QTL mapping for pre-harvest sprouting resistance in *japonica* rice varieties utilizing genome re-sequencing

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Abstract
Pre-harvest sprouting (PHS) leads to serious economic losses because of reductions in yield and quality. To analyze the quantitative trait loci (QTLs) for PHS resistance in *japonica* rice, PHS rates on panicles were measured in 160 recombinant inbred lines (RILs) derived from a cross between the temperate *japonica* varieties Odae (PHS resistant) and Unbong40 (PHS susceptible) under two different environmental conditions—field (summer) and greenhouse (winter) environments. Genome re-sequencing of the parental varieties detected 266,773 DNA polymorphisms including 248,255 single nucleotide polymorphisms and 18,518 insertions/deletions. We constructed a genetic map comprising 239 competitive allele-specific PCR and 49 cleaved amplified polymorphic sequence markers. In the field environment, two major QTLs, *qPHS-3FD* and *qPHS-11FD*, were identified on chromosomes 3 and 11, respectively, whereas three major QTLs, *qPHS-3GH*, *qPHS-4GH*, and *qPHS-11GH*, were identified on chromosomes 3, 4, and 11, respectively, in the greenhouse environment. *qPHS-11GH* and *qPHS-11FD* had similar locations on chromosome 11, suggesting the existence of a gene conferring stable PHS resistance effects under different environmental conditions. The QTLs identified in this study can be used to improve the PHS resistance of *japonica* varieties, and they may improve our understanding of the genetic basis of PHS resistance.

Keywords *Japonica* rice · Pre-harvest sprouting · Re-sequencing · Quantitative trait locus

Introduction

Pre-harvest sprouting (PHS) of rice (*Oryza sativa* L.) is a serious economic issue causing significant reductions in grain quality and yield primarily in *japonica* rice-cultivating regions. PHS involves the germination of grains on matured panicles before harvest, and it is typically promoted by frequent rainfall and high temperatures during the grain ripening or harvest season (Tejakhod and Ellis 2018). PHS is closely associated with the degree of seed dormancy, which inhibits seed germination under optimal environmental conditions (Takahashi 1980). Seed dormancy is a complex agronomic trait that is influenced by environmental factors, such as temperature and humidity (Takahashi 1980; Basbouss-Serhal et al. 2016), endogenous hormones, such as abscisic acid (ABA; promotes dormancy), and gibberellin acid (promotes germination) (Graeber et al. 2012; Ye et al. 2015), and some biological tissues, such as the embryo, endosperm, and maternal tissues (Gu et al. 2015). Because high levels of dormancy

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in seeds can cause undesirable results, such as preventing post-harvest germination, the balance between seed dormancy and its release must be maintained to enhance the yield and quality of rice.

The degree of seed dormancy is affected by genetic factors controlling germination (Gu et al. 2003; Li and Foley 1997). Regarding PHS and seed dormancy in cultivated and wild rice, several quantitative trait loci (QTLs) have been reported in the progenies of crosses between japonica and indica varieties (Lin et al. 1998; Miura et al. 2001; Dong et al. 2003; Guo et al. 2004; Wan et al. 2005, 2006; Chen et al. 2006; Jiang et al. 2006; Gao et al. 2008; Ji et al. 2009; Xie et al. 2011; Marzagui et al. 2012; Li et al. 2013; Sasaki et al. 2013; Wang et al. 2014; Lee and Kwon 2015) between indica varieties (Li et al. 2011), between japonica varieties (Fujino et al. 2004; Hori et al. 2010), between weedy rice lines and cultivars (Gu et al. 2004, 2005; Ye et al. 2010; Subudhi et al. 2012), between upland and lowland rice varieties (Mizuno et al. 2018), and between a wild rice (Oryza rufipogon) line and a cultivar (Cai and Morishima 2000). These QTLs were distributed across all 12 chromosomes, and among them, a few genes have been identified via map-based cloning (Ye et al. 2015; Fujino et al. 2008; Sugimoto et al. 2010). qSD1-2 on chromosome 1, which is associated with seed dormancy, was delimited to a 20-kb region containing OsGA20ox2, and the naturally occurring or induced loss-of-function mutations of this gene enhance seed dormancy (Ye et al. 2015). qLTG3-1, a QTL for low-temperature germinability on chromosome 3, was found to encode a protein of unknown function, and it is strongly expressed in the embryo during seed germination (Fujino et al. 2008). It was suggested that qLTG3-1 causes tissue weakening of the epiblast covering the coleoptile and aleurone layers, resulting in reduced mechanical resistance to the growth potential of the coleoptile (Fujino et al. 2008). A rice QTL on chromosome 7, named Seed dormancy 4 (Sdr4), was identified and revealed to substantially contribute to differences in seed dormancy between japonica and indica varieties. Sdr4 expression is positively regulated by OsVPI1, a global regulator of seed maturation; further, Sdr4 positively regulates potential regulators of seed dormancy and represses the expression of post-germinative genes, suggesting that this gene acts as an intermediate regulator of dormancy in the seed maturation process (Sugimoto et al. 2010). More recently, 10 SNP loci were identified as significantly affecting PHS through re-sequencing 21 representative rice accessions (10 PHS resistant and 11 PHS susceptible), and a regression equation for evaluating PHS based on the genotypes of eight significant loci was constructed, which accounted for an $R^2$ value of 0.401 in japonica rice (Lee et al. 2017). Better understanding of the complexity of genetic factors for PHS and seed dormancy is required to accelerate the development of PHS-resistant varieties via marker-assisted breeding.

Generally, most japonica rice varieties have weaker seed dormancy than indica rice varieties, although relatively high PHS resistance has been detected in several japonica rice varieties (Lee et al. 2016). Despite the detection in the relatively high PHS resistance and seed dormancy among japonica rice varieties, genetic analyses including QTL mapping have been limited in japonica rice varieties because of their low frequencies of polymorphisms in traditional markers, such as restriction-fragment length polymorphism (RFLP) and simple sequence repeats (Zhang et al. 1996; Mackill 1995). Recently, next-generation sequencing (NGS) technology has enabled the large-scale detection of sequence variations among japonica rice varieties (Hori et al. 2017; Jeong et al. 2015; Cheon et al. 2018) and has facilitated the development of high-throughput single nucleotide polymorphism (SNP) marker and QTL mapping in japonica varieties and populations. Among the high-throughput SNP markers, the competitive allele-specific PCR (KASP) marker system has the advantages of improved cost efficiency and time-effectiveness (Semagn et al. 2014; Yuan et al. 2014). Previously, we analyzed genome sequence data from 13 Korean japonica rice varieties and discovered 740,566 SNPs among these varieties. Based on this result, we developed 771 KASP markers for japonica rice varieties (Cheon et al. 2018, 2019).

In this study, KASP and cleaved amplified polymorphic sequence (CAPS) markers were used to construct a genetic map using 160 recombinant inbred lines (RILs) derived from a cross between the japonica rice varieties Odae (PHS-resistant) and Unbong40 (PHS susceptible). Five QTLs for PHS resistance were identified in this RIL population under two environmental conditions. The QTLs of qPHS-11GH and qPHS-11FP which were identified under greenhouse and field conditions, respectively, had similar locations on chromosome 11, suggesting the existence of a gene conferring stable PHS resistance effects under different environmental conditions, and will be useful in molecular breeding of PHS-resistant rice varieties.

Results

Phenotypic variations of PHS

We cultivated an F$_9$ RIL population from a cross between Odae and Unbong40 in two environmental conditions (greenhouse in winter and field in summer) and measured PHS rates. The mean PHS rate of Unbong40 was higher than that of Odae in both environments (Fig. 1a), and the average PHS rates of Odae and Unbong40 were 1.5% and 36.4%, respectively, in the field and 19.5% and 69.3%, respectively.
in the greenhouse (Fig. 1b). The mean PHS rate of the 160 RILs ranged continuously from 0 to 100% under both conditions, and a frequency of 0–10% class was the highest in both environmental conditions (Fig. 1b). The analysis of variance of the means of the PHS rates under both conditions was performed to test the significance of differences between lines and replications (Table 1). The differences between lines and replications were both highly significant.

Re-sequencing analysis of parental varieties

The parental Odae and Unbong40 varieties were re-sequenced, and the obtained raw sequence data for the varieties were approximately 14.55 and 15.06 Gbp, respectively (Table 2). After quality trimming and read mapping onto the Nipponbare reference genome sequence (IRGSP-1.0, https://rapdb.dna.affrc.go.jp/download/irgsp1.html), $106.07 \times 10^6$ reads containing 10.37 Gbp were mapped with an average mapping depth of $27.28 \times$ for the Odae variety, whereas $103.99 \times 10^6$ reads containing 10.16 Gbp were mapped with a mapping depth of $27.22 \times$ for the Unbong40

![Fig. 1 Phenotypic variations of pre-harvest sprouting (PHS) in the parental varieties under two environmental conditions. a PHS of parental varieties. Panicles were harvested 35 days after flowering and incubated for 7 days under 25 °C and 100% humidity. Each bar indicates 3 cm. b The distribution of the PHS rates of 160 recombinant inbred lines derived from a cross between Odae and Unbong40. FD and GH denote field and greenhouse conditions, respectively. Solid inversed triangles on the histogram indicate the PHS rates of the parental varieties Odae and Unbong40](image)

Table 1  Analysis of variance of the pre-harvest sprouting rates of 160 recombinant inbred lines

| Environmental conditions | Source   | df* | Sum of square | Mean square | F value | Probability |
|--------------------------|----------|-----|---------------|-------------|---------|-------------|
| Field                    | Line     | 159 | 420,034.22    | 2641.72     | 90.09   | 7.46E−282   |
| Field                    | Replication | 3   | 18,681.56     | 6227.19     | 212.36  | 1.81E−87   |
| Greenhouse               | Line     | 159 | 530,578.80    | 3336.97     | 128.34  | 0           |
| Greenhouse               | Replication | 3   | 18,363.88     | 6121.29     | 235.43  | 1.06E−93   |
| Greenhouse               | Error    | 477 | 12,402.16     | 26.00       |         |             |

*df degree of freedom
variety. Between Odae and Unbong40, 266,773 DNA polymorphisms including 248,255 SNPs and 18,518 insertions/deletions (indels) were detected (Table S1). The number of DNA polymorphisms detected varied greatly from chromosome to chromosome from a high of 82,593 on chromosome 8 to a low of 3267 on chromosome 5. Regions of high and low SNP density were distributed unevenly over all chromosomes (Fig. S1).

Genetic map construction and QTL mapping

Using 242 KASP markers selected from the previous studies (Cheon et al. 2018, 2019), we genotyped 160 RIL plants derived from a cross between Odae and Unbong40. Reliable genotypic data were obtained from 239 KASP markers with three markers producing unusable data owing to poor allelic discrimination. An initial genetic map comprising 239 KASP markers was constructed (Fig. S2). CAPS markers developed (Table S2) based on the detected SNPs between Odae and Unbong40 through the aforementioned re-sequencing analysis were used to genotype the RILs to fill in gaps. A final genetic map comprising 288 markers including 239 KASP and 49 CAPS markers was constructed (Fig. 2). The total length of this genetic map was 1651.5 cM, and the average interval between markers was 5.98 cM.

Using the genetic map and phenotypic data of 160 RILs from the Odae/Unbong40 cross, QTL mapping of the PHS rate was performed (Fig. S2). The QTL analysis with PHS field data revealed two major QTLs, qPHS-3FD and qPHS-11FD, located at 0 cM on chromosome 3 and 129.91 cM on chromosome 11, respectively, with logarithm of the odds (LOD) scores of 13.86 and 13.96, respectively. The LOD threshold was calculated to be 6.8 through a 1000 × permutation with a probability level of 0.05 (Fig. S3). The additive effects of these QTLs were -14.02, 9.50, and -11.52, respectively, with \( R^2 \) of 0.222, 0.108, and 0.152, respectively. The Odae alleles of qPHS-3GH and qPHS-11GH decreased PHS rate, whereas that of qPHS-dGH increased PHS rate. The closest markers of these QTL were KJ03_068, KJ04_068, and OUC11_11310, respectively, and the QTL intervals at 95% probability were 132.1–135.2, 129.5–132.7, and 134.7–137.3 cM, respectively (Table 3).

### Table 2 Summary of the sequencing data of parental varieties

| Varieties | No. of reads (×10⁶) | Nucleotides (Gb) | Sequencing depth (×) | No. of reads (×10⁶) | Nucleotides (Gb) | Average mapping depth (×) |
|-----------|---------------------|-----------------|---------------------|---------------------|-----------------|-----------------------------|
| ‘Odae’    | 144.07              | 14.55           | 125.95              | 12.30               | 106.07          | 10.37                       | 27.28                       |
| ‘Unbong40’| 149.13              | 15.06           | 130.15              | 12.70               | 103.99          | 10.16                       | 27.22                       |

*The “Q20” value indicates an accuracy of 99% for the base called.*

The qLTG3-1 genotype analysis

The qLTG3-1 gene (Os03g0103300) controlling low-temperature germinability is located in the 219,979–220,919-bp region on chromosome 3. Italicica Livorno exhibiting greater low-temperature germinability has a functional allele of qLTG3-1, whereas Hayamasari exhibiting weaker low-temperature germinability has a loss-of-function allele (Fujino et al. 2008). It was proposed that the allelic difference in qLTG3-1 between Nipponbare and Koshihikari is likely associated with differences in PHS resistance (Hori et al. 2010). Interestingly, qLTG3-1 is located within the 0–1.5-Mb genomic region of qPHS-3FD detected in the present study. To reveal the relationship between PHS phenotype and genotype of this QTL, we investigated the genomic sequences of the qLTG3-1 genes in Odae and Unbong40 and found a 71-bp deletion in the qLTG3-1 gene.
Fig. 2 Construction of a genetic map using 239 competitive allele-specific PCR and 49 cleaved amplified polymorphic sequence markers with genotypes of 160 recombinant inbred line plants derived from a cross between the varieties Odae and Unbong40. The chromosome number is indicated at the top of each chromosome, the name of each marker is indicated on the right side of each chromosome, and the genetic distance of each marker from the first marker at the top of each chromosome is shown on the left side. Genetic distances, measured in cM, were calculated using the Kosambi function. The quantitative trait locus interval at 95% probability is indicated by the filled black box.

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sequences in the Odae allele (Fig. S4a). This deletion has also been found in Koshihikari (Hori et al. 2010). This 71-bp indel of the qLTG3-1 gene was validated by PCR using the indel marker qLTG3-1ind (Fig. S5). Under the field cultivation condition, only two major QTLs, namely qPHS-3FD and qPHS-11FD, were found, indicating that these QTLs might be useful for discriminating PHS-resistant and PHS-susceptible plants in field conditions.

We classified the RILs into four classes according to the genotype combinations of the two markers for these QTLs (qLTG3-1ind and KJ11_090) and investigated the PHS rate distributions in each class. The group with Odae alleles at both QTLs displayed the lowest PHS rate, whereas the group with Unbong40 alleles at these loci had the highest PHS rate (Fig. 3). This indicates that selecting the Odae genotypes at these two QTLs may enable the selection of PHS-resistant plants under field conditions.

In addition, we analyzed genotypes of the two markers with 29 Korean japonica rice varieties known to be resistant or susceptible to PHS (Kang et al. 2018; Ko et al. 2005; Park 2009; Park and Kim 2009). Among 15 PHS-resistant varieties, six varieties had Odae alleles at both markers, seven had an Odae allele and an Unbong40 allele, and two had Unbong40 alleles at both markers. Among 14

| Environmental condition | QTL name | Chr. | Location (cM) | LOD** | Closest marker | QTL interval*** | Additive effect | R² | References |
|------------------------|----------|------|---------------|-------|---------------|----------------|----------------|----|-----------|
| Field                  | qPHS-3FD | 3    | 0             | 12.33 | KJ03_004      | 0–1.2         | −11.72         | 0.206 | qSdnj-3 (Wan et al. 2006) |
|                        |          |      |               |       |               |                |                |     | qSD-3 (Miura et al. 2002) |
|                        |          |      |               |       |               |                |                |     | qLTG3-1 (Fujino et al. 2004) |
|                        |          |      |               |       |               |                |                |     | qLTG3-1 gene (Fujino et al. 2008) |
|                        | qPHS-11FD| 11   | 130.91        | 12.28 | KJ11_090      | 129.5–133.5   | −11.76         | 0.205 | qLTG-11 (Miura et al. 2001) |
|                        |          |      |               |       |               |                |                |     | qSD-11 (Miura et al. 2002) |
|                        |          |      |               |       |               |                |                |     | qLTG-11–1 and -2 (Jiang et al. 2006) |
|                        |          |      |               |       |               |                |                |     | qSD11BR (Subudhi et al. 2012) |
|                        |          |      |               |       |               |                |                |     | qDOR-11–4, -5, and -6 (Cai and Morishima 2000) |
| Greenhouse             | qPHS-3GH | 3    | 133.21        | 18.12 | KJ03_068      | 132.1–135.2   | −14.02         | 0.222 | qLTG-3–2 (Fujino et al. 2004) |
|                        | qPHS-4GH | 4    | 78.61         | 10.34 | KJ04_068      | 77.7–82.0     | 9.50           | 0.108 | qLTG-4–2 (Miura et al. 2001) |
|                        |          |      |               |       |               |                |                |     | qSD-4 (Gu et al. 2004) |
|                        |          |      |               |       |               |                |                |     | qSD4 (Gu et al. 2005) |
|                        | qPHS-11GH| 11   | 135.21        | 14.32 | OUC11_11310   | 134.7–137.3   | −11.52         | 0.152 | qLTG-11 (Miura et al. 2001) |
|                        |          |      |               |       |               |                |                |     | qSD-11 (Miura et al. 2002) |
|                        |          |      |               |       |               |                |                |     | qLTG-11–1 and -2 (Jiang et al. 2006) |
|                        |          |      |               |       |               |                |                |     | qSD11BR (Subudhi et al. 2012) |
|                        |          |      |               |       |               |                |                |     | qDOR-11–6 (Cai and Morishima 2000) |

*Chr. Chromosome
**LOD logarithm of the odds
***Interval at 95% probability
PHS-susceptible varieties, nine varieties had Unbong40 alleles at both markers, two had an Odae allele and an Unbong40 allele, and two had Odae alleles at both markers (Table S3). Therefore, PHS responses of 15 out of 29 varieties coincided with the genotype combinations of the two markers. This indicates that the genotype combination of these markers might considerably correlate with PHS response of japonica rice varieties and that there are other genes regulating PHS response in these japonica rice varieties.

Analysis of candidate genes for the QTL on chromosome 11

Because $qPHS-1^{FD}$ (22.89–24.03 Mb) and $qPHS-1^{GH}$ (24.03–24.81 Mb) were detected in similar regions on chromosome 11, a common gene with a highly stable effect under extremely different environmental conditions might underlie these two QTLs. To identify candidate genes for these QTLs, annotations of DNA polymorphisms between Odae and Unbong40 were investigated in the 22.0–25.0-Mbp region on chromosome 11. In this region which harbors 155 genes, 2493 DNA polymorphisms including 2330 SNPs and 163 indels were detected. Based on the gene description information of the Nipponbare reference genome annotation database, we searched for genes potentially associated with seed germination, seed dormancy, or PHS among these genes. In this manner, 47 genes were selected. Among these genes, 34 carry non-synonymous SNPs (nsSNPs) and are regarded as potential candidate genes for PHS resistance (Table S4), whereas the remaining 13 genes are not considered likely candidates because their SNPs were located in untranslated regions (UTRs) or introns or they were synonymous SNPs (sySNPs) in protein coding sequences.

Discussion

PHS is a quantitative trait, and many factors can contribute to PHS resistance, including seed dormancy. Environmental factors, such as temperature and moisture during the ripening period, also can affect the development of PHS. Moreover, seed dormancy is strongly affected by environmental conditions during seed development (Godinez-Palma et al. 2013), and the broad-sense heritability of seed germination on the day of harvest for the trait was low (Gu et al. 2015). Therefore, repeated experiments under different conditions are critical for increasing the accuracy of PHS resistance estimation. To identify QTLs highly related to the genetic control of PHS resistance, we performed cultivation under two different environmental conditions using the same RIL population derived from a cross between Odae and Unbong40. The differences between the phenotypes of parental varieties and the RIL population under two environmental conditions might be attributable to genotype–environment interactions considering the different conditions during the ripening periods between the field and greenhouse. For example, the average daily mean temperature varied over the range of 19.6–30.4 °C during the period from flowering to harvest in the field condition, whereas it remained relatively constant over the range of 25.4–26.8 °C in the greenhouse condition (Fig. S6). Moreover, natural rainfall affected RILs in the field condition but not the greenhouse condition. These differences might be major factors for detecting different QTLs under the two conditions.

The reported QTLs for PHS, seed dormancy, and low-temperature germinability were co-located with detected QTLs in this study. On chromosome 3, the $qPHS-3^{FD}$ region (0–1.5 Mb) overlapped with QTLs that were previously detected in different backgrounds. $qSD-3$ was detected at the marker interval C515–C25 using 98 backcrossed inbred lines (BILs) derived from a cross between the japonica variety Nipponbare and indica variety Kasalath (Miura et al. 2002). $qSdnj-3$ was detected at the marker interval RM132–RM231 using a BC1 population derived from Nanjing35 (japonica)//N22 (indica) (Wan et al. 2006). $qLTG-3–1$ for low-temperature germinability was located in the marker interval GBR3001–GBR3002 using 122 BILs derived from a cross between the japonica varieties Italica Livorno and Hayamasari (Fujino et al. 2004), and the low-temperature germinability gene $qLTG-3-1$ was
identified at the QTL (Fujino et al. 2008). Another QTL on chromosome 3, the qPHS-3GH region (28.53–29.30 Mb), co-localized with qLTG-3–2 detected in the marker interval RM3436–RM2593 with the BILs from a cross between Italic Livorno and Hayamasari (Fujino et al. 2004). Regarding the QTL detected on chromosome 4, the qPHS-4GH region (22.89–24.03 Mb) was co-located with three previously reported QTLs. qSD4 for seed dormancy was mapped to the marker interval RM564A–RM252 using 204 BC1 individual plants derived from crosses between the weedy rice line SS18-2 and indica type breeding line EM93-1 (EM93-1/EM93-1/SS18-2) (Gu et al. 2005). qLTG-4–2 for low-temperature germinability was detected in the marker interval R93–C513 using 98 BILs derived from a cross between Nipponbare and Kasalath. The effect of the Kasalath allele on qLTG-4–2, which suppressed germination, was temporary, disappearing after 7–8 months of storage, suggesting that the lack of suppression of germination was attributable to the breaking of a residual effect of dormancy and that qLTG-4–2 might control seed dormancy (Miura et al. 2001). On chromosome 11, qSD-11 was detected in the marker interval S2260–G376 using 98 BILs derived from a cross between Nipponbare and Kasalath (Miura et al. 2002). qLTG-11–1 and qLTG-11–2 at the marker interval RM202–RM254 for low-temperature germinability were detected on chromosome 11 using 148 F2 population derived from a cross between the Bengal variety and weedy rice line PSRR-1 (Subudhi et al. 2012). These reported QTLs overlapped with both the qPHS-11FD (22.89–24.03 Mb) and qPHS-11GH (24.03–24.81 Mb) regions. In addition, the qPHS-11FD region overlapped with qDOR-11–4, qDOR-11–5, and qDOR-11–6 located at the marker intervals CDO365–C6a, R1465–RG1109, and RG1109–RZ536, respectively, which were identified using 125 RILs derived from a cross between the indica variety Pei-kuh and wild rice line W1944 (O. rufipogon Griff.) (Cai and Morishima 2000). In contrast, qPHS-11GH only co-located with qDOR-11–6. Hori et al. (2010) reported that a QTL including the qLTG3-1 gene on chromosome 3 was detected with BILs of Nipponbare/Koshihikari/Nipponbare and chromosome segment substitution lines derived from crosses between Nipponbare and Koshihikari, and the level of germinability under low temperature was strongly correlated with the level of PHS resistance. Iwata and Fujino (2010) reported that qLTG3-1 and qLTG11 were effective for controlling low-temperature germinability in the Hoshinoyme and Koshihikari backgrounds, whereas only qLTG3-1 was effective in the Hayamasari background. Moreover, the genotypes of the qLTG3-1ind marker in the qPHS-3FD region and KJ11_090 marker in the qPHS-11FD region largely corresponded with the PHS rates of RILs in field environment. From these findings, it is expected that qPHS-3FD and qPHS-11FD play important roles in PHS resistance in japonica rice and that the qLTG3-1ind and KJ11_090 markers, which are most tightly linked with qPHS-3FD and qPHS-11FD, will have utility in marker-assisted breeding for reduced PHS in japonica rice varieties.

Subsequently, because qPHS-11FD and qPHS-11GH were located in similar regions and these two QTLs overlapped with most of the reported QTL regions on chromosome 11 in different rice varieties, we investigated gene annotation in the 22.0–25.0 Mb region containing qPHS-11FD and qPHS-11GH. In total, 34 genes carrying nsSNPs were identified as possible candidate genes for qPHS-11FD and qPHS-11GH associated with PHS resistance. For example, Os11g0600500 carried 11 nsSNPs in the protein-coding sequences (CDSs) of the calcium-binding EF-hand domain containing protein. Arabidopsis calmodulin-like 24 (CML24) containing a calcium-binding EF-hand domain encodes a potential Ca2+ sensor that functions in response to ABA, and CML24-underexpressing transgenics are resistant to ABA-mediated inhibition of germination (Deld et al. 2005). The contents of and sensitivity to ABA were considered major variables in the regulation of dormancy and germination; specifically, ABA positively regulates dormancy induction and maintenance (Finch-Savage et al. 2017). In this context, Os11g0586001 with three nsSNPs and one indel in the CDS might also be a strong candidate because it encodes a protein phosphatase 2C that plays a key role in the ABA signal transduction pathway and that can positively regulate seed germination (Bhatnagar et al. 2017). In addition, Os11g0587600 can be a strong candidate because it encodes an ABC transporter that may be required for fatty acid transport during suberin deposition in the seed coat, which is necessary for seed dormancy (Fedi et al. 2017). Moreover, Os11g0622800 can be another strong candidate because two significant SNPs associated with PHS in 80 japonica accessions, Chr11_24303075 and Chr11_24307321 (Lee et al. 2017), were located in this gene, and were also polymorphic between Odae and Unbong40.

Further fine mapping of the QTL region including both qPHS-11FD and qPHS-11GH on chromosome 11 and functional analysis of candidate genes using transgenic plants are required for the exact identification of genes and development of selection markers within these QTL regions.
Materials and methods

Plant growing and samplings

In total, 160 F$_9$ RILs derived from a cross between Odae and Unbong40, which have early heading dates among japonica rice varieties, were cultivated in the National Institute of Agricultural Sciences of the Rural Development Administration (Jeonju, Republic of Korea) in the 2018–2019 season. The RILs were grown during winter in a greenhouse with maximum/minimum temperatures of 32 °C/22 °C and light/dark lengths of 14 h/10 h. Seeds of the two parental varieties and 160 F$_9$ RILs were sown in early December 2018, and these seedlings were transplanted at three seedlings per growth pot of 150-mm diameter and 125-mm height (two pots per line) in late December 2018. During the cultivation of seedlings, a main culm was left in each seedling, and all tillers were removed to keep good growth under dense planting condition. In the field, the seeds of two parental varieties and 160 F$_9$ RILs were sown in mid-May 2019, and these seedlings were transplanted at 20 seedlings in a row per line and 30 cm × 15 cm space at early June 2019. The tillers of plants in the field were not removed. For genotyping, genomic DNA from more than 10 seedlings per line was extracted using a Biomedic Plant gDNA Extraction Kit (Biomedic, Bucheon, Korea).

Evaluation of PHS rates

PHS rates were evaluated according to the method by Kang et al. (2018) with slight modifications. The heading panicles were tagged by wrapping their culms with color tape. Their heading dates were recorded on the tape and data sheet. Six panicles per each line from six plants (one panicle from one plant) were harvested at approximately 35 days after heading. Harvested panicles were put on paper towels on stainless steel trays of 465 × 365 × 35-mm size. Tap water was added to the tray to slightly immerse panicles, and the panicles were covered with paper towels. The tray was covered with plastic wrap to prevent water loss through evaporation and put in an incubator at 25 °C. After 7 days of incubation, the numbers of germinated seeds and not-germinated seeds per panicle were counted. Seeds with shoots just emerged from a break in the husk were regarded to have germinated. The PHS rate (%) was calculated as (germinated seeds /total filled seeds) × 100%. The average PHS rates of RILs were calculated from four panicles per line removing maximum and minimum PHS rate panicles and used for QTL analysis.

Parental variety re-sequencing and SNP analysis

We extracted genomic DNA from Odae and Unbong40 seedlings using a DNeasy Plant mini kit (Qiagen, Hilden, Germany). Sequencing libraries were created according to the manufacturer’s protocols using a TruSeq DNA PCR-free kit (Illumina, Inc., San Diego, CA, USA). The libraries were paired-end sequenced using the HiSeq 2000 system (Illumina, Inc.). The raw readouts that were graded as high-quality using Phred quality values > Q20 were used to analyze genetic variations between the Odae and Unbong40 varieties. As a reference genome sequence of rice, the Oryza sativa L. cv. Nipponbare sequence (IRGSP-1.0, https://rapdb.dna.affrc.go.jp/download/irgsp1.html) was used. We used the CLC Assembly Cell program (ver. 3.2.2, https://www.clcbio.com) mainly for read mapping and SNP detection. The generated reads were mapped on to the Nipponbare reference sequence using the clc-mapper command with the following parameters: alignment mode, local; similarity, 95%; gap cost, 3; deletion cost, 3; mismatch cost, 2; length fraction, 1.0; and repeat, ignore. DNA polymorphisms between each parental variety and Nipponbare were detected using the clc_find_variation command. Thus, DNA polymorphisms including SNPs and small indels up to 5 bp in length were detected. DNA polymorphisms between Odae and Unbong40 were analyzed using Python programs developed in-house using the detected DNA polymorphisms between Nipponbare and Odae and between Nipponbare and Unbong40. The detected DNA polymorphisms were annotated as genic and intergenic based on positional information obtained from the Rice Annotation Project Database (RAPDB, https://rapdb.dna.affrc.go.jp/index.html) using a Python program developed in-house. Further, DNA polymorphisms in genic regions were classified as CDSs, UTRs, non-coding exons, and introns, and those in CDSs were separated as sySNPs and nsSNPs on the basis of amino acid substitutions.

Genetic map construction and QTL mapping

Overall, 160 F$_9$ RILs derived from a cross between Odae and Unbong40 were genotyped using 242 KASP markers, which showed polymorphisms between parental varieties among 771 previously developed KASP markers (Cheon et al. 2018, 2019). After genetic map construction using KASP markers and primary detection of QTLs to determine the PHS rate, CAPS markers were developed on all chromosomes to fill gaps in the first genetic map on the basis of SNPs detected via the re-sequencing of Odae and Unbong40 sequences. Among the detected SNPs from Odae and Unbong40 sequences, those located in restriction enzyme sites were extracted, and CAPS markers were designed as follows: 500-bp sequences flanking the left and right of SNPs located in
restriction enzyme sites were used to design PCR primers using the BatchPrimer3 1.0 primer design program (https://probes.pw.usda.gov/batchprimer3/). The designed CAPS markers were used to test parental polymorphisms. PCR products amplified using the primers and parental DNA were digested using restriction enzymes and separated via electrophoresis on 1.2% agarose gels. The selected CAPS markers were used to analyze the genotypes of RIL plants.

Based on the genotypes of 160 RIL plants, a genetic map was constructed using the MapDisto 1.7 program (Lorieux et al. 2012) together with the MapChart program (Voorrips et al. 2002), and the Kosambi function was used for mapping. QTL analysis was performed via composite interval mapping (CIM) using the Windows QTL Cartographer ver. 2.5 program (Basten et al. 1996). Through a 1000× permutation with a probability level of 0.05, the LOD threshold was calculated. CIM was performed with the default condition of the Windows QTL Cartographer ver. 2.5 program.

Analysis of the QTL closest to the qLTG3-1 gene

To clarify sequence differences within the qLTG3-1 gene, which is located within the same genomic region as the target QTL on chromosome 3, we assembled genomic sequence reads of Odae and Unbong40 using the clc_assembler command of the CLC Assembly Cell program. Among the produced contigs after assembly, the contigs containing the qLTG3-1 gene were identified using BLAST. From these contigs, the qLTG3-1 sequences of Odae and Unbong40 were extracted and compared. A primer set was designed to validate an indel found between Odae and Unbong40 in the qLTG3-1 gene (Fig. S4a). The developed indel marker was named qLTG3-1ind and was used to genotype 160 RILs.

Analysis of candidate genes on chromosome 11

The physical intervals of QTL regions including qPHS-11FD and qPHS-11GH on chromosome 11 were deduced from the positions of the left and right flanking markers of the QTL intervals at 95% probability. The DNA polymorphisms located in this target interval were extracted. The polymorphisms located in the same genomic regions were selected according to the annotation of DNA polymorphisms. The gene description information from RAP-DB for the genes harboring DNA polymorphisms between Odae and Unbong40 were used as queries together with the terms “seed germination” or “seed dormancy” or “pre-harvest sprouting” to search related literature in the PubMed database (https://www.ncbi.nlm.nih.gov/pubmed). Through this strategy, the genes with DNA polymorphisms and molecular functions that are possibly related with PHS resistance were selected. Among these genes, those with nsSNPs or indels in CDSs were selected as final candidate genes, whereas those with sySNPs or DNA polymorphisms in introns and UTRs were excluded.

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