 μg of GST, 0.5 μg of GST-OsKID, and/or 0.5 μg of MBP-OsTKD, as indicated above the lanes. The reaction was allowed to proceed for 20 min at room temperature. The lower bands are proteolytic degradation products. Right, Autoradiogram of the same gel.



tion assay with MBP-OsTKD and GST-OsKID in the presence of excess GST-OsKID, GST, or BSA was performed (Fig. 8B). Phosphorylation of MBP-OsTKD and GST-OsKID were both reduced at higher GST-OsKID concentrations (Fig. 8B, compare lanes 1 and 4). Inhibition of MBP-OsTKD activity was also observed in the presence of BSA or GST (Fig. 8B, lanes 2 and 3, respectively), indicating that in vitro inhibition of OsTMK activity is not specific to its substrate and that the kinase assay is sensitive to protein concentration.

**Expression of OsTMK and OsKAPP in Rice**

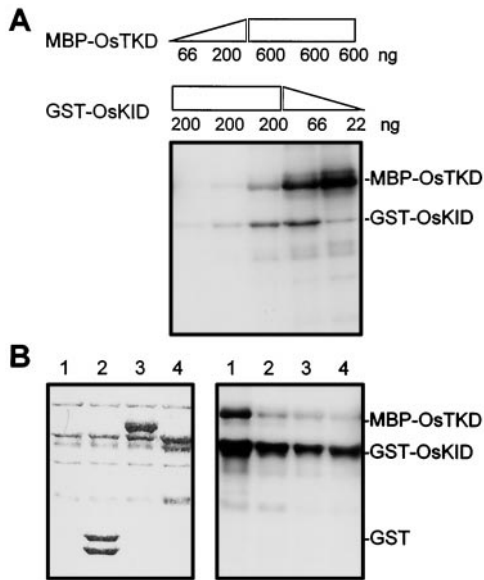
KAPP was shown to interact in vitro with several RLKs (Braun et al., 1997). To determine whether OsTMK and OsKAPP may interact in vivo, the expression patterns of these genes were investigated by RNA gel-blot analysis. High transcript levels of *OsTMK* were detected in many tissues of rice, particularly in regions containing dividing and elongating cells (Fig. 9). Lower levels of expression were found in the basal part of the second youngest leaf blade and in the oldest part of the internode; these tissues show reduced growth or no growth, respectively. The expression of *OsKAPP* showed a similar trend (Fig. 9). Whereas the transcript levels of *OsTMK* clearly increased during GA treatment of stem sections (Fig. 2), the GA-induced accumulation of *OsKAPP* mRNA was small, reaching only a 3-fold increase after 24 h (data not shown).

**DISCUSSION**

**Amino Acid Sequence Comparisons of OsTMK with TMK1 and Other LRR-RLKs**

We identified an LRR-RLK that was highly expressed in growing regions of deepwater rice. The gene encoding this kinase was named *OsTMK* for several reasons: (a) it encodes a putative TMK; (b) its highest amino acid sequence identity is to TMK1 from Arabidopsis (Chang et al., 1992); and (c) the expression pattern of *OsTMK* resembles that of *TMK1* in Arabidopsis (Chang et al., 1992). DNA gel-blot analysis indicated that *TMK1* is a unique gene in Arabidopsis. However, a related gene (AC000103) showing 53.3% amino acid identity to TMK1 was identified in the Arabidopsis genome sequence database. DNA gel-blot analysis of rice genomic DNA and the region corresponding to the OsTMK kinase domain as a probe also indicated the presence of only one gene in the rice genome. However,





**Figure 8.** Phosphorylation activity of MBP-OsTKD. A, Phosphorylation assay in the presence of different amounts of fusion proteins as indicated above the lanes. The reaction mixtures were incubated at room temperature for 20 min. B, Inhibition of MBP-OsTKD activity as shown by a phosphorylation assay with 500 ng of MBP-OsTKD and 500 ng of GST-OsKID. Lane 1, No additions; lane 2, 3  $\mu$ g of GST added; lane 3, 2.5  $\mu$ g of BSA added; lane 4, additional 750 ng of GST-OsKID added. The reaction mixtures were incubated at 30°C for 20 min. Left, Coomassie Blue-stained gel; right, corresponding autoradiogram.

a rice expressed sequence tag (D39936) with high sequence similarity to part of the LRR domain of OsTKM was identified recently. Therefore, it is possible that homologs of *OsTKM* exist in rice as well.

Arabidopsis AC000103 and *TMK1* are likely to be evolutionarily related. The position of a unique intron (84 nucleotides in both *TMK1* and AC000103) is conserved between the first and the second nucleotide of the codon corresponding to Val at position 766 in *TMK1*, immediately following kinase subdomain VIII (Chang et al., 1992). For *OsTKM*, the intron position(s) has not been determined. However, the lane containing *Hind*III-digested genomic DNA was expected to show a band of 1.7 kb based on the cDNA sequence. Instead, the band detected was 2 kb, indicating the presence of an intron in this fragment that spans the region containing the intron in AC000103 and *TMK1*.

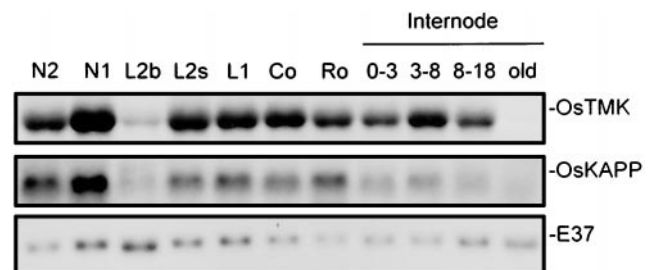
Based on amino acid sequence similarity, *TMK1* is more related to *OsTKM* than to AC000103. Several gaps exist in the sequence alignment of *TMK1* and AC000103. In Arabidopsis, *TMK1* is found in the membrane fraction and is glycosylated in vivo (Schaller and Bleecker, 1993). Six of the nine potential glycosylation sites in the presumed extracellular domain are conserved between *OsTKM* and *TMK1*. Between AC000103 and *OsTKM* or *TMK1*, only one and two of the nine potential glycosylation sites, respectively, are conserved. Furthermore, phylogenetic analysis using the Clustal W method (Thompson et al., 1994) with the PAM250 residue weight table showed that *TMK1* and

*OsTKM* are more related to each other than to AC000103 (data not shown). The high sequence conservation and similar expression pattern between two RLKs from a monocot and a dicot species indicate an important role and functional relatedness for this gene.

### Potential Role of *OsTKM* in GA-Mediated Growth

Treatment with GA increased the transcript level of *OsTKM* in deepwater rice internodes. The expression of this gene was particularly high in all regions undergoing cell division and elongation and low in the nongrowing region of the internode. This suggests a role for this gene in plant growth. Recently, BRI1, an LRR-RLK that plays a role in brassinosteroid signaling, was identified (Li and Chory, 1997). Because both GA and brassinosteroids are terpenoids, the intriguing possibility arises that an LRR-RLK such as *OsTKM* may also be involved in GA signal transduction, as is the case in brassinosteroid signaling.

Hormones and external stimuli regulate the transcript levels of several genes encoding RLKs in plants. The mRNA encoding SFR, an *S*-locus-like RLK of cauliflower, accumulates after wounding and bacterial infection (Pastuglia et al., 1997). Similarly, the transcript levels of *RPK1*, which codes for an LRR-RLK in Arabidopsis, increases after ABA treatment and in response to a variety of environmental stresses (Hong et al., 1997). Inducible expression of some receptor kinase genes has been observed in animals as well. The epidermal and platelet-derived growth factors cause an increase in the transcript levels of their respective receptors (Clark et al., 1985; Erickson et al., 1991). Ligand-induced changes in receptor transcript levels have also been observed in plants. The mRNA levels for the putative ethylene receptors NR in tomato (Wilkinson et al., 1995) and ERS1, ERS2, and ETR2 in Arabidopsis (Hua et al., 1998) increase in response to ethylene. Therefore, there is



**Figure 9.** Tissue-specific expression of *OsTKM* and *OsKAPP* in rice. N2, Second highest node; N1, highest node containing the shoot apex; L2b, basal 2 cm of second youngest leaf blade; L2s, basal 2 cm of second youngest leaf sheath; L1, youngest leaf; Co, coleoptile 3 d after germination; Ro, root 3 d after germination; 0-3, internodal region 0 to 3 mm above N2 containing the intercalary meristem; 3-8, internodal region 3 to 8 mm above N2 containing the elongation zone; 8-18, internodal region 8 to 18 mm above N2 containing the upper part of the elongation zone and the differentiation zone; old, oldest part of the internode. Top, Hybridization signals with *OsKIN*, which corresponds to the kinase domain of *OsTKM*, as a probe; middle, hybridization signals with *OsKAPP*; bottom, hybridization signals with *E37*, a truncated cDNA corresponding to a constitutively expressed chloroplast inner membrane protein.

precedence for ligands changing the transcript level of their respective receptors. To elucidate the function of OsTMK, it will be necessary to determine whether this RLK is indeed involved in mediating growth and, if so, whether it is a component of the GA transduction pathway. If the latter turns out to be the case, it will be interesting to find out whether GA by itself or GA with an accessory protein can bind to OsTMK and, as a ligand, induce the accumulation of *OsTMK* mRNA.

### Phosphorylation Characteristics of MBP-OsTKD and the Potential in Vivo Role of OsKAPP

OsTMK is an active protein kinase that autophosphorylates primarily on Thr residues and to a lesser extent on Ser residues, as has been shown for TMK1 (Chang et al., 1992). The autophosphorylation mechanism of MBP-OsTKD is complex and appears to occur via a higher-order reaction mechanism. Complex autophosphorylation was found for RLK5 as well (Horn and Walker, 1994). Phosphorylation of the inactive RLK5 kinase domain by active RLK5 kinase showed that RLK5 uses primarily an intermolecular mechanism. In animals, it is known that inactive receptor monomers are in equilibrium with active receptor dimers and that ligand binding stabilizes the active dimeric form (Ullrich and Schlessinger, 1990). Dimerization (or higher-order oligomerization) is responsible for activation of the intrinsic protein kinase activity and for autophosphorylation; both processes are mediated by an intermolecular mechanism (Lemmon and Schlessinger, 1994).

One candidate for a signal transduction component downstream of several RLKs is KAPP. We isolated a rice ortholog of *KAPP* using a heterologous cloning approach. Similar to *Arabidopsis* (Stone et al., 1994) and maize (Braun et al., 1997) *KAPP*, the rice gene encodes a putative carboxy-terminal 2C type protein phosphatase with a potential N-terminal membrane anchor and a centrally located KID. Because the phosphatase domain of OsKAPP carries the 11 structurally conserved motifs of type 2C protein phosphatases, it is likely to be an active phosphatase. Previous studies have shown that *Arabidopsis* and maize KIDs interact in vitro with the protein kinase domain of several RLKs, including RLK5 and TMK1, but not with ZmPK1 (Braun et al., 1997). Similarly, rice KID interacts with the kinase domain of RLK5 and OsTMK but not with the kinase domain of ZmPK1 and Xa21. These results suggest that KAPP-mediated signaling is conserved across dicot and monocot plant species. To date, the in vivo role of these interactions is unknown. In the CLV1-signaling pathway, KAPP appears to interact with this LRR-RLK in vivo (Williams et al., 1997; Stone et al., 1998). However, *CLV1* is expressed only in the shoot and floral meristems (Clark et al., 1997). Because of its ubiquitous expression in the plant and its interaction with several RLKs, KAPP is likely to function in a number of other signaling pathways. The expression pattern of *OsKAPP* showed a trend similar to that of *OsTMK*, which, along with the in vitro phosphorylation of GST-OsKID by MBP-OsTKD, may be indicative of in vivo interaction of these two proteins and a role for OsKAPP in OsTMK-mediated signaling.

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