

# A Subset of *OsSERK* Genes, Including *OsBAK1*, Affects Normal Growth and Leaf Development of Rice

Hye Sun Park, Hee Young Ryu, Beg Hab Kim, Sun Young Kim, In Sun Yoon<sup>1</sup>, and Kyoung Hee Nam\*

Since the identification of BRI1-Associated receptor Kinase 1 (BAK1), a member of the Somatic Embryogenesis Receptor Kinase (SERK) family, the dual functions of BAK1 in BR signaling and innate immunity in *Arabidopsis* have attracted considerable attention as clues for understanding developmental processes that must be balanced between growth and defense over the life of plants. Here, we extended our research to study cellular functions of *OsSERKs* in rice. As it was difficult to identify an authentic ortholog of AtBAK1 in rice, we generated transgenic rice in which the expression of multiple *OsSERK* genes, including *OsBAK1*, was reduced by *OsBAK1* RNA interference. Resulting transgenic rice showed reduced levels of *OsBAK1* and decreased sensitivity to BL, leading to semi-dwarfism in overall growth. Moreover, they resulted in abnormal growth patterns, especially in leaf development. Most of the *OsBAK1RNAi* transgenic rice plants were defective in the development of bulliform cells in the leaf epidermal layer. They also showed increased expression level of pathogenesis-related gene and enhanced susceptibility to a rice blast-causing fungal pathogen, *Magnaporthe oryzae*. These results indicate that *OsSERK* genes, such as *OsBAK1*, play versatile roles in rice growth and development.

## INTRODUCTION

SERK proteins, which are members of one of the subfamilies of leucine rich-repeat receptor-like kinases (LRR-RLKs), are involved in somatic embryogenesis, an artificial stimulation of natural embryogenesis (Toonen et al., 1993). *Arabidopsis* SERK proteins are encoded by a gene family consisting of five close homologs (*AtSERK1-5*) and are important not only as molecular markers in somatic embryogenesis but also because of their pleiotropic roles in plant development (Albrecht et al., 2008; Hecht et al., 2001). In these respects, BAK1 has received the most attention since identified as a co-receptor of BRI1, part of the plasma membrane localized brassinosteroid (BR) receptor that mediates BR signaling independent of somatic embryogenesis (Li et al., 2002; Nam and Li, 2002). BRs play critical roles in various aspects of plant growth and development,

including germination, photomorphogenic responses, cell elongation, xylem differentiation, and male fertility (Bajguz, 2007; Kim et al., 2009). BAK1 was found to be identical to AtSERK3, and it is now known that other BAK1 homologs also belong to AtSERK family, including AtSERK2/BAK2, AtSERK4/BAK7, and AtSERK5/BAK8 (Jeong et al., 2010). AtSERK1 and AtSERK2 have been pulled down in complexes with BRI1 in total protein fractions from *Arabidopsis* (Karlova et al., 2006), and AtSERK4 rescues *bri1-5* and *bri1-301* mutants (Albrecht et al., 2008), further indicating that these proteins are involved in BR signaling and are redundant with BAK1/AtSERK3.

SERK proteins in various species have been reported to play roles in plant innate immunity and senescence, too. BAK1 can form a complex with FLS2, an another LRR-RLK, initiating PAMP triggered immunity (PTI) with flagellin binding, and the *bak1* mutant showed reduced responsiveness to flagellin-triggered immunity (Chinchilla et al., 2007). BAK1-deficient plants exhibited broader necrotic region upon microbial infection, accompanied by increase of ROS production (Kemmerling et al., 2007). Interestingly, a different set of genes seem to be engaged in BAK1-mediated BR signaling and innate immunity, as genes induced by brassinolide (BL) do not overlap with the genes induced by infection (Kemmerling et al., 2007). Double mutant plants lacking *BAK1* and *BKK1/AtSERK4/BAK7* displayed a spontaneous cell death phenotype with constitutive defense gene expression (He et al., 2007), and transgenic plants expressing *BAK7* RNA interference (RNAi) constructs showed early senescence with premature expression of senescence-associated genes (Jeong et al., 2010), suggesting additional function of SERK family proteins. Taken together, the pleiotropic functions of BAK1 open new possibilities for genetic engineering, because manipulation of a single gene such as *BAK1* may result in the production of plants with both higher yield and improved disease resistance.

Although most of studies on BAK1 have been in *Arabidopsis*, studies of SERK proteins, including an ortholog of AtBAK1, are currently being extending to monocotyledonous plants. The first *SERK* gene in rice, *OsSERK1*, was screened from the rice cDNA library using a rice EST-clone probe derived from the amino acid sequence of DcSERK1 as a query (Ito et al., 2005), and it was also independently identified as a gene induced by the infection of *Magnaporthe grisea* (Hu et al., 2005). *OsSERK2*

Department of Biological Science, Sookmyung Women's University, Seoul 140-742, Korea, <sup>1</sup>Bio-Crops Development Division, National Academy of Agricultural Science, Rural Development Administration, Suwon 441-857, Korea

\*Correspondence: khnam514@sookmyung.ac.kr

Received August 31, 2011; revised October 3, 2011; accepted October 11, 2011; published online November 3, 2011

**Keywords:** BR signaling, bulliform cell, *Magnaporthe Oryzae*, *OsBAK1*, *OsSERKs*, RNA interference

was found in the data-base using *OsSERK1* as a query (Ito et al., 2005). *OsSERK1* shows more than 85% homology with *OsBISERK1*, benzothiadiazole (BTH)-induced *SERK1* (Song et al., 2008). In addition to *OsSERK1* and *OsSERK2*, *OsSERK3* and *OsSERK4* were also identified as homologs of *BAK1/AtSERK3*. The *OsSERK1-4* genes all rescued *bri1-5* mutant phenotypes, indicating that these proteins are functionally redundant with *AtSERKs*, including *BAK1*. Because the degree of phenotypic rescue of *bri1-5* was highest with *OsSERK1*, *OsSERK1* is believed to be the functional Arabidopsis *BAK1/AtSERK3* ortholog in rice and has been renamed *OsBAK1* (Li et al., 2009). However, phylogenetic analysis shows that *OsSERK1/OsBAK1* is an ortholog of *AtSERK1*, not *BAK1/AtSERK3*. Moreover, recent sequence analysis has revealed nine more *SERL (SERK-like)* genes in rice (Singla et al., 2009). Therefore, it has been suggested that gene duplication occurred in genes encoding *OsSERKs* after the evolutionary divergence of monocots and dicots. As there are still many annotated partial cDNAs encoding only kinase domains that are very similar to the kinase domain of *OsSERKs* and *OsSERLs* in rice, it may be difficult to gain insight into the specific functions of each *OsSERK* gene.

In this study, our aim was to functionally characterize *OsSERKs*. Because we could not determine which gene is the true ortholog of *AtBAK1* in rice, we generated transgenic rice transformed with *OsBAK1RNAi* construct to investigate the function of the *OsSERK* family, including *OsBAK1*. We found that most of the *OsBAK1RNAi* transgenic plants showed defective development of bulliform cells in the leaf epidermal layer and increased susceptibility to the rice blast-causing fungal pathogen *Magnaporthe oryzae*. These results indicate that a subset of *OsSERK* genes, including *OsBAK1*, play multiple roles in growth and development as well as in defense signaling in rice.

## MATERIALS AND METHODS

### Plant materials and growth conditions

Rice seeds (*Oryza sativa*, japonica cv. Nakdong) were used for transformation of all the genes in this study. Transgenic rice plants were grown in square containers with Murashige and Skoog (Duchefa) agar medium in growth chambers set with 16 h/8 h (L/D) photoperiod at 28°C/25°C and 80% relative humidity with/without phosphinothricin selection. Then, 15-day-grown plants were transferred to large containers with rice paddy soil and grown in the same conditions or under sunlight.

### Plasmid constructions and rice transformation

To generate the *OsBAK1RNAi* construct, a 250-bp genomic fragment that encodes the kinase domain of *OsSERK1* was PCR-amplified using primer pairs of *RBAK1CF/RBAK1BR* and *RBAK1KF/RBAK1XR* to fit in the RNA interference cassette *pHannibal*. When designing the primers, we added *NotI* and *XmaI* to the *RBAK1BR* and *RBAK1XR*, respectively, to clone them into *pSB505* binary vector later. The resulting plasmid, *pHannibal-OsBAK1RNAi*, was digested with *NotI* and *XmaI* and ligated into *pSB505* binary vector to generate *pSB505-OsBAK1RNAi*. All fragments produced by PCR were confirmed by sequencing. Detailed primer sequences are listed in Supplementary Table 1.

All constructs including the *pSB505* vector plasmid alone were introduced into wild-type rice (*Oryza sativa*, japonica cv. Nakdong) by Agrobacterium-mediated transformation as described by Hiei et al. (1994).

### Expression by RT-PCR analysis

RNA was purified from the frozen tissues with extraction buffer (0.2 M Tris-Cl, pH 9.0, 0.4 M LiCl, 25 mM EDTA, 1% SDS) and aqua phenol (Q-biogene), and treated with RNase-free RQ1 DNase (Promega). First-strand cDNA synthesis was performed with SuperscriptIII-MMLV reverse transcriptase (Invitrogen) using oligo d(T)<sub>15</sub> as a primer. The same aliquot of first-strand cDNA was then used as a template for the second PCR step using Real Taq polymerase (RBC). The sequences of the primers and use of them are described in Supplementary Table 1.

### Lamina joint assay

Imbibed seeds were sown on plates containing 1/2 MS media (100 mg/L myo-inositol, 125 mg/L cefotaxime, 0.6% agar, pH 5.6) and grown for 6 days described as above. Then, 1 µl of 70% ethanol containing 0, 1, 10, or 100 ng of BL was applied to the lamina joint of the second leaf. After incubation for 3 days, the bending angles between the leaf blade and leaf sheath were measured.

### Treatment of rice blast fungus

Inoculation with *Magnaporthe oryzae* was performed as described (Park et al., 2009). The *M. oryzae* strain KJ201 was obtained from the Center for Fungal Genetic Resources. Conidia were harvested from fungal cultures grown on V8 agar medium (V8 80 ml/L, agar 15 g/L, pH 6.5) for 7 days under constant fluorescent light at 25°C. After counting the number of conidia in a small aliquot of conidial suspension using a hemocytometer, the concentration was adjusted to 4 × 10<sup>4</sup> spores/ml. The final conidial suspensions were sprayed onto the young leaves of transgenic plants grown for 7 weeks. Infected plants were incubated in a chamber with 100% humidity in the dark for 24 h and then, were placed in a growth chamber with normal conditions. Leaf samples were analyzed after four days.

### Microscopic observation

A stereo microscope (Leica MZ 12.5) and Image Manager 5.0 software were used to observe the surfaces and internal structure of the leaves of transgenic plants.

## RESULTS

### Reduced expression of *OsBAK1* by RNA interference caused inhibition of growth, abnormal development, and loss of BL sensitivity in rice

As there are no reports of natural mutants for *OsBAK1*, we searched for *AtBAK1* orthologs among the rice genes that were reported to be as *OsSERKs* or *OsSERLs* in the public database using the amino acid sequence of *AtBAK1* as a query. It was difficult to determine which gene was an exact *AtBAK1* ortholog, because many similar genes were identified. *OsSERKs* and *OsBISERK1* cluster together, closer to each other than to any other *AtSERKs*, based on amino acid sequence homology (Fig. 1A). However, *OsSERK1*, *OsSERK2*, and *OsBISERK1* still showed the considerably high amino acid sequence homology to *AtBAK1* (77%) and *AtSERK4/AtBAK7* (71-72%). The amino acid sequence homologies between *AtBAK1* and other *OsSERKs* or *OsSERLs* were in broad ranges (Fig. 1A and Table 1). Therefore, we used an RNA interference approach to knock down *OsBAK1* and highly homologous genes simultaneously.

To clone the *OsBAK1* RNAi construct, we first amplified a 250-bp fragment encoding part of the *OsBAK1* kinase domain based on its ability to rescue *bri1-5* (Li et al., 2009). The resulting 250-bp amplified region was identical to the corresponding

**Table 1.** Comparison of amino acids homologies among SERK and SERK-like proteins in rice and *Arabidopsis*

	OsSERK1	OsSERK2	OsSERK3	OsSERK4	OsSERL1	OsSERL2	OsSERL3	OsSERL4	OsSERL5	OsSERL6	OsSERL7	OsSERL8	OsSERL9	OsBISERK1	AtSERK1	AtSERK2	AtSERK3	AtSERK4	AtSERK5
OsSERK1	100	87	60	53	46	26	30	45	52	60	9	56	53	99	83	85	77	72	68
OsSERK2		100	58	54	46	28	31	47	52	58	6	55	54	87	82	81	77	72	67
OsSERK3			100	42	41	25	29	38	44	100	5	43	42	60	57	59	56	55	52
OsSERK4				100	56	23	27	42	48	42	11	87	99	53	53	52	49	48	47
OsSERL1					100	25	25	40	44	41	9	57	56	46	46	45	45	43	43
OsSERL2						100	61	29	33	25	10	23	23	26	29	26	26	23	23
OsSERL3							100	34	42	29	7	22	27	30	31	30	30	30	26
OsSERL4								100	54	38	10	45	42	45	45	44	44	42	42
OsSERL5									100	44	7	50	48	52	52	51	49	48	48
OsSERL6										100	5	43	42	60	57	59	56	55	52
OsSERL7											100	8	11	9	6	9	8	9	2
OsSERL8												100	86	55	55	55	52	51	49
OsSERL9													100	53	52	52	49	48	47
OsBISERK1														100	83	84	77	71	67
AtSERK1															100	89	77	73	67
AtSERK2																100	77	74	69
AtSERK3																	100	81	78
AtSERK4																		100	85
AtSERK5																			100

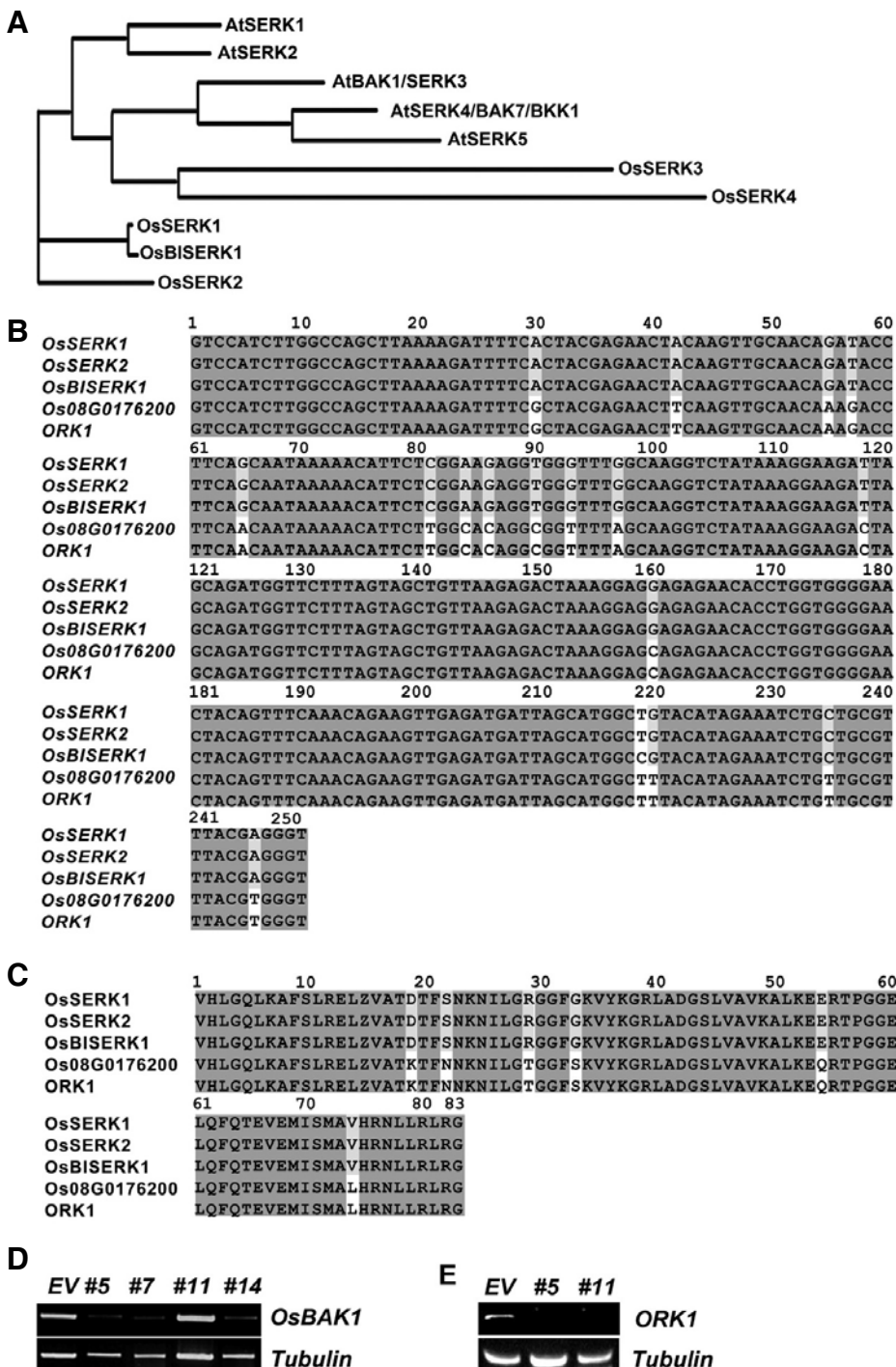
Full length of amino acids sequences of OsSERK1 (624 aa), OsSERK2 (628 aa), OsSERK3 (616 aa), OsSERK4 (607 aa), OsSERL1 (608 aa), OsSERL2 (644 aa), OsSERL3 (324 aa), OsSERL4 (620 aa), OsSERL5 (628 aa), OsSERL6 (616 aa), OsSERL7 (453 aa), OsSERL8 (543 aa), OsSERL9 (607 aa), OsBISERK1 (624 aa), AtSERK1 (625 aa), AtSERK2 (628 aa), AtSERK3 (615 aa), AtSERK4 (620 aa), and AtSERK5 (601 aa) were analyzed by pair-wise alignment using the CLUSTAL 2.1 Multiple Sequence Alignments. Amino acid sequences of OsSERK1-OsSERK4 were pulled out Li et al. (2009), and those of OsSERL1-OsSERL9 were from Single et al. (2009). All other amino acid sequences obtained from the data base NCBI.

region of *OsSERK1* and *OsSERK2* (Fig. 1B). Another three genes, *OsBISERK1*, *Os08g0176200*, and *ORK1*, showed 99%, 93%, and 93% identity in the corresponding regions, respectively (Fig. 1C). We inserted this fragment into the *pSB505* binary vector with an inverted position to generate *pSB505-OSBAK1RNAi* and transformed into rice (Supplementary Fig. 1). We obtained 43 primary transformants in the T0 generation. Even in this T0 generation, most of the transgenic plants showed abnormal growth patterns, both in vegetative and reproductive growth. These disrupted growth patterns reappeared in the next generation (data not shown). We performed RT-PCR analysis from the RNA of several independent T1 transgenic lines. While the expression level of *OsBAK1* in *OsBAK1RNAi #11* plants was similar to that of control plant containing empty vector plasmid, the *OsBAK1* expressions level of *OsBAK1RNAi #5*, *#7*, and *#14* plants were dramatically reduced (Fig. 1D). We selected two lines, one showing approximately normal *OsBAK1* expression, *#11*, and another showing greatly reduced expression of *OsBAK1*, *#5*, for further analyses. In these two lines of transgenic plants, we tried to confirm the down-regulation of other homologous genes, *OsBISERK1* and *ORK1*. However, it is very hard to design the primer set of *OsBISERK1* due to high nucleotide sequence homology to *OsSERK1*. Only *ORK1* was confirmed to be inhibited its expression in both transgenic rice plants (Fig. 1E).

Although there were some variations in growth, growth in most of the *OsBAK1RNAi #11* plants were not much reduced in several criteria including total height and leaf elongation (Figs. 2A-2C). However, many *OsBAK1RNAi #5* plants exhibited broad ranges of growth defects, severe to mild (Fig. 2A). Throughout the vegetative growth period, *OsBAK1RNAi #5* trans-

genic plants displayed semi-dwarf stature, with fewer internodes than control plants containing the empty vector (Figs. 2A and 2B). Leaf elongation was clearly reduced in the *OsBAK1RNAi #5* transgenic plants. However, leaf width of the *OsBAK1RNAi #5* leaves showed no significant reduction compared with that of controls, but clearly wider than that of *OsBAK1RNAi #11* plants (Fig. 2C). In addition, we observed several other unusual phenotypes in the *OsBAK1RNAi #5* line, including corrugated leaves at the early stage of growth (Figs. 2D and 2L). Most of the other leaves of the *OsBAK1RNAi #5* plants were dry and twisted (Figs. 2E and 2F), and the leaves were easily bent (Fig. 2H). We often observed that parts of the blades in the whole leaves were not fully opened and that side edges of leaf were confronted and fused together (Figs. 2G and 2I). We also observed abnormal plait-like lumps in the stem, which may indicate a failure in new leaf initiation (Figs. 2J and 2K). These abnormally looking phenotypes in leaves were not only limited in the *OsBAK1RNAi #5* plants. Although the degree of them were weaker than in *OsBAK1RNAi #5* plants, many of the leaves of the *OsBAK1RNAi #11* plants also displayed dry and twisted as well as easily bent leaf shape (data not shown).

We next examined whether reduced *OsBAK1* expression caused decreased sensitivity to BL in *OsBAK1RNAi* transgenic rice (Fig. 3). We observed that the *OsBAK1RNAi #11* plants exhibited little bit less degrees of laminar inclination to the control plants with the increasing concentrations of BL. On the contrary, we detected little change in the angle even at high concentrations of BL in the *OsBAK1RNAi #5* plants, indicating that these plants had dramatically reduced sensitivity to BL. Taken together, these results suggest that the reduced expression of *OsBAK1* led to phenotypic alterations, including inap-



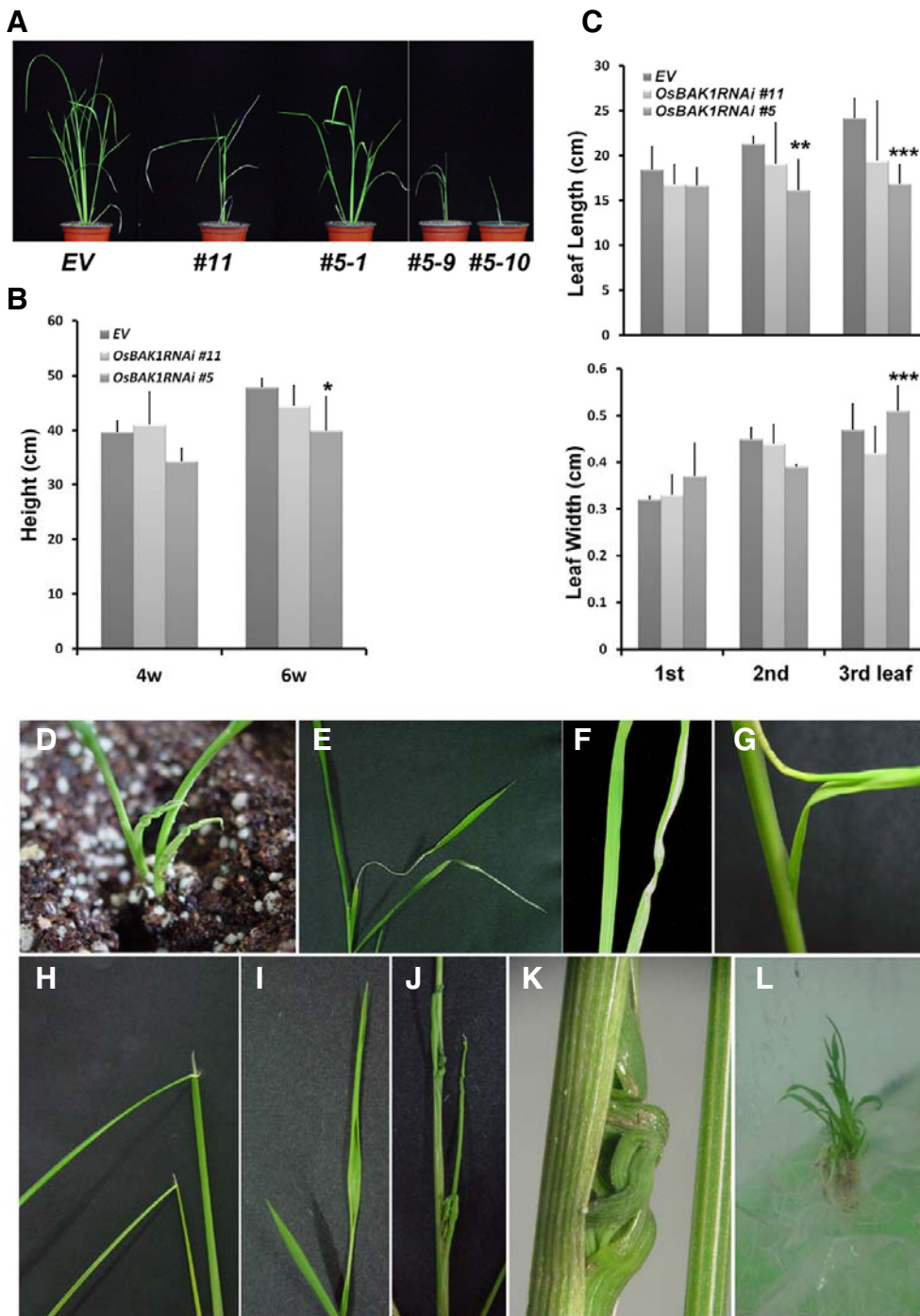
**Fig. 1.** Sequences and constructs for RNA interference for *OsBAK1*. (A) Phylogenetic analysis of SERK proteins in *Arabidopsis* and rice. Amino acid sequences encoded by *AtSERK1* (At1g71830), *AtSERK2* (At1g34210), *AtBAK1/AtSERK3* (At4g33430), *AtSERK4/BAK7/BKK1* (At2g13790), *AtSERK5* (At2g13800) in *Arabidopsis* and *OsSERK1* (Os08g0174700), *OsBISERK1* (AY463361), *OsSERK2* (Os04g0457800), *OsSERK3* (Os06g0225300), *OsSERK4* (Os02g0283800) in rice (*Oryza sativa*) were used for CLUSTAL 2.1 multiple sequence alignment. Phylogenetic trees were generated by PHYML and MEGA (Guindon and Gascuel, 2003). (B, C) The nucleotide (B) and amino acid (C) sequences of a partial kinase domain encoded by *OsSERKs* for RNA interference for *OsBAK1*. Alignments were drawn by the ClustalW program. (D) RT-PCR analysis showing reduced expression of *OsBAK1* by *OsBAK1RNAi* in several independent transgenic lines. (E) RT-PCR analysis showing reduced expression of *ORK1* in the *OsBAK1RNAi#5* and *OsBAK1RNAi#11* transgenic rice plants.

appropriate leaf development, growth inhibition over the whole life time of rice plants, and BL insensitivity.

#### Reduced *OsBAK1* expression caused impairment in leaf development and increased susceptibility to biotic stress in rice

Abnormal leaf development and growth were distinct phenotypes in most of the transgenic plants expressing the *OsBAK1RNAi* construct, including the #5 lines. We next focused

on characterizing the effect of *OsBAK1* knockdown leaves. We first examined the surface of the *OsBAK1RNAi #5* leaves using a stereomicroscope, and observed that the color of the leaves was pale green with dried, yellowish regions and necrotizing spots, which were not observed on the surface of wild-type rice leaves (Fig. 4A). We also examined the internal leaf structure by performing transverse sectioning of the leaves of wild-type and *OsBAK1RNAi #5* plants. The leaves of the *OsBAK1RNAi #5* were much thicker and contained more mesophyll cells



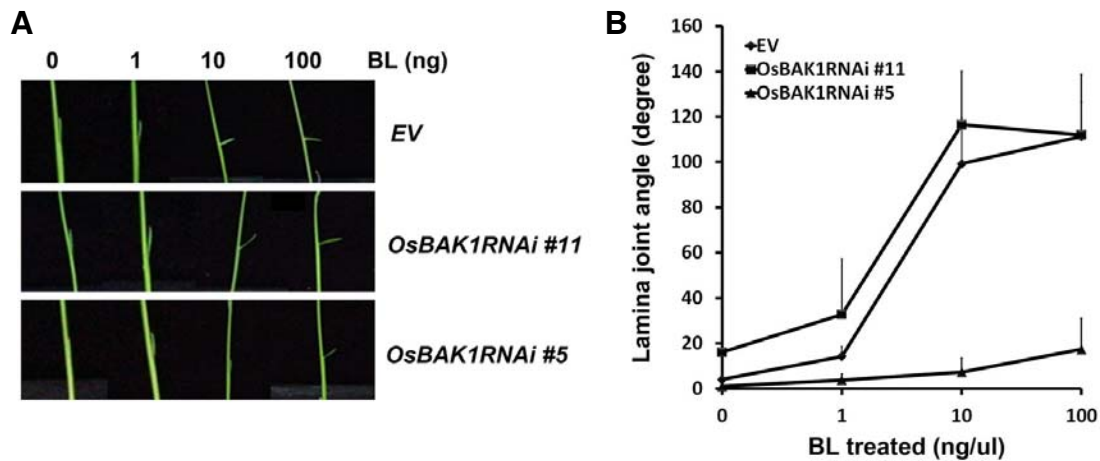
**Fig. 2.** Morphological analyses of transgenic plants expressing the *OsBAK1RNAi* construct. (A) Gross morphology of transgenic plants grown for 6 weeks in the greenhouse. EV empty vector containing transgenic plant, #11 *OsBAK1RNAi* #11 plant, and three representative *OsBAK1RNAi* #5 transgenic plants (#5-1, #5-9, and #5-10) showing different degrees of growth defects. (B) Plant height measured at the 4<sup>th</sup> week and 6<sup>th</sup> week of growth. (C) The length and width of each leaf were measured in the transgenic plants grown for 4 weeks. Data presented in (B) and (C) are averages for four different plants in each specific line. In (B) and (C), statistical test was performed by ANOVA analysis and significance in differences was indicated by P value (\*;  $P \leq 0.01$ , \*\*;  $P \leq 0.05$ , and \*\*\*,  $P \leq 0.005$ ). (D-L) Abnormal leaf features seen in the *OsBAK1RNAi* #5 plants. Corrugated leaves appeared at an early stage (D) and remained through later stages of development, as well as when grown in tissue culture media (L). Dried and twisted leaves (E, F), folded or fused leaf blades (G, I), bent leaves (H), and plait-like lumps in the nodes of stem (J, K) were also frequently observed.

which extended in the abaxial side. The aerenchyma in the leaves of *OsBAK1RNAi* transgenic plants was also larger than in wild-type plants. Moreover, whereas the flanking sides between the longitudinal veins were flat in wild-type leaf blades, the blades of the *OsBAK1RNAi* #5 leaves were not fully opened. This feature explained why we observed that parts of the blades in the *OsBAK1RNAi* #5 leaves were not fully opened (Figs. 2H and 2J). Interestingly, we noticed that there were no bulliform cells in the adaxial surface of the *OsBAK1RNAi* #5 leaves, unlike the wild-type leaves (Fig. 4B). Bulliform cells are

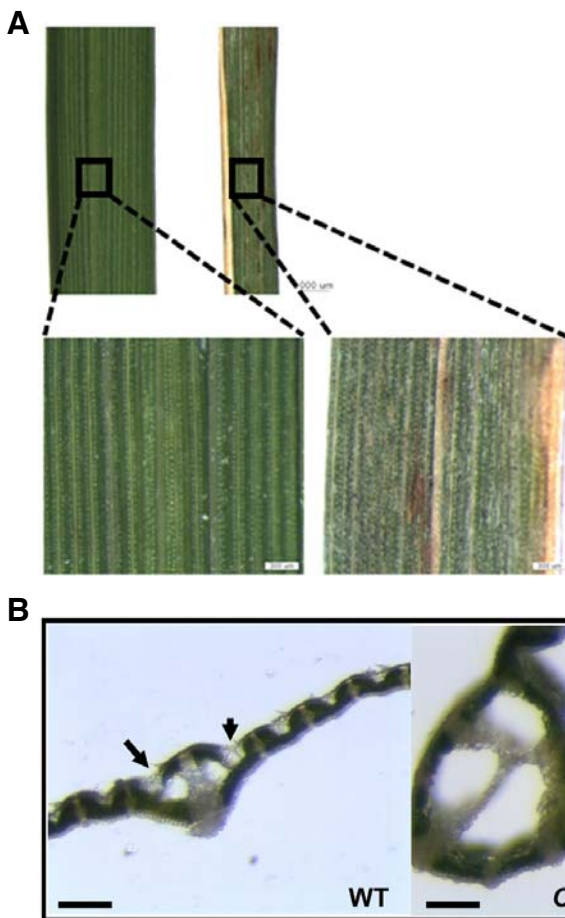
larger than typical epidermal cells and are colorless, because they contain highly developed vacuoles and no chloroplasts (Becraft, 1999). The appropriate distribution of bulliform cells is known to be important for leaf curling in monocot plants (Becraft et al., 2002; Zhang et al., 2009).

Furthermore, we observed that the leaves of *OsBAK1RNAi* #5 transgenic plants contained several necrotic spots, reminiscent of being infected, even under normal growth conditions (Fig. 5A). We cannot exclude the possibility that excessive dryness of the leaves, as shown in Fig. 4A, was the ultimate cause





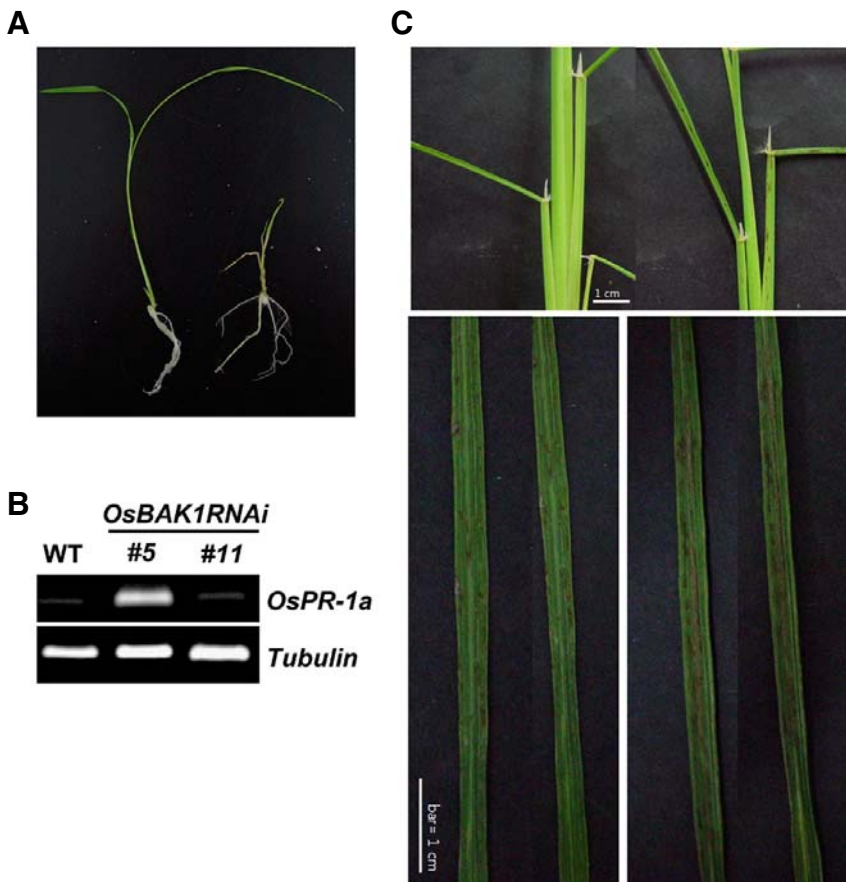
**Fig. 3.** BL responsiveness of *OsBAK1RNAi* transgenic plants. (A) Lamina joint assay. The 1  $\mu$ l of BL containing indicated amount was applied to the lamina joint of the second leaf of each transgenic plant. Pictures were taken after three days. (B) Quantification of lamina joint assays in (A). The experiment was repeated four times.



**Fig. 4.** Defective leaf development of *OsBAK1RNAi* #5 plants. (A) Surface of leaves of an *OsBAK1RNAi* #5 plants (right) and control plant (left) grown for four weeks. Pictures in the lower panel show higher magnification of the boxed region. White bars indicate 300  $\mu$ m. (B) Internal structures of leaves of an *OsBAK1RNAi* #5 plant (right) and a wild-type plant (left) grown for five weeks. Leaves containing veins were transversely sectioned by hand and observed under a compound microscope. Bulliform cells shown in wild-type plants are marked with arrow heads. Bar indicates 500  $\mu$ m.

of this phenotype. However, it is also possible that *OsBAK1RNAi* #5 transgenic rice is less resistant to infections that would be easily overcome under normal conditions by plants. To test this we examined basal expression level of *PR-1a*, one of the marker genes of the defense for pathogenesis in rice as well as in *Arabidopsis* (Park et al., 2009). We found that the

expression of *PR-1a* was greatly increased in the leaves of *OsBAK1RNAi* #5 plants, while it was similar in the leaves of *OsBAK1RNAi* #11 plants compared in wild type plants (Fig. 5B). To continuously know the defense capacity of the rice to disease-causing pathogen *in vivo*, we sprayed a conidial suspension of *Magnaporthe oryzae*, known as rice blast fungus onto



**Fig. 5.** Higher susceptibility to biotic stress of leaves of *OsBAK1RNAi* #5 plants. (A) The outer appearance of *OsBAK1RNAi* #5 line leaves (right) showing constitutive sickness compared with those of control plants (left) grown for two weeks. (B) RT-PCR analysis showing increased expression of PR-1a in the *OsBAK1RNAi* transgenic lines. (C) Extent of *OsBAK1RNAi* #5 line (right) infection with *Magnaporthe oryzae* compared to wild-type plant (left) grown for 7 weeks. Enlarged leaf features of them are shown in the lower panel.

*OsBAK1RNAi* #5 and control transgenic rice. At five days after infection, we observed that the rice blast disease had been spread more severely throughout the leaves and culms in *OsBAK1RNAi* #5 plants compared to the control plants, indicating that the reduced level of *OsBAK1* also affects plant immunity (Fig. 5C). Taken together, these results suggested that reduced *OsBAK1* expression caused increased susceptibility to biotic stress in rice as well as impairment in leaf development.

## DISCUSSION

It seems that more *OsSERKs* and *OsSERLs* were generated by gene duplication after the evolutionary divergence of dicots and monocots. Although we cannot exclude the possibility that each gene has specific functions in the developmental process of rice, it is possible that each gene functions redundantly with other genes, making it difficult to investigate the specific roles of each gene by mutant analysis. The RNA interference approach to knock-down several related-genes together is often the most effective strategy in such studies (Blokland et al., 1994; Ecker and Davis, 1986). In this investigation, more than three *OsBAK1*-related genes, including *OsSERK1*, *OsSERK2*, and *OsBISERK1*, were targeted by our *RNAi* construct (Fig. 1). The resulting *OsBAK1RNAi* transgenic rice plants with reduced levels of *OsBAK1* (Fig. 1D) exhibited intermediate growth retardation compared to strong BR-biosynthetic mutants, such as *brd1-1* and *brd1-2* (Hong et al., 2002). Similarly, BR-biosynthetic or BR-signaling mutants, such as *d2*, *d11*, *brd2*, and *d61* display mild dwarfism in the vegetative stage but are still fertile in reproductive development (Jeon et al., 2010). These results

indicate that a subset of *OsSERK* genes, including *OsBAK1* is important for proper growth in rice mediated by BR signaling.

Another interesting feature of the *OsBAK1RNAi* transgenic plants is abnormal leaf development. Leaf morphology is one of the important parameters of rice architecture. In addition to growth inhibition, several abnormalities in leaf development (Figs. 2E-2L), was reported in the *d1/d61* double mutant, in which rice *Gα* (*RGA1*) and *OsBRI1* are defective, whereas this finding was not observed in the *d61* single mutant (Oki et al., 2009). These data indicate that the reduced expression of *OsBAK1* may modulate the expression of *RGA1*. Fused blades in several regions of a leaf, very dried and yellowish parts, and a constitutively wilted appearance, were often observed in the leaves of the *OsBAK1RNAi* transgenic plants. These defects could be partly attributable to the lack of bulliform cells as shown in Fig. 4B. Although there is some controversy about the exact function of bulliform cells in the grass family, their rapid expansion during a specific stage of leaf development has been proposed to be responsible for the unfolding of the leaf blade (Alvarez et al., 2008). The size and placement of bulliform cells are important factors that determine the direction of leaf curling. Usually, bulliform cells exist mainly in the adaxial surface of the leaves and are larger than neighboring epidermal cells. Increased numbers of bulliform cells in the abaxial side of *slf1* (*shallow-like1*) mutant leaves and smaller adaxial bulliform cells in the leaves of *nr7* (*narrow leaf7*) and *nrl1-1* (*narrow and rolled leaf 1*) mutants have been shown to affect adaxial leaf curling (Fujino et al., 2008; Hu et al., 2010; Zhang et al., 2009). In comparison, overexpression of *ACL1* (*abaxially curled leaf 1*) produced more and larger bulliform cells, leading to the abaxial

leaf curling (Li et al., 2010). Therefore, it is possible that the fused and confronted leaf blades in the *OsBAK1RNAi* transgenic plants represent an extreme case, in which the lack of bulliform cells in the adxial side of the leaf, led to tight folding of leaf blade. In mature leaves, bulliform cells can act as motors adjacent to the mid-vein of leaf through changes in turgor. This mechanism provides plants with an advantage in resisting environmental stresses, especially drought and heat conditions. The constitutively wilted leaves of *OsBAK1RNAi* transgenic plants may be due to malfunction of bulliform cells at maturity.

In *Arabidopsis*, spontaneous cell death phenotypes were observed only in the double mutant plants lacking both *BAK1* and *BKK1/AtSERK4/BAK7* (He et al., 2007), or transgenic plants expressing *BAK7* RNA interference (RNAi) constructs (Jeong et al., 2010). There are no reports about these kinds of phenotypes in single mutants of *BAK1* or *BKK1/AtSERK4/BAK7*. In this sense, several necrotic spots in the leaves of *OsBAK1RNAi* #5 transgenic plants in normal condition (Fig. 5A) might be caused by the reduced expression of *OsBAK1* and its homologs together through the RNA interference, resulting in higher level of basal of *PR-1a* expression (Fig. 5B). There are many reports to show induced expressions of pathogenesis-related (PR) genes upon various stressful stimuli including infection of pathogens, wounding, and treatment of hormones, such as salicylic acid or jasmonate in rice as well as in *Arabidopsis* (Mitsuhara et al., 2008). However, no consistent correlation exists whether the higher expression of *PR* gene means the plants to have higher capacity to overcome these stresses or not. In our result, *OsBAK1RNAi* #5 transgenic rice plants, showing increased level of *PR-1a*, exhibited increased susceptibility to infection by *M. oryzae* KJ201 (Fig. 5C). The fungal pathogen *M. oryzae* uses a hemibiotrophic strategy for initial infection and proliferation and then switches to necrotrophy, resulting in necrotic lesions on the infected rice leaves (Park et al., 2009). Similar phenomena were reported in many cases. In *Arabidopsis*, although *bak1* mutant showed increased expression of pathogen infection and senescence-related genes, such as *PR1*, *PR2*, *PR5*, *Cf-like*, and *PTR3*, it also showed enhanced susceptibility to necrotropic fungal infection (Kemmerling et al., 2007). It was also reported that lesion-like necrosis along the central leaf vein was developed in the transgenic rice overexpressing (*At*)*NPR1* in which *PR-1b* transcript abundance was increased (Fitzgerald et al., 2008).

*OsSERK1* has been independently isolated from a rice cDNA that was inducible by blast fungus infection. Expression of *OsSERK1* is also activated by defense molecules, including salicylic acid, jasmonic acid, and abscisic acid (Hu et al., 2005). Another homolog, *OsBISERK1*, identified as a BTH-inducible gene is also induced by *M. grisea* (Song et al., 2008). Lettuce plants, in which the *LsSERK* was knocked down by post-transcriptional gene silencing, were also reported to have reduced somatic embryogenesis ability as well as increased susceptibility to *Sclerotinia* (Santos et al., 2009). These studies and our current findings raise the possibility of expanding the range of *OsSERK* gene function to include responses to biotic and abiotic stresses, as well as developmental processes, such as somatic regeneration and leaf cell specification.

*Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).*

#### ACKNOWLEDGMENTS

This work was supported by a grant from the Next-Generation BioGreen 21 Program (SSAC, grant #: PJ008003), Rural Development Administration, Republic of Korea, and by the SRC

Research Center for Women's Diseases of Sookmyung Women's University (2009) (grant # 3-0903-0022 to K.H.N.).

#### REFERENCES

- Albrecht, C., Russinova, E., Kemmerling, B., Kwaaitaal, M., and de Vries, S.C. (2008). *Arabidopsis* somatic embryogenesis receptor kinase proteins serve brassinosteroid-dependent and independent signaling pathways. *Plant Physiol.* 148, 611-619.
- Alvarez, J.M., Rocha, J.F., and Machado, S.R. (2008). Bulliform cells in *Loudetiopsis chrysothrix* (Nees) Conert and *Tristachya leiostachya* Nees (Poaceae): structure in relation to function. *Braz. Arch. Biol. Technol.* 51, 113-119.
- Bajguz, A. (2007). Metabolism of brassinosteroids in plants. *Plant Physiol. Biochem.* 45, 95-107.
- Becraft, P.W. (1994). Development of the leaf epidermis. *Curr. Top. Dev. Biol.* 45, 1-40.
- Becraft, P.W., Li, K., Det, N., and Asuncion-Crabb, Y. (2002). The maize *dek1* gene patterns in embryonic pattern formation and cell fate specification. *Development* 129, 5217-5225.
- Chinchilla, D., Zipfel, C., Robatzek, S., Kemmerling, B., Numberger, T., Jones, J.D., Felix, G., and Boller, T. (2007). A flagellin-induced complex of the receptor FLS2 and BAK1 initiates plant defense. *Nature* 448, 497-500.
- Ecker, J.R., and Davis, R.W. (1986). Inhibition of gene expression in plant cells by expression of antisense RNA. *Proc. Natl. Acad. Sci. USA* 83, 5372-5376.
- Fitzgerald, H.A., Chern, M.S., Navarre, R., and Ronald, P.C. (2008). Overexpression of (*At*)*NPR1* in rice leads to a BTH-and environmental-induced lesion-mimic/cell death phenotype. *Mol. Plant Microbe Interact.* 17, 140-151.
- Fujino, K., Matsuda, Y., Ozawa, K., Nishimura, T., Koshihara, T., Fraaije, M., and Sekiguchi, H. (2008). *NARROWLEAF 7* controls leaf shape mediated by auxin in rice. *Mol. Genet. Genomics* 279, 499-507.
- Guindon, S., and Gascuel, O. (2003). A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. *Syst. Biol.* 52, 696-704.
- He, K., Gou, X., Yuan, T., Lin, H., Asami, T., Yoshida, S., Russell, S.D., and Li, J. (2007). *BAK1* and *BKK1* regulate brassinosteroid-dependent growth and brassinosteroid-independent cell-death pathways. *Curr. Biol.* 17, 1109-1115.
- Hecht, V., Veille-Calzada, J.P., Hartog, M.V., Schmidt, E.D., Boutilier, K., Grossniklaus, U., and de Vries, S.C. (2001). The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR KINASE 1 gene is expressed in developing ovules and embryos and enhances embryogenic competence in culture. *Plant Physiol.* 127, 803-816.
- Hiei, Y., Ohta, S., Komari, T., and Kumashiro, T. (1994). Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* 6, 70-282.
- Hong, Z., Ueguchi-Tanaka, M., Shimizu-Sato, S., Inukai, Y., Fujioka, S., Shimada, Y., Takatsuto, S., Agetsuma, M., Yoshida, S., Watanabe, Y., et al. (2002). Loss-of-function of a rice brassinosteroid biosynthetic enzyme, C-6 oxidase, prevents the organized arrangement and polar elongation of cells in the leaves and stem. *Plant J.* 32, 495-508.
- Hu, H., Xiong, L., and Yang, Y. (2005). Rice SERK1 gene positively regulates somatic embryogenesis of cultured cell and host defense response against fungal infection. *Planta* 222, 107-117.
- Hu, J., Zhu, L., Gao, Z., Guo, L., Fang, Y., Zhang, G., Dong, G., Yan, M., Liu, J., and Qian, Q. (2010). Identification and characterization of *NARROW AND ROLLED LEAF 1*, a novel gene regulating leaf morphology and plant architecture in rice. *Plant Mol. Biol.* 73, 283-292.
- Ito, Y., Takaya, K., and Kurata, N. (2005). Expression of SERK family receptor-like protein kinase genes in rice. *Biochim. Biophys. Acta* 1730, 253-258.
- Jeon, J.S., Jung, K.H., Kim, H.B., Suh, J.P., and Khush, G.S. (2011). Genetic and molecular insight into the enhancement of rice yield potential. *J. Plant Biol.* 54, 1-9.
- Jeong, Y.J., Shang, Y., Kim, B.H., Kim, S.Y., Song, J.H., Lee, J.S., Lee, M.M., Li, J., and Nam, K.H. (2010). *BAK7* displays unequal genetic redundancy with *BAK1* in brassinosteroid signaling and early senescence in *Arabidopsis*. *Mol. Cell* 29, 259-266.



- Karlova, R., Boeren, S., Russinova, E., Aker, J., Vervoort, J., and de Vries, S.C. (2006). The *Arabidopsis* SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE1 protein complex includes BRASSINOSTEROID-INSENSITIVE1. *Plant Cell* **18**, 625-638.
- Kemmerling, B., Schwedt, A., Rodriguez, P., Mazzotta, S., Frank, M., Qamar, S.A., Mengiste, T., Betsuyaku, S., Parker, J.E., Mussig, C., et al. (2007). The BRI1-associated kinase 1, BAK1, has a brassinolide-independent role in plant cell-death control. *Curr. Biol.* **17**, 1116-1122.
- Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tand, W., Shang, J.X., Sun, Y., Burlingame, A.L., and Wang, Z.Y. (2009). Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. *Nat. Cell Biol.* **11**, 1254-1260.
- Li, J., Wen, J., Lease, K.A., Doke, J.T., Tax, F.E., and Walker, J.C. (2002). BAK1, an *Arabidopsis* LRR receptor-like protein kinase, interacts with BRI1 and modulates brassinosteroid signaling. *Cell* **110**, 213-222.
- Li, D., Wang, L., Wang, M., Xu, Y., Liu, Y., Xu, Z., Li, J., and Chong, K. (2009). Engineering of *OsBAK1* gene as a molecular tool to improve rice architecture for high yield. *Plant Biotech. J.* **7**, 791-806.
- Li, L., Shi, Z., Li, L., Shen, G., Wang, X., An, L., and Zhang, J. (2010). Overexpression of *ACL1* (*abaxially curled leaf 1*) increased bulliform cells and induced abaxial curling of leaf blades in rice. *Mol. Plant* **3**, 807-817.
- Mitsuhashi, I., Iwai, T., Seo, S., Yanagawa, Y., Kawahigashi, H., Hirose, S., Ohkawa, Y., and Ohashi, Y. (2008). Characteristic expression of twelve rice *PR1* family genes in response to pathogen infection, wounding, and defense-related signal compound (121/180). *Mol. Genet. Genomics* **279**, 415-427.
- Nam, K.H., and Li, J. (2002). BRI1/BAK1, a receptor kinase pair mediating brassinosteroid signaling. *Cell* **110**, 203-212.
- Oki, K., Inaba, N., Kitagawa, K., Fujioka, S., Kitano, H., Fujisawa, Y., Kato, H., and Iwasaki, Y. (2009). Function of the  $\alpha$ -subunit of rice heterotrimeric G protein in brassinosteroid signaling. *Plant Cell Physiol.* **50**, 161-172.
- Park, J.Y., Jin, J., Lee, Y.W., Kang, S., and Lee, Y.H. (2009). Rice blast fungus (*Magnaporthe oryzae*) Infects *Arabidopsis* via a mechanism distinct from that required for the infection of rice. *Plant Physiol.* **149**, 474-486.
- Santos, M.O., Romano, E., Vieira, L.S., Baldoni, A.B., and Aragao, F.J.L. (2009). Suppression of SERK gene expression affects fungus tolerance and somatic embryogenesis in transgenic lettuce. *Plant Biol.* **11**, 83-89.
- Singla, B., Khurana, J., and Khurana, P. (2009). Structural characterization and expression analysis of the SERK/SERL gene family in rice (*Oryza sativa*). *Int. J. Plant Genomics* **539402**.
- Song, D., Li, G., Song, F., and Zheng, Z. (2008). Molecular characterization and expression analysis of OsBISERK1, a gene encoding a leucine-rich repeat receptor-like kinase, during disease resistance responses in rice. *Mol. Biol. Rep.* **35**, 75-283.
- Toonen, M., Schmidt, E., Hendriks, T., Verhoeven, H., and de Vries, S.C. (1993). Identification of single embryo-forming cells in carrot suspension cultures. *Acta Bot. Neerl.* **42**, 518-519.
- Van Blokland, R., Van der Geest, N., Mol, J.N.M., and Kooter, J.M. (1994). Transgene-mediated suppression of chalcone synthase expression in *Petunia hybrida* results from an increase in RNA turnover. *Plant J.* **6**, 61-877.
- Zhang, G., Xu, Q., Zhu, X., Qian, Q., and Xue, H. (2009). SHALLOT-LIKE1 is a KANADI transcriptional factor that modulates rice leaf rolling by regulating leaf abaxial cell development. *Plant Cell* **21**, 719-735.

