**Letter**

# An unconventional NOI/RIN4 domain of a rice NLR protein binds host EXO70 protein to confer fungal immunity

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A subset of plant nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins carry extraneous integrated domains that have been proposed to mediate pathogen effector recognition. The current view is that these unconventional domains function by directly binding or serving as substrates for pathogen effectors, yet only a few domains have been functionally characterized to date. Here we report that the integrated NOI domain of the rice NLR protein Pii-2, together with its partner Pii-1, mediates immunity to the rice blast fungus *Magnaporthe oryzae* by indirect recognition of the AVR-Pii effector. We discovered that the Pii-2 NOI domain does not physically interact with the effector itself but instead binds the host protein OsExo70-F3, which is a target of AVR-Pii. We further identified mutations within the NOI core motif (PxFGxW) of Pii-2 that abolish both OsExo70-F3 binding and *Pii*-mediated resistance to *M. oryzae* expressing AVR-Pii. This led us to propose a novel conceptual model in which an NLR-integrated domain functions to detect host proteins targeted by pathogen effectors, in a framework that extends classical indirect recognition models.

(174 words)

Plants have evolved immune receptors to defend against pathogen infection. These include cytosolic nucleotide-binding domain and leucine-rich repeat-containing (NLR) proteins that recognize pathogen avirulence (AVR) effectors and activate a complex immune response that includes hypersensitive cell death. Plant NLRs have a modular architecture comprising either an N-terminal coiled-coil (CC) or a Toll/interleukin-1 receptor (TIR) homology domain, followed by nucleotide binding (NB) and leucine rich

23 repeat (LRR) domains. To date, several reports have shown that genetically-linked NLR
24 pairs (e.g., *RPS4*/*RPS1*, *RGA4*/*RGA5* and *Pikp-1*/*Pikp-2*) are required to act together for
25 the recognition of cognate AVRs. Of these pairs, one NLR is a sensor NLR that
26 perceives pathogen effectors while the other is a helper NLR that is necessary to
27 activate immune signaling (ref. 1,2). A number of sensor NLRs, perhaps as many as
28 10%, have recently been reported to contain additional unconventional domains called
29 integrated domains (IDs) that appear to have originated from host targets of effectors
30 (ref. 3,4). The current view is that NLR-integrated domains function as baits or decoys
31 that bind pathogen effectors or serve as substrates of effectors, in order to detect
32 invading pathogens. One example is *Arabidopsis* RRS1, an NLR that carries a
33 C-terminal domain with similarity to WRKY transcriptional factors. RRS1 is required
34 for the perception of bacterial effectors PopP2 and AvrRps4, which are bacterial
35 effectors that target *Arabidopsis* WRKY proteins to enhance virulence. Therefore, Le
36 Roux *et al*. (2015) concluded that a WRKY domain targeted by pathogen effectors has
37 integrated into RRS1 to facilitate recognition of the bacterial effectors (ref. 5,6). Other
38 examples include the rice NLR pairs Pia (RGA4/RGA5) and Pikp (Pikp-1/Pikp-2),
39 which recognize the effectors AVR-Pia and AVR-PikD of the rice blast fungus,
40 *Magnaporthe oryzae* (syn. *Pyricularia oryzae*), respectively. Remarkably, both RGA5
41 and Pikp-1 include a Heavy Metal-Associated (HMA) domain integrated in different
42 positions in these NLRs. Binding of the integrated HMA domain to the corresponding
43 AVR effectors triggers the hypersensitive response and immunity to the blast fungus
44 (ref. 7,8). These findings let to the proposal that unconventional integrated domains of

45 NLRs have evolved to directly bind pathogen effectors or serve as a substrate for the
46 effectors's activity (ref. 9). As yet, the degree to which IDs associate with host proteins
47 is largely unknown.
48
49 The *Pii* locus in rice encodes a pair of CC-NLR proteins, Pii-1 and Pii-2, that detect
50 the *M. oryzae* effector AVR-Pii and mount an effective immune response against races
51 of this pathogen that express AVR-Pii (ref. 10,11). Despite repeated attempts using
52 various protein-protein interaction methods, we did not detect direct interaction between
53 AVR-Pii and either one of Pii-1 and Pii-2 (ref. 12). Instead, we discovered that AVR-Pii
54 binds two members of the large EXO70 protein family of rice, OsExo70F-2 and
55 OsExo70F-3 (ref. 12). EXO70 is a subunit of the exocyst, an evolutionarily conserved
56 vesicle tethering complex that functions in the last stage of exocytosis (ref. 13,14).
57 Although we do not understand the degree to which AVR-Pii binding to EXO70
58 contributes to virulence, we found that *OsExo70-F3* is genetically required for
59 *Pii*-mediated immunity to *M. oryzae* expressing *AVR-Pii* (ref. 12). This led us to
60 propose that Pii response to AVR-Pii follows the indirect recognition model, in which
61 the NLR protein recognizes modifications of a host protein targeted by the effector (ref.
62 12).
63 Here we report that Pii recognition of AVR-Pii follows a novel conceptual model of
64 NLR perception of plant pathogens. We found that the 280 C-terminal amino acids of
65 Pii-2 (Pii-2-CT) bind the rice protein OsExo70-F3 that is targeted by the AVR-Pii
66 effector (Fig. 1, Supplementary Fig. 1, 2 and 3). We further delineated the interaction

67 region to the 103 amino acids at the C-terminus of the protein (Pii-2-CTC)
68 (Supplementary Fig. 1, 2 and 3). Interestingly, this region of Pii-2 contains a short
69 sequence with similarity to a core motif of nitrate-induced (NOI) domain (PxFGxW)
70 that is also conserved in the *Pi5-2* which is allelic to *Pii-2* (Supplementary Fig. 3 and ref.
71 3,15). The NOI domain also defines the *Arabidopsis* RPM1-INTERACTING
72 PROTEIN4 (RIN4), which is known to negatively regulate plant defense and is targeted
73 by bacterial effectors AvrRpt2, AvrB and AvrRpm1 (ref. 16). In fact, the protease
74 effector AvrRpt2 cleaves RIN4 protein at the NOI motif PxFGxW. To further
75 investigate the importance of the Pii-2 NOI motif, we introduced multiple mutations in
76 this region and tested the resulting mutants for binding to OsExo70-F3 (Fig.1,
77 Supplementary Fig. 4). Mutations in the NOI conserved residues, Pro(P), Phe(F) and
78 Trp(W) abolished binding to OsExo70-F3 (Fig. 1, Supplementary Fig. 4 and 5). This
79 indicates that the Pii-2 NOI core motif is required for OsExo70-F3 binding.
80
81 Next, we investigated the role of the NOI motif in *Pii*-mediated immunity. We
82 determined the effect of mutations within the NOI core motif on *Pii*-mediated resistance
83 to *M. oryzae* using genetic complementation of a *pii-2* loss-of-function rice line with
84 *Pii-2* transgenes carrying the mutations in the NOI motif described above. To obtain a
85 *pii-2* loss-of-function mutant rice, we screened 5,600 ethyl methanesulfonate (EMS)
86 mutant lines of rice cultivar Hitomebore, which carries the *Pii* gene (ref. 17). This led to
87 the identification of two *pii-2* loss-of-function mutants, Hit5882 and Hit13701, carrying
88 an amino acid substitution (V371D) and a nonsense mutation (Q247stop) in the Pii-2

89 NB-domain, respectively (Supplementary Fig. 3; ref. 11). A full-length cDNA of
90 wild-type *Pii-2* under the control of the 35*S* promoter (Hit5882+WT-Pii-2) transformed
91 in Hit5882 complemented *Pii*-mediated resistance (Fig. 2). In contrast, transformation
92 of Hit5882 with a full-length cDNA of *Pii-2* carrying the NOI-m5 mutation failed to
93 confer *Pii*-dependent resistance in all transgenic lines tested (Hit5882+Pii-2-NOI-m5;
94 Fig. 2). The Pii-2-NOI-m5 mutant also failed to complement the second
95 *Pii*-loss-of-function rice line Hit13701 (Supplementary Fig. 6). Overall, our data
96 genetically link Pii-2 NOI motif binding to OsExo70-F3 with *Pii*-mediated resistance to
97 *M. oryzae* expressing the AVR-Pii effector. This suggests that NOI binding to EXO70
98 is crucial for Pii function.
99
100 The current view is that integrated domains of NLR immune receptors function by
101 directly binding or serving as substrates for pathogen effectors. Our findings suggest
102 that NLR-integrated domains may function by detecting host proteins targeted by
103 pathogen effectors in a novel model in which an integrated domain of an NLR mediates
104 indirect recognition of a pathogen effector (ref. 18,19) (Fig. 3). We hypothesize that the
105 rice NLR Pii-2 has integrated a NOI domain originating from a host NOI-EXO70
106 complex targeted by AVR-Pii. This NOI integration has enabled Pii to monitor
107 OsExo70-F3 *via* physical interaction, which allowed indirect detection of AVR-Pii (Fig.
108 3). Recent reports showing RIN4-EXO70 interactions (ref. 20,21) support this
109 hypothesis.
110

111 Our work also points to the NOI-EXO70 complex as a major target of plant pathogen
112 effectors. These domains have therefore been acquired as sensor domains by NLR
113 immune receptors, and both NOI and EXO70 are commonly seen integrated into NLRs
114 (ref. 22, 23). Whether integrated NOI and EXO70 domains can mediate direct
115 recognition of effectors remains to be determined. Nonetheless, our finding that
116 NLR-integrated domains can indirectly detect AVR effectors expands the mechanistic
117 view of pathogen detection by immune receptors—that the interactors of
118 NLR-integrated domains can be either pathogen effectors, or host proteins that are
119 targeted by effectors. This work shows the first example of indirect pathogen
120 recognition *via* an integrated NLR, highlighting how the arms race between plants and
121 pathogens has driven the emergence of a wealth of molecular interactions and
122 mechanisms in the fight for survival.
123

124 **Methods**

125 **Plasmids and Plants**

126 The primers and their sequences used for plasmid constructions are listed in
127 Supplementary Table 1. For Y2H assay, OsExo70-F3 in pGADT7 (prey vector),
128 *Pii-1-CC*, *Pii-1-NB*, *Pii-1-LRR+CT, Pii-2-CC*, *Pii-2-NB* and *Pii-2-LRR+CT* in
129 pGBKT7 (bait vector) have been previously constructed (ref. 17). To construct
130 pGBKT7-Pii-2-CT and pGBKT7-Pii-2-CTC, *Pii-2-CT* and *Pii-2-CTC* fragments were
131 amplified by PCR using primer sets: KF825f/KF826r and KF827f/KF826r, respectively,
132 and inserted into pGBKT7 vector by utilizing *Eco*RI and *Bam*HI sites. To prepare bait
133 vectors of the *Pii-2-CT* mutant, *Pii-2-CT-m5*, the N-terminal half and the C-terminal
134 half of *Pii-2-CT* fragment were amplified by using primer sets (KF825f/KF831r and
135 KF830f/KF826r, respectively), and resulting PCR products were mixed and used as
136 DNA template for second PCR using a primer set (KF825f /KF826r) to amplify
137 *Pii-2-CT-m5* fragment. Similarly, to construct other pGBKT7-Pii-2-CT mutants, primer
138 sets (1$^{st}$ PCR : KF825f/KF833r for *Pii-2-CT-VA-N* and KF832f/KF826r for
139 *Pii-2-CT-VA-C*; KF825f/KF835r for *Pii-2-CT-PA-N* and KF834f/KF826r for
140 *Pii-2-CT-PA-C*; KF825f/KF837r for *Pii-2-CT-FA-N* and KF836f/KF826r for
141 *Pii-2-CT-FA-C*; KF825f/KF839r for *Pii-2-CT-GA-N* and KF838f/KF826r for
142 *Pii-2-CT-GA-C*; KF825f/KF841r for *Pii-2-CT-WA-N* and KF840f/KF826r for
143 *Pii-2-CT-WA-C*) were used for PCR to amplify the N- and C-terminal half fragments of
144 *Pii-2-CT* mutants. The N- and C-terminal half fragments of each *Pii-2-CT* mutants were
145 mixed and used as DNA template for second PCR using a primer set (KF825f /KF826r).

146 After the second PCR, the resulting 0.85 kb PCR products were inserted into pGBKT7
147 vector by utilizing *Eco*RI and *Bam*HI sites.

148 For preparing the expression vectors in rice, full-length coding sequences of *Pii-2*
149 was amplified from cDNA by PCR using primer set, KF828f/KF829r, and was
150 introduced into pCAMBIA1300S vector (Cambia, www.cambia.org) by utilizing *Kpn*I
151 and *Xba*I sites (pCAMBIA-Pii-2). To construct expression vector for Pii-2 mutant
152 (Pii-2-NOI-m5), two *Pii-2* fragments were amplified by using primer sets
153 (KF828f/KF831r and KF830f/KF829r, respectively), and resulting PCR products were
154 mixed and used as DNA template for the second PCR using a primer set (KF828f
155 /KF829r) to amplify *Pii-2-NOI-m5* fragment. The *Pii-2-NOI-m5* fragment was inserted
156 into pCAMBIA1300S vector (Cambia, www.cambia.org) by utilizing *Kpn*I and *Xba*I
157 sites (pCAMBIA-Pii-2-NOI-m5).

158 To prepare transgenic rice plants expressing wild-type *Pii-2* and mutant *Pii-2*
159 (Pii-2-NOI-m5), pCAMBIA-Pii-2 and pCAMBIA-Pii-2-NOI-m5 were introduced into
160 *Agrobacterium tumefaciens*, which were used for transformation of Hit5882 and
161 Hit13701 that are pii-2 deficient mutants of the rice cultivar Hitomebore (ref. 11).

162

163 **Yeast two-hybrid assay**

164 Y2H assay was performed as described previously (ref. 24). Ten-times dilution series
165 [OD600 = 3.0 (x1), 0.3 ($x10^{-1}$) and 0.03 ($x10^{-2}$)] of yeast cells were prepared and spotted
166 onto quadruple dropout medium (QDO); basal medium lacking Trp, Leu, Ade and His
167 but containing 5-Bromo-4-Chloro-3- indolyl a-D-galactopyranoside (X-a-gal)

168 (Clontech). To detect interactions, both QDO medium with or without 10 mM
169 3-amino-1,2,4-triazole (3AT) (Sigma) was used. Yeast cells were also spotted onto
170 double dropout medium (DDO); basal medium lacking Trp, Leu to test cell viability. To
171 check protein accumulation in yeast cells, yeast cells were propagated in liquid DDO at
172 30°C overnight. Forty mg yeast cells were collected and resuspended with 160 μl GTN
173 + DC buffer [10% glycerol, 25mM Tris-HCl (pH 7.5), 150mM NaCl, 1 mM DTT and 1
174 tablet of complete EDTA-free (Roche, Basel Switzerland)]. Then, 160 μl of 0.6 N
175 NaOH was added, mixed gently, and incubated at room temperature for 10 min. Next,
176 160 μl of gel sample buffer [40%(w/v) glycerol, 240 mM Tris-HCl pH 6.8, 8% (w/v)
177 SDS, 0.04% (w/v) bromophenol blue, 400 mM DTT] was added and incubated at 95 °C
178 for 5 min. After 20,000 x g centrifugation for 5 min, the supernatant was subjected to
179 SDS-PAGE. Proteins expressed from bait and prey vectors were immunologically
180 detected by using anti-Myc antibody (MBL, Nagoya, Japan) and anti-HA 3F10 (Roche),
181 respectively.
182
183 **Assays for fungal pathogenicity and gene expression**
184 Wild-type *M. oryzae* isolate Sasa2 (no AVR-Pii) is stored at Iwate Biotechnology
185 Research Center, and a transgenic *M. oryzae* strain (Sasa2+AVR-Pii-s) was previously
186 established as Sasa2(+22p:pex33) (ref. 17). Rice leaf blade spot inoculation with
187 conidial suspension ($5 \times 10^5$ conidia/ml) was performed as described previously (ref.
188 24). Disease lesions were photographed at 10 days after inoculation, and vertical length
189 of the lesions was measured. To check *Pii-2* expression in transgenic rice plants,

190 semi-quantitative RT-PCR was performed as described previously (ref.12). Messenger
191 RNA from *Pii-2* transgene but not from intrinsic gene was specifically detected by
192 using a primer set (5'-CTTTCGCGAGCTCGGTACCAACAATG-3' and
193 5'-ATCCACATCATTTTGTAACAATAG-3'). As a control, rice *Actin* was detected by
194 using a primer set (5'- CTGAAGAGCATCCTGTATTG -3' and 5'-
195 GAACCTTTCTGCTCCGATGG -3').
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294

295 **Contributions**

296 K.F. S.K. and R.T. initiated, conceived and coordinated the project; K.F. identified and
297 characterized NOI core motif in Pii-2; E.K. generated transgenic rice plants; K.F., Y.A.
298 and H.U. prepared plamid vectors and performed fungal infection assay and RT-PCR.
299 K.F. and K.I. performed yeast two hybrid assay. H.S., M.B., S.K. and R.T. supervised
300 the project; K.F., A.B., S.K., and R.T. wrote the manuscript

301

302 **Competing interests**

303 The authors declare no competing financial interests.

304

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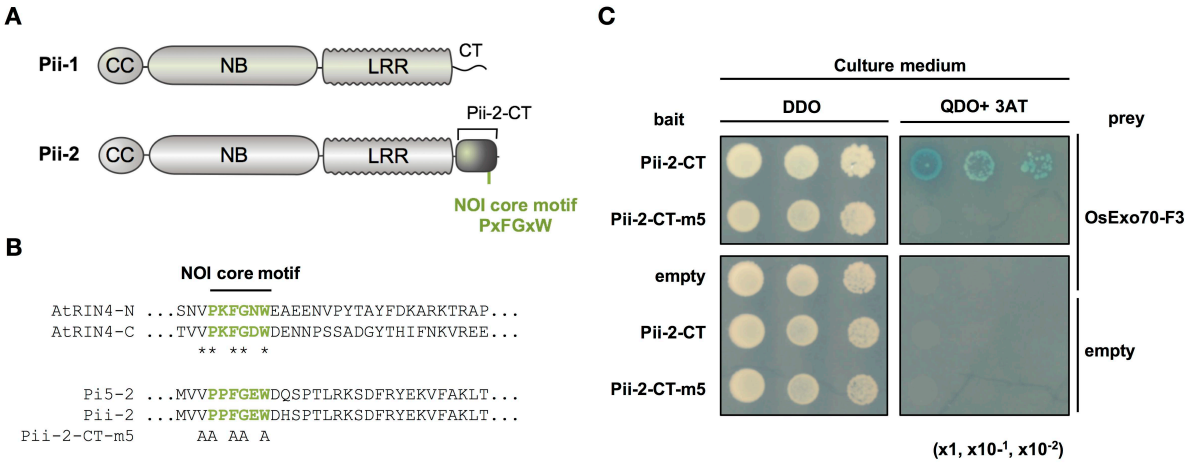


**Figure 1. OsExo70-F3 binds the NOI domain of Pii-2 C-terminal region (Pii-2-CT).**

(A) A schematic diagram of Pii-1 and Pii-2 NLRs. Coiled-coil (CC), nucleotide-binding (NB) and leucine rich repeat (LRR) domains are shown in light grey. OsExo70-F3 binds the C-terminus of the Pii-2 (Pii-2-CT) shown in dark grey (Pii-2-CT) that contains the core motif (PxFGxW) of NOI domain.
(B) Alignment of amino acid sequences around the NOI core motif of *Arabidopsis* RIN4, rice Pi5-2 and Pii-2 proteins. Residues of the NOI core motif are shown in green. Asterisks indicate conserved amino acids among *Arabidopsis* RIN4, Pi5-2 and Pii-2 proteins, which are substituted for Ala (A) in the Pii-2-CT-m5 mutant.
(C) Yeast two hybrid (Y2H) assay showing the interaction of OsExo70-F3 with the wild-type Pii-2-CT but not with the Pii-2-CT-m5 mutant. Protein interactions were tested in the yeast grown on Quadruple dropout (QDO) + 3AT medium ($Trp^-Leu^-Ade^-His^-$ X $\alpha$ $gal^+$ +10 mM 3AT : right panels). Viability of all transformed yeast cells were demonstrated by growing them on double dropout (DDO) medium ($Trp^-Leu^-$ : left panels). Yeast cells were spotted onto the media as tenfold dilution series (x1, $x10^{-1}$, $x10^{-2}$). Empty vectors (labeled as "empty") were used as negative controls.

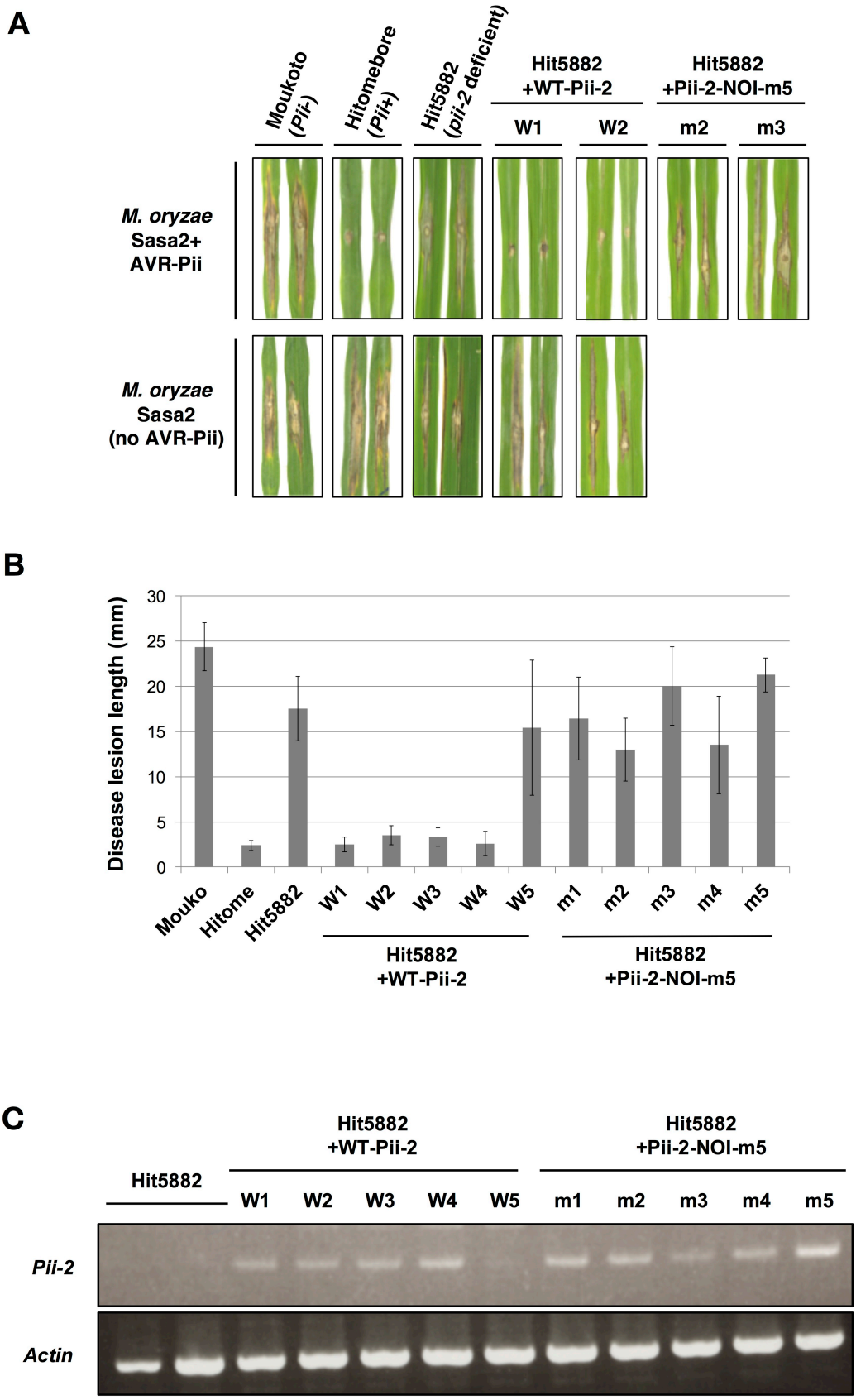
A
Moukoto (Pii-)
Hitomebore (Pii+)
Hit5882 (pii-2 deficient)
Hit5882 +WT-Pii-2
Hit5882 +Pii-2-NOI-m5
W1
W2
m2
m3
M. oryzae Sasa2+ AVR-Pii
M. oryzae Sasa2 (no AVR-Pii)
B
Disease lesion length (mm)
30
25
20
15
10
5
0
Mouko
Hitome
Hit5882
W1
W2
W3
W4
W5
m1
m2
m3
m4
m5
Hit5882 +WT-Pii-2
Hit5882 +Pii-2-NOI-m5
C
Hit5882
Hit5882 +WT-Pii-2
Hit5882 +Pii-2-NOI-m5
W1
W2
W3
W4
W5
m1
m2
m3
m4
m5
Pii-2
Actin

**Figure 2. *AVR-Pii* recognition of *Pii* requires Pii-2 NOI core motif.**

(A) Results of rice leaf blade spot inoculation assay of *M. oryzae*. Two *M. oryzae* isolates, with (transgenic Sasa2+AVR-Pii; top) or without (Sasa2; bottom) *AVR-Pii* were spot inoculated onto the leaves of rice lines Moukoto (Pii-), Hitomebore (Pii+), Hit5882, a *pii-2* Hitomebore mutant, and transgenic Hit5882 lines expressing a full-length cDNA of the wild-type *Pii-2* (Hit5882+WT-Pii-2) and those expressing the mutant version of *Pii-2* with m5 mutation in the NOI core motif (Hit5882+Pii-2-NOI-m5). Representative data of two line each of Hit5882+WT-Pii-2 (W1 and W2) and Hit5882+Pii-2-NOI-m5 (m2 and m3) are shown.
(B) Bar graphs showing disease lesion lengths after inoculation of an *M. oryzae* isolate Sasa2+AVR-Pii onto rice lines from panel A, labelled on the bottom. Here, five lines each from Hit5882+WT-Pii-2 (W1 to W5) and Hit5882+Pii-2-NOI-m5 (m1 to m5) were tested. The graph illustrates average lesion lengths and standard deviations from six inoculated spots per line.
(C) Expression of *Pii-2* transgenes in the tested rice plants. Messenger RNA from *Pii-2* transgene but not from intrinsic gene was specifically detected by semi-quantitative RT-PCR using a combination of primers derived from *Pii-2* gene and the binary vector sequences. Rice *Actin* was used as control.

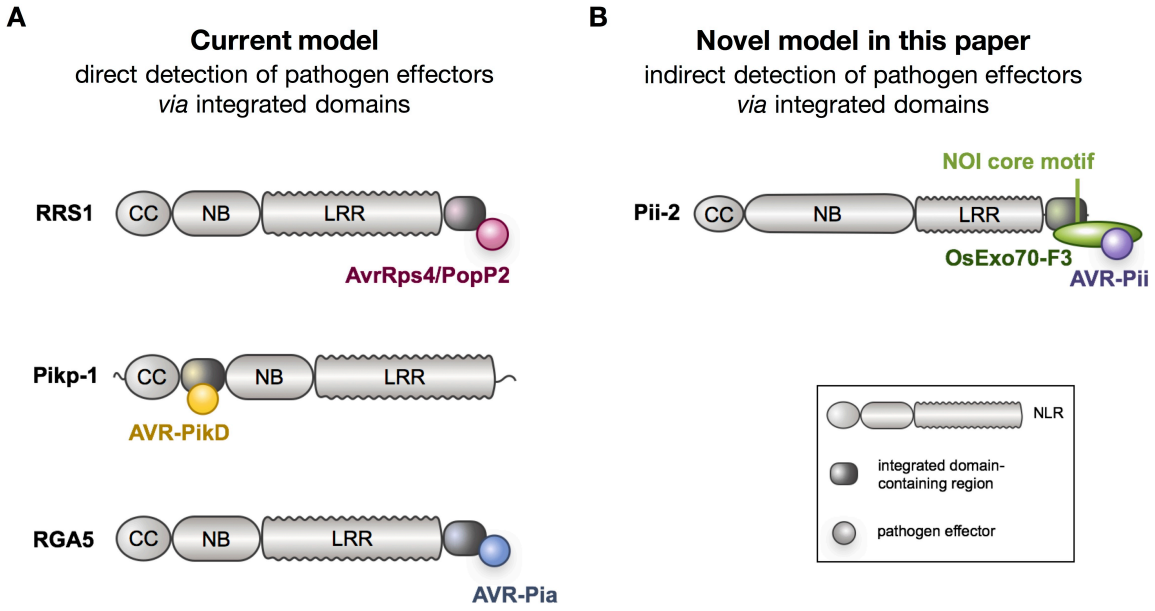


**Figure 3. A scheme showing possible roles of NLR-integrated domains in the effector recognition.**

(A) A widely accepted model of NLR-ID function. NLR directly interacts with pathogen effector *via* its integrated domain, and subsequently activates immune response. An *Arabidopsis* sensor NLR, RRS1 (top), has the WRKY domain at the C-terminus, and bacterial effectors AvrRps4/PopP2 directly bind to the RRS1 WRKY ID (ref. 5,6). The rice sensor NLRs, Pikp-1 (middle) and RGA5 (bottom), carry integrated HMA domain. Pathogen AVR effectors AVR-PikD and AVR-Pia, respectively, directly bind to their cognate HMA (ref. 7,8). In the current model (ref. 1,2), these sensor NLRs trigger ETI together with their NLR helpers.
(B) A novel model of ID function in indirect detection of pathogen effectors. Pii-2 is an NLR with an ID, NOI core motif (PxFGxW), residing in its C-terminal region. Pii-2-ID directly binds OsExo70-F3, which is necessary for AVR-Pii recognition. OsExo70-F3 also binds AVR-Pii. We propose that Pii-2 monitors OsExo70-F3, the status of which is altered upon binding of AVR-Pii. Thus, Pii-2 ID guards a host protein, OsExo70-F3, that is targeted by a pathogen effector, AVR-Pii.