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A Rice Kinase-Protein Interaction Map

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ABSTRACT

Plants uniquely contain large numbers of protein kinases and, for the vast majority of the 1429 kinases predicted in the rice (*Oryza sativa*) genome, little is known of their functions. Genetic approaches often fail to produce observable phenotypes, thus new strategies are needed to delineate kinase function. We previously developed a cost-effective high-throughput yeast two-hybrid system. Using this system, we have generated a protein interaction map of 116 representative rice kinases and 254 of their interacting proteins. Overall, the resulting interaction map supports a large number of known or predicted kinase-protein interactions from both plants and animals and reveals many new functional insights. Notably, we found a potential widespread role for E3 ubiquitin ligases in pathogen defense signaling mediated by receptor-like kinases, particularly by the kinases that may have evolved from recently expanded kinase subfamilies in rice. We anticipate that the data provided here will serve as a foundation for targeted functional studies in rice and other plants. The application of yeast two-hybrid and TAPtag analyses for large-scale plant protein interaction studies is also discussed.

INTRODUCTION

Protein kinases play a key role in regulating nearly all aspects of cellular processes, including growth, differentiation, and defense. After sequencing the genomes of *Arabidopsis* (*Arabidopsis thaliana*) and rice (*Oryza sativa*), 1027 and 1429 protein kinases, respectively, have been identified, representing more than 3% of the annotated proteins encoded by each genome (<http://plantsp.genomics.purdue.edu/>; <http://rkd.ucdavis.edu/>). The number of protein kinases identified in rice alone is almost three times the number of those found in the human (*Homo sapiens*) genome (Manning et al., 2002), and is similar to the combined total of the 1800 protein kinases found in human, mouse (*Mus musculus*), fruit fly (*Drosophila melanogaster*), nematode (*Caenorhabditis elegans*), and yeast (*Saccharomyces cerevisiae*) genomes (<http://kinase.com/>). Therefore, rice offers a rich source of material for studying kinase-regulated cell signaling as well as for the molecular mechanisms of kinome expansion (Shiu et al., 2004). Although plant kinomes are large, only a limited number of kinases have been functionally characterized. Examples include a number of receptor-like kinases (RLKs) and receptor-like cytoplasmic kinases (RLCKs) which control pathogen recognition and plant development (Morris and walker, 2003; Morillo and Tax, 2006; Chinchilla et al., 2007a; Afzal et al., 2008). Other examples are several SNF1-related kinases (SnRKs) involved in the regulation of metabolism, development, and stress responses (Gong et al., 2004; Polge and Thomas, 2007; Lu et al., 2007); and a few mitogen-activated protein kinases (MAPKs) and their regulatory kinases (Jonak et al., 2002; Hamel et al., 2006; Colcombet and Hirt, 2008).

It has been well documented that many kinases function through their interactions with other proteins. For instance, the *Arabidopsis* receptor kinases BRI1 and BAK1 bind to each other and both are involved in brassinosteroid signal transduction (Li et al., 2002; Nam and Li, 2002). In addition to BRI1, BAK1 forms a complex with the receptor kinase FLS2 in response to the stimulation of bacterial flagellin (Chinchilla et al., 2007b; Heese et al., 2007). In the resistance to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), XA21-mediated resistance is regulated by a number of the XA21 binding proteins (Wang et al., 2006; Peng et al., 2008; Park et al., 2008;). During salt stress, the SnRK protein SOS2 interacts with SOS3, a novel EF-hand Ca^{2+} sensor with a sequence similar to the regulatory calcineurin B subunit from yeast (Halfter et al., 2000; Gong et al., 2004). In *Brassica*, the ARC1 protein interacts with the *S*

receptor kinase to control self-incompatibility (Gu et al., 1998). Aside from these studies, the precise function and the protein binding partners for the vast majority of plant kinases are unknown.

A major focus of functional genomics is mapping cellular protein-protein interaction networks on a large scale. The information generated from these studies not only establishes links between well characterized proteins, but also reveals potential functions of uncharacterized proteins when their interacting partners have known functions: the “guilt by association” principle (Hazbun and Fields, 2001). To generate such databases of protein interactions, a number of high throughput approaches have been developed (Phizicky et al., 2003; Zhu et al., 2003). Among them, yeast two-hybrid analysis is a robust method for detecting pair-wise protein-protein interactions in a cellular setting (Nodzon and Song, 2004; Parrish et al., 2006). It has been estimated that over half of the protein interactions reported in the literature were originally identified by yeast two-hybrid analyses (Xenarios et al., 2002). Notably, all the protein-kinase interactions mentioned above can be detected by yeast two-hybrid analysis. The viability of this approach relies on low costs, simple manipulation, and sensitive detection. To date, yeast two-hybrid analysis has been used to systematically analyze protein-protein interactions in several model organisms, including yeast, bacteria, *Drosophila*, *Caenorhabditis elegans*, *Plasmodium falciparum*, and humans (Uetz et al., 2000; Ito et al., 2001; Rain et al., 2001; Giot et al., 2003; Li et al., 2004; LaCount et al., 2005; Rual et al., 2005; Lim et al., 2006; Parrish et al., 2007; Shimoda et al., 2008; Sato et al., 2008). The resulting databases of protein-protein interactions continue to be a valuable resource for understanding signaling networks in various organisms.

Unlike gene expression studies, protein-protein interaction datasets are notoriously difficult to verify; yeast two-hybrid being no exception. This has been attributed in part to the complexity and sometimes transient nature of protein-protein interactions themselves and not necessarily inherent error in the methods used. Yeast two-hybrid screening is particularly notorious for producing high rates of false positives based on both the inability to reproduce identified interactions upon yeast re-transformation and confirm those using alternative methods such as co-immunoprecipitation. While the former can be controlled through additional

screening steps, the later poses a practical limitation that hinders calculation of actual false positive rates. There, it is critical to eliminate as many false positives as possible by testing the reproducibility of all candidate interactions in retransformed yeast cells (Vidalain et al., 2004). However, many large-scale yeast two-hybrid analyses do not include these verification steps because the yeast plasmid isolation, propagation in *E. coli*, and re-transformation into yeast is costly and time-consuming when performed on a large scale. We previously developed a cost-effective yeast two-hybrid recovery/retransformation system using rolling circle amplification (RCA) (Ding et al., 2003; Ding et al., 2004; Ding et al., 2006). This system allows one to inexpensively and efficiently test reproducibility and specificity of the interactions on a large scale in yeast. We have conducted two-hybrid analyses with 204 representative rice kinases using this system. Among them, 22 kinases auto-activated the *His3* reporter in the absence of a prey plasmid and 66 kinases yielded no interactors. Here we report a yeast two-hybrid-based protein interaction network for 116 rice kinases consisting of 378 kinase protein interactions. These interactions support a number of hypotheses for the functions of these kinases and their interacting partners.

RESULTS AND DISCUSSION

Selection of Kinase Targets and Bait Design

The rice kinome is composed of kinases from six of the seven known protein kinase groups: AGC (PKA, PKG, and PKC kinases), CAMK (calcium/calmodulin-dependent protein kinase), CK1 (casein kinase 1), CMGC (containing the CDK, MAPK, GSK3, and CLK families), STE (homologs of yeast sterile 7, sterile 11, and sterile 20 kinases), TKL (tyrosine kinase-like which includes MLKs [mixed lineage kinases], transforming growth factor- β receptor kinases, and Raf kinases), and other kinases (Manning et al., 2002; Shui et al., 2004; Dardick and Ronald, 2006). Plants lack known members of the TK (tyrosine kinases) group. Approximately 75% (1068) of rice kinases, within the TKL group, are members of the RLK/Pelle family that are related to the *Drosophila* Pelle and the human interleukin-1 receptor-associated kinases (IRAK) (Shui et al., 2004). The rice RLK/Pelle family (also called the IRAK family) includes both RLKs and RLCKs, which have been further subdivided into nearly 70 subfamilies based on conserved domains and phylogenetic relationships (Shui et al., 2004; Dardick and Ronald, 2006). A total of 204 representative kinases (15% of the kinome) were selected from all 6 kinase groups

based, in part, on their proportion to the entire kinome and on their expression in leaves (Table 1). Among these 204 kinases, 130 belong to the RLK/Pelle family (Table 1).

These kinase-encoding open reading frames were RT-PCR amplified and cloned into the Gateway® entry vector pDONR207 as described previously (Rohila et al., 2006). For RLKs, only the intracellular kinase domains (including the juxtamembrane [JM] domain) were cloned. Through recombination, the cDNAs were individually fused in-frame to the GAL4 DNA binding domains in the bait vectors pXDGATcy86 and pXDGATU86 (see Ding et al., 2006, for vector details).

RCA-Based Yeast Two-Hybrid Screening

We conducted a large-scale yeast two-hybrid analysis using 204 representative rice kinases. Among the 204 rice kinases, 22 auto-activated the *His3* reporter in the absence of a prey plasmid (Table 1, Supplemental Table 1). Half of the 22 kinases (11) are members of the RLK/Pelle family. These 22 bait kinases were removed from further yeast two-hybrid analysis, and the remaining 182 were subjected to screening rice cDNA libraries to determine their interacting proteins (see Materials and Methods). To verify interactors identified from the initial library screening, we amplified the bait (on the pXDGATcy86 vector) and prey plasmids from the yeast cells capable of growing on selective media using RCA. The amplified DNA was retransformed into fresh yeast cells containing either the empty pXDGATU86 vector or the same bait kinase carried by pXDGATU86. Both 2-amino-5-fluorobenzoic acid and the cycloheximide counterselections were used to eliminate the original pXDGATcy86-derived bait constructs (Ding et al., 2006). Only the prey that specifically bound to a bait kinase, not the empty GAL4 activation domain (AD), was scored as a positive interactor. By using these criteria, almost two-thirds of the initial interactions were removed. Most of the discarded clones (~80%) contain a rice cDNA that is not in-frame fused to the activation domain of the GAL4 transcription factor in the prey vector. Therefore, they were unlikely to be genuine interactors. About 15% of these clones were putative DNA binding proteins or transcription factors. The remaining 5% of the clones did not fall into either category. Among the 182 kinases used for library screenings, 116 yielded at least one interactor (Table 1, Supplemental Table 2). The other 66 kinases that generated no interactors are listed in Supplemental Table 3.

A Rice Kinase-Protein Interaction Map

A total of 378 interactions composed of 254 distinct kinase interactors were identified (Supplemental Table 2). A kinase-protein interaction map was generated using the software “Cytoscape 2.3.2” (<http://www.cytoscape.org/>) (Supplemental Fig. 1A). The number of interactors for each bait kinase varies considerably, ranging from those having just a single interactor to those having up to 26 for a single bait kinase. For example, we identified 24 and 26 interactors for the SnRKs Os03g17980 and Os05g45420, respectively. Through a putative transcription factor (Os03g08460) and a putative DNA binding protein (Os02g57200), these two SnRKs were linked to a network composed of 68 bait kinases and their 166 interacting proteins. The other 48 bait kinases and their 89 partners form discrete interaction networks. Many interactions in this map are supported by previous studies in rice and in other heterologous systems (see below for more discussion). Additional interactions include functionally uncharacterized kinases and/or unknown interacting proteins which provides a resource to explore potentially novel associations in kinase-mediated signaling in the future. The sequences of the identified interacting partners also provide information on the domains that are responsible for binding to the kinases (Supplemental Table 2). Among the 254 interactors, 15% are putative DNA binding/transcription factors, 11% are protein kinases, and 9% are various metabolic enzymes (Supplemental Fig. 1B), suggesting broad roles for rice kinases in the regulation of gene expression, kinase cascades, and metabolic pathways. Three percent of the identified interactors were categorized as E3 ubiquitin ligases (Supplemental Fig. 1B), some of which interacted with multiple members of the RLK/Pelle family (see below for more discussion).

In an attempt to assess the quality of our map, we examined six of the kinase-protein interactions in rice cells using a biomolecular fluorescence complementation (BiFC) system. This system is based on the formation of a fluorescent complex through the interaction of two proteins that are fused to nonfluorescent fragments of the yellow fluorescent protein (YFP) (Hu and Kerppola, 2003). The BiFC system has been used to monitor protein-protein interactions in living plant cells in real time with high specificity and low background (Bracha-Drori et al, 2004; Bhat et al., 2006). Table 2 summarizes the BiFC results for the six kinase-interactor pairs that we selected for testing. Each protein was fused downstream (N-terminal fusions) to one of the

two halves of YFP (YFPN-term and YFPC-term) and transiently expressed in protoplasts derived from etiolated rice seedlings (Bart et al. 2006). Complex formation between the two interacting proteins is indicated by reconstitution of YFP. As shown in Supplemental Fig. 2, we detected interactions between four out of the six pairs. Different pairs required different amounts of time to mature and give a clear YFP signal. For example, Pair #1 showed a very strong signal in the cytoplasm and around the nucleus by 24 hours after transformation, whereas Pair #4 showed a weak interaction only after 24 hours post-transformation. For negative controls, we tested each fusion protein in the presence of the other half of YFP alone. That is, each YFPN-term-interactor fusion construct was tested with YFPC-term (no fusion), and each YFPC-term-interactor fusion construct was tested with YFPN-term (no fusion). Only one of the two negative controls is shown in Supplemental Fig. 2, but, for the pairs shown, no interactions between the unfused YFP segments were detected with either interactor. For Pair #5 (Table 2), we were unable to test the interaction since the YFPC-term-bait fusion gives a YFP signal with the unfused YFPN-term construct, albeit a less intense one than when both interactors are present (data not shown). For Pair #6 (Table 2), we were unable to detect an interaction between this bait and prey with the N-terminal split-YFP fusions (data not shown). We tested both orientations without detecting an interaction, i.e., YFPN-term- and YFPC-term-bait with YFPC-term- and YFPN-term-prey. While these results do not necessarily provide an accurate estimate of the error in our interaction maps, they do provide evidence that a significant number of kinase-protein interactions identified by our yeast two-hybrid system are likely to also occur in rice cells.

RLK/Pelle Family Member-E3 Ubiquitin Ligase Interactions in Rice

The RLK/Pelle family represents the largest group of kinases in rice and Arabidopsis. The number of predicted rice RLK/Pelle family members, however, is nearly twice as many as that of Arabidopsis. The additional rice members are thought to result from recent lineage-specific expansions of resistance/defense-related genes (Shiu et al., 2004; Dardick and Ronald, 2006). Part of the support for this hypothesis comes from the fact that three cloned rice disease resistance genes, *Xa21*, *Xa3/Xa26*, and *Pi-d2*, encode RLKs (Song et al., 1995; Sun et al., 2004; Chen et al., 2006; Xiang et al., 2006). It has been recognized that these three resistance proteins fall into the non-RD class of kinases that lack the conserved arginine in kinase subdomain VI. An association between the non-RD motif and a role in pathogen recognition has been

demonstrated, although some RD RLKs are also involved in resistance/defense response (Scheer and Ryan, 2002; Godiard et al., 2003; Diener and Ausubel, 2005; Hu et al., 2005; Llorente et al., 2005; Dardick and Ronald, 2006). To study RLK/Pelle-mediated signaling in rice, we selected 130 kinases representing 47 RLK/Pelle subfamilies and conducted two-hybrid screenings with 119 kinases (the remaining 11 can auto-activate the reporter) (Table 1). A total of 170 interactions were detected (Table 1).

One pattern that emerged from the identified interactions is that four phylogenetically distant RLKs (Os04g38480, Os07g35580, Os07g35260, and Os08g03020) bind to two closely related U-box/ARM proteins (Os08g01900 and Os01g66130) (Fig. 1A). These RLKs contain extracellular leucine-rich repeat (Os04g38480), Duf26 (Os07g35580 and Os07g35260), and lectin (Os08g03020) domains, respectively. Most of their intracellular kinase domains share less than 46% amino acid identity, with the exception of 66% and 69% identities between those of Os07g35580/Os07g35260 and Os04g44910/Os07g38800, respectively (Supplemental Table 4). The U-box/ARM proteins interacting with these RLKs belong to the Plant U-Box (PUB) family of putative E3 ubiquitin ligases (Mudgil et al., 2004). The overall identity between these two proteins is 50% (66% similarity), but their ARM repeats, a well-known protein-protein interaction domain (Andrade et al., 2001; Samuel et al., 2006), are much more conserved, with a 74% identity (83% similarity). A truncated version of Os08g01900 containing mainly the ARM domain was recovered from the library screening using the kinase domain of Os08g03020 as bait (Supplemental Table 2). This is consistent with the previous observation that the ARM domain of the *Brassica* ARC1 protein is sufficient for binding to the *S* receptor kinase that controls self-incompatibility (Gu et al., 1998). Thus, the same kinase-binding specificity of the PUB-ARM proteins can be attributed to their conserved ARM domains. The PUB motif of these two proteins is also highly conserved (78% identity, 86% similarity). Such conservation does not seem to be necessary for supporting E3 activity only, because the PUB motif in many other functional E3 ubiquitin ligases appears to be divergent, with the exception of a few conserved cysteine and histidine residues. It would be interesting to determine if the PUB motifs of Os08g01900 and Os01g66130 have a role in specific protein-protein interactions.

It has been reported that Arabidopsis PUB-ARM proteins interact with Arabidopsis and *Brassica* S domain receptor kinases and some of these interacting modules have been implicated in hormone response (Samuel et al., 2008). The function of the rice PUB-ARM proteins Os08g01900 and Os01g66130 is still unclear, however they also interacted with the kinase domain of Pi-d2 (Fig. 2A). Pi-d2 confers resistance to rice blast disease caused by the fungal pathogen *Magnaporthe grisea* (Chen et al., 2006). Parallel to these interactions, we previously found that the rice ankyrin-RING protein XB3 interacts in yeast and in planta with XA21, which confers resistance to the bacterial pathogen *Xoo* (Wang et al., 2006). The ankyrin domain of XB3 alone is sufficient for binding to the kinase domain of XA21, whereas the RING finger motif in XB3 can mediate auto-ubiquitination (Wang et al., 2006). We therefore propose that the PUB-ARM proteins Os08g01900 and Os01g66130 may function equivalently to XB3 in the Pi-d2-mediated immune response. Interestingly, all six E3 binding RLKs (XA21, Pid2, Os04g38480, Os07g35580, Os07g35260, and Os08g03020) contain a putative proteolytic cleavage motif (P/GX₅₋₇P/G) in their intracellular JM domain (Fig. 1B). This motif has been suggested as a cause of XA21 protein instability (Xu et al., 2006). Because instability appears to be a common feature of a number of plant disease resistance proteins (Mackey et al., 2002; Tornero et al., 2002; Hubert et al., 2003; Bieri et al., 2004; Holt et al., 2005; Xu et al., 2006; Wang et al., 2006), it is attractive to speculate that the RLKs Os04g38480, Os07g35580, Os08g03020, and Os07g35260 may be involved in rice immunity. Indeed, RLK Os04g38480 (OsSERK1, rice somatic embryogenesis receptor-like kinase 1) is known to be associated with the processes of embryogenesis and defense response (Hu et al., 2005). The *OsSERK1* gene is significantly induced at the RNA level by rice blast fungus with higher levels during the incompatible interaction and by a number of defense signaling molecules, including salicylic acid, benzothiadiazole (BTH), jasmonic acid, and ABA. Downregulation of *OsSERK1* expression in rice calli reduces the shoot regeneration rate (Hu et al., 2005). By contrast, overexpression of *OsSERK1* enhances rice shoot regeneration and resistance against the blast fungus. These observations reinforce our hypothesis that the PUB-ARM proteins Os08g01900 and Os01g66130 may function as convergence points for various signals perceived and transduced by corresponding RLKs, including Pi-d2 and OsSERK1, in antifungal immunity.

In addition to the PUB-ARM proteins, our yeast two-hybrid screens identified two BTB/POZ (bric a brac, tramtrack, and broad complex/pox virus and zinc finger) domain-containing proteins (Os06g31100 and Os03g57854) as common interactors of two sets of RLKs/RLCKs (Fig. 1A). The Os03g57854 protein possesses an additional protein-protein interaction domain, MATH (meprin and TRAF homology). Emerging evidence suggests that BTB domain-containing proteins may generally be involved in E3 ubiquitin ligase complexes (van den Heuvel, 2004; Gingerich et al., 2007). For instance, in the CUL-3 ubiquitin ligase complex of *Caenorhabditis elegans*, the BTB protein MEL-26 functions as a substrate-specific adaptor that interacts with the substrate MEI-1 through its N-terminal MATH domain (Furukawa et al., 2003; Pintard et al., 2003; Xu et al., 2003). To test whether these two rice BTB domain-containing proteins also form protein complexes, we performed pair-wise yeast two-hybrid analyses and found that Os06g31100 interacted with the RING finger-containing protein XB3, whereas Os03g57854 bound to the PUB-ARM proteins Os08g01900 and Os01g66130 (Fig. 1A). These results suggest that Os06g31100 and Os03g57854 might be involved in E3 ubiquitin ligase complexes that function in rice RLK/Pelle-mediated immunity. Strikingly, the observed RLK-E3 ubiquitin ligase interactions in rice appear to be parallel to the proximal portion of the conserved innate immunity pathway in animals. Here the RING finger-containing E3 ubiquitin ligase TRAF6 functions downstream of the receptor/kinase complex that consists of the interleukin-1 receptor, Myd88, and IRAK (Fig. 1A; Wu and Arron, 2003). Although the mechanism of how the E3s function in rice immunity remains to be determined, two possible models have been proposed previously (Wang et al., 2006).

Taken together, we have identified 11 E3 binding RLK/Pelles (excluding XA21), which represent more than 9% of the 119 rice RLK/Pelle family members that have been subjected to our yeast two-hybrid screening in this study. Notably, 8 of these 11 (73%) belong to recently expanded rice kinase subfamilies (RERKs) as compared with the fact that less than 43% of the 119 RLK/Pelles are RERKs (Fig. 1A; Supplemental table 2; see Table S6 in Dardick and Ronald (2006) for RERKs). Given that the rice kinome contains a total of 1069 RLK/Pelle family members, we propose that a large number of RLK/Pelles may interact with a limited number of E3 ubiquitin ligases, including XB3, Os06g31100, Os08g01900, Os01g66130, and Os03g57854.

Most of these kinases may have evolved from recent lineage-specific expansions, and many of them may be related to rice immunity.

Interactions between Components in the Rice MAP kinase Cascade

The MAP kinase cascade is composed of three sequentially activated kinases: MAPKKK, MAPKK, and MAPK (Hamel et al., 2006). We found that the MAPKK OsMKK6 (Os01g32660) interacted with OsMPK4 (Os10g38950) and OsMPK6 (Os06g06090), that belong to groups A and B of the MAPK family, respectively (Fig. 2). The OsMKK6 and OsMPK4 interactions were further confirmed by reciprocal screening and by the BiFC system *in vivo* (Table 2; Supplemental Fig. 2). Notably, the results from BiFC assays suggest that these interactions occur in nucleus. OsMPK6 is orthologous to the Arabidopsis AtMPK6 that has been linked to defense signal transduction pathways (Fig. 2; Asai et al., 2002; Menke et al., 2004). In cell culture suspensions, OsMPK6 was activated by the treatment of a sphingolipid elicitor isolated from the fungal pathogen *Magnaporthe grisea* (Lieberherr et al., 2005). It is possible that OsMKK6 is an upstream kinase that activates OsMPK4 and OsMPK6 in the elicitor-induced defense response.

In a separate yeast two-hybrid screen, OsMKK4 (Os02g54600) was linked to OsMPK3 (Os03g17700) that has been implicated in stress tolerance and host resistance (Fig. 2; Xiong and Yang, 2003). Thus, OsMKK4 might function as an upstream regulator for OsMPK3. In support of this hypothesis, OsMKK4 is orthologous to the Arabidopsis AtMKK4, which have been identified as the upstream kinases of AtMPK3 and AtMPK6 in the flagellin-triggered pathway (Fig. 2; Asai et al., 2002). Given that OsMPK3 is orthologous to AtMPK3 (Fig. 2; Hamel et al., 2006), the proposed OsMKK4-OsMPK3 cascade in rice appears to be parallel to the AtMKK4-AtMPK3/AtMPK6 pathway that may function in Arabidopsis innate immunity (Asai et al., 2002).

A Cyclin-Dependent Kinase Cascade in Rice

Cyclin-dependent kinases (CDK) are key molecules in mediating cell cycle progression (Potuschak and Doerner, 2001). CDK activity is regulated by multiple mechanisms, including phosphorylation by CDK-activating kinases (CAK), association with cyclin subunits, and degradation of CDK inhibitors (CKIs) that associate with CDKs. We found that rice R2

(Os05g32600), the first plant CAK isolated (Hata, 1991), interacted with the rice kinase Os06g22820, which is orthologous to the Arabidopsis kinase CAK1At (Fig. 3; Umeda et al., 1998). These interactions were confirmed *in vivo* (Table 2; Supplemental Fig. 2). Both Os06g22820 and CAK1At contain a unique insertion sequence (amino acids 178-289 in Os06g22820), present in the T-loop region (Supplemental Fig. 3A; Umeda et al., 1998). It has been demonstrated that CAK1At can phosphorylate the T-loop of CAK2At and CAK4At *in vitro* and activates the CTD-kinase activity of CAK4At in root protoplasts, indicating that CAK1At may be a CAK-activating kinase (Shimotohno et al., 2004). Therefore, the rice kinase Os06g22820 might function as an R2 activating kinase (Fig. 3).

R2 can specifically phosphorylate Thr161 and Thr160 in the T-loop of the rice CDK Cdc2Os1 (Os03g02680) (Yamaguchi et al., 1998; Vandepoele et al., 2002). When Cdc2Os1 and a nearly identical kinase (Os03g01850, 98% identity, 98% similarity) were used as bait, a rice protein (Os03g05300) orthologous to the evolutionarily conserved cyclin-dependent kinase subunits (CKS) was identified to interact with these two kinases (Fig. 3; Supplemental Fig. 3B). We were unable to confirm the Cdc2Os1-Os03g05300 interactions by the BiFC system because Cdc2Os1 is capable of generating an YFP signal (Table 2). However, previous studies in animals have demonstrated that Cks1 directs the ubiquitin-mediated degradation of the CDK2-associated inhibitor protein p27^{Kip1} by the ubiquitin ligase complex SCF^{skp2} (Ganoth et al., 2001; Spruck et al., 2001). Given that two CKIs (Os02g52480 and Os10g33310) have been identified in the Cdc2Os1 complex by using the TAPtag approach (Rohila et al., 2006), we propose that the CKS protein Os03g05300 might play a similar role in regulating Cdc2Os1 kinase activity through mediating the degradation of the Cdc2Os1-bound inhibitors (Os02g52480 and Os10g33310) (Fig. 3). Taken together, by combining the yeast two-hybrid and TAPtag data, a large portion of the putative components in the rice R2-mediated gene expression signaling have been identified (Fig. 3).

Proteins Associated with Rice SnRKs

The yeast SNF1 and its mammalian homolog AMP-activated protein kinase (AMPK) function as metabolic sensors to protect cells from environmental and nutritional stresses (Polge and Thomas, 2007). SNF1 and AMPK form stable heterotrimeric complexes consisting of a

catalytic subunit (SNF1/AMPK α) and two regulatory subunits (SIP1/SIP2/GAL83/AMPK β and SNF4/AMPK γ). Unlike yeast and mammals, plants carry a large family of SNF1-related kinases classified into three subgroups (SnRK1, SnRK2, and SnRK3) (Hrabak et al., 2003). Eighteen rice SnRKs were used for library screening and yielded 82 interactions (Fig. 4).

As expected, two members of the SnRK1 family, Os03g17980 and Os05g45420, interacted with two proteins related to the SNF1/AMPK/SnRK1 β -subunits (Os05g41220 and Os09g20010) and the interactions between Os05g45420 and Os05g41220 were confirmed *in vivo* (Fig. 4; Table 2; Supplemental Fig. 2). Os05g41220 is homologous to Arabidopsis AKIN β 1 (At5g21170, 50% identity, 67% similarity) with the conserved KIS (kinase interaction sequence) and ASC (association with the SNF1 complex) domains, whereas Os09g20010 is similar to Arabidopsis AKIN β 3 (At2g28060, 61% identity, 68% similarity), lacking the N-terminal region of typical β -subunits (Supplemental Fig. 4). Despite the truncated structure, AKIN β 3 can interact with the other subunits of the SnRK1 complex and complement the yeast β -subunit deletion mutant (*sip1 Δ sip2 Δ gal83 Δ*) (Gissot et al., 2005). Consistent with these observations, the β -subunit Os09g20010 has also been identified in the Os05g45420 kinase complex by TAPtag analyses (J.S. Rohila and M.E. Fromm, unpublished), suggesting that the rice kinases Os03g17980 and Os05g45420 function redundantly as the α -subunits in the conserved SNF1-like complexes.

Os03g17980 and Os05g45420 also interacted with a protein (Os03g63940) homologous to the Arabidopsis AKIN $\beta\gamma$ (At1g09020, 67% identity, 80% similarity) and maize (*Zea mays*) ZmAKIN $\beta\gamma$ -1 (AF276085, 85% identity, 93% similarity) proteins that form a plant-specific class of regulator proteins (Fig. 4; Lumberras et al., 2001). This class of proteins is characterized by the presence of an N-terminal KIS domain (characteristic of β -subunits), and a C-terminal region related to the animal SNF4/AMPK γ protein (Supplemental Fig. 4). The *ZmAKIN $\beta\gamma$ -1* gene is capable of complementing the yeast *Δ snf4-2* mutant. It has been proposed that plants may possess two distinct types of SNF1 complexes with two or three subunits (Lumberras et al., 2001). In line with our observation, the $\beta\gamma$ subunit Os03g63940 has been associated with the Os05g45420 kinase *in planta* by TAPtag analyses (J.S. Rohila and M.E. Fromm, unpublished).

Additionally, AMPK α can be dephosphorylated by a type 2C protein phosphatase (Davies et al., 1995). The interaction between a putative type 2C protein phosphatase (Os02g38780), and Os03g17980 or Os05g45420 offers the possibility that Os02g38780 is involved in dephosphorylation of these two rice kinases (Fig. 4).

Ten rice SnRK2s are known as osmotic stress/ABA-activated protein kinases (SAPKs) 1-10 (Kobayashi et al., 2004). We found that SAPK4 and SAPK6 interacted with OREB1, a protein related to bZIP transcription factors/ABA-responsive element (ABRE)-binding factors (ABFs), and the SAPK4-OREB1 interactions were confirmed to occur in the cytoplasm of rice mesophyll protoplasts by the BiFC system (Fig. 4; Table 2; Supplemental Fig. 2). Moreover, the SAPK6 (also known as OSRK1)-OREB1 interactions have recently been demonstrated in yeast by Chae et al (2007). They showed that OREB1 can be strongly phosphorylated by SAPK6 and that both proteins can co-localize in nuclear bodies in onion epidermal cells. This discrepancy in subcellular localizations of SAPK4 and OREB1 may reflect the differences of the cell systems used in the study and the potential subcellular translocations of these proteins under distinct conditions. A similar phenomenon has been observed when the subcellular localization of SAPK4 was determined in onion epidermal cells and Arabidopsis mesophyll protoplasts (Diédhiou et al., 2008). Our results suggest that OREB1 might be regulated by multiple SAPKs.

Nine kinases of the rice SnRK3 subfamily were used as bait to screen for binding proteins. Among them, four (Os05g04550, Os11g02240, Os01g60910, and Os01g10890) interacted with one or both of two closely related rice proteins (Os12g06510 and Os12g40510) that belong to the family of the SOS3-like Ca²⁺ sensor/binding proteins (ScaBPs, also referred to as Calcineurin B-like proteins, CBLs) (Fig. 4; Supplemental Fig. 5; Gong et al., 2004). In Arabidopsis, there are 24 SnRK3s (also known as protein kinases related to SOS2 (PKSs) or CBL-interacting protein kinases (CIPKs)) and nine ScaBPs. The interactions between specific PKS and ScaBPs have been shown to regulate various targets, including potassium transporter, the plasma membrane H⁺-ATPase, and those involved in salt, drought and cold stresses, and ABA signaling (Li et al., 2006; Fuglsang et al., 2007; Halfter et al., 2000; Liu et al., 2000; Luan et al., 2002; Gong et al., 2004). Thus, rice might also utilize PKS-ScaBP complexes in stress signaling.

Interactions with a rice LAMMER kinase

LAMMER kinases are one of two families of kinases that can phosphorylate and regulate SR proteins in RNA splicing (Reddy, 2004; Isshiki et al., 2006). We found that a rice LAMMER kinase (Os01g62080) interacted with four Ser/Arg-rich (SR) proteins (SCL30a, SCL30b, RSZp23, and Os06g50890) that all possess RNA recognition (RRM) and Arg/Ser-rich (RS) domain (Fig. 5). SCL30a and SCL30b are members of the SC35-type group, whereas OsRSZp23 belongs to the 9G8-type family containing a single CCHC-type zinc knuckle motif between the RRM and the RS domains (Isshiki et al., 2006). Differing from many other SR proteins, the RS domain of Os06g50890 is located at the N-terminus (Fig. 5). The rice LAMMER kinase Os01g62080 is similar to the tobacco ethylene-induced LAMMER kinase PK12 (AAC04324, 56% identity, 65% similarity). It has been reported that PK12 and its Arabidopsis homolog, AFC2, can interact with and phosphorylate several plant and animal SR proteins (Golovkin and Reddy, 1999; Savaldi-Goldstein et al., 2000; Savaldi-Goldstein et al., 2003). Therefore, the rice kinase Os01g62080 likely regulates its interacting SR proteins SCL30a, SCL30b, RSZp23, and Os06g50890 in RNA splicing.

The CK2 Protein Complex

CK2 (formerly known as casein kinase II), a kinase essential for cell viability, forms a tetrameric complex with two catalytic (α and α') subunits and a dimeric β subunit (Meggio and Pinna, 2003). The rice CK2 kinase II alpha subunit-like protein Os07g02350 contains the conserved HIKE domain (Olsten et al., 2005). As expected, a CK2 β -subunit (Os07g31280) was identified as an interactor of Os07g02350 (Supplemental Table 2). This observation has been supported in a TAPtag study *in planta* (Rohila et al., 2006).

Kinases Associated with Putative Transcription Regulators

We have previously shown that the XA21 RLK binds to a WRKY type transcription factors suggesting that XA21 may be directly involved in regulating gene expression (Peng et al., 2008). Interestingly, among the 254 identified kinase interactors, 15% appear to be putative DNA binding/transcription factors further supporting a role for kinase regulation of transcription factors (Supplemental Fig. 1B). Examples from our dataset include the OREB1-SAPKs interactions described above and the interactions between the rice TOUSLED-like kinase

Os03g53880 and the protein Os01g08680 (Supplemental Table 2). The Arabidopsis TOUSLED kinase, required for leaf and flower development, is capable of oligomerizing and interacting with the C-terminal portion of the SANT/myb domain protein TKI1 (Roe et al., 1997; Ehsan et al., 2004). Os03g53880 exhibits 62% identity (74% similarity) to TOUSLED, whereas Os01g08680 shares homology to the C-terminal half (amino acids 210-741) of TKI1 (AF530160, 28% identity, 40% similarity). Our interaction results support that Os03g53880 may be orthologous to the Arabidopsis TOUSLED kinase.

Comparison of the Yeast Two-Hybrid and TAPtag Approaches

We used both yeast two-hybrid and TAPtag methods to identify proteins associated with the same set of rice kinases (Rohila et al., 2006; this study). In addition to the interactions described above, the resulting information obtained also allows assessment of these two methods to study protein interactions in plants. TAPtag identified a greater number of proteins when a protein complex is relatively abundant and stable. For example, in the broadly conserved CDK (Os03g02680) and CAK (Os05g32600) complexes, 17 components were found by TAPtag analyses (Rohila et al., 2006). In contrast, the yeast two-hybrid approach identified only three interactors for these two kinases (Fig. 3). Another drawback to the yeast two-hybrid approach is that further experiments are required to confirm that interactions take place *in planta*. However, the yeast two-hybrid assay is a more practical technique for use in high throughput studies. It can also detect both stable and transient interactions often missed by TAPtag. This aspect is particularly important for those kinase-protein interactions where the kinases form weak and/or transient contacts with their substrates, or when the kinase complexes are sensitive to the purification procedure. In support of this reasoning, a number of interactions involved in the MAP kinase cascade were identified, supporting a greater degree of sensitivity of yeast two-hybrid assays. Moreover, no interactors were found when the RLK protein XA21 was subjected to TAPtag analyses (W.-H. Xu, Fromm, M.E., and W.-Y. Song, unpublished data). In contrast, more than 20 XA21 binding proteins were identified by yeast two-hybrid screens (L.-Y. Pi, X.-D. Ding, and W.-Y. Song, unpublished data; T. Richter and P.C. Ronald, unpublished data), and at least four of them have been confirmed to be associated with XA21 *in planta* or *in vivo* (Wang et al., 2006; Park et al., 2008; Peng et al., 2008; Y.-N. Jiang and W.-Y. Song, unpublished data).

Comparative analysis reveals that there is little overlap between the two protein-kinase interaction datasets generated by the yeast two-hybrid and TAPtag assays. Among the 93 kinases that have been analyzed, only four common kinase binding proteins (Os07g12780, Os09g20010, Os03g63940, and Os07g31280) were identified by these two methods (Rohila et al., 2006). This could reflect the differences between the rice tissues used for these two assays and the fact that whereas TAPtag analyses identify proteins that indirectly associate with a kinase, the yeast two-hybrid analyses detects only those proteins that interact directly (see Materials and Methods; Rohila et al., 2006). This could also be attributed to the fact that many protein-kinase interactions *in vivo* rely on the phosphorylation status of the proteins and/or the kinases, which may be regulated by developmental program and environmental stimuli. Another reason is that both assays likely miss many interactors (also known as false negatives). Similar situations could occur when BiFC assays are used to verify the interactions identified by yeast two-hybrid analyses.

There has also been little overlap in the datasets in large scale protein-protein interaction studies in yeast. For instance, of the 80,000 yeast protein associations detected or predicted by various approaches, including yeast two-hybrid and TAPtag, only ~2,400 were supported by more than one method (von Mering et al., 2002). In two genome-wide yeast two-hybrid screens using the same 6,000 yeast open reading frames, only about 10% of the interactions were identified as having common characteristics (Ito et al., 2002). When the two-hybrid datasets were compared with the protein complex datasets generated by TAPtag, only 7% of the interactions were identified by both methods (Gavin et al., 2002). Likewise, the protein complex datasets obtained from two distinct purification techniques (TAPtag vs. high-throughput mass spectrometric protein complex identification) shared only 10% of the common proteins detected (Gavin et al., 2002; Ho et al., 2002; Ito et al., 2002). It has been estimated that the false negative rate for high-throughput screens could be more than 80% (Stanyon et al., 2004). In our system, only two MAPKK-MAPK interactions were identified when eight out of 15 rice MAPKs were subjected to library screenings. If a given MAPK interacts with at least one MAPKK, the false negative rate for the system could be more than 75%. These assessments indicate the limitation of each method used for studying protein-protein interactions, thus supporting the idea that

distinct techniques are required to obtain the information necessary to unravel the complex interactome in a cell (von Mering et al., 2002; Zhu et al., 2003).

Protein kinases are involved in crucial steps along numerous cell signaling pathways, ranging from the proximal receptors that perceive external signals to the distal kinases that directly regulate transcription factors. The identification of the proteins associated with 116 kinases using yeast two-hybrid analyses is the important step toward understanding the global signaling network regulated by rice kinases. Because of the presence of false positives and negatives, and the inability to calculate reliable positive and negative discovery rates, caution is needed when using some of the data. However, the large-scale nature of the protein interaction dataset allows for exploration of the patterns of kinase-protein interactions in rice and provides a basis for future functional characterization of these interactions. This can be particularly significant because rice has a much larger kinome size and most plant RLKs have serine/threonine specificity that differs from the receptor tyrosine kinases predominately present in animals. Finally, our comparison of the overlap of the TAPtag and yeast two-hybrid methods supports the strategy of using complementary screening methods to find greater numbers of interacting proteins to more fully develop interaction networks. This strategy and the tools developed from this and previous studies (Rohila et al., 2006) should facilitate large-scale proteomics work in rice and in other plants.

MATERIALS AND METHODS

Cloning of Rice (*Oryza sativa*) Kinase Genes

Total RNA was isolated from *Oryza sativa* cv. Nipponbare leaves using Trizol reagent (Invitrogen, Carlsbad, CA). First strand cDNA was synthesized using RETROscript® reverse transcriptase (Ambion, Austin, TX). PCR was performed to quantify the amount of cDNA synthesized. The cDNA products were amplified using *LA Taq*™ DNA polymerase (LA PCR Kit, Ver. 2.1, Takara Mirus Bio, Madison, WI). The PCR primers contained the *attB1* and *attB2* sequences for cloning PCR products into pDONR207 by BP reaction (Gateway®) (Invitrogen). Primers were designed using Primer3 Input software (<http://frodo.wi.mit.edu/cgi-bin/primer3/primer3.cgi>). The forward primer contained an ATG starting codon and the reverse primer had a termination codon to synthesize a cDNA clone without a 5' untranslated region and 3' untranslated region. The 5' open reading frame was in-frame with the upstream Gateway® reading frame (*AttB1* forward: 5'-ggggacaagttgtacaaaaagcaggctcaaccATG, where ATG is the translation initiation codon). For the RLKs, extracellular and transmembrane domains were excluded and only the cytoplasmic kinase region was amplified. All amplified kinase clones were verified by sequencing.

Construction of Rice cDNA Libraries

Three rice cDNA libraries were prepared. The first was constructed from the *Japonica* cultivar Taipei309. Two to three-week-old rice seedlings were treated either by cold (4°C for 24 hours), heat (42°C for six hours), BTH (3 mM of BTH three times per 48 hours), salt (100, 200, and 300 mM NaCl for 12 hours), drought (air-dried for 2 hours), or by the plant hormone ABA (0.1 mM three times per 24 hours). Equal amounts of tissue from entire plants were harvested and combined to isolate RNA. Up to 50 µg mRNA were purified using the Poly(A)Purist™ mRNA purification Kit (Ambion). A rice cDNA library was constructed using the mRNA and the HybriZAP-2.1 XR library construction kit (Stratagene, La Jolla, CA). The titer of the library is approximately 2.8 million plaque forming units with an average insert size of 1.6 kb. The second library was made from 3 to 6-week-old Indica plants, cultivar IRBB21, carrying the resistance gene *Xa21* (Chern et al., 2001). RNA used for making the library was isolated from leaf tissue harvested after inoculation with *Xanthomonas oryzae* pv. *oryzae* Philippine race 6. The third library was generated from three-week-old leaves of *Japonica* cultivar Moroberekan.

Yeast Two-Hybrid Screens

The detailed procedure for screening the libraries is described by Ding et al (2006). Briefly, equal amounts of plasmid DNA recovered from the three cDNA libraries were combined and transformed into the yeast strain Y187. Transformants were harvested and aliquots were stored in a -80°C freezer. Rice kinases in pDONOR207 were converted into both pXDGATcy86 and pXDGATU86 vectors (Ding et al., 2006) by using the Gateway® LR Clonase Enzyme Mix (Invitrogen) according to the manufacturer's instructions. The resulting bait constructs were transformed into the yeast strain HF7c and subjected to testing for bait auto-activation. The kinases that did not auto-activate the *His3* reporter were then used to screen the cDNA libraries. HF7c cells containing a pXDGATcy86-derived rice kinase were mated with the Y187 cells transformed with the cDNA libraries. Ten to twenty million zygotes were typically obtained and selected for their interacting proteins on a SD/-Leu-Trp-His+2mM 3-AT medium. After ten days, the plasmids were rescued by RCA from the yeast colonies grown on selective media. One portion of the RCA-amplified plasmids was used for prey sequencing and the other portion was transformed into the yeast strain MaV203. After removing the pXDGATcy86-derived kinase bait by counterselection (Ding et al., 2006), the yeast cells were transformed with the same kinase cloned into the pXDGATU86 vector or with the pXDGATU86 empty vector. The transformants were then selected on a SD/-Leu-Trp-His+50mM 3-AT medium. Only a prey that showed a specific interaction with the bait, not with AD, was scored as a positive interactor.

DNA Sequencing and Analysis

Sequencing templates were prepared directly from yeast or bacterial cultures using the TempliPhi™ DNA Sequencing Template Amplification method as specified by the manufacturer (GE healthcare, Piscataway, NJ). Dye terminator DNA sequencing reactions utilized the primer SSO20 (5'-AGGGATGTTTAATACCACTAC-3') and ET-Terminators from Amersham Biosciences. Sequence reactions were desalted by ethanol precipitation, dried, and re-suspended in 10 µL of 0.06% aqueous solution of Seakem Gold agarose (Cambrex, Rockland, ME) prior to electrokinetic injection. All DNA sequencing was performed on capillary array DNA sequencing units (MegaBACE 1000, GE healthcare).

To specify the identity of each prey, the TIGR Rice Pseudomolecules database (<http://tigrblast.tigr.org/euk-blast/index.cgi?project=osa1>) was searched using the BlastP algorithm. The European Molecular Biology Open Software Suite (EMBOSS) program “needle” was used to perform sequence alignment and to calculate the percentage of sequence identity and similarity.

The PAUP (version 4.0b10) software package (Swofford, 2002) was used to conduct phylogenetic analyses. Neighbor-joining was used to reconstruct the phylogenetic relationships of these amino acids. Bootstrap values were derived from 1,000 replicates to quantify the relative support for branches of the inferred phylogenetic tree.

Split-YFP Assays

We modified the split-YFP vectors developed by Bracha-Drori and colleagues (2004) to make them compatible with the Gateway™ system of Invitrogen (Carlsbad, CA). Simultaneously, we introduced a more typical flexible linker (G-G-G-G-S-G-G-G-G-S) and, for the YFPN-term vector (p736GC), a c-myc tag, since the original poly-glutamic acid tag was reported to be ineffective (Bracha-Drori 2004). First, pSY vectors were digested with *SalI* and *BamHI* and ligated with a synthetic DNA segment, introducing a blunt ended restriction site unique to the vector (*PmeI*) and the flexible linker and tag. The resulting vectors were then cut and ligated with Gateway™ Cassette reading frame C.1. to create p735GC (YFPC-term-HA tag-2X(G4S)-Gateway Cassette) and p736GC (YFPN-term-myc tag-2X(G4S)-Gateway Cassette). Each bait and prey was previously cloned into pENTR and were recombined into the split-YFP vectors following the manufacturer’s protocol (Invitrogen, Carlsbad, CA). Unless otherwise stated, baits were recombined into p735GC and preys into p736GC. After recombination, the junction sites and inserts were sequenced.

We conducted split-YFP assays through transient transformation of protoplasts prepared from etiolated above-ground tissues of 7- to 14-day-old Kitaake rice plants, prepared essentially as described (Bart et al. 2006). To promote consistent seedling growth, rice seeds were surface sterilized for 25 minutes in 40% bleach prior to germination on media containing 0.8% agar, 1X Murashige and Skoog medium and 30% sucrose. We found that transformation of protoplasts

with the Gateway™ compatible vectors containing the chloramphenicol resistance and *ccdB* genes supported reconstitution of YFP in the absence of interactors. Therefore, we used the original vectors (pSY736 and pSY735) developed by Bracha-Drori et al. (2004) as the empty, unfused vectors for negative control experiments.

All microscopic images were generated using a Zeiss Axiovert 25 Fluorescent microscope and taken by Nikon D70s digital camera with the Nikon Capture 4 software. YFP fluorescence was visualized under the Zeiss YFP filter cube 46HE (excitation: BP500/25; beamsplitter: FT515; emission: BP535/30). YFP expression was monitored between 18 and 48 hours after rice protoplast transformation.

Accession Numbers

The accession numbers for the protein from the other species are: ZmSNF1, AAS59400; AKIN11, NP_974375; AKIN α 1, P92958; PpSNF1b, AAR03831; SNF1, A26030; MtSK1, AAV41843; SOS2, AAF62923; SOS3 AF060553; AtFHA1, AAF20220; AtFHA2, AAF20224; NtFHA1, AAL05884; FHL1, P39521; Cks-1, P61025; SUC1, CAA21308; and AHK3, NP_564276.

SUPPLEMENTAL MATERIALS

Supplemental Table S1. List of the 22 kinases that can auto-activate the *His3* reporter.

Supplemental Table S2. List of the 378 rice kinase-protein interactions identified by the yeast two-hybrid screen.

Supplemental Table S3. List of the 66 kinases that yielded no interactors.

Supplemental Table S4. Amino acid sequence comparisons between the kinase domains of the 12 rice RLK/Pelles described in Fig. 1A.

Supplemental Figure S1. A rice kinase-protein interaction map.

Supplemental Figure S2. Bimolecular fluorescence complementation assay to visualize protein-protein interaction in rice protoplasts.

Supplemental Figure S3. Components in the rice CDK signaling pathway.

Supplemental Figure S4. Structure comparison of the β - and $\beta\gamma$ -subunits of rice SNF1-like complexes.

Supplemental Figure S5. Sequence alignment of Os12g06510, Os12g40510, and the Arabidopsis SOS3 protein.

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Table 1. Summary of the rice kinases chosen and the interactions identified by yeast two-hybrid screen.

Table 2. BiFC results for six kinase-interactor pairs identified by yeast two-hybrid screen.

FIGURE LEGENDS

Figure 1. Interactions between RLK/Pelle kinases and putative E3 ubiquitin ligase complexes in rice. **(A)** Connectivity of 12 rice RLK/Pelle kinases with their binding E3 ubiquitin ligases. Interacting proteins (filled circles) are connected with solid lines to their bait kinases (open squares). Domains of the proteins are indicated. For comparison, part of the conserved interleukin-1-mediated pathway in animal innate immunity is adapted from Wu and Arron (2003). The interaction between XA21 and XB3 is from Wang et al. (2006). Recently expanded rice kinases (RERKs) and RD kinases (RDKs) are indicated by “+”, whereas non-RERKs and non-RDKs are labeled by “-”. IL-1R, interleukin-1 receptor. **(B)** JM sequences of the six rice RLKs containing a putative proteolytic cleavage motif (P/GX₅₋₇P/G) in **(A)**. The motifs are underlined and the conserved proline (P) and glycine (G) residues are in bold red (X denotes any amino acids). Numbers indicate amino acid positions in the full-length proteins.

Figure 2. Interactions between MAPKKs and MAPKs. Interacting kinases (filled circles) are connected with solid lines to their bait kinases (open squares). Dashed lines denote the connections of Arabidopsis MAPKKs and MAPKs described by Asai et al., 2002. Phylogenetic trees of rice, Arabidopsis, and poplar MAPKKs (groups A and C) and MAPKs (groups A and B) are adapted from Hamel et al. (2006).

Figure 3. Model for CAK- and CDK-mediated signaling pathways in rice. The R2 and Cdc2Os1 kinase complexes (grey shadowed ovals) and phosphorylation of Cdc2Os1 and TFIIH by R2 are based on the studies by Rohila et al. (2006) and Yamaguchi et al. (1998), respectively. The protein Os07g12780 (dotted oval) was identified by both TAPtag and yeast two-hybrid analyses. A hypothetical SCF E3 ubiquitin ligase complex, presumably recruited by the conserved CKS protein Os03g05300 to the Cdc2Os1 kinase complex, is shown as a dashed oval.

Protein phosphorylation (P) and ubiquitination (Ub) events are also indicated. Os06g22820 is a putative kinase orthologous to the Arabidopsis CAK1At (Umeda et al., 1998).

Figure 4. Proteins associated with rice SnRKs. A phylogenetic tree for 17 rice SnRKs and their related kinases (ZmSNF1 from maize; AKIN11 and AKIN α 1 from Arabidopsis; PpSNF1b from moss; SNF1 from *Saccharomyces cerevisiae*; MtSK1 from *Medicago truncatula*; SOS2 from Arabidopsis). Classification of these kinases is indicated on the left. The interacting proteins (filled circles) are connected by solid lines with their corresponding kinases (open squares).

Figure 5. Protein interactions with the rice LAMMER-type kinase Os01g62080. Schematic representation of Os01g62080 (*upper*) and comparison of four interacting proteins (*lower*). In Os01g62080, the characteristic sequence motif (boxed) for the LAMMER-type is indicated. In the Os01g62080-binding proteins, the RNA recognition motif (RRM) and the arginine/serine-rich (RS) region are shown. The dark box in RSZp23 denotes a zinc finger motif. Numbers above domains indicate amino acid positions in the full-length proteins.

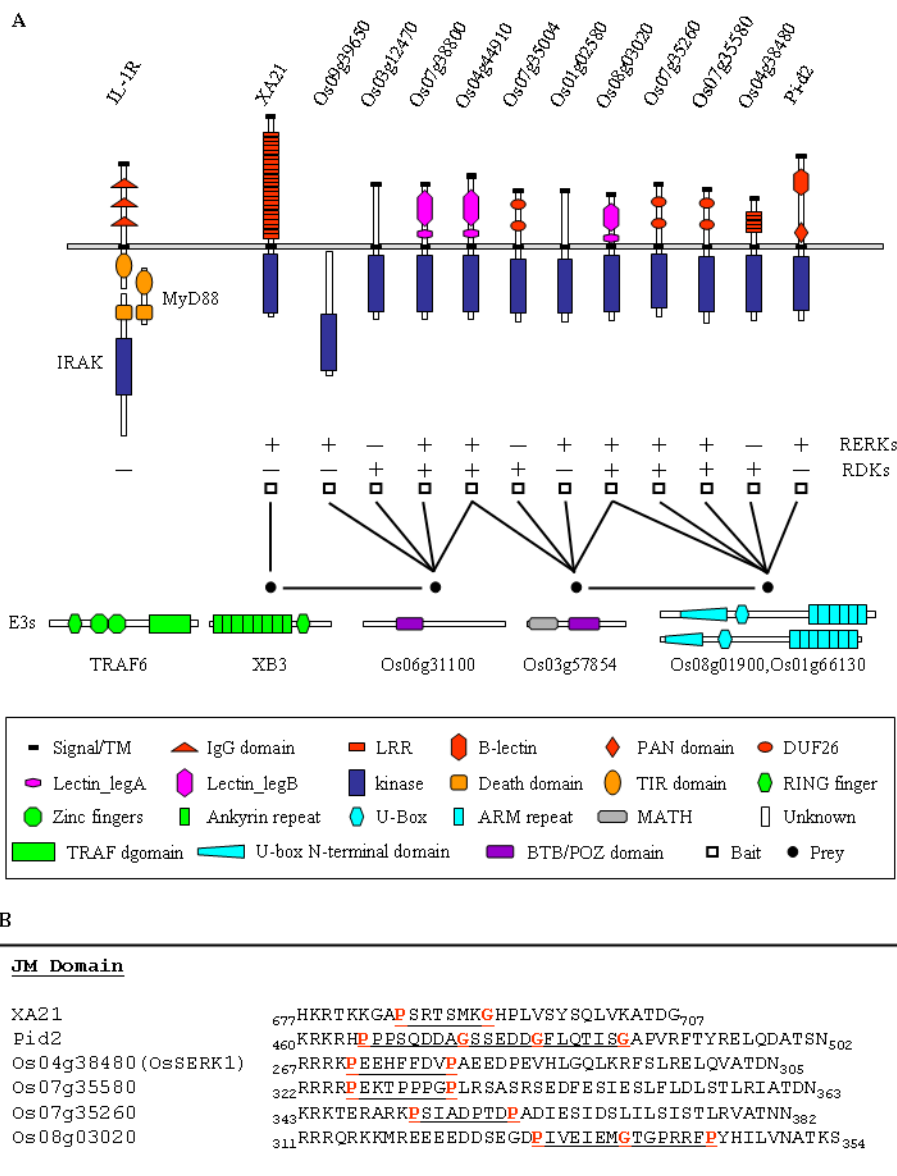


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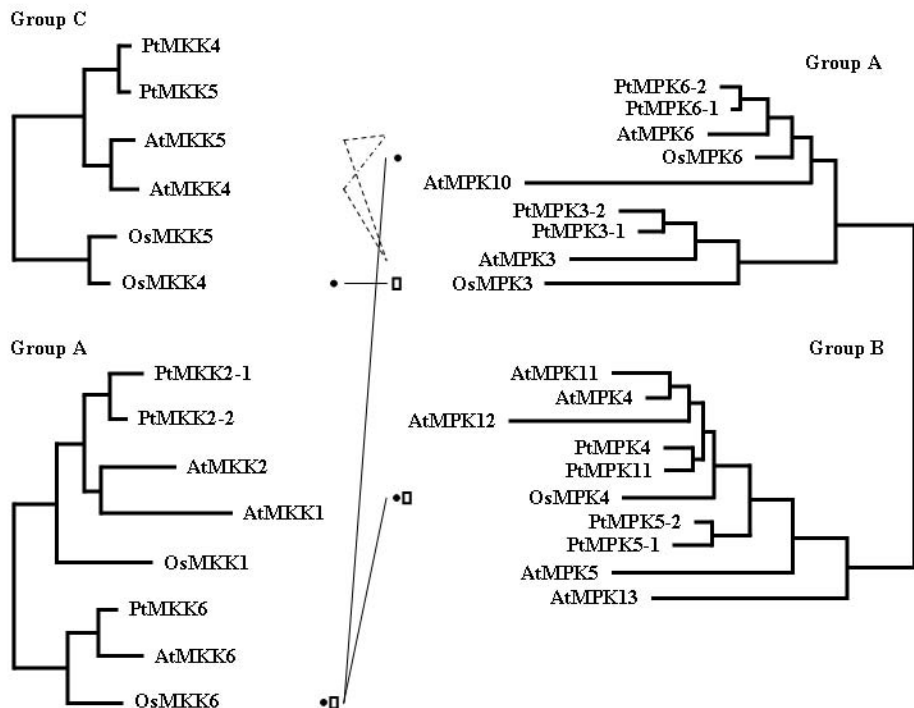


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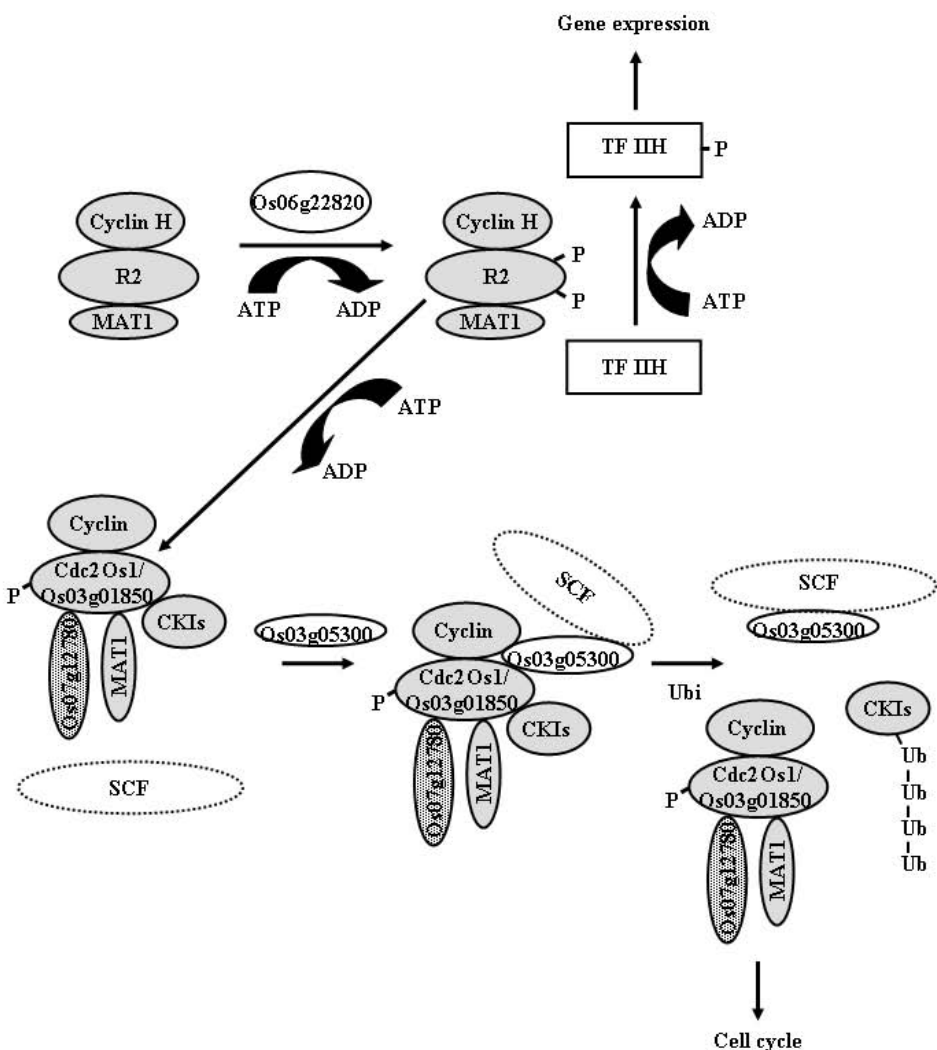


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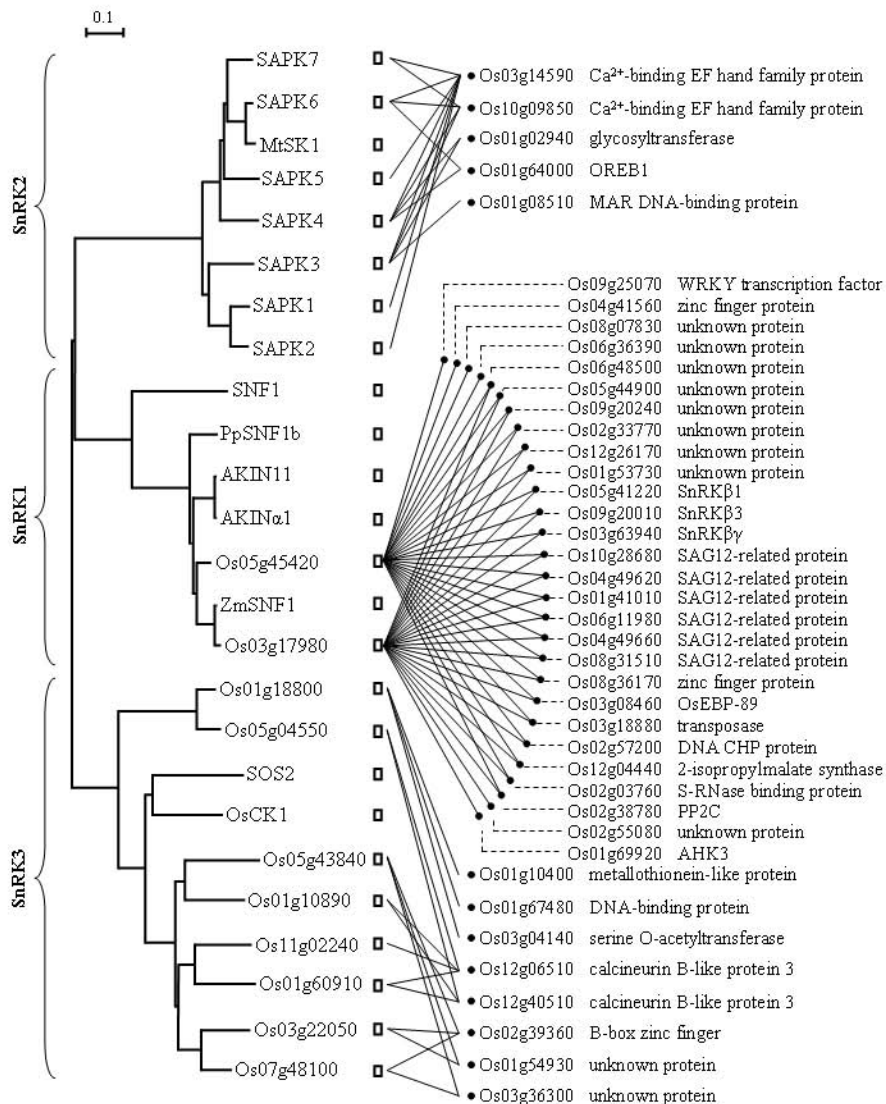


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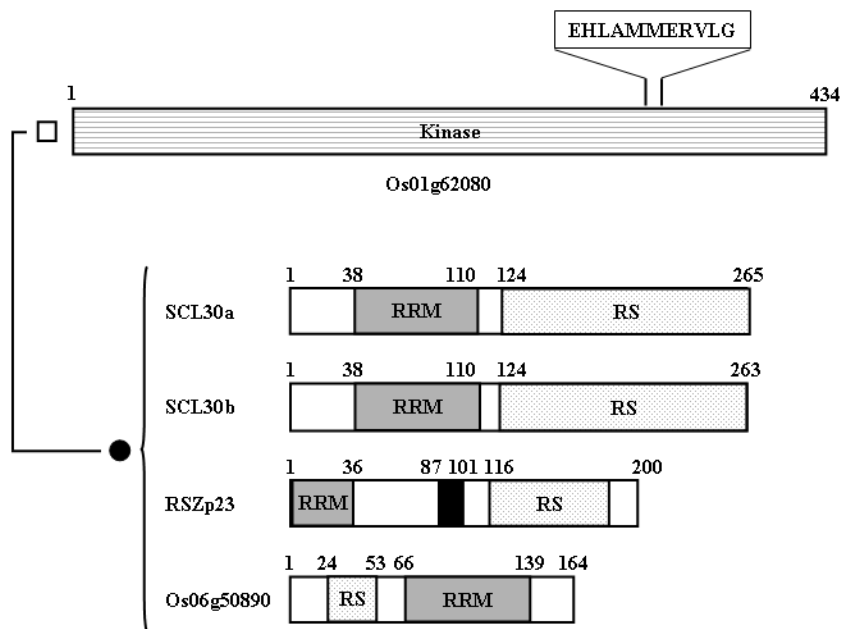


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