Transcriptional insights into the pyramided resistance to rice bacterial blight

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The pyramiding of resistance (R) genes provides broad-spectrum and durable resistance to plant diseases. However, the genetic basis for bacterial blight (BB) resistance remains unclear. The BB R gene pyramided line IRBB54, which expresses xa5 and Xa21, possessed a higher level of resistance than both single R gene lines. Large-scale genotyping of genetic markers in this study revealed similar genetic backgrounds among the near-isogenic lines (NILs), suggesting that resistance in the resistant NILs was mainly conferred by the individual R genes or the interaction between them. Transcriptome analysis demonstrated that more than 50% of the differentially expressed genes (DEGs), and more than 70% of the differentially expressed functions, were shared between IRBB54 and IRBB5 or IRBB21. Most of the DEGs in the resistant NILs were downregulated and are predicted to function in cellular and biological process. The DEGs common among the resistant NILs mainly showed non-additive expression patterns and enrichment in stress-related pathways. The differential expression of agronomic trait-controlled genes in the resistant NILs, especially in IRBB54, indicated the existence of potential side-effects resulting from gene pyramiding. Our findings contribute to the understanding of R gene pyramiding, as well as its effects on targeted and non-targeted trait(s).

Rice (*Oryza sativa* L.) is the staple food of more than half of the world’s population. Bacterial blight (BB), caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), is one of the most serious rice diseases affecting major rice-growing regions globally. Host plant resistance is currently the most effective and economical way to control BB1. To date, approximately 40 BB-resistance (R) genes (*Xa* genes) have been identified in rice, 10 of which (*Xa1*, *Xa3/Xa26*, *Xa4*, *xa5*, *Xa10*, *Xa13*, *Xa21*, *Xa23*, 8, and *Xa27*) have been cloned. These R genes have been widely deployed in breeding programs, but some pathogen strains have overcome plant resistance conferred by a single R gene. For example, strains with mutated *avrXa4* overcame the resistance mediated by *Xa4* and *xa5* is ineffective against strains that possess the transcription activator-like (TAL) effector gene *phXo1*14.

The pyramiding of multiple genes into a single genotype is considered an effective way to develop offspring with elite performance in target traits. The typical application of gene pyramiding is to breed varieties with broad-spectrum and durable disease resistance; for example, the pyramided rice lines harboring the R genes *Xa4 + Xa21, xa5 + Xa21, Xa4 + xa5 + Xa21, xa5 + xa13 + Xa21*, or *xa5 + Xa4*14 have a wider spectrum or higher level of resistance than each R-gene line alone. Interestingly, the line with *xa5* and *Xa27* stacked had attenuated BB resistance as compared to the line harboring only *Xa27*17. Similarly, both *avrXa10*-dependent *Xa10* expression and *Xa10*-mediated resistance to *PXO99a* were partially suppressed in *xa5* and *Xa10* double homozygous plants16; in addition, *AvrXa23*-induced *Xa23* expression was abolished, and the level of *Xa23*-mediated resistance weakened, in *xa5* homozygous plants18.

Gene pyramiding is a time-consuming process and hence understanding the underlying molecular mechanisms is necessary to avoid invalid or antagonistic gene combinations. For this, near-isogenic-lines (NILs) with pyramids of the R genes, developed by multi-generations backcrossing, are valuable tools for studying the effects of single R genes and R gene combinations13,19; high-throughput sequencing is also a powerful tool that can be used to analyze the transcriptional effects of gene pyramiding, providing expression information on a genome-wide scale. In this study, we profiled the transcriptomes of two lines, each with a single R gene, and a
to answer the following questions: Are the effects of R genes accumulative or synergistic in the pyramided line? Can the accumulative effect of the induced genes explain the broad and durable resistance that is widely observed in pyramided lines? Finally, does the pyramiding strategy promote side effects, in addition to the advantages related to BB resistance?

Results

Validation of the resistant NILs. Genetic background can influence BB resistance, as was shown in the pyramided line with both R genes, to answer the following questions: Are the effects of R genes accumulative or synergistic in the pyramided line? Can the accumulative effect of the induced genes explain the broad and durable resistance that is widely observed in pyramided lines? Finally, does the pyramiding strategy promote side effects, in addition to the advantages related to BB resistance?

Validation of the resistant NILs. Genetic background can influence BB resistance, as was shown in the pyramided line with \( xa5 + xa13 + xa21 \)20. Through backcrossing, the R genes \( xa5, xa21, \) and \( xa5 + xa21 \) were introduced into the same genetic background of IR24 to develop the BB resistant NILs of IRBB5, IRBB21 and IRBB54. The introduction of R genes into the three resistant NILs was validated through linked molecular markers (Fig. 1a) and the relative expression of R genes in the resistant NILs, with respect to the recipient line IR24 was measured by qRT-PCR using the ubiquitin gene as an internal control21 (Fig. 1b). As many as 3,105 simple sequence repeats (SSRs), including forty-eight SSRs listed in the National Agricultural Standard of China (NY/T 1433–3014) and 3,057 randomly-selected SSRs from the japonica reference genome (irgsp1.0), were chosen as target SSRs to evaluate the similarity in the genetic background of the three resistant NILs. The Ampseq-SSR genotyping method, developed in our previous study22, was used to genotype the 3105 SSRs in the NILs. Through strict quality control of the raw reads (see methods), we found that the qualified reads for each sample were low, leading to a low coverage of the 3105 target SSRs. However, we then searched for comparable SSRs for the three groups of samples (IRBB5 vs. IR24, IRBB21 vs. IR24, and IRBB54 vs. IR24) following the criterions for valid SSR and comparable SSR (see methods), and identified 349, 235, and 332 comparable SSRs in sample groups IRBB5 vs. IR24, IRBB21 vs. IR24, and IRBB54 vs. IR24, respectively, which were more than the 48 SSRs adopted by the National Agricultural Standard of China to identify rice varieties (NY/T 1433–3014) (Fig. 1c). The genotypes of over 94% of the comparable SSR markers in the resistant NILs were identical to the IR24 donor (Fig. 1c, Supplementary Table S1), suggesting the similarity of the genetic backgrounds between the resistant NILs and IR24. The region linked to \( xa5 \) covered approximately seven megabase pairs in IRBB5 and IRBB54, as revealed by the peak of single nucleotide polymorphism (SNP) distribution of the expressed genes identified from the transcriptome profiles of the resistant NILs (Supplementary Fig. S1). The SNPs outside the R gene-linked regions were sparsely distributed, reinforcing the conclusion that the genetic backgrounds were similar among the three resistant NILs. Therefore, the three resistant NILs and the susceptible line IR24 formed an optimal system to compare the resistance mechanisms from the single R genes and R gene combinations.

Figure 1. Validation of the three resistant NILs, IRBB5, IRBB21, and IRBB54. (a) Validation of \( Xa21 \) and \( xa5 \) in the genomes of the resistant NILs using molecular markers. Full-length gels are presented in Supplemental Fig. 4. (b) The relative expression level of \( Xa21 \) and \( xa5 \) in the resistant NILs identified by RNA-seq and qPCR. IR24 was the reference sample and rice ubiquitin gene was the internal control. (c) Genetic background analysis of the three resistant NILs by SSR markers. (d) Lesion lengths of the three resistant NILs after inoculation with eight \( Xoo \) strains. P1–P10: \( Xoo \) strains. Represent the outliers. * Represents a significance level of \( p < 0.01 \) in IRBB5, IRBB21, and IRBB54 with respect to IR24 (Student’s t test, two-tailed). # Represents a significance level of \( p < 0.01 \) in IRBB5 and IRBB21 with respect to IRBB54 (Student’s t test, two-tailed).
Broader and stronger resistance of the pyramided line. The ten commonly used Xoo strains from the Philippines were selected for resistance evaluation. Because the morphology of the P5 and P9 strains was abnormal, only the remaining eight strains were used. Among them, P1, P2, and P6 have traditionally been used for the functional identification of Xa4\(^5\), xa5\(^4\), and Xa21\(^6\), respectively. IR24 was susceptible to all eight tested Xoo strains and developed the longest lesions with the P8 strain (Fig. 1d, Supplementary Fig. S2), indicating that these tested strains were virulent and effective for resistance testing. IRBB5 was resistant to six strains, but only moderately resistant to strains P4 and P6 (Fig. 1d, Supplementary Fig. S2) which harbor the pthXo1 effector\(^24\) and therefore, partially overcame the resistance conveyed by xa5\(^4\).

Xa21 was the first BB resistance gene to be cloned and has been found to confer resistance to a wide spectrum of Xoo strains\(^9\). In the present study, however, IRBB21 exhibited moderate resistance to P2, P3, P4, and P6; moderate susceptibility to P1, P7, and P10; and full susceptibility to the P8 strain, with a mean lesion length of approximately 13 cm (Fig. 1d, Supplementary Fig. S2). Compared to the two lines with a single R gene, the pyramided line IRBB54 showed a higher level and wider spectrum of resistance to the eight Xoo strains (Fig. 1d, Supplementary Fig. S2), which is consistent with a previous report\(^15\).

DEG pyramiding and BB resistance. As shown by the qRT-PCR analysis (Fig. 1b) and previous reports\(^6\)^\(^25\), both xa5 and Xa21 were constitutively expressed, suggesting that their resistance mechanisms could be detected before Xoo infection. In the present study, the transcriptomes of IRBB5, IRBB21, IRBB54, and the susceptible line IR24 were analyzed by RNA-seq, before Xoo infection. Through quality control for all raw reads (see methods), the clean reads that mapped to the reference genome for each sample ranged from 2,472,094 to 4,783,943. A total of 55,779 genes were covered. Only 2.3–9.6% of the total mapped reads were multiple alignments. This indicated that a reliable result could be achieved based on these data.

The RNA-seq data also indicated that Xa21 and xa5 were constitutively expressed (Fig. 1b). Compared to the susceptible line IR24, 2,367, 2,412, and 3,596 DEGs were identified from IRBB21, IRBB5, and IRBB54, respectively, before Xoo infection. The data also showed that the R genes tended to suppress rather than upregulate the expression of their downstream genes (Fig. 2a). Xa21 and xa5 regulated almost the same number of DEGs (2,367 and 2,412, respectively), of which 1,136 (~50%) were common between them, indicating the functional redundancy and independence of the two R genes (Fig. 2b).

The pyramiding of Xa21 and xa5 induced substantially more DEGs than either of the two R genes alone (Fig. 2a), providing a larger transcriptional pool with which to resist various Xoo strains. Interestingly, 1,420 (60%) and 1,390 (58%) of the DEGs induced by xa5 and Xa21, respectively, were also induced in the pyramided line, including 543 xa5-specific and 573 Xa21-specific DEGs (Fig. 2b). These observations indicated that the transcriptional mechanisms adopted by the single R genes were partially combined in the pyramided line. Except for the DEGs common among the NILs, 1,633 (45%) of the 3,596 DEGs were specific to the pyramided line (Fig. 2b), suggesting that new BB resistance mechanisms were acquired through interactions between xa5 and Xa21.

DEF pyramiding and BB resistance. The GO functions of the upregulated and downregulated DEGs were analyzed separately\(^26\). For convenience, the up-/downregulated DEGs were defined as up-/down-DEGs and the enriched GO functions of up-/down-DEGs were defined as up-/down-DEFs. The up- and down-DEGs were assigned to three GO classes: biological process, cellular component, and molecular function. The up-DEFs and down-DEFs in the three resistant NILs can be found as Supplementary Table S2. As shown in Fig. 2a, the number of down-DEGs in the three resistant NILs was higher than the number of up-DEGs. We found that the number of down-DEGs in the three resistant NILs was also higher than the number of up-DEFs, e.g., 33 down-DEFs vs. 9 up-DEFs in IRBB5, and 32 down-DEFs vs. 24 up-DEFs in both IRBB21 and IRBB54 (Fig. 3a, Supplementary Table S2). Interestingly, we found that 100% (9 vs. 9) of the up-DEFs and 90.6% (29 vs. 32) of the down-DEFs in IRBB5 overlapped with the up-DEGs and down-DEGs in IRBB21. Moreover, 88.9% (8 vs. 9) and 70.8% (17 vs. 24) of the up-DEGs and down-DEGs in IRBB54 overlapped with the up-DEGs and down-DEGs in IRBB21.
vs. 24) of the up-DEFs in IRBB5 and IRBB21, respectively, overlapped with the 24 up-DEFs detected in IRBB54 (Fig. 3b, Supplementary Table S2). The down-DEFs in the pyramided line had an 81.8% (27 vs. 33) and 84.4% (27 vs. 32) overlap with the down-DEFs in IRBB5 and IRBB21, respectively (Fig. 3b, Supplementary Table S2). These results suggested that the Xa21 gene adopted most of the BB resistance characteristics derived from the xa5 gene and most of the BB resistance characteristics derived from xa5 and Xa21 were transmitted to, and pyramided in, IRBB54. On the other hand, eight novel up-DEFs and 3 down-DEFs were observed in the pyramided line, suggesting that novel BB resistance mechanisms may be generated through interaction between xa5 and Xa21 (Fig. 3b, Supplementary Table S2).

Additionally, we noticed that 8 up-DEFs and 25 down-DEFs were common among the three resistant NILs (Fig. 3b). Only one up-DEF (structural molecule activity) and two down-DEFs (kinase activity and nucleotide binding) were assigned to the molecular function GO class; most of the common DEFs were assigned to the other two GO classes, indicating that the R genes mainly regulate genes associated with cellular components and biological processes (Fig. 3c).

Expression patterns and functions of common DEGs. The pyramided line was superior to either of its donor parents for BB resistance. Therefore, the gene expression pattern of the pyramided line is of interest. DEGs that showed differential expression not only between resistant and susceptible lines, but also between any two resistant NILs, were used to determine the gene expression pattern in the pyramiding line (see methods). Surprisingly, an additive expression pattern, which is often used to explain heterosis in hybrids27, was not observed in the pyramided line (Table 1). Instead, eight novel up-DEFs and 3 down-DEFs were observed in the pyramided line, suggesting that novel BB resistance mechanisms may be generated through interaction between xa5 and Xa21 (Fig. 3b, Supplementary Table S2).

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Three pathways were enriched in the common DEGs, including phenylpropanoid biosynthesis, S-adenosyl-L-methionine (SAM) cycle, and spermidine biosynthesis (Fig. 4b,c). The expression levels of these common DEGs in the enriched pathways were differentially regulated in the three resistant NILs (Fig. 4b,c), suggesting that they might play different roles in different R gene-mediated resistance responses.

**Effects of gene pyramiding on agronomic traits.** Constitutive expression of *xa5* and *Xa21* may have deleterious effects on host growth, especially in the pyramided line that possesses a large number of DEGs. Among the 2,367, 2,412, and 3,596 DEGs in IRBB5, IRBB21, and IRBB54, 365 (98 upregulated and 267 downregulated) genes, respectively, had the R-motif typically found in the 5′ leader sequence of genes with altered translational efficiency during plant immune responses28 (Dataset 1). When we investigated further the GO functions of all these up-DEGs and down-DEGs, we found that in the three resistant NILs, the down-DEGs were significantly enriched in GO terms, e.g., 14 in IRBB5, 17 in IRBB21, and 15 in IRBB54, whereas only the up-DEGs in IRBB21 were enriched in 3 GO terms. The common enriched GO terms of the three down-DEGs lists were belong to the biological process GO class (Supplementary Table S4). These results suggested that the disease-resistance genes tend to suppress the function of genes that regulate basic biological process.

To further analyze potential side-effects of disease resistance on the important agronomic traits of hosts, we analyzed the expression levels of 223 curated agronomic trait-controlled genes (downloaded from http://www.ricedata.cn/gene/) in the three resistant NILs (Supplementary Table S5). These 223 genes included 33 genes that function in plant architecture, 50 genes for leaf color and leaf shape, 5 genes for leaf aging and necrosis, 2 genes for leaf inclination, 5 genes for flowering phase, 9 genes for pollen development, 19 genes for floral organ development, 21 genes for heading date, 4 genes for spike sprouting, 14 genes for spike shape, 2 genes for cold tolerance of seed germination, 5 genes for seed shattering, 22 genes for grain shape and grain weight, 2 genes for grain number per panicle, and 8 genes for grain quality. We found that 23, 17, and 34 genes that controlled plant architecture, leaf color and leaf shape, flowering, floral development, male sterility, heading date, grain number, seed shattering, low-temperature germinability, grain quality, and fragrance were differentially expressed in IRBB5, IRBB21 and IRBB54, respectively (Table 2). However, the expression of 73.9% (17 vs. 23), 76.5% (13 vs. 17), and 85.3% (29 vs. 34) of the DEGs was downregulated in IRBB5, IRBB21 and IRBB54, respectively (Table 2). More importantly, only 3 (13.0%), 3 (17.6%) and 5 (14.7%) of the DEGs in IRBB5, IRBB21 and IRBB54, respectively, had R-motifs in their 5′ leader sequence, indicating that side-effects from gene pyramiding on the agronomic traits of plants are minor (Table 2).

**Discussion**

The exploration of gene function is at the core of the post-genomic era. NILs, which ideally have identical genetic backgrounds except for the target gene, provide an ideal system to study the function of a gene of interest, where any functional or phenotypic difference between two NILs can be attributed to the target gene.

A simple way to create NILs is backcrossing, followed by selection toward the target phenotype. Indeed, backcrossing may be the only option for creating NILs when the target gene has not been cloned. For genomic regions unlinked to the target gene, backcrossing can rapidly eliminate different genetic backgrounds; however, backcrossing is less efficient for genomic regions linked to target genes. If the target gene has been cloned, transgene technology can be a better option to create NILs with more similar genetic backgrounds, helping to avoid linkage drag in backcrossing. For example, our previous study revealed that the introduction of the *Xa21* gene into rice plants by transgene technology resulted in substantially fewer DEGs than by backcrossing29. However, the integration site of a target gene is uncontrollable in transgene technology, complicating analysis of target gene function. However, the recent advent of the CRISPR/Cas9 system has facilitated the accurate and high-throughput editing of target genes *in situ*, thus avoiding complications associated with random integration30,31; for example, a herbicide-resistant rice variety was developed recently by editing a single base32. Nevertheless, tissue culture procedures are necessary for both the transgene technique and CRISPR/Cas9 system and random mutations in
the host genome are common during tissue culture, which hampers the analysis of target gene function. Under such scenarios, traditional backcrossing can be used after the transgene or gene editing procedure to eliminate the mutations among NILs.

The inoculation experiments showed that the rice line harboring both the *Xa21* and *xa5* genes exhibited a stronger level of resistance, and wider resistance spectrum, to *Xoo* strains than the lines with a single R gene, suggesting that there is a positive interaction between the two R genes. However, the combination of *xa5* with *Xa27*, *Xa10*, or *Xa23* failed to promote BB resistance17,18, highlighting the need to understand the underlying molecular mechanisms of R gene pyramiding so as to predetermine its validity. *AvrXa27*, *avrXa10*, and *avrXa23*, the bacterial avirulence (*avr*) genes of *Xa27*, *Xa10*, and *Xa23*, respectively, are transcription activator-like (TAL) effectors7,12,33. The dominant *Xa5* gene, coding for the basal transcription factor TFIIA gamma subunit, is a nuclear target of several bacterial TAL effectors18, suggesting that *Xa5* might play a role in the resistance expression of *Xa27*, *Xa10*, and *Xa23*. When *Xa5* is replaced with a recessive *xa5*, *Xa27*, *Xa10*, and *Xa23* may not be activated in the pyramided lines of *xa5*+*Xa27/Xa10/Xa23*, resulting in the invalidation of the R gene combinations. Unlike *Xa27*, *Xa10*, and *Xa23*, the expression of *Xa21* is constitutive25 (Fig. 1b) and independent of *Xa5*. Therefore, it is not surprising to observe that the DEGs and DEFs from *xa5* and *Xa21* were pyramided in IRBB54 (Figs 2b and 3b), which suggested the pyramiding of resistance mechanisms from the two R genes and subsequent enhanced BB resistance in the pyramided line, which was observed in this (Fig. 1d) and previous studies15. As such, the

![Figure 4. Functional enrichment of common DEGs. (a) Enriched GO terms of the common DEGs. P, biological process; C, cellular component; F, molecular function. (b) Expression patterns of detected DEGs that are involved in phenylpropanoid biosynthesis, the S-adenosyl-L-methionine (SAM) cycle, and spermidine biosynthesis. (c) Reaction step in the biosynthesis pathway in which each of the detected DEGs in (b) is involved.](image-url)
| Trait                        | Gene symbol          | MSU_Locus          | Log2Fold change | IRBB5 vs. IR24 | IRBB21 vs. IR24 | IRBB54 vs. IR24 |
|-----------------------------|----------------------|--------------------|-----------------|----------------|----------------|-----------------|
| **Plant architecture**      | d61; OsBR11          | LOC_Os01g52050     | −1.09138        |                |                |                 |
|                            | d35; OsKO2; OsKOL2;  | LOC_Os06g37364     | 5.19089         | 3.82001        |                |                 |
|                            | OsKO53               |                    |                 |                |                |                 |
|                            | d18; OsGA3ox2        | LOC_Os01g08220     | −1.15146        |                |                |                 |
|                            | d505Pase             | LOC_Os02g27620*    | −1.88278        | −2.45244       |                |                 |
|                            | D53                  | LOC_Os11g01330     |                | −1.32443       |                |                 |
|                            | sdn; Osmr156h        | LOC_Os06g44034     | −2.62083        | −2.4453        | −2.57991       |                 |
|                            | DGL1                 | LOC_Os01g49000     | −1.03082        | −2.39966       | −1.55808       |                 |
|                            | OsDWARF4; CYP90B2    | LOC_Os03g12660     | 1.9424          | 1.60517        |                |                 |
| **Leaf color and leaf shape** | WSL12; OsNDPK2      | LOC_Os12g36194     | −2.45442        | −2.23626       | −3.42447       |                 |
|                            | WSP1                 | LOC_Os04g51280     |                | −1.23409       |                |                 |
|                            | ASL2; RPL21c         | LOC_Os02g15900     | 1.04275         | 1.06502        |                |                 |
|                            | Se5; OsHY1; OsHO1;  | LOC_Os06g44034     | −2.62083        | −2.4453        | −2.57991       |                 |
|                            | yg2; grc1            | LOC_Os06g00080     | −1.19716        |                |                |                 |
|                            | YL1                  | LOC_Os01g12710     | −1.31067        | −1.54862       | −1.24634       |                 |
|                            | BGL11(t)             | LOC_Os06g45194     | −1.50762        | −1.4619        | −2.24912       |                 |
|                            | NOL                  | LOC_Os09g21250     | −1.1087         | −2.32767       | −2.74288       |                 |
|                            | NAL9; YL1; ClpP      | LOC_Os03g29810     |                | −1.47524       |                |                 |
|                            | SPL28                | LOC_Os01g5070      | −2.01764        | −2.6691        |                |                 |
|                            | OsHdA4d; Sp7         | LOC_Os05g45410     | −1.39258        | −1.2356        |                |                 |
|                            | sp5; SFB3; OsSLS      | LOC_Os07g10390     | −1.0955         |                |                |                 |
|                            | SPL3; OsEDR1; OsACDR1; OsMAPRK1 | LOC_Os03g06410 | −1.55476 | −1.37346 |                 |                 |
|                            | SRL2; AVR; NRL2      | LOC_Os10g40960     | −1.48141        |                |                |                 |
|                            | SLR1                  | LOC_Os04g43170     | 3.65825         | 1.94451        |                |                 |
| **Leaf aging and necrosis** | OsNaPRT1; LTS1       | LOC_Os03g62110     | −1.93727        |                |                |                 |
|                            | NLS1                 | LOC_Os11g13480*    |                | −1.47394       |                |                 |
| **Flowering phase**         | OsFKF1               | LOC_Os11g34460*    | −1.3657         |                |                |                 |
|                            | OsCO3                | LOC_Os09g066464*   | −1.29873        |                |                |                 |
| **Pollen sterility**        | COX11                | LOC_Os02g09940     | −1.14302        |                |                |                 |
|                            | UbL404               | LOC_Os04g31031     | 2.70476         |                |                |                 |
| **Floral organ development**| FON1                 | LOC_Os06g50340     | 2.48489         |                |                |                 |
|                            | OsMADS1; LHS1; AFO   | LOC_Os03g11614*    | −2.90314        | −3.40898       | −3.08375       |                 |
| **Heading date**            | SDG272; lip1; OsSET3.4 | LOC_Os09g13740 | −2.66602       | −1.02506       |                |                 |
|                            | Hd17; E7; OsELF3; OsELF3-1; OsELF3-1; Hid-q | LOC_Os06g50506 | −1.02506 |                |                |                 |
|                            | Hdb; CK2α            | LOC_Os03g55389*    | −1.39385        |                |                |                 |
|                            | Hd16; CK1; EL1       | LOC_Os03g57940     | −1.28229        |                |                |                 |
| **Spike sprouting**         | B3-OsLCY; zebra524   | LOC_Os02g08750     | −1.2212         |                |                |                 |
|                            | OsPDS                | LOC_Os03g08570     | −1.32846        |                |                |                 |
| **Spike shape**             | LF; EP3              | LOC_Os02g15950     | −1.37519        |                |                |                 |
| **Seed shattering**         | qSH1                 | LOC_Os01g62920*    | 1.4227          |                |                |                 |
| **Grain shape and grain weight** | SRS5; TID1         | LOC_Os11g14220     | −1.20487        |                |                |                 |
|                            | D2; CYP90D2; smgl1   | LOC_Os01g10040     | 1.84531         |                |                |                 |
|                            | G55                  | LOC_Os05g06660     | −1.90103        | −2.62763       |                |                 |
| **Grain number per panicle**| Gn1a; OsCKX2        | LOC_Os01g10110     | −1.3632         |                |                |                 |
| **Fragrance**               | BADH2 (fgr)          | LOC_Os08g32870     | 1.03846         | 1.67364        |                |                 |
| **Low-temperature germinability** | qL13G3-1          | LOC_Os03g01320     | −1.40969        | 1.02667        |                |                 |

Table 2. Differentially expressed genes related to agricultural traits. Pleiotropic genes list only one trait they control. Genes that have the R-motif within their 5′ leader sequence, a typical motif of genes with altered translation during plant immune responses, were marked with *.
pyramiding of independent R genes is expected to be effective; alternatively, a case-by-case analysis may be needed to predict the changes to resistance mechanisms for pyramiding of inter-dependent R genes.

At the transcriptional level, protein processes; they were not directly related to stress, suggesting that the R genes tended to suppress basic energy and normal growth, especially in the pyramided line that exhibited a large number of DEGs. When we performed plant, which is an energy-consuming BB resistance tactic. Therefore, these genes might have deleterious effects and using marker-assisted selection toward plants with both Xa21 genes15.

Genetic background analysis. Genomic DNA was isolated from fresh rice leaf tissue using the cetyltrimethyl ammonium bromide protocol. The integration of Xa21 and xa5 genes in the resistant NILs was validated by polymerase chain reaction (PCR) based on molecular markers U1 and I2, and xa5/XhoIF and xa5/ XhoIR, respectively (Supplementary Table S6). The genetic background analysis of the three resistant NILs with respect to susceptible IR24 were performed based on SNPs derived from transcriptome data and SSRs derived from the AmpSeq-SSR genotyping data 22. For SNP genotyping, all the sequence reads were first aligned with the japonica reference genome (irgsp1.0) with Bowtie2 (version 2.1.0) and effective SNPs were identified using SAMtools mpileup (version 1.2) and BCFTools (version 1.3.1) with default parameters. To achieve high accuracy in SNP calling, only consistent SNP sites between the two replicates of each rice line were kept and potential differences in genetic backgrounds between each NILs and IR24 were estimated based on these highly reliable SNPs. AmpSeq-SSR genotyping, which combined super multiplex-PCR and high-throughput sequencing, was used to calculate the similarity in the genetic backgrounds of the resistant NILs 22. Details for AmpSeq-SSR genotyping can be seen in the authors’ previous report22. Libraries for AmpSeq-SSR genotyping were constructed according to the user guide for the Ion AmpliSeq™ Library Kit 2.0 (CatNo. 4475345, Thermo Fisher Scientific, Waltham, MA, USA). 3105 SSRs, including forty-eight SSRs that are listed in the National Agricultural Standard
of China (NY/T 1433–3014), and 3057 randomly selected SSRs from the *Japonica* reference genome (igsp1.0) were chosen as target SSRs. The primers for target SSRs were designed at https://ampliseq.com/ and synthesized by Thermo Company, USA. The full list of primers has been reported previously22. All primers were pooled and 14 PCR cycles were performed for DNA amplification. The resulting libraries were sequenced on the Ion S5™ next-generation sequencing system (Cat. No. A27212, Thermo Fisher Scientific, Waltham, MA, USA) using single-end sequencing with a length of 300bp. Strict quality control was conducted for the raw reads of each sample. All reads shorter than 100bp and that could not be mapped to the targeted regions were discarded. Moreover, only SSRs with a coverage of at least 20 reads and a stutter ratio no greater than 0.5 were regarded as valid SSRs22. The genotype represented by the most number of reads is recorded as the major genotype of the SSR locus and the stutter ratio of the SSR locus is the ratio between the number of reads of the second genotype and major genotype. Based on the results of SSR genotyping, all sites consistent between IR24 and each NIL were recorded as comparable SSRs and used to compare genetic backgrounds.

**Xoo cultivation, inoculation, and analysis of resistance level.** Eight representative *Xoo* strains from the Philippines, including P1 (PXO61), P2 (PXO86), P3 (PXO79), P4 (PXO71), P6 (PXO99), P7 (PXO145), P8 (PXO280), and P10 (PXO341) were used in this study. Each *Xoo* strain was suspended in sterile water at a concentration of 10^8 cells/ml and inoculated at similar positions on three to five leaves using the leaf clipping method47 at the maximum tillering stage. Lesion lengths of 10 inoculated leaves from each tested rice line were measured 15 days after inoculation. Lesion lengths of ≤3 cm, 3–6 cm, 6–10 cm, and ≥10 cm were respectively determined as resistant (R), moderately resistant (MR), moderately susceptible (MS), and susceptible (S) based on the standard disease rating system for lesion length43.

**Library preparation and high-throughput sequencing.** Ten leaves were randomly harvested from 10 individuals of each rice line and pooled for RNA extraction. Total RNA was isolated using TRIZol reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer’s instructions. The RNA integrity number was evaluated with an Agilent® 2100 Bioanalyzer® instrument. Only RNA with an integrity number >7 was used for library construction. mRNA was purified from 20 μg of total RNA using the NEB next poly(A) mRNA magnetic isolation module (Cat. No. E7490; New England Biolabs, Ipswich, MA, UK). Approximately 100 ng of mRNA was used to construct RNA-seq libraries using the Ion Total RNA-Seq Kit v2 (Cat. No. 4479789, Thermo Fisher Scientific, Waltham, MA, USA) following the manual. Each sample performed two biological replicates. The yield and size distribution of the libraries were assessed with the Agilent® 2100 Bioanalyzer® and Agilent® High Sensitivity DNA Kit (Cat. No. 5067-4626; Agilent Technologies, Santa Clara, CA, USA). Sequencing chips were prepared on the Ion Chef™ system and sequencing was carried out on the Ion S5™ next-generation sequencing system (Cat. No. A27212, Thermo Fisher Scientific, Waltham, MA, USA).

**Differential expression analysis.** Quality check was conducted on all raw data. Reads shorter than 50bp, with adapter sequences, or with poly-N sequences were discarded. The remaining reads were mapped to the rice reference genome (MSU 7.0) using TopHat (version 2.0.13). Cufflinks (version 2.0.2) was used to assemble the mapped reads with default parameters and estimate the expression of each transcript49. The number of qualified reads for each gene was normalized to TPM (number of transcripts per million qualified reads), which was then used as the digital gene expression abundance of the gene. The Benjamini & Hochberg method was used to adjust the P-value for multiple tests50. Significant DEGs across two samples were determined with the P value cut-off of less than 0.05 and an absolute value of log2 fold change ≥1. DEGs that showed differential expression not only between resistant and susceptible lines, but also between any two resistant NILs, were used to determine gene expression patterns in the pyramided line.

**R-motif analysis.** R-motifs within 5’ leader sequences of DEGs were scanned by the online FIMO tool, with default settings, in the MEME suits51. The R-motif frequency matrix was provided by Xu28.

**Quantitative reverse-transcription (qRT-) PCR.** Two micrograms of total RNA was extracted for first-strand cDNA synthesis in a 20-μL reaction volume using M-MLV reverse transcriptase (Promega) and oligo (dT) 18 primer according to the manufacturer’s protocol. The reaction mixture contained 0.3 μL cDNA, 0.2 μM primers (Supplementary Table S4), 10 μL TranStart® TipTop Green qPCR SupMix reagent, and 0.4 μL ROX as a passive reference dye (Cat. No. AQ141; TransGen Biotech. China). The mixture was loaded on the Applied Biosystems StepOne™ Real-Time PCR machine for real-time PCR detection using a procedure of 30 s at 95 °C, 40 cycles of 5 s at 95 °C and 30 s at 60 °C, followed by melting analysis. The relative expression levels of *Xa21* and *xa5* were analyzed by qPCR using IR24 as a reference sample and the rice *ubiquitin* gene as the internal control gene44. The primers for the *ubiquitin* gene were synthesized based on a previous study41. The 2^(-ΔΔCT) method was used to estimate the relative expression changes of target genes44. Three biological replicates were included in this experiment. The primers for qPCR analysis are listed in Supplementary Table S6.

**Gene Ontology and pathway enrichment analysis.** Gene Ontology were assigned to DEGs using the bio tool agrIGOV2 v2.0.14. Plant GoSlim was selected for GO enrichment analysis. A Hypergeometric test was used to calculate the enrichment of GO terms26 and the GO terms with an FDR less than 0.05 after multi-test adjustments by the Yekutieli method (FDR under dependency) were considered significantly enriched44. Genes were associated with metabolic pathways using the RiceCyc pathway database (version 3.3, http://pathway.gramene.org/ricecyc.html). Pathways with P < 0.05 were considered enriched.
Data availability. The datasets generated during the current study are available in the GenBank repository https://trace.ncbi.nlm.nih.gov/Traces/sra_sub/sub.cgi?acc=SRP108493&focus=SRP108493&from=submission&action=show:STUDY. The datasets will be publicly available upon acceptance of the manuscript.

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Author Contributions
L.F.G., W.X.Z.H. and H.P. designed the experiments, Z.W.F. performed the transcriptome data analysis and the genetic background analysis, J.F.Z.H. performed the sequencing experiments, and L.L. and L.L. helped with the R-motif analysis of DEGs and pathway enrichment analysis. T.T.L. and L.H.C.H. measured the lesion length. L.L.L. performed the gene expression validation. L.F.G. wrote the manuscript. H.P. and W.X.Z.H. revised the manuscript.

Additional Information
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