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Loss of Function of a Proline-Containing Protein Confers Durable Disease Resistance in Rice

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Blast disease is a devastating fungal disease of rice, one of the world's staple foods. Race-specific resistance to blast disease has usually not been durable. Here, we report the cloning of a previously unknown type of gene that confers non-race-specific resistance and its successful use in breeding. *Pi21* encodes a proline-rich protein that includes a putative heavy metal-binding domain and putative protein-protein interaction motifs. Wild-type *Pi21* appears to slow the plant's defense responses, which may support optimization of defense mechanisms. Deletions in its proline-rich motif inhibit this slowing. *Pi21* is separable from a closely linked gene conferring poor flavor. The resistant *pi21* allele, which is found in some strains of *japonica* rice, could improve blast resistance of rice worldwide.

The use of resistance genes (*R* genes) that confer race-specific resistance is a cost-effective strategy for the control of disease in crops. *R* genes are a key component of disease

resistance and often are associated with a hypersensitive response (HR), according to the gene-for-gene concept (1). Most *R* genes cloned in plants contain conserved gene structures (2), and mutation of the pathogen effectors that trigger *R* gene-mediated resistance leads to a loss of resistance, suggesting a substantial vulnerability of this defense mechanism (3). In contrast, resistance controlled by quantitative trait loci (QTLs) is usually non-race-specific (4), and its durability might be a consequence of decreased selective pressure for pathogens to overcome host resistance. Despite the importance of resistance QTLs in crops, their molecular basis remains largely unknown, except *Lr34* and *Yr36*, which house genes that differ structurally from previously reported *R* genes (5, 6).

The resistance to blast disease caused by *Magnaporthe oryzae* conferred by QTLs in rice

(*Oryza sativa* L.) lacks HR, yet it restricts the development of lesions (7). Some cultivars carrying resistance QTLs, such as the *Pi21* locus (8), have maintained resistance throughout a century of cultivation. However, the reintroduction of resistance and undesirable agricultural traits, including grain characteristics from donors, has prevented the use of potential genetic resources for the development of elite cultivars for the past 80 years (9). The causes of the troublesome association have long been debated and could include either tight linkage of genes that control independent traits, known as "linkage drag," or pleiotropic effects of the target gene on other traits (10). Such factors pose penalties on crop production, yet research on the cost to agricultural crops is limited (10). To identify the molecular basis of *Pi21*-mediated resistance and to settle the long-term debate about the cause of associated undesirable characteristics, we undertook map-based cloning of *pi21*, a recessive allele conferring resistance to rice blast.

Leaves of AA-*pi21*, a near-isogenic line (NIL) carrying *pi21* in the genetic background of a susceptible cultivar, had smaller lesions than did the susceptible cultivar (AA) in both a field evaluation and a greenhouse inoculation test (Fig. 1, A and B, and figs. S1A and S2). The resistant *pi21* allele had a consistent effect against all 10 of the widely distributed races of *M. oryzae* studied, although the resistance was incomplete as compared with that triggered by *R* genes (Fig. 1C). Histological examination showed that cells infected with a virulent race were still alive and intact in both AA and AA-*pi21* 40 hours after inoculation (Fig. 1D and table S1), in contrast to those infected with an avirulent race, which induces *R* gene-dependent hypersensitive cell death (table S1). However, cytoplasmic granules, the first sign of cell death, were frequently observed in AA-*pi21* 96 hours after inoculation (Fig. 1E).

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The rate of penetration of hyphae into host cells in AA-pi21 did not differ from that in AA (Fig. 1F), but the rate of invasion of hyphae from penetrated

cells into adjacent cells, as an indicator of hyphal growth, was significantly lower in AA-pi21 (Fig. 1G), which is consistent with the observations in

the greenhouse inoculation test (fig. S1, A and B). These observations suggest that *pi21* confers non-race-specific resistance, which is possibly associated with a slow induction of resistance (11). The resistant *pi21* allele did not affect resistance to other fungal and bacterial pathogens (fig. S1, C and D).

By means of high-resolution mapping, we delimited the *Pi21* locus to a 1705-base pair (bp) region containing a single gene, Os04 g0401000 (Fig. 2, A to C, and fig. S3, A and B). The deduced amino acid sequence forms a protein containing a heavy metal-transport/detoxification protein domain in the N-terminal region (fig. S4), implying that metal transport by Pi21 might be associated with defense as previously reported (12, 13). Comparing the 1705-bp region among two susceptible and one resistant cultivars, we identified seven nucleotide polymorphisms, two of which were located in the open reading frame associated with the phenotype (Fig. 2C and table S2). The resistant *pi21* allele had deletions of 21 and 48 bp from one susceptible *Pi21* allele and of 12 and 48 bp from the other allele in the proline-rich region (fig. S4A).

Transforming a 4.7-kb genomic fragment containing the candidate gene from the resistant cultivar into the susceptible cultivar did not confer resistance, whereas transforming the susceptible *Pi21* allele into AA-pi21 increased susceptibility to blast in T₁ progeny (table S3). These results suggest that the susceptible allele negatively regulates resistance and that the resistant allele carries a loss-of-function mutation. We confirmed that suppression of susceptible *Pi21* expression by means of RNA interference (RNAi) increased resistance (Fig. 3, A to C), providing evidence that a gene that encodes a protein with a heavy metal-transport/detoxification protein domain (fig. S6) has a role in plant defense. T₂ plants that showed increased susceptible *Pi21* expression also showed enhanced susceptibility to the virulent race, but

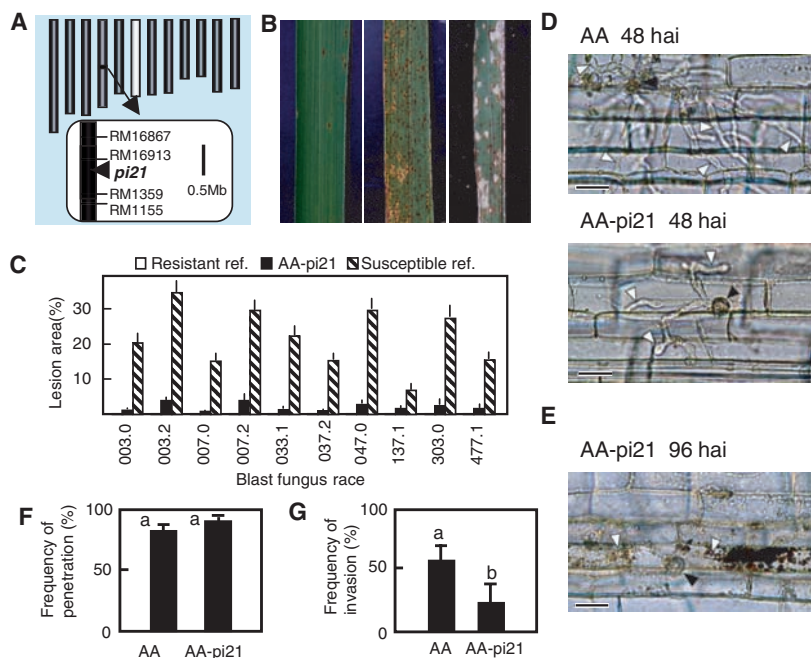
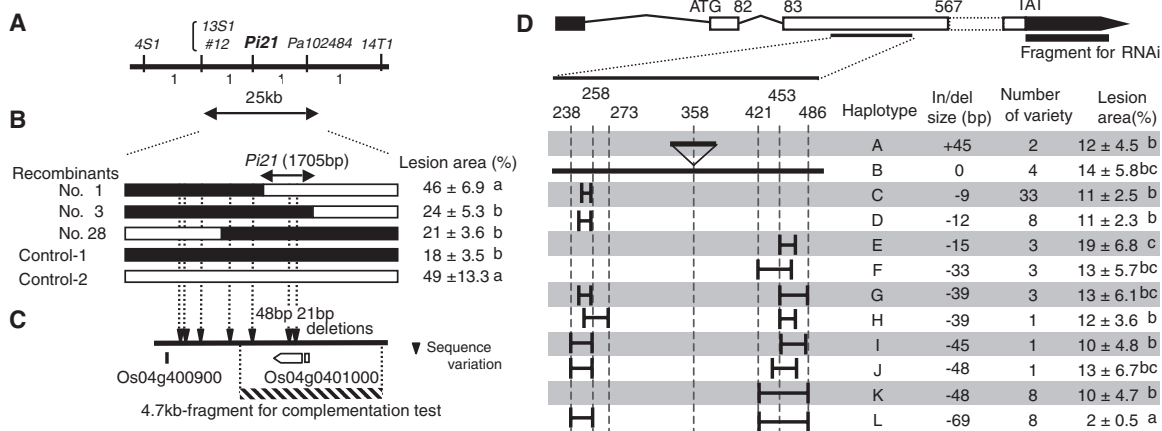


Fig. 1. Phenotypic characterization of the resistant *pi21* allele. (A) Chromosome map of NIL AA-pi21. Open boxes indicate chromosomes from susceptible cultivar Aichiasahi (AA); the red box indicates chromosomes from donor cultivar Owarihatamochi. (B) Diseased leaves of 60- to 70-day-old plants from the field evaluation for resistance to blast: Owarihatamochi (left), AA-pi21 (middle), and AA (right). (C) Lesion area of 30-day-old plants, each inoculated with one of 10 races of blast. The lesion area of the resistant reference was near 0 for all races tested. (D) Infected cells 48 hours after inoculation: AA (top) and AA-pi21 (bottom). (E) Infected cells 96 hours after inoculation (AA-pi21). Black triangles indicate appressoria; white triangles indicate invading hyphae. Scale bars, 20 μ m. Cells challenged by avirulent races are shown in fig. S1E. (F) Frequency of successful penetration of hyphae into cells 48 hours after inoculation. (G) Frequency of invasion of hyphae from penetrated cells into adjacent cells 48 hours after inoculation; a and b are significantly different according to Student's *t*-test ($P = 4.9 \times 10^{-4}$). In (D) to (G), leaf sheath epidermal tissue of 48-day-old plants and blast race 007.0 are shown. Bars in (C), (F), and (G) indicate SE.

Fig. 2. Genetic and physical maps of *Pi21*. (A) Genetic map produced from 1014 F₂ plants of AA crossed with AA-pi21. Numbers below the horizontal line indicate recombinants between *Pi21* and flanking markers. (B) Additional genetic mapping of *Pi21* and progeny testing. Recombinants between marker loci 12 and *Pa102484* were selected from 2703 F₂ plants in which resistant and susceptible alleles from Kasalath segregated. Black bars indicate homozygous resistant; white bars indicate homozygous susceptible. (C) Physical map around *Pi21*. The arrowheads above the horizontal line indicate positions of sequence variations between Owarihatamochi (resistant allele) and Aichiasahi and Kasalath (susceptible alleles). Open boxes represent genes described in the Rice Annotation Project Database (RAP-DB). The hatched box represents the fragment used for the complementation test. (D) Natural



variations in *Pi21*. Indels in *Pi21* identify 12 haplotypes among cultivated rice. The size difference of each haplotype from Nipponbare is shown. The average lesion areas in backcrossed lines carrying the respective *Pi21* haplotypes are indicated. The cultivars used as donors are listed in table S4. The lesion area data in (C) and (D) followed by different letters differ significantly according to Tukey's post hoc test at 5%.

these plants all showed resistance with clear HR against an avirulent race (Fig. 3, D to F), suggesting the involvement of *Pi21* in basal resistance.

We identified 12 variants (haplotypes A to L) in a set of cultivars that represent the genetic variation within cultivated rice (14) on the basis

of insertion–deletion polymorphisms at three positions in a proline-rich region (Fig. 2D and table S4). The resistant *pi21* allele carrying 18- and 48-bp deletions (haplotype L) was found only in *japonica* rice, possibly originating from additional deletion mutation in haplotype K (Fig. 2D and figs. S4B

and S5). We developed a series of backcrossed lines, each possessing one of the *Pi21* haplotypes in the genetic background of a susceptible cultivar, and evaluated their blast resistance (Fig. 2D). Only the line carrying haplotype L showed improved resistance to blast; the rest showed similar susceptibility to the recipient cultivar. Thus, we hypothesized that the defect in *pi21* function is due to the deletion of both the 18- and 48-bp sequences, which house the consensus motif sequence PxxPxxP (fig. S4B), the “core motif” for protein–protein interaction in multicellular organisms (15, 16). A proline-rich protein was suggested to be involved in a defense mechanism of human neutrophils, possibly through competitive inhibition of protein–protein interaction of the proline-rich motif and its counterpart (17). A similar scenario may operate in *pi21*-mediated resistance. Because most rice cultivars carry susceptible *Pi21* alleles (Fig. 2D), the *Pi21* gene is a component associated with susceptibility that can be replaced with the resistant *pi21* allele.

We monitored gene expression in resistant and susceptible lines challenged by either a virulent or an avirulent race of *M. oryzae*. The transient decrease at 3 to 6 hours after the inoculation of all plants implies that *Pi21* responds to stress resulting from inoculation or from incubation in a humidity chamber so as to promote infection (fig. S7A). Plants with the resistant *pi21* allele showed a higher expression of pathogenesis-related genes 3 to 6 hours after inoculation with the virulent race than did plants with the susceptible *Pi21* allele, but not with the avirulent race. This implies that *Pi21*

Fig. 3. Molecular characterization of *Pi21*. (A) Lesions of RNAi lines (AND14 and AND19) 7 days after inoculation with virulent blast race 007.0; T₁ Aichiasahi plants carrying an RNAi construct or an empty vector were used. (B) Lesion areas of empty vector control, RNAi lines, and AA-*pi21* 7 days after inoculation (fig. S2). (C) Reverse transcription polymerase chain reaction (RT-PCR) analysis of *Pi21* in RNAi lines. (D) Lesions of *Pi21*-overexpressing transgenic lines 7 days after inoculation with virulent or avirulent races of blast. Shown are 30-day-old plants of empty vector control, two independent T₃ lines (7-2 and 10-2) carrying multiple copies of the susceptible *Pi21* allele in AA-*pi21* (derived from independent T₀ plants), and Aichiasahi. (E) Lesion area of susceptible *Pi21*-introduced transgenic lines 7 days after inoculation. F₁ of AA crossed with AA-*pi21* is also shown. (F) RT-PCR analysis of *Pi21* in *Pi21*-introduced transgenic lines. In (C) and (F), total RNA was isolated from 24-day-old plants 1 week before the inoculation test. Bars in (B) and (E) indicate SE, and those indicated by different letters differ significantly according to Tukey’s post hoc test at 5%.

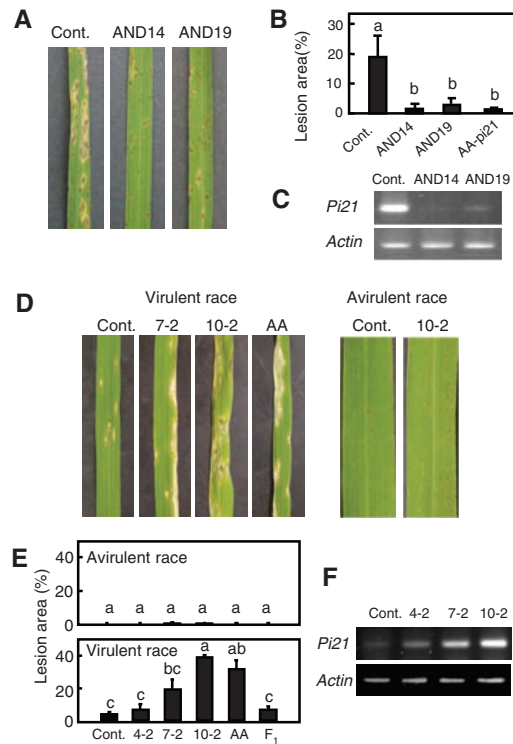
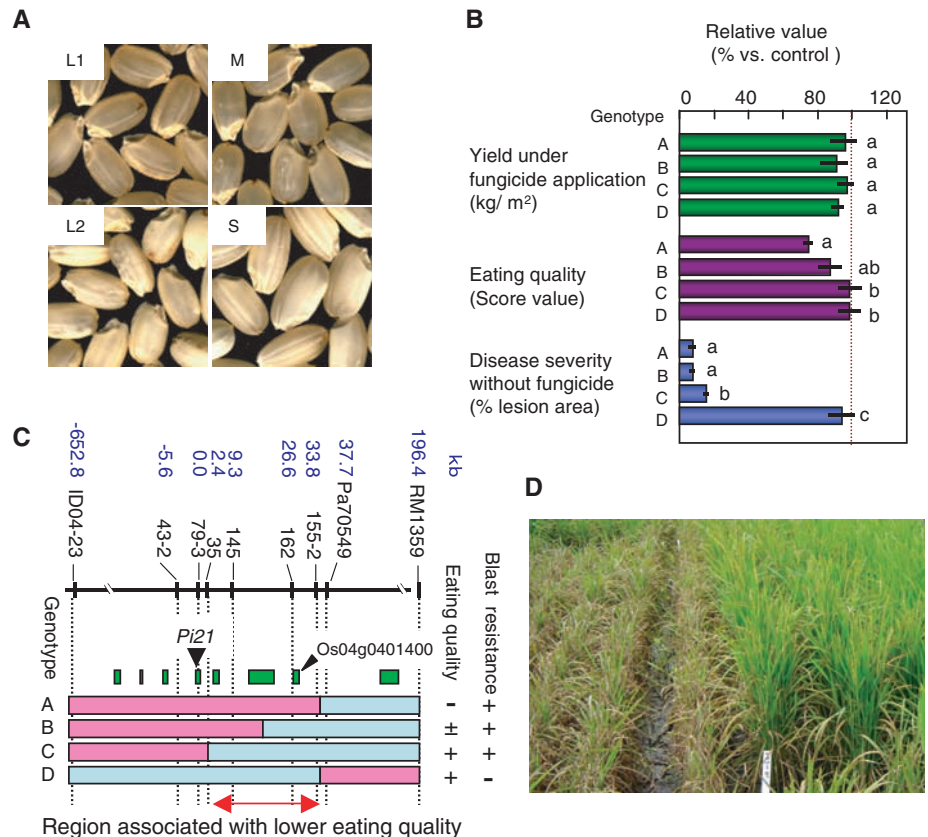


Fig. 4. Successful introduction of resistant *pi21* allele into an elite cultivar. (A) Hulled seed of backcrossed line carrying susceptible *Pi21* allele (L1) and resistant *pi21* allele (L2) in the genetic background of elite rice cultivar Mineasahi. L2 and the donor parent (S) show poor grain quality, which is characterized by a chalky appearance as compared with that of L1 and the recurrent parent (M). (B) Agronomic traits of progeny; the chromosomal structure of each genotype is shown in (C). Bars followed by the same letters are not significantly different according to Tukey’s post hoc test at 5%. (C) Map of genomic region associated with eating quality. Physical distance from the *Pi21* locus is shown in kilobases. Pink indicates chromosomes from donor (Sensho); blue indicates chromosomes from recipient elite cultivar (Koshihikari). Phenotype classes are indicated as + (favorable), – (not favorable), and ± (intermediate) and are based on the data in (B). The green boxes indicate positions of genes in RAP-DB. (D) Field trial showing severe infection by blast. (Left) Recurrent parent Koshihikari. (Right) Line carrying the resistant *pi21* allele with improved eating quality (genotype C).



has a role in early response to a virulent race (fig. S7B). Cytoplasmic localization of resistant and susceptible Pi21–green fluorescent protein (GFP) fusion protein accorded with the absence of a nuclear localization signal in the gene sequences (fig. S8).

We introduced the resistant *pi21* allele into an elite cultivar in order to see its effect on other agronomic traits. The plants carrying *pi21* showed undesirable grain characteristics (Fig. 4A and fig. S9), confirming the strong association between blast resistance and poor eating quality that might be contributed by the lower stickiness and hardness of cooked rice. The eating quality of plants carrying the elite cultivar's chromosomal sequence from a point less than 2.4 kb downstream of the *Pi21* locus was equivalent to that of the elite cultivar, and the plants showed a high level of blast resistance (Fig. 4, B to D). In contrast, plants carrying the donor chromosomal sequence up to 37 kb downstream of the *Pi21* locus showed inferior eating quality (Fig. 4, B and C); this sequence carries a gene, Os04.g0401400 (LOC_Os04.g32890), that is highly expressed only at seed maturation, according to the Rice Functional Genomic Express Database. These results clearly show that the resistant *pi21* allele does not penalize agronomic traits and that the cause of the association is tight linkage with genes that cause undesirable effects. A promising line with improved blast resistance (genotype C in Fig. 4) has been evaluated in the field at several locations. Negative regulators of defense, such as *pi21*, may reduce yield owing to constitutive activation of defense responses and

other secondary effects, as barley *Mlo* does (10, 18). However, our data imply that the slow induction of defense by *pi21* will contribute to pathogen control without penalty on yield and other agronomic traits.

Incompleteness, as well as non–race specificity, may be a component of durable resistance. Two QTLs for disease resistance cloned so far in wheat confer resistance that is dependent on temperature or growth stage (5, 6). The response in resistant *pi21* plants after pathogen attack is not as fast or as strong as the *R* gene response. This slower induction of defense may be another type of incompleteness that may contribute to the durability of a plant's resistance. The durability of a resistance gene needs to be proved when cultivars carrying that gene alone maintain prolonged resistance under natural field conditions (19). Monitoring of a newly released cultivar obtained from our study will provide further evidence to confirm or deny the durability of *pi21*-mediated resistance.

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Bcl6 Mediates the Development of T Follicular Helper Cells

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A fundamental function of CD4⁺ helper T (T_H) cells is the regulation of B cell–mediated humoral immunity. Development of T follicular helper (T_{FH}) cells that provide help to B cells is mediated by the cytokines interleukin-6 and interleukin-21 but is independent of T_H1, T_H2, and T_H17 effector cell lineages. Here, we characterize the function of Bcl6, a transcription factor selectively expressed in T_{FH} cells. Bcl6 expression is regulated by interleukin-6 and interleukin-21. Bcl6 overexpression induced T_{FH}-related gene expression and inhibited other T_H lineage cell differentiation in a DNA binding–dependent manner. Moreover, Bcl6 deficiency in T cells resulted in impaired T_{FH} cell development and germinal center reactions, and altered production of other effector T cell subsets. Our data thus illustrate that Bcl6 is required for programming of T_{FH} cell generation.

A critical function of CD4⁺ helper T (T_H) cells is to provide “help” to B cells, especially in the germinal center structures where activated B cells proliferate and undergo

antibody affinity maturation. Recently, T follicular helper (T_{FH}) cells have been characterized by their expression of chemokine (C-X-C motif) receptor 5 (CXCR5) (1–3). We, as well as others, recently reported that T_{FH} cell development is mediated by interleukin (IL)–6 or IL–21 but is independent of T_H1, T_H2, and T_H17 cells (4, 5).

The B cell lymphoma 6 (Bcl6) transcription factor is selectively expressed by T_{FH} cells (2, 3). Bcl6 was previously shown to be inhibitory to

T_H2 responses by blocking signal transducer and activator of transcription 6 (STAT6) binding to DNA (6, 7), whereas Bcl6-deficient mice developed multiorgan inflammatory diseases, enhanced immunoglobulin E (IgE) production, and defective germinal center reaction (6, 8). It is not clear whether the germinal center defect in these mice is caused by lack of proper T and/or B cell function because Bcl6 is also expressed by germinal center B cells (9). To analyze the function of Bcl6 in T_{FH} cells, we activated naïve CD4⁺ T cells (CD44^{low}CD62L^{high}CD25[–]) from C57BL/6 mice with antibodies to CD3 and CD28 in the presence or absence of various cytokines for 1 or 2 days, and Bcl6 mRNA expression was assessed by real-time reverse transcription polymerase chain reaction (RT-PCR) analysis (10). Treatment with IL-6 or IL-21 significantly up-regulated Bcl6 expression, which was strongly inhibited by the addition of exogenous transforming growth factor beta (TGFβ) (Fig. 1A). These results correlate with our previous observations that IL-6 or IL-21 alone induces T_{FH} cell development and Bcl6 expression, whereas treatment, together with TGFβ, promotes T_H17 differentiation instead (4). To determine whether IL-21 is necessary for IL-6–induced Bcl6 expression, we activated naïve wild-type and IL-21– or IL-21 receptor (IL-21R)–deficient CD4⁺ T cells in the presence of IL-6. IL-21– and

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