Introduction

Closed related species of domesticated crop possesses potentially valuable genetic resources that are not present in the gene pools of cultivated species. Harlan and de Wet (1971) suggested three sources of gene pools, which they called primary (via intraspecific hybridization among cultivated species: GP1), secondary (via interspecific hybridization with closely related compatible species: GP2), and tertiary (via interspecific hybridization with more distantly related species through radical artificial treatment such as embryo rescue: GP3). A breeder has made extensive efforts to exploit wild genetic resources, mainly for pest and disease resistance; more than 80% of the favorable traits conferred by gene transfer from wild species involve resistance to pests and diseases in major crops (reviewed by Hajjar and Hodgkin 2007, Prescott-Allen and Prescott-Allen 1988). Genes that improve drought and salinity tolerance, yield components, and grain and fruit quality also have been introgressed from wild germplasms (Dandan et al. 2007, Lippman et al. 2007, Nevo and Chen 2010, Zhang et al. 2014). However, unfavorable traits or genes, selected out through domestication and breeding, are frequently transferred also. Therefore, the utility of wild germplasm as a genetic resource has both benefits and drawbacks.

Cultivated rice, *Oryza sativa* L., a staple food in much of the world, was domesticated from the ancestral species *O. rufipogon* Griff. The AA genome consist of two cultivated species, *O. sativa* L. and *O. glaberrima* Steud., and six wild species, *O. rufipogon*, *O. nivara* Sharma et Shastry, *O. barthii* A. Chev., *O. longistaminata* A. Chev. et Roehr., *O. glumaepatula* Steud., and *O. meridionalis* Ng (Vaughan et al. 2008). Although chromosome pairing in *F*1 hybrids among AA genome species is normal and gene exchange is possible in hybrid progeny, interspecific hybrids show reproductive isolation. With the progress of Next Generation Sequencing, public databases have rapidly accumulated reference sequences (Reuscher et al. 2018, Sakai et al. 2014, Schatz et al. 2014, Stein et al. 2018), haplotype maps (Alexandrov et al. 2015, Huang et al. 2012, McCouch et al. 2016, Meyer et al. 2016, Wang et al. 2014), and RNA transcription profiles (Childs et al. 2011, Sato et al. 2013, Tian et al. 2015). However, the use of wild genetic sequences in hybrid progeny is hindered by many unfavorable traits including seed dormancy, short-day requirement, lodging, seed shattering at harvest, and various maladaptive phenomena such as hybrid sterility, lethality, and breakdown. So far, genes conferring tolerance or resistance to abiotic and biotic stresses from wild species have been incorporated into cultivated species (Khush 1997, Sanchez et al. 2013). However, the vast array of allelic variations in wild germplasm has not
been exploited to accelerate rice breeding and to deepen our understanding of the genetic architecture of wild species.

A chromosome segment substitution line (CSSL) is a line carrying several chromosome segments derived from a donor parent in the genetic background of a recurrent parent. A full set of CSSLs covers the whole genome. Using CSSLs, we can evaluate minor allelic differences conferred by additive quantitative trait loci (QTLs) in a uniform genetic background. Their high detection power makes it possible to manipulate a QTL as a simple Mendelian factor, subsequently allowing gene isolation by positional cloning. In addition, they offer the potential for favorable genes hidden in the genetic background of related species to be discovered in the genetic background of cultivated species (Arbelaez et al. 2015, Bessho-Uehara et al. 2017, Cheema et al. 2008, Doi et al. 1997, Furuta et al. 2014, Gutiérrez et al. 2010, He et al. 2017, Hirabayashi et al. 2010, Qiao et al. 2016, Ramos et al. 2016, Rangel et al. 2008, Shim et al. 2010, Tian et al. 2006, Yang et al. 2016). Therefore, the genetic resources of related species in GP1 or GP2 could be transferred into the genetic background of cultivated species to form a foundation for studies of genetic variation of closed related rice.

We have created chromosome segment substitution lines (CSSL) of *O. glumaepatula*, designated GLU-ILs, and *O. meridionalis*, designated MER-ILs, using the term ‘introgression lines’ (ILs) to refer to CSSLs based on intraspecific hybridization (Yoshimura et al. 2010). Here we offer new ILs of *O. rufipogon*, *O. nivara*, and *O. glaberrima* in the genetic background of the *O. sativa* ssp. *japonica* type cultivar Taichung 65 (T65). Applications for seed sharing are accepted through Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/).

### Plant materials

The *O. glaberrima*, *O. rufipogon*, and *O. nivara* accessions were kindly provided by the International Rice Research Institute (IRRI), Manila, the Philippines (‘IRGC’ accessions), and the National Institute of Genetics, Mishima, Japan (‘W’ accessions). Line IRGC 103777 (*O. glaberrima*) originated from Mali, IRGC 105715 (*O. nivara*) from Cambodia, and W1962 (*O. rufipogon*) from China. Their derived isolates were respectively designated WK18, WK56, and WK1962. F1 hybrids carrying either T65 or *O. glaberrima* cytoplasm were obtained from reciprocal crosses between T65 and WK18. F1 hybrids carrying T65 cytoplasm were also obtained by pollination with either *O. rufipogon* or *O. nivara* pollen. T65 was used as the recurrent male parent to develop BC1F1, BC2F1, BC3F1, and BC4F1 plants. F1, BC1F1, and BC2F1 plants were grown in pots under short-day treatment (10 h dark, 14 h light) to promote heading and the later generation were grown in paddy field at the Harumachi farm of Kyushu University, Fukuoka, Japan.

### Genotyping

Genomic DNA was extracted from freeze-dried leaves according to Dellaporta et al. (1983) with minor modifications. Simple-sequence-repeat (SSR) markers were used for genotyping of the whole genomic region (Supplemental Tables 1–3). PCR reaction mixtures (15 μL) contained 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 200 μM each dNTP, 0.2 μM each primer, 0.75 units of GoTaq polymerase (Promega), and template DNA (~5 ng) in a GeneAmp PCR system 9700 (Applied Biosystems, CA, USA). Thermal cycling for PCR started with 95°C for 5 min, followed by 35 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. PCR products were run in 4% agarose gel.

**Fig. 1.** Development of introgression lines of *Oryza glaberrima*, *O. rufipogon*, and *O. nivara* in the genetic background of *O. sativa* ssp. *japonica* cv. Taichung 65. (A, B) Breeding of WK18ILs for *O. glaberrima* with (A) T65 and (B) WK18 cytoplasm. (C) Breeding of WK1962ILs for *O. rufipogon*. (D) Breeding of WK56ILs for *O. nivara*.
Introgression lines of AA genome rice species

agarose gels (Amresco, OH, USA) in 0.5× TBE buffer to separate polymorphic DNA bands.

Results and Discussion

**WK18ILs (O. glaberrima)**

We developed 3 BC₁F₁, 23 BC₂F₁, 52 BC₃F₁, and 11 BC₄F₁ lines with T65 cytoplasm from an F₁ of T65 × WK18 by recurrent backcrossing with T65 pollen (Fig. 1A). We similarly developed 2 BC₁F₁, 9 BC₂F₁, 43 BC₃F₁, and 20 BC₄F₁ lines with O. glaberrima cytoplasm from an F₁ of WK18 × T65 (Fig. 1B). To develop the ILs of O. glaberrima in the T65 genetic background (WK18ILs), we conducted whole-genome genotyping using 121 SSR markers evenly distributed across the 12 chromosomes (Supplemental Table 1) with 136 plants of all 31 BC₄F₁ lines from both crosses. From these 31 BC₄F₁ lines, we selected 11 BC₄F₁ plants with T65 cytoplasm and 15 with O. glaberrima cytoplasm so as to select the minimum set of ILs that covers the whole genomic region (Fig. 2A, Supplemental Fig. 1). We grew BC₂F₂, BC₂F₃, and BC₃F₄ plants and genotyped the targeted chromosome regions so as to fix them as homozygous for O. glaberrima (red lines in Fig. 2A and Supplemental Fig. 1). Chromosome segments of O. glaberrima were not retained in the WK18 ILs on Chr. 1 (markers RM246, egt710, RM3709, RM265, RM1361, RM3362), Chr. 4

Fig. 2. Graphical representation of chromosome introgression of Oryza glaberrima, O. rufipogon, and O. nivara in genetic background of O. sativa ssp. japonica cv. Taichung 65. Blue, green, and purple represent introgression of O. glaberrima, O. rufipogon, and O. nivara on homozygous condition. Missing genotypes at markers showing heterozygous genotypes at BC₂F₁ generations are indicated by grey. Heterozygous genotypes are indicated by yellow. The chromosome region for a minimal set of introgression for alien chromosomes indicated in red underlines were target for population maintenances. Blue circles and white circles represent the lines with WK18 and T65 cytoplams, respectively.
(RM567), Chr. 5 (RM3620, RM6346), Chr. 6 (RM5463), or Chr. 7 (RM3394, RM5436, RM1306). The 26 ILs cover 89.3% (108/121 markers) of the O. glaberrima genome. At the distal end of the short arm of Chr. 7 around RM1306, hybrid pollen sterility caused by S2I in heterozygous condition can reduce transmission of O. glaberrima alleles (Doi et al. 1998). This is the likely cause of non-introgression on this region in our results.

**WK1962ILs (O. rufipogon)**

We developed 3 BC1F1, 21 BC2F1, 51 BC3F1, and 58 BC4F1 lines from an F1 of T65 × WK1962 by recurrent backcrossing with T65 pollen (Fig. 1C). At the BC4F2 generation, we conducted whole-genome genotyping using 101 SSR markers (**Supplemental Table 2**) with 113 bulked DNA derived from BC4F1 plants (1 line per BC4F1 plants) to select a minimum set of ILs. To fix target chromosome segments and eliminate retained chromosome segments in the background of the selected lines, we performed marker-assisted selection (MAS) in the BC4F2, BC5F3, and BC6F4 generations. We selected 44 BC4F1 lines, designated ‘WK1962ILs’, with O. rufipogon segments fixed as homozygous (red lines in **Fig. 2B** and **Supplemental Fig. 2**). Chromosome segments of O. rufipogon were not retained in the WK1962 ILs on Chr. 1 (RM3148, RM5552), Chr. 6 (RM7023, RM3567, RM1031), or the short arm of Chr. 12 (RM3483). The 44 ILs cover 94.1% (95/101 markers) of the O. rufipogon genome in the T65 genetic background.

**WK56ILs (O. nivara)**

We developed 1 BC1F1, 5 BC2F1, 20 BC3F1, and 109 BC4F1 lines from an F1 of T65 × WK56 by recurrent backcrossing with T65 pollen. The BC4F1 plants were self-pollinated, and the bulked DNA of BC4F1 line derived from each of BC4F1 plant was genotyped using 107 SSR markers (**Supplemental Table 3**). In the BC4F4 generation we selected 33 lines carrying WK56 chromosome segments in the T65 genetic background, which we designated ‘WK56ILs’. Targeted chromosome segments for O. nivara introgressions were fixed in homozygous condition (red lines in **Fig. 2C** and **Supplemental Fig. 3**). The 33 ILs cover 69.2% (74/107 markers) of the O. nivara genome. Chromosome segments of O. nivara were not retained at many SSR markers on Chrs. 1 (RM272, RM3235, RM6642, RM5385, RM5638, RM3362), 2 (RM7562, RM6853, RM6611, RM5472), 4 (RM3367, RM1735, RM6089, RM3836, RM1113), 5 (RM3695, RM6841), 6 (RM7399), 7 (RM5508), 8 (RM7356, RM6976, RM3155), 10 (RM6370, WGS11, RM1375, RM4771), 11 (RM3717, RM1124, RM4504, RM4112), and 12 (RM3483, RM6296, RM7003). These omissions would have been due to the population bottleneck at BC3F1 (5 lines).

**Distribution of materials via National Bioresource Project (Rice) in Japan**

WK18ILs (O. glaberrima), WK1962ILs (O. rufipogon), and WK56ILs (O. nivara) are available through Oryzabase (https://shigen.nig.ac.jp/rice/oryzabase/).

**Author Contribution Statement**

YY, KTW, YM, CO, and HY conducted development of plant materials and genotyping. AY and YY design the experiment.

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