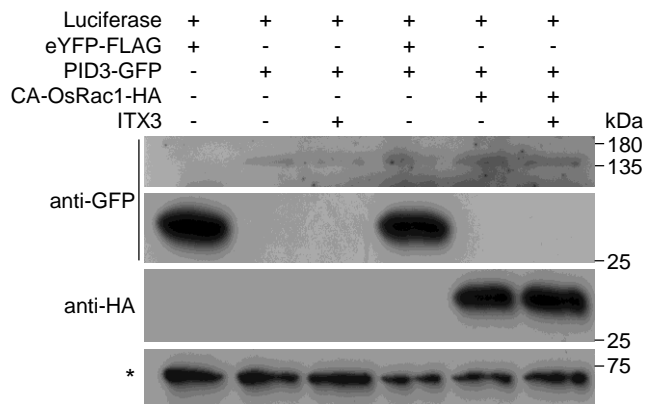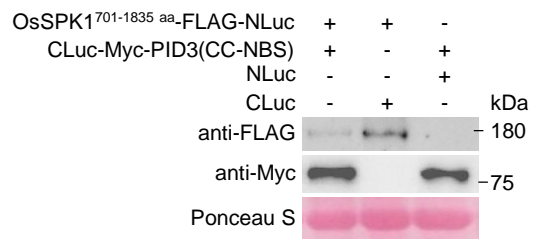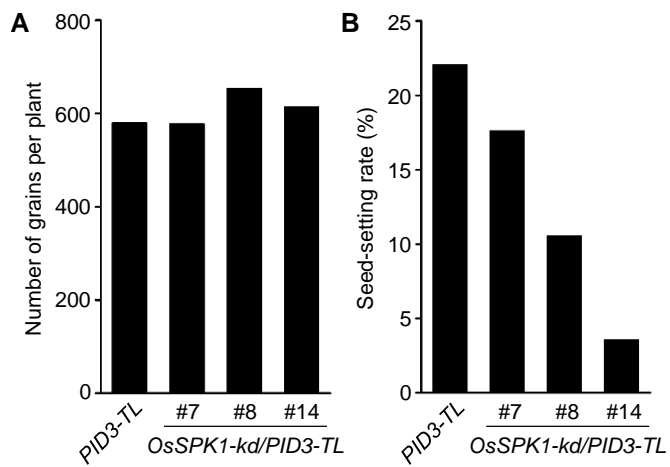The OsSPK1–OsRac1–RAI1 defense signaling pathway is shared by two distantly related NLR proteins in rice blast resistance

Luciferase	+	+	+	+	+	+	
eYFP-FLAG	+	-	-	+	-	-	
PID3-GFP	-	+	+	+	+	+	
CA-OsRac1-HA	-	-	-	-	+	+	
ITX3	-	-	+	-	-	+	kDa

Supplemental Figure S1. Detection of the expressed proteins in Fig. 2A. Non-specific hybridized bands (*), ~ 70 kilo-Dalton (kDa) in size, were used as equal-loading controls.

| OsSPK1[701-1835 aa]-FLAG-NLuc | + | + | - | |
CLuc-Myc-PID3(CC-NBS)	+	-	+	
NLuc	-	-	+	
CLuc	-	+	-	kDa
anti-FLAG				— 180
anti-Myc				—75
Ponceau S				

Supplemental Figure S2. Detection of the expressed proteins in Fig. 2C. The levels of Rubisco are shown as equal loading controls.

Supplemental Figure S3. Comparison of grain numbers and seed-setting rate between *PID3-TL* and *OsSPK1-kd/PID3-TL* plants (T_0 generation).

A

Pb1
Pik-2
Pi1-6
Pikm2-TS
Pikp-2
Pi7-J-2
Pi-ta
Pi-a (RGA4)
Pikp-1
Pi7-J-1
Pik-1
Pikm1-TS
Pi1-5
Piks-1
Pi36
Pib
Pid4
Pi9
Pi2
Piz-t
Pi50
PlgmR
PID3
Pi25
Pit
Pi5-1
Pi5-2
Pi56(t)
Pi63
Xa1
Pi64
Pi37
Pish
Pi35
Pish-J
Pikh

0.2

67
100
69
47
37
78
99
100
27
90
71
34
96
100
39
100
56
69
100
49
64
51
94
99
85
93
70
100
100

B

```
                    40                          80                              120
Pi9: ----- MAETVLSMARSLVGSAISKAASAANETSLLLGVEKDIWYIKDELKTMQAFLRAAEVMKKKDELLKVWAEQIRDLSYDIEDSLDEFKVHIES---------QTLFRQLVKLRE  104
PID3: MAEGVVGSLIVKLGDALASEAVEVAKSLLGLEGSALKRLFSEIGEVKGELESIHAFLQAAERFKDADETTSAFVKQVRSLALSIEDVVDEFTYELGEGDGR----MGMAVALKRVCKNGT  116

                    160                         200                             240
Pi9: RHRIAIRIHNLKSRVEEVSSRNTRYNLVEPISSGTEDDMDSYAEDIRNQSARNVDEAELVGFSDSKKRLLEMIDTNANDGPAKVICVVGMGGLGKTALSRKIFESEEDIRKNFPCIAWIT  224
PID3: WSRLAGNLQDIKVNLKNAAERRIRYDLKG-VERGAKSTAGRRSSNWRSDSVLFKREDELVGIE--KKRDLLMKWVKDEIQRRMVSVWGMGGIGKTALVANYYN---AIKADFDTCAWIT  230

                    280                         320                             360
Pi9: VSQSFHRIIELLKDMIRQLLGPSSLDQLLQEIQGKVVVQVHHLSEYLIEELKEKRYFVILDDLWILHDWNMINEIAFPKNIKKGSRIVITTRNVDLAEKCATASLVYHLDFLQMNDAITLL  344
PID3: VSQSYEADDLLRRTAQEFRKNDRKKDFPVDVD---ITNYRGLVETTRSYLENKRYVLVLDDVWNANVW-FDSKDAFEDGNIG--RIILTSRNYDVALLAHETHIIN-LQPLEKHHAWDLF  343

                    400                         440                             480
Pi9: LRKTNKNHEDMESNKNMQKMVERIVNKCGRLPLAILTIGAYLATK--HVSEWEKFYEQLPSELEINPSLEALRRMVTLGYNHPSHLKPCFLYLSIFPEDFEIKRNRLVGRWIAEGFVRP  462
PID3: CKEAFWKNEIRNCPPELQPWANNFVDKCNGLPIAIVCLGRLLSFQGSTYSDWEKVYKNLEMQLTNNSIMDMWNIILKISLEDLPHIKNCFLYCSMFPENYVMKRKSLVRLWWAEGFIEE  463

                    520                         560                             600
Pi9: KVSMTTKDVGESYFNELINRSMIQRSRVGIAGKIKTCRIHDIIRDITVSISRQENFVLLPMGDGSDLVQENTRHIAFHGSMSCKTGLDWS-IIRSLAIFGDRPKSLAHAVCLDQLRMLRV  581
PID3: TEHRTLEEVAEHYLTELVNRCLLLVKRNEAGHYHEVQMHDILRVLALSKAREQNFCIVVNHSRSTHLIGEARRLSIQRGDFAQLADHAP-HLRSLLLFQSSPNVSSLHSLPXSVKLLSV  582

                    640                         680                             720
Pi9: LDLEDVTFLITQKDFDRIALLCHLKYLSIGYSSSIYSLPRSIGKLQGLQTLNMLRTY-IAALPSEISKLQCLHTLRCSRKFVYDNFSLNHPMKCLTNTICLPKVFTPLVSRDDRAKQIAE  700
PID3: LDLTDSSVDRLPKEVFGLFNLRFLGLR----RTKISKLPSSIGRLKNLLVLDAWCK-IVKLPLAITKLCKLTHLIVT--------------SKAVVVSKQFVPSVG-  670

                    760                         800                             840
Pi9: LHMATKSCWSESFGVKVPKGIGKLRDLQVLEYVDIRRTSSRAIKELGHLSKLRKLGVITKGSTKEKCKILYAAIEKLSSLQSLYYNAALLSDIETLECLDSISSPPPLLRTLGLNG--SL  818
PID3: ------------YPAPLRICSMTTLQTLLLMEAS--SQMVHHLGSLVELRTFRISKVRS--CHCEQLFMAITNMIHLTRLGIQADSSQEVLHLESLK----PPPLLQKLFLQGTLSH  767

                    880                         920                             960
Pi9: EEMPNMIEQLTHLKKIYLLRSKLKEGKTMLILGALPNLMVLYLYWNAYLGEKLVFKTGAFPNLRTLKIYELDQLREMRFEDG----------SSPLEXIEISCQR-LESGIIGIHLPR  927
PID3: ESLPHFVSVSNLNNLTFLRLAGSRIDENAFLNLEGLQQLVKLQLYDAFDGMNIYFHENSFPKLRILKIWGAPHLNEIKMTKG---------AVASLTHLKFLLCPNLKQLPCGIEHVRT  877

                    1000                        1040
Pi9: LKEISLEYKSKVARLGQLEGEVNTHPNRPVLRMDSDRRDHDLGAEAEGSSIEVQTADPVPDAEGSVTVAVEATDPLPEQEGESSSQSVITLTTNDSEELGTAQAG-- 1032
PID3: LEELTLDHT---------AEELVDRVRRKKERMICDVQRVYVGFIRNG----YLAAERIQ----------------------------------------- 924
```

Supplemental Figure S4. Evolutionary relationships of Pi9 with other NLR proteins. A, Analysis of evolutionary relationships among the majority of known NLR proteins in rice. The phylogenetic tree was generated by MEGA6 software using a neighbor-joining method. Numbers at the nodes show bootstrap values (1000 replicates). The distance scale indicates nucleotide substitutions per site. Pi9 and PID3 are highlighted in bold. B, Sequence alignment between Pi9 and PID3 by ClustalW. Black and gray shadows represent highly and moderately conserved residues, respectively. Dashes indicate gaps.

cgctgccgaagaaatcacttcctctgctccttcctatccccatccacaggattccagctattcgacaacccttctctcttttctttatctcttggctttttttcctt
actgtcactctctttctctctctctctgtcttccttctagtcattcctttggcaaagttctttttttccttccagcccaacATGCAGTTCTCTCAGATCC
TCACCGTCTTGTTCCTTGGCGTCTCCGTCAGCGCCCTTCCCGCCGGCGGTCTGCCCGGCAGCCCTG
GCAGCGCTGTCCAGAGGTGCCACTGCCCCCCCTCGTGGCTCCCACGCCCACGGCTCCCTCGCCGCT
CGGGAGGAAGCGCCCGAGGCCGAAGGTGACGCCAAGATTTCCGCCCGCTACACCTGCCCCAACTG
CCACAAGACGGGCAAAGGCTGCGATGATGGgtaaataagcaaccccccatccccttgaaatccttttgcggtcatgttgctga
cgtctttgatagCTGGTGCCAAGTCGAAAAGACGCACTGGTAGaggaaaagtcaatcgagtaattatacaggttagtgcttgta
ctagtgctctttctaataacaatccatagtatattatactaagtagtaagcacattcgcctttaattaattctggtatcagtaatgcgcagaagggtgttgat
gctggggcttcaaaagtcgggccactcgcattatcgcataattg

Supplemental Figure S5. Sequence for the *AvrPi9* locus identified in the rice blast isolate RB-22.
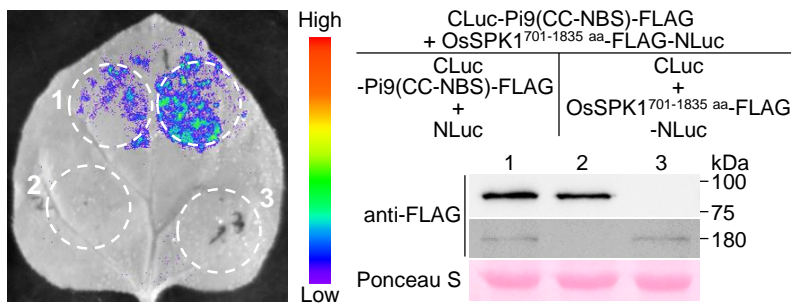
Supplemental Figure S6. Pi9 interacts with OsSPK1 as detected by LUC assay. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium*-containing construct combinations as indicated. The luminescence images were captured using a cooled CCD imaging apparatus. Immunoblotting on the right panel showed the levels of the expressed proteins. Ponceau S staining indicates equal sample loading.
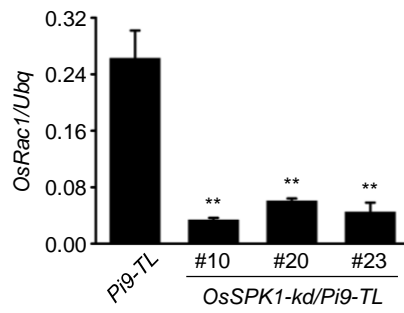
Supplemental Figure S7. *OsSPK1* knockdown affects *OsRac1* transcripts in rice plants. RT-qPCR data are shown as means±SD (*n* = 3), and the difference significance was calculated from *Pi9-TL*. *Ubq*, *UBIQUITIN*. **, *P* < 0.01 (Student's *t*-test).

High

CLuc-Pi9(CC-NBS)-FLAG + OsRac1-Myc-NLuc

CLuc-Pi9(CC-NBS)-FLAG + NLuc	CLuc + OsRac1-Myc-NLuc

	1	2	3	kDa
anti-FLAG				−100 −75
anti-Myc				−75
Ponceau S				

Low
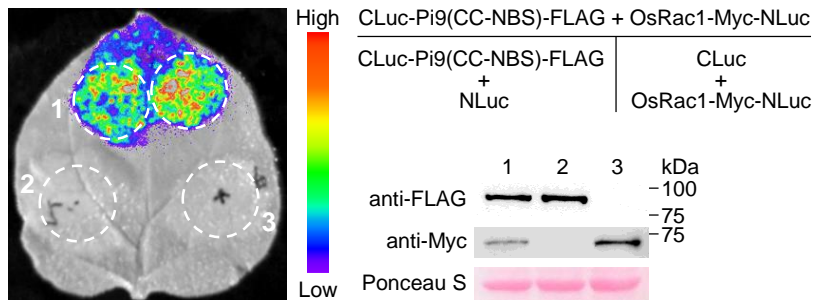
Supplemental Figure S8. Pi9 interacts with OsRac1 as detected by LUC assay. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium*-containing construct combinations as indicated. The luminescence images were captured using a CCD imaging system. Immunoblotting on the right panel showed the levels of the expressed proteins. The levels of Rubisco are shown as equal loading controls.
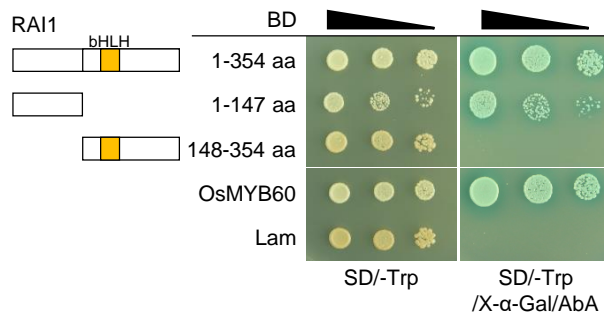
Supplemental Figure S9. Determination of RAI1 transactivation activity in yeast cells. In this experiment, the truncated regions of RAI1, or full-length RAI1, were each fused to the carboxyl end of the GAL4 DNA binding domain (BD). Yeast cultures transfected with OsMYB60 were used as positive controls while those with Lam as negative ones. Pictures taken at 5 d after the transformation.
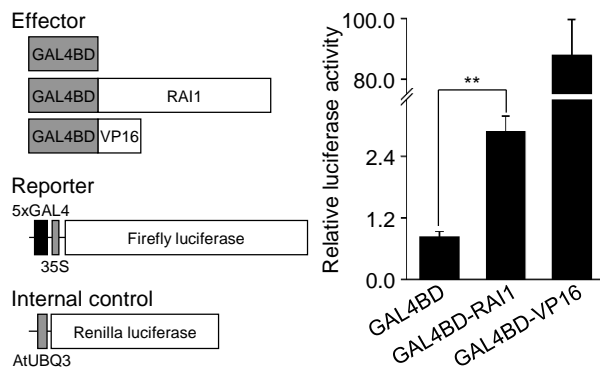
Supplemental Figure S10. Determination of RAI1 transactivation activity with a dual-luciferase reporter system. Schematic representation of each plasmid structure is shown (left panel). The plasmids of effector, reporter, and internal control were co-transfected into rice protoplasts for overnight incubation before detection. Cell cultures transfected with GAL4BD-VP16 were used as positive controls while those with GAL4BD as blank ones. Data are shown as mean$\pm$SD (n = 5). **, P < 0.01 (Student's t-test).
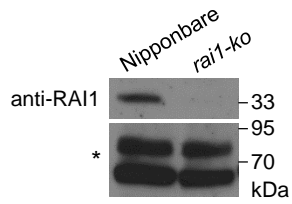
Supplemental Figure S11. Performance of anti-RAI1 in Western blotting analysis. The *rai1* knock-out mutant (*rai1-ko* for short) was created based on the Nipponbare background. Total protein was extracted and analyzed by immunoblotting. Non-specific bands (*) confirmed equal loading.

Myc-RAI1-NLuc	+	+	-	
CLuc-Pi9(CC-NBS)-FLAG	+	-	+	
NLuc	-	-	+	
CLuc	-	+	-	kDa

anti-Myc	−75
anti-FLAG	−75
Ponceau S	
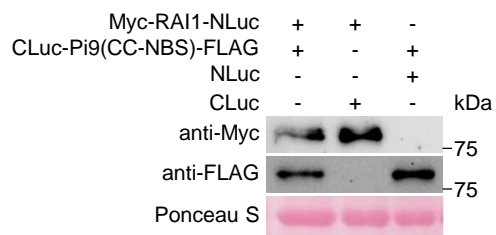
Supplemental Figure S12. Detection of the expressed proteins in Fig. 6D. Rubisco blots stained by Ponceau S are used as an equal loading control.

A

OsRPT2a

Construct	AD → RAI1 (SD/-Leu-Trp)	AD → RAI1 (SD/-Ade-His-Leu-Trp/X-α-Gal/AbA)
Prot ATP ID OB AAA AAA lid 3	1-448 aa	
	1-229 aa	
	230-363 aa	
	364-448 aa	
	385-429 aa	
	1-384 aa	
	230-448 aa	
OsMAPK3		
Empty		

SD/-Leu-Trp   SD/-Ade-His-Leu-Trp/X-α-Gal/AbA

B

Myc-OsRPT2a-NLuc + CLuc-Myc-RAI1   Myc-OsRPT2a⁣364-448 aa-NLuc + CLuc-Myc-RAI1

Myc-OsRPT2a-NLuc + CLuc | NLuc + CLuc-Myc-RAI1   Myc-OsRPT2a364-448 aa-NLuc + CLuc | NLuc + CLuc-Myc-RAI1

C

	1	2	3	4	5	6	kDa
anti-Myc							— 135
							— 100
							— 75
							— 65
Ponceau S							

Supplemental Figure S13. OsRPT2a associates with RAI1. A, Yeast two-hybridyzation (Y2H) assay. The full-length RAI1 was fused to the carboxyl end of the GAL4 activation domain (AD). The indicated truncated regions of OsRPT2a and the full-length OsRPT2a were each fused to the carboxyl end of the BD. Yeast cultures co-transfected with BD-OsMAPK3 and AD-RAI1 were used as positive controls. Pictures taken at 5 d after the transformation. B, LUC assay. *N. benthamiana* leaves were co-infiltrated with *Agrobacterium*-containing construct combinations as indicated. The luminescence images were captured using a CCD imaging system. C, Levels of the expressed proteins shown in (B), as detected by immunoblotting. Ponceau S staining indicated equal loading.
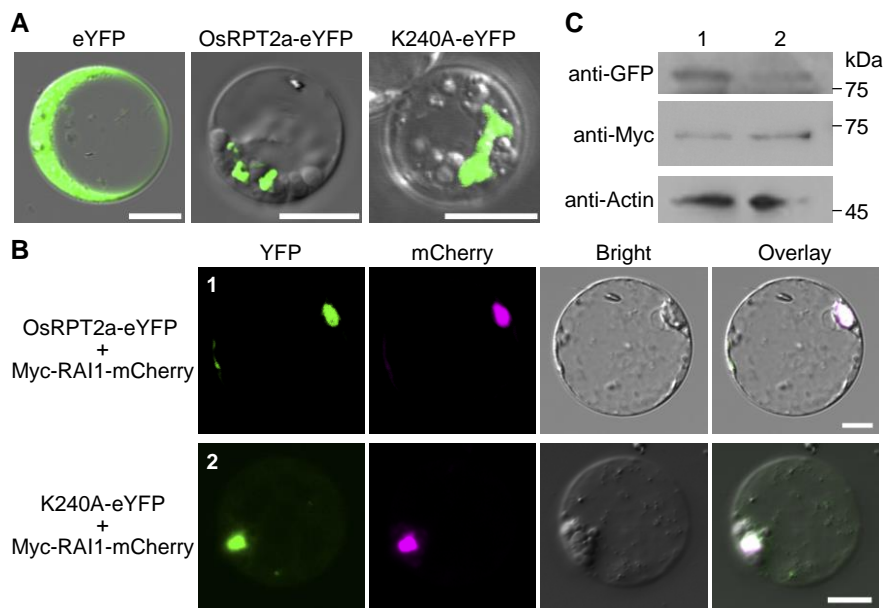
Supplemental Figure S14. OsRPT2a^{K240A} has little effect on its subcellular distribution or co-localization with RAI1. A, Subcellular distribution eYFP fusions of OsRPT2a and OsRPT2a^{K240A} (K240A). B, Co-localization of RAI1 and OsRPT2a or K240A in rice protoplasts. For A and B, fluorescent signals were captured with a confocal fluorescence microscope. Scale bars, 10 μm. C, Detection of the expressed proteins shown in (B). Actin levels are shown as equal loading controls.

Supplemental Table S1 Primers used in this study.

Primers for RNA interference		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
OsSPK1	GTAGAAGAGGTACCCGGGATGTGTTCAGAGAGCCAAGAGATG	GCATGCCTGCAGGTCGACATGTGTTCAGAGAGCCAAGAGATG
Primers for transient expression tests in rice protoplasts		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
PID3-GFP	AGAACACGGGGGACTCTAGAATGGCGGAGGGTGTTGTGGG	CTTGCTCACCATGGATCCTTGAATCCTTTCTGCAGCCA
OsRPT2a-FLAG/K240A-FLAG	GAACACGGGGGACGAGCTATGGGGCAGGGCACCCCGGG	TAGTCTTCGAACTCGAGGCACATGTAAAGACCCTCCGG
eYFP-FLAG	GAACACGGGGGACGAGCTATGGTGAGCAAGGGCGAG	TAGTCTTCGAACTCGAGGCACTTGTACAGCTCGTCCAT
Primers for BiFC		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Yn-Pi9(CC)	GAGCTCAAGCTTGAATTCATGGCGGAGACGGTGCTG	CTCTAGATCAGGTGGATCCTCAAACAACACAGATTACCT
Yc-RAI1	GAGCTCAAGCTTGAATTCATGGAGCTTGACGAGGAGTC	CTCTAGATCAGGTGGATCCCTACAGACACCTTCCGCCAT
Primers for subcellular localization		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
OsRPT2a-eYFP/K240A-eYFP	AGCTCAAGCTTCGAATTCATGGGGCAGGGCACCCCGGG	TTGCTCACCATCAGGATCCACATGTAAAGACCCTCCGGC
Myc-RAI1-mCherry	AGCTCAAGCTTCGAATTCATGGAGCAAAAGCTCATTTCT	TGCTCACCATCAGGATCCACAGACACCTTCCGCCATA
Primers for RT-qPCR		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
Ubq	AACCAGCTGAGGCCCAAGA	ACGATTGATTTAACCAGTCCATGA
OsLOX3	CCGTACCAGCTGATGAAGC	TTTTGGAGCGTTTTGTCTCA
OsPR5	CGCTGCCCCGACGCTTAC	ACGACTTGGTAGTTGCTGTTGC
OsRac1	GTGTTCATCCTGTCCTTCTCC	AGGCCCTATCTTCACGGAG
OsSPK1	GGATACTCCAAGGAAGTGTGG	GGCCATGAATTCCAGCAATG
OsWRKY45	CGGGTAAAACGATCGAAAGA	TTTCGAAAGCGGAAGAACAG
Primers for semi-quantitative RT-PCR		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
RAI1	CGGCGACAAGGCGCTGCTCCA	CTCCTGAACAATGTCTGCTTG
OsActin1	GGAACTGGTATGGTCAAGGC	AGTCTCATGGATACCCGCAG

Supplemental Table S1 (continued)

Primers for genomic sequencing		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
AvrPi9	AGGCCGCCTTTCGCTGCCGA	TTAACTTGGTCGCAATTATG
Primers for Co-IP		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
PID3(CC-NBS)-GFP	AGAACACGGGGGACTCTAGAATGGCGGAGGGTGTTGTGGG	CTTGCTCACCATGGATCCATGGTCTGCAAGTTGTGCAAA
OsSPK1$^{701-1835\ aa}$-FLAG	AGAACACGGGGGACTCTAGAATGTTTCGAGCTATGGTCAAT	CATATGGTCGACGGATCCTAGCTCTGAAAGAATTGCAG
Primers for LUC assay		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
OsSPK1$^{701-1835\ aa}$-FLAG-NLuc	GCTCGGTACCCGGGATCCATGTTTCGAGCTATGGTCAATA	GTACGAGATCTGGTCGACCTTATCGTCATCGTCCTTGTAA
Myc-RAI1-NLuc	GACGAGCTCGGTACCCGGATGGAGGAGCAGAAGCTGAT	CGTACGAGATCTGGTCGACCAGACACCTTCCGCCATAGC
OsRac1-Myc-NLuc	GCTCGGTACCCGGGATCCATGGAGGAGCAGAAGCTGATC	GTACGAGATCTGGTCGACCGCGAAACAAGCGCTTCCGCA
Myc-OsRPT2a-NLuc	GACGAGCTCGGTACCCGGATGGAGGAGCAGAAGCTGAT	CGTACGAGATCTGGTCGACCATGTAAAGACCCTCCGGC
Myc-OsRPT2a$^{364-448\ aa}$-NLuc	GACGAGCTCGGTACCCGGATGGAGGAGCAGAAGCTGAT	CGTACGAGATCTGGTCGACCATGTAAAGACCCTCCGGC
CLuc-Myc-PID3(CC-NBS)	GGGCGGTACCCGGGATCCAGAGGAGCAGAAGCTGATC	GAAAGCTCTGCAGGTCGACCTAATGGTCTGCAAGTTGTGCAAA
CLuc-Pi9(CC-NBS)-FLAG	GGGCGGTACCCGGGATCCAATGGCGGAGACGGTGCTGAGC	GAAAGCTCTGCAGGTCGACCTACTTATCGTCATCGTCCTT
CLuc-Myc-RAI1	GGCGGTACCCGGGATCCAGAGCAAAAGCTCATTTCTGAA	AAAGCTCTGCAGGTCGACCTACAGACACCTTCCGCCATA
Primers for yeast-related assays		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
AD-OsSPK1$^{1002-1835\ aa}$	ATGGCCATGGAGGCCAGTGACGACATGACTCTGATCAA	GCTCGAGCTCGATGGATCTTATAGCTCTGAAAGAATTG
AD-RAI1	ATGGCCATGGAGGCCAGTATGGAGCTTGACGAGGAGTC	GCTCGAGCTCGATGGATCCTACAGACACCTTCCGCCAT
BD-PID3(CC)	CATATGGCCATGGAGGCCATGGCGGAGGGTGTTGTGGGG	GCTGCAGGTCGACGGATCCTACCTCCTTCCCGCTGTCG
BD-PID3(NBS)	CATATGGCCATGGAGGCCAGCTCAAACTGGAGATCTGA	GCTGCAGGTCGACGGATCCTAATGGTCTGCAAGTTGTGC
BD-Pi9(CC)	CATATGGCCATGGAGGCCATGGCGGAGACGGTGCTG	GCTGCAGGTCGACGGATCTCAAACAACACAGATTACCT

Supplemental Table S1 (continued)

BD-Pi9(NBS)	CATATGGCCATGGAGGCCGGGATGGGTGGTTTAGGCAA	GCTGCAGGTCGACGGATCTCAACAAACTGCATGTGCTAGA
BD-Pi9(LRR)	CATATGGCCATGGAGGCCCTAGATCAATTGAGGATGTT	GCTGCAGGTCGACGGATCTCATCCTTCGGCTTCAGCCCCC
BD-RAI1	CATATGGCCATGGAGGCCATGGAGCTTGACGAGGAGTC	GCTGCAGGTCGACGGATCCTACAGACACCTTCCGCCAT
BD-RAI1[1-147 aa]	CATATGGCCATGGAGGCCATGGAGCTTGACGAGGAGTC	GCTGCAGGTCGACGGATCCTAGAACGTCGGCGACGACGA
BD-RAI1[148-354 aa]	CATATGGCCATGGAGGCCATGTTTGGAGGTGGCGCC	GCTGCAGGTCGACGGATCCTACAGACACCTTCCGCCAT
BD-OsRPT2a	CATATGGCCATGGAGGCCATGGGGCAGGGCACCCCGGG	GCTGCAGGTCGACGGATCTCACATGTAAAGACCCTCCG
BD-OsRPT2a[1-229 aa]	CATATGGCCATGGAGGCCATGGGGCAGGGCACCCCGGG	GCTGCAGGTCGACGGATCTCATCCCTTGGGTGGCCTGAT
BD-OsRPT2a[230-363 aa]	CATATGGCCATGGAGGCCGTCATATTATACGGAGAACC	GCTGCAGGTCGACGGATCTCACAGAGGAAATTCTATCT
BD-OsRPT2a[364-448 aa]	CATATGGCCATGGAGGCCCCAGATATTAAAACTAGGCG	GCTGCAGGTCGACGGATCTCACATGTAAAGACCCTCCG
BD-OsRPT2a[385-429 aa]	CATATGGCCATGGAGGCCGATGTGAACCTGGAAGAGTT	GCTGCAGGTCGACGGATCTCACTTCTTGAAGTCAGCAT
BD-OsRPT2a[1-384 aa]	CATATGGCCATGGAGGCCATGGGGCAGGGCACCCCGGG	GCTGCAGGTCGACGGATCTCAATCTGCCAATGTCATCT
BD-OsRPT2a[230-448 aa]	CATATGGCCATGGAGGCCGTCATATTATACGGAGAACC	GCTGCAGGTCGACGGATCTCACATGTAAAGACCCTCCG

Primers for dual luciferase reporter assay		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
GAL4BD-RAI1	CCGTCTAGAACTAGTGGAATGGAGCTTGACGAGGAGTC	TAAGCTTGATATCGAATTCTACAGACACCTTCCGCCAT
Primers for ATPase activity assays		
Gene name	Forward primer (5'-3')	Reverse primer (5'-3')
OsRPT2a-GST/K240A-GST	GGTGGTGGTGGAATTCCAATGGGGCAGGGCACCCCGGG	TCACGATGAATTAAGCTTCATGTAAAGACCCTCCGGCA