Correlation of Genomic Compartments with Contrastive Modes of Functional Losses of Host Specificity Determinants During Pathotype Differentiation in *Pyricularia oryzae*

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The specificity between pathotypes of *Pyricularia oryzae* and genera of gramineous plants is governed by gene-for-gene interactions. Here, we show that avirulence genes involved in this host specificity have undergone different modes of functional losses dependent on or affected by genomic compartments harboring them. The avirulence of an *Eleusine* pathotype on wheat is controlled by five genes, including *PWT3*, which played a key role in the evolution of the *Triticum* pathotype (the wheat blast fungus). We cloned another gene using an association of its presence or absence with pathotypes and designated it as *PWT6*. *PWT6* was widely distributed in a lineage composed of *Eleusine* and *Eragrostis* isolates but was completely absent in a lineage composed of *Lolium* and *Triticum* isolates. On the other hand, *PWT3* homologs were present in all isolates, and their loss of function in *Triticum* isolates was caused by insertions of transposable elements or nucleotide substitutions. Analyses of whole-genome sequences of representative isolates revealed that these two genes were located in different genomic compartments; *PWT6* was located in a repeat-rich region, while *PWT3* was located in a repeat-poor region. These results suggest that the course of differentiation of the pathotypes in *P. oryzae* appears to be illustrated as processes of functional losses of avirulence genes but that modes of the losses are affected by genomic compartments in which they reside.

Keywords: avirulence factors, fungal effectors, *PWT3*, *Pyricularia oryzae*

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Sequence data for the genes described in the present study can be found in the GenBank/EMBL database under accession numbers LC574008 for *PWT6*, LC574010 for *PWT6^{Era}*, and LC574009 for *PWT6^{Ory}*.

*The e-Xtra logo stands for “electronic extra” and indicates there is supplementary material published online.

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Filamentous plant pathogens secrete small proteins called effectors, which promote their colonization of host tissues by manipulating host cells, repressing host defense reactions, or shielding themselves (Sánchez-Vallet et al. 2018). Recent accumulation of whole-genome sequences of filamentous plant pathogens led to the two-speed genome model, in which they have a bipartite architecture (Dong et al. 2015). One compartment comprises gene-rich and repeat-poor regions that evolve slowly while the other comprises gene-poor and repeat-rich regions that evolve rapidly (Seidl and Thomma 2017). Effector genes tend to be located in the latter compartment, in which abundant repetitive sequences cause frequent chromosomal rearrangements, such as translocation, duplication, or deletion of genes, which facilitate adaptive evolution of pathogens (Dong et al. 2015; Seidl and Thomma 2017). Against these pathogens, host plants evolved resistance genes whose products recognize effectors directly or indirectly to induce defense reactions. Such effectors are called avirulence factors (Fouché et al. 2018) encoded by AVR genes. Gene-for-gene interactions between AVR genes and resistance genes (Flor 1956) govern not only race-cultivar specificity but also some taxonomically higher levels of specificity, such as the forma specialis–genus specificity in powdery mildews of cereals (Bourras et al. 2019; Matsumura and Tosa 1995; Tosa 1989, 1994). The AVR genes involved in this specificity are considered to be host specificity determinants. Tosa (1992) proposed a model to illustrate the course of evolution of the formae speciales as processes of functional losses of AVR genes. However, it has not been elucidated which compartments these host specificity determinants reside in or what kinds of functional losses they suffered.

Blast diseases of monocots are caused by several genera of family Pyriculariaceae (Klaubauf et al. 2014). The most familiar genus, *Pyricularia*, includes some cryptic species that are morphologically indistinguishable (Hirata et al. 2007; Klaubauf et al. 2014). *Pyricularia oryzae* (syn. *Magnaporthe oryzae*), the most prevailing species among them, is composed of several pathotypes such as *Oryza*, *Setaria*, *Eleusine*, and *Lolium* pathotypes, which are pathogenic on rice, foxtail millet, finger millet, and ryegrass, respectively (Kato et al. 2000; Tosa et al. 2004). The host range of each pathotype is roughly restricted to a plant genus. This pathotype-genus specificity is governed by gene-for-gene interactions (Takabayashi et al. 2002), implying that the evolutionary course of pathotype differentiation may be revealed by tracing dynamics of AVR genes involved in the specificity.

The wheat blast fungus is a new pathotype (*Triticum* pathotype) (Cruz and Valent 2017) that emerged in 1985 (Urashima et al. 1993). Since its first emergence in Brazil, this devastating

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pathogen has spread to neighboring countries in South America, such as Bolivia, Paraguay, and Argentina, and caused a severe decrease of wheat production (Ceresini et al. 2018). In 2016, it spread to Asia and caused a severe outbreak of wheat blast in Bangladesh (Callaway, 2016; Islam et al. 2016; Malaker et al. 2016). In 2018, it caused an outbreak of wheat blast in Zambia (Tembo et al. 2020). This was the first report of this disease in Africa. To elucidate processes of evolution of the *Triticum* pathotype, we first focused on avirulence of *Lolium* and *Avena* isolates (isolates from genera *Lolium* and *Avena*, respectively) on common wheat because they are phylogenetically very close to *Triticum* isolates (Gladieux et al. 2018). Inoue et al. (2017) found that the resistance of common wheat to *Lolium* isolates is mainly controlled by *Rwt3*, which recognizes *PWT3*, an AVR gene shared by *Lolium* isolates. Based on their distributions in the host and pathogen populations and historical data on wheat cultivation in Brazil, they concluded that the *Triticum* pathotype emerged due to widespread deployment of one or more wheat cultivars lacking *Rwt3* and subsequent functional losses of *PWT3* caused by insertions of transposable elements or nucleotide substitutions.

Secondly, we focused on avirulence of *Eleusine* isolates on common wheat, because they have been considered to maintain characteristics of a common ancestor of *P. oryzae*, such as high fertility (Kato et al. 2000). The *Eleusine* pathotype consists of EC-I (pathogenic on finger millet and weeping lovegrass) and EC-II (pathogenic on finger millet alone) (Asuke et al. 2020b). Asuke et al. (2020a) crossed an EC-II isolate (MZ5-1-6) with a *Triticum* isolate (Br48), produced 80 F₁ cultures, and inoculated common wheat cultivars Norin 4 (N4), Chinese Spring (CS), and Hope with those cultures. A segregation analysis revealed that the avirulence of MZ5-1-6 on Hope (lacking *Rwt3*) and N4 and CS (carrying *Rwt3*) was conditioned by two and (at least) five genes, respectively. The two AVR genes detected on Hope were commonly involved in the avirulence on N4 and CS. These two genes were tentatively designated as *eA1* and *eA2*, while the three genes detected on N4 and CS but not on Hope were designated as *eA3*, *eA4*, and *eA5*. One of the three AVR genes in the latter group proved to be *PWT3* and was assigned to *eA3*.

In the present study, we cloned *eA5* and designated it as *PWT6*. In contrast to *PWT3*, *PWT6* was completely absent in *Triticum* isolates and their relatives, suggesting that *PWT6* has lost its function through deletions of entire fragments during the course of pathotype differentiation in *P. oryzae*. These contrastive modes of functional losses in *PWT3* and *PWT6* were inferred to be associated with characteristics of the genomic compartments they resided in; *PWT3* was located in a repeat-poor region while *PWT6* was located in a repeat-rich region.

RESULTS

Cloning of *PWT6*.

Yoshida et al. (2016) sequenced the whole genome of Z2-1 (accession number DRR059888), an EC-II *Eleusine* isolate collected in Japan, and predicted 1,796 genes encoding signal peptides as candidates for effector genes. Of these putative genes, 12 genes showed presence/absence polymorphisms between Z2-1 and *Triticum* isolate Br48 (Yoshida et al. 2016). These genes were subjected to Southern analysis with a panel of 75 *P. oryzae* isolates including various pathotypes (Supplementary Table S1). A host specificity gene controlling avirulence of *Eleusine* isolates on common wheat may be present in all *Eleusine* isolates and absent in all *Triticum* isolates. *NM11*, one of the candidates for effector genes, showed this type of distribution (Fig. 1).

A 946-bp fragment containing *NM11* and its putative promoter was amplified from genomic DNA of MZ5-1-6, an EC-II *Eleusine* isolate, with NM11 forward and reverse primers (Fig. 2A;

Supplementary Table S2) and was inserted into pBluescript SK(+). The recombinant plasmid obtained was introduced into Br48 through cotransformation with a plasmid carrying a hygromycin-resistance gene. Of 12 transformants carrying the transgene, nine gained avirulence on N4 and CS without losing virulence on Hope. One (number 15) of the nine transformants is shown as an example in Figure 2B. This result indicates that *NM11* conditions the avirulence of MZ5-1-6 on N4 and CS. We designated this gene as *PWT6* (accession number LC574008). At the stage of prediction, *NM11* was supposed to contain two exons and one intron. To confirm this structure, primary leaves of barley cultivar Nigra (Ngt), which is susceptible to all pathotypes of *P. oryzae* (Hyon et al. 2012), were inoculated with MZ5-1-6, and transcripts were extracted from the infected leaves 30 h after inoculation. A comparison of their complementary DNA (cDNA) sequences with the whole-genome sequence of MZ5-1-6 (Gómez Luciano et al. 2019) revealed that *PWT6* had two exons and one intron, as predicted (Fig. 2A). The putative *PWT6* protein consisted of 83 amino acids and contained a signal peptide (Fig. 2C).

As mentioned above, the avirulence of MZ5-1-6 on common wheat is conditioned by five genes. Among them *eA1* and *eA2* are recognized by both N4 and CS and by Hope, while *eA3* (*PWT3*), *eA4*, and *eA5* are recognized by N4 and CS but not by Hope (Asuke et al. 2020a). *PWT6* should be one of the latter three genes because it is recognized by N4 and CS but not by Hope. To check an association of *PWT6* with the three genes, we examined the presence or absence of *PWT6* in the 80 F₁ cultures derived from MZ5-1-6 × Br48 (Asuke et al. 2020a), using NM11-ORF forward and reverse primers (Fig. 2A; Supplementary Table S2). *PWT6* carriers and noncarriers segregated in a 1:1 ratio (38:42, $\chi^2 = 0.20$, $P = 0.66$). Data from 18 F₁ cultures virulent on Hope (noncarriers of *eA1* and *eA2*) are shown in Figure 3. The segregation pattern of *PWT6* was different from those of *eA3* (*PWT3*) and *eA4*. Moreover, the avirulence of 200R30 and 200R79 (lacking *eA3* and *eA4*) on N4 and CS could be explained by the presence of *PWT6* in these cultures. Based on these results, we concluded that *PWT6* is *eA5*.

Distribution of *PWT6* homologs in family Pyriculariaceae.

To clarify the distribution of *PWT6* in family Pyriculariaceae, genomic DNA of 98 isolates (the 75 isolates of *P. oryzae* mentioned above, 11 isolates of other cryptic species of *Pyricularia*, and 12 isolates belonging to other genera in this family) (Supplementary Table S1) were hybridized with a fragment amplified from MZ5-1-6 with NM11-ORF forward and reverse primers. *PWT6* homologs were widely distributed in *P. oryzae* but were sporadic in *Oryza* and *Setaria* pathotypes and absent in *Triticum* and *Lolium* pathotypes (Fig. 1; Supplementary Table S1). They were also found in some isolates belonging to cryptic species of *Pyricularia* but were absent in genera other than *Pyricularia* (Supplementary Table S1). Taken together with results from a homology search in public databases, *PWT6* was considered to be a *Pyricularia*-specific secreted protein.

Functional analyses of *PWT6* homologs in *P. oryzae*.

The *PWT6* homologs detected in the Southern hybridization were characterized further. *Eleusine* isolates are very close to *Eragrostis* isolates (from *Eragrostis* spp. such as weeping lovegrass and lehman lovegrass) (Asuke et al. 2020b). The 75 isolates of *P. oryzae* in the panel (Fig. 1) contained 13 isolates from *Eleusine* spp. and one isolate from *Eragrostis lehmanniana* (Supplementary Table S1). The 13 *Eleusine* isolates were composed of two EC-I isolates (SZEC1-1-1, GFEC1-5-1) and 11 EC-II isolates (the others including MZ5-1-6). In the Southern analysis, the 11 EC-II isolates shared an approximately 6-kb fragment (Fig. 1). Sequence analysis revealed that all these fragments of the EC-II isolates contained *PWT6*, which was 100%

identical to that of MZ5-1-6 at the nucleotide level. On the other hand, the two EC-I isolates shared an approximately 7-kb fragment with the *Eragrostis* isolate (Fig. 1). Sequence analysis revealed that these fragments contained the same *PWT6*

homolog (tentatively designated as *PWT6^{Era}*, accession number LC574010), with two nucleotide substitutions compared with the authentic *PWT6* of MZ5-1-6. These nucleotide substitutions caused two amino-acid substitutions (Fig. 2C). To check its

Fig. 1. Distribution of *NM11* in 75 isolates of *Pyricularia oryzae*. Genomic DNA was digested with *SalI* and was electrophoresed, blotted, and hybridized with a *NM11*-open reading frame (ORF) fragment amplified with *NM11*-ORF forward and reverse primers.

function as an AVR gene, a fragment containing *PWT6^{Era}* was amplified from UG77-15-1-1, which had been used as a representative of EC-I *Eleusine* isolates (Asuke et al. 2020b), with PWT6hEra forward and reverse primers (Supplementary Table S2). The amplicon was cloned into pBluescript SK(+) and was introduced into Br48 through cotransformation. Of six transformants carrying the transgene, five gained avirulence on N4 and CS without losing virulence on Hope. One (number 7) of the five transformants is shown as an example in Figure 2B. This result indicates that *PWT6^{Era}* maintains its function in spite of the two amino-acid substitutions.

Among the 30 *Oryza* isolates in the panel, 22 isolates had positive fragments with various sizes (Fig. 1). *PWT6* homologs were

amplified from Ina168 collected in Japan and PO12-7301-2 collected in Indonesia. Sequence analysis revealed that these two *Oryza* isolates shared the same *PWT6* homolog (tentatively designated as *PWT6^{Ory}*, accession number LC574009) with 20 nucleotide substitutions compared with the authentic *PWT6* of MZ5-1-6. These nucleotide substitutions caused 14 amino-acid substitutions (Fig. 2C). To check its function as an AVR gene, a fragment containing *PWT6^{Ory}* was amplified from PO12-7301-2 with PWT6hO forward and reverse primers (Supplementary Table S2), was cloned into pBluescript SK(+), and was introduced into Br48 through cotransformation. Of eight transformants carrying the transgene, seven gained avirulence on both N4 and CS without losing virulence on Hope. One (number 16) of the seven

A

B

C

PWT6	M	Q	L	S	K	I	Y	L	L	A	L	W	A	T	G	A	T	A	L	F	S	E	E	E	N	L	H	K	I	K	[30]		
PWT6 ^{Era}	M	Q	L	S	K	I	Y	L	L	A	L	W	A	T	G	A	T	A	L	F	S	E	E	E	N	L	H	K	I	K	[30]		
PWT6 ^{Ory}	M	Q	L	S	K	I	Y	L	L	A	L	W	A	T	G	A	T	A	L	F	S	E	E	E	N	L	H	M	I	K	[30]		
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PWT6	D	G	E	I	P	P	D	T	K	D	Y	R	G	K	P	L	F	K	L	P	G	N	A	H	C	Y	L	I	R	H	[60]		
PWT6 ^{Era}	N	G	E	I	P	P	D	T	K	D	Y	R	G	K	P	L	F	K	L	P	G	N	A	H	C	Y	L	I	R	H	[60]		
PWT6 ^{Ory}	N	G	E	I	P	P	D	T	K	D	Y	H	G	N	P	L	L	K	L	P	G	N	A	H	C	Y	L	I	R	H	[60]		
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PWT6	A	E	F	C	D	K	I	R	S	C	V	W	V	P	P	L	G	C	R	V	K	D	Y	*	[83]								
PWT6 ^{Era}	A	E	F	C	D	K	I	R	T	C	V	W	V	P	P	L	G	C	R	V	K	D	Y	*	[83]								
PWT6 ^{Ory}	P	Q	F	C	D	K	I	I	P	C	V	W	A	G	P	L	G	C	I	I	Q	D	Y	*	[83]								

Fig. 2. Structure and function of *PWT6* and its homologs. **A**, Structure of *NM11*. Black arrowheads represent primers. **B**, Complementation tests of *PWT6* (*NM11*) and its homologs. Eight-day-old primary leaves of three wheat cultivars (Hope, Norin 4 [N4], Chinese Spring [CS]) were inoculated with *Eleusine* isolate MZ5-1-6, *Triticum* isolate Br48 (WT), and its transformants carrying an empty vector (+EV), *PWT6* (+*NM11*), *PWT6^{Era}* (+*NM11* Era type), and *PWT6^{Ory}* (+*NM11* O type). Barley cultivar Nigrate (NgT) was used as a susceptible control. Phenotypes were evaluated 4 days after inoculation. **C**, Amino acid alignments of the three types of *PWT6* in *Pyricularia oryzae*. The predicted signal peptide is indicated by a blue bar.

transformants is shown as an example in Figure 2B. This result indicates that *PWT6^{Ory}* is also recognized by N4 and CS. However, they produced some sporadic lesions (Fig. 2B), suggesting that its amino-acid substitutions gave some negative effects on the strength of recognition.

Comparison of the distribution of *PWT6* and *PWT3* in family Pyriculariaceae.

Now, we have two host-specificity determinants, *PWT6* and *PWT3*, that condition the avirulence of *Eleusine* isolates on wheat. Comparison of these genes may reveal common characteristics or variation in *AVR* genes involved in the host genus specificity. To clarify the distribution of *PWT3* in family Pyriculariaceae, the membrane with genomic DNAs of the 98 isolates mentioned above was reprobated with a fragment amplified from *Avena* isolate Br58 with *PWT3_CDS* forward and reverse primers (Supplementary Table S2). *PWT3* signals were detected in all isolates of *P. oryzae* and *P. grisea* (Supplementary Table S1), suggesting that *PWT3* is more stable than *PWT6*.

To compare their distribution further, we downloaded whole-genome sequences of *Oryza* isolates from databases and checked the presence/absence of *PWT6* and *PWT3*. Among the 146 *Oryza* isolates examined, 87 isolates (approximately 60%) carried *PWT6* homologs (Supplementary Table S3) that were almost identical to *PWT6^{Ory}* in nucleotide sequences. The other 59 isolates lacked *PWT6* homologs. On the other hand, *PWT3* homologs were detected in all the 146 isolates (Supplementary Table S3). These results again suggest that *PWT3* is more stable than *PWT6*.

Inoue et al. (2017) classified *PWT3* homologs of *P. oryzae* and *P. grisea* isolates into several types (A, B, C, and D) based on amino-acid sequences of their open reading frame (ORF). The A type was further divided into the Ao type (functional), the Ao' type (functional) with one base substitution in the upstream region, the Atm type (nonfunctional) with an insertion of a LINE-like element, MGR583 (Hamer et al. 1989), into the upstream region, the Atc type (nonfunctional) with a complex

insertion of Pyret and RETRO5 (Farman 2002) into the ORF, and the Atp type (nonfunctional) with an insertion of an inverted repeat transposon, Pot3 (Farman et al. 1996), into the upstream region. A homolog detected in an *Oryza* isolate (Ina168) was the C type, which had seven amino-acid substitutions compared with the functional Ao type detected in *Lolium*, *Avena*, and *Eleusine* isolates (Inoue et al. 2017). All the 146 *Oryza* isolates mentioned above carried the C type *PWT3* with a 100% identical nucleotide sequence. To check if the C type is functional, primary leaves of Ngt were inoculated with PO12-7301-2 (carrying the C type) or MZ5-1-6 (carrying the Ao' type as a control) and transcripts were extracted from the infected leaves. A quantitative PCR (qPCR) analysis revealed that the C type *PWT3* in PO12-7301-2 was not expressed even 48 h after inoculation (Supplementary Fig. S1). In the upstream region of the ORF of the C type *PWT3* in PO12-7301-2, a long terminal repeat of retrotransposon Pyret (Nakayashiki et al. 2001) was detected (Supplementary Fig. S2), suggesting that this insertion disrupted the promoter of the C type in this isolate.

Evolutionary dynamics of *PWT6* and *PWT3*.

To reveal evolutionary dynamics of *PWT6* and *PWT3*, a maximum likelihood tree of 33 representative isolates (Supplementary Table S4) was constructed, using single nucleotide polymorphisms (SNPs) in their whole-genome sequences (Fig. 4). Its topology was consistent with the previous reports (Asuke et al. 2020b; Gladieux et al. 2018) and suggested that the *P. oryzae* population first differentiated into two groups, i.e., one composed of *Oryza* and *Setaria* isolates (OS group) and the other composed of *Eragrostis*, *Eleusine*, *Lolium*, *Triticum* isolates (TELE group). An *Avena* isolate, Br58, was clustered with *Lolium* isolates. Then, the TELE group differentiated into a lineage containing *Eragrostis* and *Eleusine* isolates (EE lineage) and another containing *Lolium* and *Triticum* isolates (LT lineage). *PWT6* and *PWT3* homologs were plotted on this tree with color codes showing their function (functional or nonfunctional) as avirulence genes. Their function was inferred from nucleotide sequences and results from complementation tests or expression assays. For example, a *PWT6* homolog in Guy11 (*Oryza* isolate) was inferred to be functional because nucleotide sequences of its ORF and putative promoter region were identical to those of *PWT6^{Ory}* in PO12-7301-2. *PWT6* homolog in NRS12-2-2 (*Setaria* isolate) was not determined to be functional or nonfunctional because its nucleotide sequence was split into two contigs.

The distribution of *PWT6* in the OS group was sporadic; the *PWT6* homolog (*PWT6^{Ory}*) was present in one isolate but absent in another (Fig. 4). In the TELE group, *PWT6* or the *PWT6* homolog (*PWT6^{Era}*) were ubiquitous in the EE lineage but completely absent in the LT lineage.

By contrast, *PWT3* homologs were ubiquitous irrespective of the groups or lineages. As mentioned above, the C type *PWT3* in PO12-7301-2 (*Oryza* isolate) was not expressed and was, therefore, considered to be nonfunctional as an *AVR* gene. The C type *PWT3* was shared not only by the other *Oryza* isolates but also by *Setaria* isolates (Supplementary Fig. S2). Moreover, Pyret or its remnants were recognized in their upstream regions as in PO12-7301-2 (Supplementary Fig. S2). These results suggest that these *PWT3* homologs in the OS group are nonfunctional. In the TELE group, most isolates in the EE lineage carried the functional types (Ao or Ao'). *Avena* and *Lolium* isolates in the LT lineage also carried the functional type (Ao). From this lineage *Triticum* isolates evolved through the loss of function of *PWT3* caused by insertion of transposable elements (in the Atm, Atc, and Atp types) or a gain of a chromosomal fragment containing *PWT3* with base substitutions (the B type) (Inoue et al. 2017). The Atm and Atc types may also have been introduced into the ancestral population of *Triticum* isolates

No.	F ₁	Phenotype		Genotype		
		Hope	N4/CS	<i>PWT3</i> (<i>eA3</i>)	<i>eA4</i>	<i>PWT6</i> (<i>eA5</i>)
1	200R35	V	A	+	+	+
2	200R71	V	A	+	+	-
3	200R73	V	A	+	-	+
4	200R22	V	A	+	-	-
5	200R2	V	A	+	-	-
6	200R52	V	A	+	-	-
7	200R20	V	A	-	+	+
8	200R17	V	A	-	+	+
9	200R7	V	A	-	+	-
10	200R72	V	A	-	+	-
11	200R9	V	A	-	ND	-
12	200R30	V	A	-	-	+
13	200R79	V	A	-	-	+
14	200R29	V	V	-	-	-
15	200R16	V	V	-	-	-
16	200R31	V	V	-	-	-
17	200R51	V	V	-	-	-
18	200R54	V	V	-	-	-

Fig. 3. Segregation of phenotypes (avirulence [AVR] on wheat cultivars Hope, Norin 4 [N4], Chinese Spring [CS]) and genotypes at three AVR loci in F₁ cultures derived from a cross between MZ5-1-6 and Br48. Eighteen cultures virulent on Hope were extracted from a total of 80 F₁ cultures. A = avirulent, V = virulent, + = the MZ5-1-6 type, - = the Br48 type, ND = not determined. Data on phenotypes and genotypes at the *eA3* and *eA4* loci is from Asuke et al. (2020a).

through gains of chromosomal fragments containing them. These functional losses of *PWT3* were necessary for an adaptation to common wheat carrying its corresponding resistance gene, *Rwt3*.

Comparison of genomic regions harboring *PWT6* and *PWT3*.

One prominent difference of the distribution of these genes is that *PWT6* shows presence/absence polymorphism while *PWT3* does not. Why these AVR genes show such a contrastive distribution in spite of being involved in the same incompatibility, i.e., the avirulence of *Eleusine* isolates on wheat cultivars N4 and CS? To answer this question, the genomic region harboring *PWT6* was extracted from the whole-genome sequence of MZS-1-6 (Gómez Luciano et al. 2019). *PWT6* was located in a

telomeric, repeat-rich region of chromosome 3 (Fig. 5). Comparison with the whole-genome sequence of *Triticum* isolate B71 (Peng et al. 2019) revealed that B71 did not have a syntenic region but had its own specific region in the corresponding telomere of chromosome 3 (Fig. 5). On the other hand, *PWT3* was located on an internal, repeat-poor region of chromosome 5 in both isolates (Fig. 5). Similar results were obtained from whole-genome sequences of other isolates in databases (Supplementary Figs. S3 and S4). These results suggest that *PWT6* and *PWT3* are located in different compartments of the two-speed genome of *P. oryzae*; *PWT6* is in a repeat-rich region, while *PWT3* is in a repeat-poor region. It should be noted that a transition from a repeat-poor region to a repeat-rich region seems to have started in the *PWT3* compartment in some isolates, such as *Setaria* isolate US71 (Supplementary Fig. S4).

Fig. 4. Distribution of *PWT3* and *PWT6* on a phylogenetic tree of *Pyricularia oryzae*. A maximum likelihood tree was constructed using single nucleotide polymorphisms in whole-genome sequences of representative isolates. The blue arrow indicates the node to the outgroup taxon (*Pyricularia grisea* Dig41) inferred by Asuke et al. (2020b). Numbers at nodes indicate bootstrap values from 100 replications. Functional and nonfunctional homologs are shown in red and green, respectively. The asterisk indicates a *PWT6* homolog whose function was not determined. *PWT3* symbols (circles) in *Avena*, *Lolium*, and *Triticum* isolates are shown with their types (Ao, Atm, Atc, and B) as reported by Inoue et al. (2017).

Is the loss of *PWT6* in the LT lineage adaptation to perennial ryegrass?

All *Triticum* isolates were noncarriers of *PWT6* (Fig. 1), which is reasonable because they must evade the recognition by common wheat. All *Lolium* isolates were also noncarriers of *PWT6*. This raised a hypothesis that the loss of *PWT6* in *Lolium* isolates might be an adaptation to ryegrass. To test this hypothesis, *PWT6* was introduced into *Lolium* isolate TP1 (L-1J), resulting in seven transformants carrying the transgene. They were all virulent on the two cultivars of perennial ryegrass tested (Supplementary Fig. S5), suggesting that these perennial ryegrass cultivars do not carry a resistance gene recognizing *PWT6*.

DISCUSSION

In the present study, we cloned *PWT6*, a gene involved in the host specificity, using the association of its presence or absence with pathotypes. The association fulfilled a requirement for genes controlling the avirulence of the *Eleusine* pathotype on common wheat—presence in all *Eleusine* isolates and absence in all *Triticum* isolates. On the other hand, *PWT3* was isolated through map-based cloning (Inoue et al. 2017). It should be noted that *PWT3* would not have been isolated using association with presence or absence because its homologs were present in

all *P. oryzae* isolates (Supplementary Table S1). In addition, *PWT3* would not have been isolated using association with functional/nonfunctional types (polymorphisms) because ancestral *Triticum* isolates, which colonized wheat cultivars lacking *Rwt3*, carries the functional Ao type (Fig. 4) (Inoue et al. 2017).

Effector genes are known to be often located in repeat-rich genomic compartments, but not all effector genes are located in such rapidly evolving regions (Fouché et al. 2018). *PWT6* was located in a repeat-rich region while *PWT3* was located in a repeat-poor region (Fig. 5). In addition, the compartment harboring *PWT6* was located on a telomeric region of chromosome 3 of *Eleusine* isolate MZ5-1-6, which had no synteny with a corresponding chromosomal region of *Triticum* isolate B71 (Fig. 5). This appears to be lineage-specific regions (de Jonge et al. 2013; Faino et al. 2016; Seidl and Thomma 2017) or orphan regions (Plissonneau et al. 2016), which have been suggested to act as a source for genetic variation to mediate aggressiveness or virulence (de Jonge et al. 2013). By contrast, the compartment harboring *PWT3* in MZ5-1-6 was located on an internal region of chromosome 5, which had a synteny to a corresponding region of B71 (Fig. 5).

The difference of genomic compartments harboring these genes and their chromosomal locations may have led to the difference of their distribution in the *P. oryzae* population. *PWT6*

Fig. 5. Chromosomal locations of *PWT3* and *PWT6* and structures of genomic compartments harboring them in MZ5-1-6 (*Eleusine* isolate). Corresponding regions in B71 (*Triticum* isolate) are shown for comparison. The red and green bars indicate MZ5-1-6-specific and B71-specific regions, respectively. Gray rectangles represent single-copy genes.

signals were completely absent in *Triticum* and *Lolium* pathotypes (Fig. 1). Furthermore, *PWT6* showed sporadic distribution in *Oryza* and *Setaria* pathotypes (Fig. 1). It is likely that these deletion events have been associated with repetitive elements in the compartment harboring *PWT6*. The course of the *PWT6* loss can also be traced within the *Oryza* isolates listed in Supplementary Table S3. Latorre et al. (2020) found four genetic lineage (I, II, III, and IV) in the rice blast isolates and suggested that group I, mainly located in Southeast Asia, was an ancestral population with genetic diversity for all clonal lineages. In *Oryza* isolates listed in Supplementary Table S3, percentages of *PWT6* carriers were 95.5% (21 of 22) in group I, 59.3% (35 of 59) in group II, 44.2% (19 of 43) in group III, and 71.4% (5 of 7) in group IV. Apparently, *PWT6* was present at a high frequency in the ancestral population but has been lost in the process of independent clonal expansions of groups II, III, and IV.

On the other hand, *PWT3* signals were detected in all *P. oryzae* isolates, as mentioned above (Supplementary Table S1), and the loss of function of *PWT3* for adaptation to common wheat was caused not by its deletion but by nucleotide substitutions or insertion of transposable elements (Inoue et al. 2017). This may be attributable to the stable architecture of the compartment harboring *PWT3* (Fig. 5; Supplementary Fig. S4). An alternative explanation is that *PWT3* may have been very important or indispensable for high virulence of *P. oryzae*. However, this scenario seems unlikely because B71, a highly virulent wheat blast isolate collected in Bolivia, has the Atc type of *PWT3*, in which the *PWT3* function is completely disrupted by complex insertions of transposable elements (Inoue et al. 2017). Recently, a lineage with this type spread to Asia and caused a severe outbreak of wheat blast in Bangladesh (Inoue et al. 2017).

Tosa (1992) proposed a model for the evolution of formae speciales of *Blumeria graminis*. In this model differentiation of the formae speciales was illustrated as processes of (functional)

losses of AVR genes. The differentiation of the pathotypes of *Pyricularia oryzae* may be illustrated similarly. *PWT3* is an example that fit this model. Inoue et al. (2017) suggested that the *Triticum* pathotype evolved through the loss of function of *PWT3*, which was a requirement for full adaptation to wheat (Fig. 6). *PWT6* also appears to have been lost during the course of differentiation of pathotypes. This loss is considered to have been a deletion mediated by repetitive elements in the compartment. Apparently, *PWT6* was lost when the LT lineage branched from the EE lineage (Fig. 6). If this is an adaptation to *Lolium perenne*, this plant species should recognize *PWT6*. However, the two cultivars of perennial ryegrass tested were susceptible to the transformants of a *Lolium* isolate carrying *PWT6* (Supplementary Fig. S5). There seem three possible hypotheses to explain this result. The first one assumes that the loss of *PWT6* at the branching of the LT lineage was an adaptation to *Lolium* spp. but that its corresponding resistance gene has not been fixed in the current population of *Lolium* spp., as is the case of *Pi-CO39(t)* in rice corresponding to *AVR1-CO39* (Tosa et al. 2005). If this is the case, the corresponding resistance gene may be found in other cultivars of *L. perenne* or other species of *Lolium*. The second one assumes that there may be other hosts that forced these isolates to lose *PWT6* for colonizing them. If this is the case, the “other hosts” are likely to be members of subfamily Pooideae, because hosts of the LT lineage are all members of Pooideae. The third one assumes that *PWT6* may have happened to be lost simply due to the repeat-rich nature of the compartment in which it resides.

In summary, the course of differentiation of the pathotypes in *P. oryzae* appears to be illustrated as processes of the loss of function of avirulence genes, but the mode of the functional loss of each AVR gene is affected by the compartment in which it resides. Once the other three genes (*eA1*, *eA2*, and *eA4*) are cloned, the timing and mode of their losses will also be

Fig. 6. A hypothetical model for the evolutionary pathway to the *Triticum* pathotype of *Pyricularia oryzae*. Presence (+) and absence (–) of *PWT3* or *PWT6* homologs are shown on the right.

elucidated, which lead to the illustration of the entire process of evolution from the common ancestor of the TELE group to the wheat blast fungus (*Triticum* isolates). Asuke et al. (2020b) reported an exceptional case in which a gain of an AVR gene led to the differentiation of the EC-II *Eleusine* isolates. Further studies are needed to illustrate the whole process of the pathotype differentiation in *P. oryzae*.

MATERIALS AND METHODS

Fungal materials.

Ninety-eight isolates in family Pyriculariaceae, including 75 *P. oryzae* isolates collected from different host plant species (Supplementary Table S1), were employed. MZ5-1-6, UG77-15-1-1 (Asuke et al. 2020b), and PO12-7301-2 were selected as representative isolates carrying *PWT6*, *PWT6^{Era}*, and *PWT6^{Ory}*, respectively. TP1 and Br48 were chosen as representative isolates of the *Lolium* and *Triticum* pathotypes, respectively, and were used for the transformation assay. The 80 random F₁ cultures derived from MZ5-1-6 × Br48 (Asuke et al. 2020a) were used for the segregation analysis of *PWT6*.

Plant materials.

Three common wheat cultivars, *Triticum aestivum* ‘Hope’, ‘N4’, and ‘CS’, and two perennial ryegrass cultivars, *Lolium perenne* ‘Friend’ and ‘Linn’, were used for infection assays. A barley cultivar, *Hordeum vulgare* ‘Ngt’, was used as a susceptible control.

Distribution analysis of *NM11* by Southern hybridization.

Genomic DNAs were extracted from mycelia grown in complete liquid media as described by Nakayashiki et al. (1999). Extracted DNAs were digested with *SalI* (Takara, Kusatsu, Japan) at 37°C overnight, were electrophoresed on 0.7% agarose gels in Tris-borate-EDTA buffer for 12 to 16 h at 30 V, and were blotted to Hybond N+ membranes (GE Healthcare, Buckinghamshire, U.K.). A 358-bp fragment containing an ORF of *PWT6* was amplified from the genomic DNA of MZ5-1-6 with *NM11*-ORF forward and reverse primers (Supplementary Table S2) and KOD-Plus-Ver.2 (Toyobo, Osaka, Japan), using Mastercycler (Eppendorf, Hamburg, Germany) programmed for 2 min at 94°C, 30 cycles of 10 s at 98°C, and 30 s at 60°C, followed by 20 s at 68°C. The amplicon was cloned into the *EcoRV* site of pBluescript II SK(+) using the DNA ligation kit ver.2.1 (Takara). The membrane was hybridized with the labeled recombinant plasmid, using the ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, Chicago), according to manufacturer instructions.

Transformation of *P. oryzae* with *NM11* (*PWT6*) and its homologs.

Fragments containing *PWT6*, *PWT6^{Era}*, *PWT6^{Ory}* (946, 1,030, and 1,226 bp in size, respectively) were amplified from genomic DNA of MZ5-1-6, UG77-15-1-1, and PO12-7301-2, with primers shown in Supplementary Table S2 and KOD-Plus-Ver.2 (Toyobo), using Mastercycler (Eppendorf) programmed for 2 min at 94°C, 30 cycles of 10 s at 98°C, and 30 s at 60°C, followed by 1 min at 68°C. Each fragment was cloned into pBluescript II SK(+) and was established as pPWT6, pPWT6hEra, and pPWT6hO, respectively. These recombinant plasmids were introduced into Br48 following the polyethylene glycol-mediated cotransformation protocol with pSH75 (carrying a hygromycin-resistance gene), as described by Tosa et al. (2005). pPWT6 was also introduced into TP1. Copy numbers of *PWT6* in transformants were confirmed by Southern hybridization using the *PWT6*-ORF probe.

Pathogenicity assay.

Common wheat seeds were sown in vermiculite supplied with liquid fertilizer in a seeding case (5.5 × 15 × 10 cm) and were grown at 22°C in a controlled-environment room with a 12-h photoperiod of fluorescent lighting for 7 to 8 days. Primary leaves of the seedlings were fixed onto a plastic board with rubber bands just before inoculation. Conidial suspensions (1 × 10⁵ conidia per milliliter) containing 0.01% Tween20, prepared as described previously (Tagle et al. 2015), were sprayed on primary leaves using an air compressor. The inoculated seedlings were incubated at 22°C for 24 h in the dark in high humidity trays, were then transferred to a dry condition with a 12-h photoperiod of fluorescent lighting at 22°C. From 4 to 6 days after inoculation, symptoms were evaluated on the basis of the size and color of lesions. The size was rated on six grades from 0 to 5: 0 = no visible lesions, 1 = pinhead spots, 2 = small lesions (<1.5 mm), 3 = scattered lesions of intermediate size (<3 mm), 4 = large typical lesion, and 5 = complete shriveling of leaf blades. Infection types comprised a number indicating the lesion size and a letter denoting the lesion color, ‘B’ for brown and ‘G’ for green. Infection types 0 to 5 with B were considered resistant, whereas infection types 3 to 5 with G were considered susceptible.

Seeds of *Lolium perenne* ‘Friend’ and ‘Linn’ were sown in plastic pots filled with Sakata Primemix soil (Sakata Co., Yokohama, Japan) and were grown at 23°C in a growth chamber with a 12-h photoperiod of fluorescent lighting for 20 days. All leaves (the first, second, and third leaves) of the seedlings were fixed onto a plastic board with rubber bands just before inoculation. Preparation of inocula and inoculation were performed as described above. Symptoms were evaluated following the scale described by Tosa et al. (2004): 0 = no visible reaction, 1 = pinhead-sized spots, 2 small brown lesions with no distinguishable center, 3 = intermediate lesions with a gray-green center, 4 = large lesions with a gray-green center, and 5 = complete shriveling of leaf blades. All infection assays were conducted at least twice. Infection types 0 to 3 were considered resistant whereas infection types 4 and 5 were considered susceptible.

Analysis of the exon-intron structure.

To determine the exon-intron structure of *PWT6*, primary leaves of Ngt were inoculated with MZ5-1-6, UG77-15-1-1, and PO12-7301-2, were incubated at 22°C in the dark for 24 h, and were harvested. Total RNA was extracted from these leaves using Sepasol-RNA I Super G (Nakalai Tesque, Kyoto, Japan) and was treated with DNase I Amplification Grade (Thermo-Fisher Scientific, Waltham, MA, U.S.A.). cDNA was synthesized using PrimeScript first-strand cDNA synthesis kit (Takara) according to manufacturer instructions. The exon-intron structure was confirmed by Sangar sequencing with the ABI Prism Big Dye terminator cycle sequencing kit (Applied Biosystems, Foster, CA, U.S.A.) and the ABI 3100 Genetic Analyzer in Kobe University. Raw sequence data were assembled using GeneStudio version 2.2 (available online) and its assemblies were aligned with MEGA version X (Kumar et al. 2018).

Genomic DNA comparison.

To compare genomic regions with *PWT3* and *PWT6*, we used seven whole-genome sequences assembled at the chromosomal level, i.e., Guy11 (Bao et al. 2017), US71 (Win et al. 2019), LpKY97 (Rahnama et al. 2020), MZ5-1-6 (Gómez Luciano et al. 2019), CD156 (Win et al. 2019), B71 (Peng et al. 2019), and BR32 (Win et al. 2019). Approximately 40-kb regions containing *PWT3* and *PWT6* (i.e., 20 kb up- and downstream from their start codons) were extracted from each whole-genome sequence when these AVR genes were detected. Sequence similarity were calculated using the BLASTN program, implemented in

Easyfig version 2.2.3 (Sullivan et al. 2011) with option settings as follows: minimum identity value = 80%; minimum length = 500 bp. Genes and transposons in each genomic sequence were identified by BLASTN (BLAST+2.6.0, <https://www.ncbi.nlm.nih.gov/blast>) using known transposable elements and a gene set of 70-15 (Dean et al. 2005) as a query sequence.

Distribution of *PWT3* and *PWT6* in *Oryza* isolates.

To check the distribution of *PWT3* and *PWT6* in the *Oryza* pathotype of *P. oryzae*, we used total of 146 whole-genome sequence data in public databases listed in Supplementary Table S3. When whole-genome sequence data were raw fastq files, de novo assembly was performed with ABySS assembler version 2.4 (Simpson et al. 2009). Presence/absence polymorphisms of *PWT3* and *PWT6* were confirmed by BLASTN (BLAST+2.6.0).

Phylogenetic analysis.

Whole-genome sequences of 33 isolates (Supplementary Table S4) were used for phylogenetic analysis. Most of them were in databases or reported previously, while one (*Setaria* isolate NRSI2-2-2) was sequenced in the present study. Genomic DNA of NRSI2-2-2 was extracted with the DNeasy plant mini kit (Qiagen, Hilden, Germany). The sequencing library was generated using NEBNext Ultra II DNA library prep kit for Illumina (New England Biolabs, Hitchin, U.K.) and was sequenced using a Illumina NovaSeq 6000 (2 × 150-bp paired-end reads). Sequenced short reads were mapped against the chromosomal assembly of MZ5-1-6 (Gómez Luciano et al. 2019), using BWA version 0.7.17 (Li and Durbin 2010). Alignments were sorted with samtools version 1.9 (Li et al. 2009). The Genome Analysis Toolkit version 4.1.4.1 (McKenna et al. 2010) was used for calling SNPs and indels. The following settings were applied for filtering SNPs: QD < 2.0; QUAL < 30.0; SOR > 4.0; FS > 60.0; MQ < 40.0; MQRankSum < 12.5; ReadPosRankSum < -8.0. Biallelic SNPs were chosen for subsequent phylogenetic analysis. The maximum likelihood tree was constructed using the RAxML-NG version 0.9.0 and was visualized by FigTree version 1.4.4. Alleles of *PWT3* and *PWT6* in each genome was confirmed by BLAST+ 2.6.0.

Expression analysis of *PWT3*.

To verify the expression level of the C type *PWT3* in *Oryza* isolates, comparative expression analysis was performed using real-time qPCR. Eight-day-old primary leaves of NgT were inoculated with MZ5-1-6 (an EC-II *Eleusine* isolate carrying the Ao' type *PWT3*) and PO12-7301-2 (an *Oryza* isolate carrying the C type *PWT3*). At 0, 24, and 48 h after inoculation, inoculated leaves were sampled and subjected to total RNA extraction, using NucleoSpin RNA plant kit (Macherey-Nagel, Düren, Germany). cDNA was synthesized with PrimeScript RT reagent kit (Perfect Real Time, Takara). qPCR tests were performed using Quantstudio 1 (Applied Biosystems) with FastStart Universal SYBR Green Master (Rox) (Roche Diagnostics Ltd, Lewes, U.K.). Exp-*PWT3*- and Exp-Actin forward and reverse primers listed in Supplementary Table S2 were used for qPCR of *PWT3* (target gene) and the actin gene (internal control), respectively. Relative changes in *PWT3* expression values were calculated using the $2^{-\Delta\Delta C_t}$ (ΔC_t) method (Livak and Schmittgen 2001). The average ΔC_t value of MZ5-1-6 at 24 h was chosen as the calibrator.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

BLAST+2.6.0: <https://www.ncbi.nlm.nih.gov/blast>
 FigTree version 1.4.4: <https://github.com/rambaut/figtree>
 GeneStudio version 2.2: <http://www.genestudio.com>
 RAxML-NG version 0.9.0: <https://doi.org/10.5281/zenodo.3066224>

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