Gene pyramiding enhances durable blast disease resistance in rice

Shuichi Fukuoka1, Norikuni Saka2*, Yuko Mizukami2†, Hironori Koga3, Utako Yamanouchi1, Yosuke Yoshioka4††, Nagao Hayashi1, Kaworu Ebana1, Ritsuko Mizobuchi1 & Masahiro Yano1‡‡

1National Institute of Agrobiological Sciences, Kannondai 2-1-2, Tsukuba, Ibaraki 305-8602, Japan, 2Aichi Agricultural Research Center, Mountainous Region Institute, Inahasi, Toyota, Aichi 441-2513, Japan, 3Faculty of Bioresources and Environmental Sciences, Ishikawa Prefectural University, Suematsu 1308, Nonoichi, Ishikawa 921-8836, Japan, 4NARO Institute of Vegetable and Tea Science, 360 Kusawa, Ano, Tsu, Mie 514-2392, Japan.

Effective control of blast, a devastating fungal disease of rice, would increase and stabilize worldwide food production. Resistance mediated by quantitative trait loci (QTLs), which usually have smaller individual effects than R-genes but confer broad-spectrum or non-race-specific resistance, is a promising alternative to less durable race-specific resistance for crop improvement, yet evidence that validates the impact of QTL combinations (pyramids) on the durability of plant disease resistance has been lacking. Here, we developed near-isogenic experimental lines representing all possible combinations of four QTL alleles from a durably resistant cultivar. These lines enabled us to evaluate the QTLs singly and in combination in a homogeneous genetic background. We present evidence that pyramiding QTL alleles, each controlling a different response to M. oryzae, confers strong, non-race-specific, environmentally stable resistance to blast disease. Our results suggest that this robust defence system provides durable resistance, thus avoiding an evolutionary "arms race" between a crop and its pathogen.

Improving disease resistance in crops is crucial for stable food production. Although the use of race-specific resistance genes (R-genes) is a major strategy for disease control, these genes are vulnerable to counter-evolution of pathogens. New resistance genes are then needed, thus continuing a cycle referred to as an evolutionary "arms race" between crops and pathogens1–3. Quantitative trait loci (QTLs), which usually have smaller individual effects than R-genes but confer broad-spectrum or non-race-specific resistance, can contribute to durable disease resistance (DR)3–5.

DR for blast, a devastating disease of rice (Oryza sativa L.) caused by the fungal pathogen Magnaporthe oryzae, is controlled by multiple genetic loci4,5. Despite the identification of several chromosomal regions for QTLs associated with resistance to M. oryzae4–9, most of these QTLs have not been characterized for their spectrum of resistance or for their combined effects. Additionally, previous studies have used materials with heterogeneous genetic backgrounds, in which the presence of variation makes it difficult to assess the effects of the resistance alleles.

One type of DR against M. oryzae is found in the durably resistant cultivar Owarihatamochi (OW) and is controlled by four QTLs: pi21, Pi34, qBR4-2, and qBR12-1. pi21 is a loss-of-function mutation of a negative regulator of plant defence10, and the candidate genes for Pi34 encode previously uncharacterized proteins11. qBR4-2 is a complex genetic locus including three tightly linked loci: qBR4-2a, qBR4-2b, and qBR4-2c. The chromosomal regions for qBR4-2 and qBR12-1 have each been located to R-gene clusters14, P16, which is allelic to qBR4-2b, encodes a nucleotide-binding site–leucine-rich repeat (NBS–LRR) protein whose transcript expression level is associated with the level of resistance13. These data suggest that multiple biological mechanisms contribute to this type of DR, and that it would be worthwhile to examine the effects of pyramiding the component QTLs.

In the present study, we separately introgressed the resistance alleles at each of these four loci into a susceptible and well-characterized genotype, Aichiasahi (AA). Then we crossed these lines and their progeny to produce a total of fifteen genotypes containing one to four of the resistance alleles to test all possible gene combinations within a homogeneous genetic background under multiple field environments and in a glasshouse pathogen isolate challenge.

Results

Resistance to M. oryzae in four near-single-QTL near-isogenic lines. The chromosomal regions contained within four near-isogenic lines (NILs) for pi21, Pi34, qBR4-2, and qBR12-1 in the genetic background of AA have been
identified (Supp. Fig. S1). Eight field tests at two locations (Inabu and Tsukuba) revealed that the resistance conferred by each of the four alleles individually was incomplete, had varying magnitude, and was substantially sensitive to environment (coefficient of variation [C.V.] ≥ 15%) (Fig. 1a, Table 1). Nevertheless, analysis of variance (ANOVA) revealed that each allele had a significant individual effect (P < 0.001); for pi21, F(1,827) = 1483.031; for qBR4-2, F(1,827) = 91.495; for Pi34, F(1,827) = 50.319, and for qBR12-1, F(1,827) = 13.275. Similar results were obtained after a glasshouse challenge with eight races of M. oryzae that are widely distributed in Japan and a ninth race (IBOS8-1-1) that is aggressively virulent to Pi34 (Supp. Fig. S2). The resistance conferred by qBR4-2 was less effective in field trials with an average temperature above 25°C during the 2 weeks before scoring than when temperatures were lower (Table 1). In a glasshouse challenge using fungus race 007.0, the lesion area of the qBR4-2 plants was significantly smaller than that of the recurrent parent (AA) when the plants were incubated at 20°C for 1 week before inoculation, but the difference was not significant when the plants were incubated at 24 or 28°C (Supp. Fig. S3). Of the four QTLs tested in the glasshouse challenge, only Pi34 significantly reduced the lesion number per leaf when challenged with the seven of eight Japanese races of the fungus, but none of the single QTLs significantly reduced lesion number when challenged with IBOS8-1-1 (Supp. Fig. S2).

Ethylene and salicylic acid (SA) signalling are major components of disease resistance in plants16–18. We examined the responses to these signals in the pi21 and Pi34 lines because their resistance was stably detectable in the glasshouse pathogen isolate challenge. Application of an antagonist of ethylene biosynthesis, 2-aminoisobutyric acid (AIB), before inoculation did not alter average lesion area in the plants carrying pi21, whereas AA plants and plants carrying Pi34 had significantly larger lesions than the corresponding untreated controls (Supp. Fig. S4). When 500 μM SA was applied before inoculation, plants carrying Pi34 had a significantly smaller average lesion area than untreated plants, whereas the average lesion areas of AA plants and those carrying pi21 were unchanged (Supp. Fig. S4).

**Resistance to M. oryzae in QTL pyramid lines.** Combinations of resistance alleles reduced lesion area both in field tests (Fig. 1a and b, Table 1) and in glasshouse inoculation tests with the nine previously described races of M. oryzae (Fig. 2a). The pi21 resistance allele had the greatest effect on lesion area among the four QTLs when present alone (Supp. Fig. S2), and combination of pi21 with other QTLs cumulatively reduced lesion area against all nine races tested (Fig. 2a). A pyramid of the three QTLs other than pi21 had an average lesion area somewhat higher than that of pi21 alone when tested in the field (Table 1). The line with four resistance alleles (AA-4RQ), which carried only 6% of the donor (OW) genome, had an average lesion area of ≤1%, comparable to that of OW. Importantly, the C.V. for lesion area across field tests was smaller in AA-4RQ than in lines with only one or two resistance alleles (Fig. 1a, Fig. 2a, Table 1). In contrast, R-gene-mediated resistance fluctuated in field tests, possibly owing to changes in pathogen populations over time (Supp. Table S1). Lesion number per leaf was smaller in AA-4RQ than in the line with pi21 alone when the two lines were challenged with the nine races, although the difference was not significant when the lines were challenged with IBOS8-1-1 (Fig. 2b).

**Characterization of QTL pyramid lines.** In a histological analysis, cells of AA-4RQ infected with a virulent race of M. oryzae lacked the hypersensitive response (HR)19 that is often associated with R-gene-mediated resistance, as indicated by the presence of viable cells at infection sites (Supp. Table S2). But cytoplasmic granules, the first

![Figure 1](https://www.nature.com/scientificreports/srep07773/figure1)

**Figure 1 | Combining alleles of different quantitative trait loci (QTLs) enhances resistance to M. oryzae in rice.** (a) Average lesion areas of fifteen single- or multiple-QTL lines, the donor parent Owarihatamochi (OW), and the recurrent parent Aichiasahi (AA) across eight field tests. Error bars indicate SD. Genotypes are indicated below the graph. Boxes represent alleles for resistance (red) and susceptibility (white) for each QTL listed at the left. (b) Leaves of 60- to 70-day-old plants from the field evaluation for resistance to M. oryzae. Letters correspond to genotypes shown in (a). (c) Infected cells in leaf sheath epidermal tissue 45 to 48 h after inoculation. Tissue is from 48-day-old plants infected with M. oryzae race 007.0 (Ina86-137). AA-pi21 and AA-4RQ correspond to genotypes H and A, respectively, in (a) and (b). The small brown circles in the infected AA-4RQ cell are cytoplasmic granules. Black arrowhead, appressorium; white arrowheads, invading hyphae. Bars represent 20 μm.
| Genotype code\(i\) | No. of lines | Year 2004 | 2005 | 2006 | 2007 | 2008 | 2009 | 2011 | Average (2004–2011) |
|------------------|-------------|-----------|------|------|------|------|------|------|------------------|
| OW               | 3           | 0.6 ± 0.06 | 0.7 ± 0.13 | 0.7 ± 0.15 | 0.6 ± 0.05 | 0.6 ± 0.09 | 0.6 ± 0.05 | 0.6 ± 0.04 | 0.8 ± 0.13 |
| A                | 3           | 0.6 ± 0.06 | 0.6 ± 0.12 | 0.7 ± 0.12 | 0.5 ± 0.05 | 0.7 ± 0.12 | 0.6 ± 0.05 | 0.7 ± 0.08 | 0.6 ± 0.12 |
| B                | 2           | 1.7 ± 0.23 | 0.9 ± 0.26 | 1.4 ± 0.20 | 1.4 ± 0.22 | 1.9 ± 0.19 | 1.0 ± 0.14 | 2.0 ± 0.43 | 2.6 ± 0.32 |
| C                | 3           | 1.9 ± 0.24 | 1.1 ± 0.36 | 1.4 ± 0.53 | 1.4 ± 0.24 | 2.2 ± 0.61 | 1.6 ± 0.37 | 0.9 ± 0.19 | 2.7 ± 0.42 |
| D                | 3           | 2.3 ± 0.48 | 3.1 ± 0.82 | 3.8 ± 1.10 | 2.1 ± 0.39 | 2.3 ± 0.77 | 2.4 ± 0.48 | 3.1 ± 0.77 | 5.1 ± 1.26 |
| E                | 3           | 5.9 ± 1.23 | 4.3 ± 1.37 | 3.5 ± 0.72 | 3.1 ± 1.37 | 5.0 ± 0.40 | 2.6 ± 0.35 | 3.6 ± 0.95 | 5.1 ± 1.51 |
| F                | 3           | 4.0 ± 0.94 | 3.1 ± 0.92 | 2.8 ± 0.77 | 4.4 ± 1.11 | 7.9 ± 2.89 | 2.3 ± 0.49 | 5.8 ± 1.49 | 6.0 ± 1.84 |
| G                | 1           | 5.3 ± 1.31 | 4.9 ± 1.44 | 4.1 ± 1.26 | 5.1 ± 1.28 | 12.7 ± 4.98 | 4.5 ± 1.16 | 7.6 ± 2.01 | 7.3 ± 2.26 |
| H                | 1           | 12.6 ± 3.15 | 5.8 ± 1.72 | 6.4 ± 1.80 | 7.0 ± 1.69 | 15.3 ± 5.60 | 5.0 ± 1.62 | 9.4 ± 2.66 | 13.1 ± 4.09 |
| I                | 1           | 14.5 ± 3.23 | 9.1 ± 2.76 | 11.4 ± 3.89 | 17.6 ± 4.60 | 25.9 ± 6.92 | 18.2 ± 6.27 | 15.9 ± 3.94 | 16.1 ± 5.17 |
| J                | 1           | 19.7 ± 4.40 | 19.5 ± 5.79 | 13.8 ± 3.85 | 27.2 ± 7.35 | 45.1 ± 12.50 | 33.1 ± 10.36 | 22.5 ± 5.91 | 34.5 ± 6.83 |
| K                | 1           | 22.2 ± 6.20 | 20.7 ± 6.15 | 15.0 ± 4.98 | 37.6 ± 10.50 | 48.2 ± 13.51 | 59.1 ± 14.56 | 23.9 ± 6.21 | 28.4 ± 8.12 |
| L                | 3           | 292 ± 6.66 | 324 ± 9.07 | 367 ± 10.99 | 308 ± 8.07 | 56.8 ± 15.92 | 292 ± 9.45 | 37.1 ± 8.68 | 38.0 ± 7.60 |
| M                | 3           | 256 ± 7.33 | 383 ± 11.03 | 41.9 ± 11.86 | 487 ± 13.50 | 64.2 ± 16.59 | 52.2 ± 15.23 | 27.6 ± 5.97 | 37.7 ± 11.58 |
| N                | 1           | 385 ± 9.50 | 411 ± 12.21 | 58.6 ± 16.56 | 55.7 ± 14.55 | 63.6 ± 15.58 | 45.3 ± 13.90 | 31.3 ± 6.98 | 42.6 ± 8.99 |
| O                | 0           | 514 ± 13.46 | 540 ± 15.46 | 69.9 ± 15.76 | 65.4 ± 12.35 | 81.5 ± 13.94 | 56.8 ± 14.90 | 29.9 ± 7.12 | 67.4 ± 9.14 |
| AA               | 0           | 638 ± 9.42 | 827 ± 10.23 | 81.0 ± 11.42 | 73.5 ± 10.47 | 87.1 ± 11.49 | 79.0 ± 11.97 | 67.7 ± 10.18 | 76.1 ± 7.42 |
| O’               | 0           | 460 ± 10.58 | 560 ± 14.02 | 81.0 ± 11.42 | 73.5 ± 10.47 | 87.1 ± 11.49 | 79.0 ± 11.97 | 67.7 ± 10.18 | 76.1 ± 7.42 |

| Temperature      | Ave          | 23.9 ± 0.97 | 23.5 ± 1.00 | 22.6 ± 1.94 | 23.7 ± 1.21 | 25.1 ± 0.86 | 28.0 ± 1.65 | 25.0 ± 0.28 | 22.3 ± 0.35 |
| Min              | 19.5 ± 0.93 | 18.8 ± 1.19 | 19.5 ± 0.82 | 18.7 ± 1.24 | 20.5 ± 1.03 | 23.2 ± 1.32 | 21.3 ± 0.28 | 18.4 ± 0.30 |

1. resistance allele, 0, susceptibility allele
2. OW, Owarihishimachi (donor parent); AA, Aichiahi (recurrent parent). A is also referred to as AA-4RQ. All near-isogenic lines containing the resistance allele at qBR12-1 carry the donor chromosome segment shown in Fig. S1 except for O’, which carries a shorter donor chromosome segment from RM6973 to ID01-47 in Supp. Fig. S1.
3. Data in the same column followed by the same letter are not significantly different according to Tukey’s HSD test at 5%.
4. Coefficient of variation across eight trials (proc.transformed values were used for statistical testing).
5. Lesion area at test locations during the 2 weeks before scoring, provided by the Japan Meteorological Agency. Temperatures over 25°C an average and over 30°C at maximum are shown in bold.
sign of cell death, were observed as early as 45 to 48 h after inoculation in AA-4RQ plants (Fig. 1c), consistent with the higher expression of pathogenesis-related (PR) genes in AA-4RQ than in Aichiasahi or in plants with pi21 alone (AA-pi21) (Fig. 3). This induction of PR gene expression was not as great as that by R-gene-mediated resistance (Fig. 3, Avirulent) at the tested time points. Consequently, the biomass of a virulent race of M. oryzae (measured by qPCR targeting the fungal repetitive element Pot2) was dramatically lower in the AA-4RQ plants than in Aichiasahi (Fig. 4).

Discussion
Cultivars with DR against rice blast have maintained resistance over time, and multiple QTLs lie behind their high levels of resistance\(^6^-^9\). Therefore, QTL-mediated resistance is considered to be more durable than R-gene-mediated resistance. Cultivars with DR are usually unimproved, and their exotic, often heterogeneous genetic backgrounds make it difficult to predict the potential impact of their DR in the genetic background of elite cultivars. In the present study, we successfully characterized a set of four QTLs in a DR genotype conferring resistance to M. oryzae and clearly demonstrated the QTLs’ ability to confer resistance under multiple environments and against diverse isolates. The NIL with four resistant QTL alleles showed comparable resistance to the donor, and more stable resistance (i.e., lower C.V.) than lines with only one or two resistance alleles. These observations reinforce the idea that this combination of QTLs with diverse responses against the pathogen underlies DR to M. oryzae in Owarihatamochi.

Previous studies have characterized and cloned three genes for QTL-mediated resistance to M. oryzae in rice, namely, pi21, Pi63 and Pi35\(^1^1,^1^5,^2^0\); these QTLs had the largest effects in the primary genetic mapping studies\(^7,^2^1\). However, QTLs with smaller effects have not yet been characterized owing to the difficulty in controlling experimental noise caused by variation in genetic background and by genotype × environment interaction. Our study highlighted the importance of minor QTL alleles for improving the durability of resistance by using experimental lines with a highly homogeneous genetic background.

As expected, the resistance conferred by each of the four alleles individually was substantially sensitive to environment. This confirms the need to evaluate QTL effects under multiple environments to identify stable QTL alleles for use in crop improvement. Among the four QTL alleles, only Pi34 significantly reduced lesion number when present alone, implying that this QTL acts around the time of fungal penetration. Average lesion area is a function of the number of lesions per leaf and the size of each lesion. Resistance conferred by Pi34 is less effective in reducing average lesion area than that conferred by pi21, owing to the ineffectiveness of Pi34 at suppressing lesion development after infection. To elucidate the mechanism(s) of resistance, we examined the response of Pi34 and pi21 plants to altered levels of ethylene and SA, signalling compounds that modulate disease resistance in plants\(^1^6^-^1^8\). The increased resistance observed in SA-treated Pi34 plants implies that the gene at this QTL is associated with SA signalling. Ethylene induces resistance to a number of diseases when applied before infection, and inhibition
of its biosynthesis decreases resistance\(^1\). Our observations with Aichiasahi and Pi34 plants are consistent with these earlier findings. However, plants containing pi21, a loss-of-function mutant in a proline-rich-protein gene, were insensitive to this treatment, implying that pi21-mediated resistance involves ethylene signalling. We observed temperature dependence of qBR4-2, as reported in other pathosystems\(^2\)–\(^4\). Together, these findings confirm that diverse biological functions are involved in QTL-mediated resistance.

Expression analysis and histological study revealed that the four-QTL pyramid line, AA-4RQ, does not induce HR, and that induction of PR genes was not as great as that by R-gene-mediated resistance at the tested time points. These observations confirm that the resistance seen in AA-4RQ is of the “susceptible infection type”, which shows developing lesions\(^2\), but it has several characteristics that differ from the typical susceptible infection type. Around 32 or 33 h after inoculation, the hyphae of a virulent fungus race have just begun moving from the first-invaded host cell to the next cells and the first-invaded host cell is mostly intact, without any sign of defence response\(^2\)–\(^6\). Thirty-five-day-old plants were used, and leaves were sampled at 28, 32, and 36 h after inoculation. Expression of PR genes after inoculation with a virulent race (007.0, blue bars), water (open bars), or an avirulent race (001.2, green hatched bars). Genotypes were Aichiasahi, AA-pi21, and AA-4RQ (AA, H, and A in Fig. 1a and at the bottom of the figure); boxes represent alleles for resistance (red) and susceptibility (white) at pi21, qBR4-2, Pi34, and qBR12-1. Thirty-five-day-old plants were used, and leaves were sampled at 28, 32, and 36 h after inoculation. Expression of PR genes Pathogenesis-related 2 (PR2) (Os01t0940700) and Probenazole 1 (PBZ1) (Os12t00555500) was standardized to that of riboubiquitin 2 (Os02t0161900). Error bars indicate SEM (\(n = 3\)).

**Methods**

**QTLs for resistance to M. oryzae in rice.** Overahatamochi (OW) is a cultivar of rice (Oryza sativa) that has maintained resistance to blast disease caused by M. oryzae throughout a century of cultivation\(^2\) and whose resistance is controlled by several R-gene\(^2\)–\(^4\). A major QTL, pi21, explained 45.7% of the phenotypic variation, and QTLs on chromosomes 4 (qBR4-2) and 12 (qBR12-1) explained 29.4% and 13.7%, respectively\(^2\). Pi34, on chromosome 11, was detected by using a statistical test with a lower probability threshold (PROC GLM program in the Statistical Analysis Systems software, with significance at \(P = 0.05\))\(^2\). The effect of Pi34 was validated using Chubu32, a breeding line derived from a DR cultivar\(^2\).

**Development of near-isogenic lines.** By performing three to five rounds of backcrossing and marker-assisted selection (MAS), we developed NILs for qBR4-2, qBR12-1, and Pi34, each carrying the resistance allele from OW in the genetic background of Aichiasahi (AA), a highly susceptible and well-characterized cultivar used in blast resistance studies. A NIL for pi21 in the same genetic background (AA-pi21) was obtained from a previous study\(^1\). MAS was conducted to introduce 3- to 24-cM chromosomal fragments containing each QTL from the donor, by using DNA markers described in a previous study\(^2\)–\(^5\). C975 and G271 for pi21, C1016 and R738 for qBR4-2, C85 and G257 for Pi34, and OSR20 and G402 for qBR12-1. We used 118 additional DNA markers from the previous study\(^2\) to confirm that the rest of the genome of each NIL was derived from AA (Supp. Fig. S1). AA carries the R-gene Pia and shows resistance to M. oryzae races 001.0, 001.2, and 031.1, but not to the nine races shown in Fig. 2 and Supp. Fig. S2.
Development of QTL pyramids. We first crossed the four NILs pairwise, and then selected progeny homozygous for resistance alleles at two QTLs by using the PCR-based markers indicated in Supp. Fig. S1. Next, we crossed plants homozygous for resistance alleles at two QTLs. In total, we developed eleven genotypes representing all possible combinations of two or more resistance QTL alleles. We then selected two to four lines per genotype. In a glasshouse inoculation test, three QTL pyramids—one with pi21 and qBR4-2 (genotype code E in Fig. 1A), one with pi21, qBR4-2, and Pi34 (code B), and one with all four QTL resistance alleles (code A; also called AA-4RQ)—were used to test the effect of combining minor QTLs with the major QTL pi21.

Genotyping. Total DNA was extracted from leaf samples by using the CTAB method, and restriction fragment length polymorphism (RFLP) analysis was conducted as previously described. Most of the simple sequence repeat (SSR) markers were obtained from previous reports. The sequences of the other primer pairs (forward 5′–3′/reverse 5′–3′) of PCR-based markers were as follows: ID04-25, GCCAGGGACAAAGATTG/TTCGATGGTGGTTCCTC; ID04-26, TGGTTGCGGATTGCAGC/GCGTCACTGTAAATTGCCTA; and ID01-47, TTTGGGTTGAGGATTTT/GCCATTGTGGTTTCTC. All markers were assessed in 2% to 3% agarose gel.

Evaluation of resistance to M. oryzae in the field. Resistance to M. oryzae was assessed during six growing seasons (2004–2008 and 2011) in an experimental field at the Aichi Agricultural Research Center, Mountainous Region Institute (MARI) (Inabu, Toyota, Aichi, Japan). The field had high levels of M. oryzae, and the predominant fungal race was 007.0. The lesion area (percentage of total leaf area) of 60- to 70-day-old plants was scored according to a reference scale. The score of each line was standardized as described previously, and the arcsine-transformed value was used for statistical testing. Resistance was likewise tested in 2009 and 2011 in an experimental field at the National Institute of Agrobiological Sciences (Tsukuba, Ibaraki, Japan), where the predominant fungal race was 007.3.

To test the effect of ethylene and SA signalling on QTL-mediated resistance, we grew three sets of three genotypes (the recurrent parent and NILs for pi21 and qBR4-2) in the glasshouse under the conditions described above for 3 weeks. Each set was then transferred to one of three growth chambers (20, 24 or 28 °C) for 1 week before inoculation and then inoculated as described above.

To test the effect of ethylene and SA signalling on QTL-mediated resistance, we grew three sets of three genotypes (the recurrent parent and NILs for pi21 and qBR4-2) in the glasshouse under the conditions described above for 3 weeks. Each set was then transferred to one of three growth chambers (20, 24 or 28 °C) for 1 week before inoculation and then inoculated as described above.

Fungal growth on leaf sheath epidermal tissue of 48-day-old plants was histologically characterized as previously described. A previous study detected apparent differences between cells with and without pi21 at 96 h after inoculation, but not at 40 h. In a preliminary survey, we did not detect any differences among Aichiasahi, AA-pi21, and AA-4RQ at 33 h. Thus, we examined cells at 45–48 h after inoculation.
inoculation. We evaluated M. oryzae biomass in leaves after inoculation by using real-time PCR as described previously. We infected 28-day-old plants with fungal races that were either compatible (003.0) or incompatible (001.0) with the Pia genes in the genotypes used. Fungal spores were prepared at a concentration of 8–11 × 10^6 spores/mL in water containing 0.1% Tween 20. We measured the amount of fungal DNA in DNA extracted from infected leaf tissue samples 4 days after inoculation by using the fluorescent, competitive element PR2 (GenBank: AY153638); the primers were SP6R (ACGGACCCGTCTTTATCTTTTGTG) and SP8R (AAGTAGCTTGTGGGTTTGTGTTGAGG). DNA quantities were standardized against a rice ubiquitin gene (AK121590). The experiment was performed twice.

**Expression of pathogenesis-related genes.** After the inoculation test, total RNA was isolated from leaves of 35-day-old plants by using an RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed with primer dT18 and a First-Strand cDNA Synthesis Kit (GE Healthcare) according to the manufacturers’ instructions. Quantitative real-time PCR was performed by using THUNDERBIRD SYBR qPCR Mix (Toyobo) and a 7900HT Fast Real-time PCR System (Applied Biosystems) according to the manufacturers’ instructions. The genes and sequences of PCR primers (forward 5′–3′/reverse 3′–5′) were Probenazole 1 (PBZ; Os10200555500), GTTGGATGTGGATGCCCTCTCTCTCCTTTCTTCAGCATTTG, and Pathogenesis-related 2 (PR2; Os0109407000), GCCTTTCAAGAGCAAGCACGGGGCAGACCTCAAGGG. The expression level of each primer pair was determined by ANOVA. A general linear model (GLM) was used to compare means determined by ANOVA. A general linear model (GLM) was used to compare means determined by ANOVA. A general linear model (GLM) was used to compare means determined by ANOVA. A general linear model (GLM) was used to compare means determined by ANOVA.

**Statistical tests.** The individual effects of each allele and their significance levels were determined by ANOVA. A general linear model (GLM) was used to compare means among genotypes. All analyses were performed by using SPSS Statistics 19 (IBM).

1. Jones, J. D. & Dangl, J. L. The plant immune system. Nature 444, 323–239 (2006).
2. Bonman, J. M., Khush, G. S. & Nelson, R. J. Breeding rice for resistance to pests. Annu. Rev. Phytopathol. 30, 507–528 (1992).
3. Kiyosawa, S. Genetic and epidemiological modeling of breakdown of plant disease resistance. Annu. Rev. Phytopathol. 20, 93–117 (1982).
4. Pollock, A. J., Balint-Kurti, P. J., Wiss, R. J., Pratt, R. C. & Nelson, R. J. Shades of gray: the world of quantitative disease resistance. Trends Plant Sci. 14, 21–29 (2009).
5. Kou, Y. & Wang, S. Broad-spectrum and durability: understanding of quantitative disease resistance. Curr. Opin. Plant Biol. 13, 181–185 (2010).
6. Sakai, N. A rice (Oryza sativa L.) breeding for field resistance to blast disease (Pyricularia oryzae) in Mountainous Region Agricultural Research Institute, Aichhi Agricultural Research Center of Japan. Plant Prod. Sci. 9, 3–9 (2006).
7. Miyamoto, M., Yano, M. & Hirasawa, H. Mapping of quantitative trait loci affecting blast resistance in japonica rice. J. Gen. Plant Pathol. 71, 395–401 (2005).
8. Koga, H., Dohi, K., Nakayachi, O. & Mori, M. A novel inoculation method of Magnaporthe grisea for cytological observation of the infection process using intact leaf sheaths of rice plants. Plant Mol. Biol. Plant Physiol. 66, 67–72 (2004).
9. Hayashi, N. et al. Durability of partial blast-resistance gene Pi4 encodes an atypical CC-NBS-LRR protein and was generated by acquiring a promoter through local genome duplication. Plant J. 64, 498–510 (2010).
10. Berruyer, R., Poussier, S., Kankanala, P., Mosquera, G. & Valent, B. Roles for rice membrane dynamics and characterization of regions of the rice genome associated with broad-spectrum, quantitative disease resistance. Genetics 169, 2277–2793 (2005).
11. Xu, X. et al. Rice blast resistance gene Piakes1 is a member of a resistance gene cluster on chromosome 4 encodes a nucleotide-binding site and leucine-rich repeat protein. Mol. Breeding 34, 691–700 (2014).
12. van Loon, L. C., Geraads, B. P. J. & Linthorst, H. J. M. Ethylene as a modulator of disease resistance in plants. Trends Plant Sci. 11, 184–191 (2006).
13. Plot, A. C., Dempsey, D. A. & Klessig, D. F. Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annu. Rev. Phytopathol. 47, 177–206 (2009).
14. Silverman, P. et al. Salicylic Acid in Rice (Biochemistry, Conjugation, and Possible Role). Plant Physiol. 108, 633–639 (1995).
15. Greenberg, J. T. & Yao, N. The role and regulation of programmed cell death in plant-pathogen interactions. Cell. Microbiol. 6, 201–211 (2004).

**Acknowledgments**

We thank A. Takahashi, H. Inoue, E. Minami and T. Hayashi for helpful comments, and M. Matsuoka for critical reading of the manuscript. This work was supported by grants from the Ministry of Agriculture, Forestry and Fisheries of Japan (Development of Mitigation and Adaptation Techniques to Global Warming in the Sectors of Agriculture, Forestry, and Fisheries, Rice-2001; Genomics for Agricultural Innovation QT4004, GB1004, QTL2002, the Ministry of Agriculture, Forestry and Fisheries of Japan (Development of Mitigation and Adaptation Techniques to Global Warming in the Sectors of Agriculture, Forestry, and Fisheries, Rice-2001; Genomics for Agricultural Innovation QT4004, GB1004, QTL2002, and RGR1101). We thank editors from Elsevier, Inc. (http://elscs.jp/en/) for editing the manuscript.

**Author contributions**

S.F. designed the experiments and wrote the manuscript. S.F. and K.E. developed the NILs and QTL–combination lines. S.F., N.H. and Y.Y. performed phenotyping in glasshouse inoculation tests. S.F., N.Y.M. and R.M. performed phenotyping in field tests. H.K. performed histological analysis. U.Y. performed expression analysis. M.Y. provided advice on the experiments and the manuscript.

**Additional information**

**Supplementary information** accompanies this paper at http://www.nature.com/scientificreports

**Competing financial interests:** The authors declare no competing financial interests.

**How to cite this article:** Fukuoka, S. et al. Gene pyramiding enhances durable blast disease resistance in rice. Sci. Rep. 5, 7773; DOI:10.1038/srep07773 (2015).