Impact of rice *GENERAL REGULATORY FACTOR14h* (*GF14h*) on low-temperature seed germination and its application to breeding

Yusaku Sugimura[1], Kaori Oikawa[1], Yu Sugihara[2,¤a], Hiroe Utsushi[1], Eiko Kanzaki[1], Kazue Ito[1], Yumiko Ogasawara[1], Tomoaki Fujioka[3], Hiroki Takagi[1,¤b], Motoki Shimizu[1], Hiroyuki Shimono[4,5], Ryohei Terauchi[1,2], Akira Abe[1]

[1] Iwate Biotechnology Research Center, Kitakami, Iwate, Japan

[2] Crop Evolution Laboratory, Kyoto University, Muko, Kyoto, Japan

[3] Iwate Agricultural Research Center, Kitakami, Iwate, Japan

[4] Faculty of Agriculture, Iwate University, Morioka, Iwate, Japan

[5] Agri-Innovation Center, Iwate University, Morioka, Iwate, Japan

[¤a] Current address: The Sainsbury Laboratory, University of East Anglia, Norwich, United Kingdom

[¤b] Current address: Faculty of Bioresources and Environmental Science, Ishikawa Prefectural University, 1-308, Suematsu, Nonoichi, Ishikawa, 921-8836, Japan

* Corresponding author

E-mail: a-abe@ibrc.or.jp (AA)

ORCIDs

Yusaku Sugimura, 0000-0001-7271-7703

Yu Sugihara, 0000-0001-6042-1091

Hiroki Takagi, 0000-0002-8576-9892
Motoki Shimizu, 0000-0002-5622-5554
Hiroyuki Shimono, 0000-0002-7328-0483
Ryohei Terauchi, 0000-0002-0095-4651
Akira Abe, 0000-0002-0344-2643

Abstract

Direct seeding is employed to circumvent the labor-intensive process of rice (*Oryza sativa*) transplantation, but this approach requires varieties with vigorous low-temperature germination (LTG) when sown in cold climates. To investigate the genetic basis of LTG, we identified the quantitative trait locus (QTL) *qLTG11* from rice variety Arroz da Terra, which shows rapid seed germination at lower temperatures, using QTL-seq. We delineated the candidate region to a 52-kb interval containing *GENERAL REGULATORY FACTOR14h* (*GF14h*) gene, which is expressed during seed germination. The Arroz da Terra *GF14h* allele encodes functional GF14h, whereas Japanese rice variety Hitomebore harbors a 4-bp deletion in the coding region. Knocking out functional *GF14h* in a near-isogenic line (NIL) carrying the Arroz da Terra allele decreased LTG, whereas overexpressing functional *GF14h* in Hitomebore increased LTG, indicating that *GF14h* is the causal gene behind *qLTG11*. Analysis of numerous Japanese rice accessions revealed that the functional *GF14h* allele was lost from popular varieties during modern breeding. We generated a NIL in the Hitomebore background carrying a 172-kb genomic fragment from Arroz da Terra including *GF14h*. The NIL showed superior LTG compared

to Hitomebore, with otherwise comparable agronomic traits. The functional *GF14h* allele from Arroz da Terra represents a valuable resource for direct seeding in cold regions.

Author Summary

Rice serves as a fundamental crop sustaining over half of the global population. With the rapid growth of the world's population, it will become increasingly important to improve rice productivity. On the other hand, the aging of rice farmers in Japan has resulted in a constant labor shortage. To address this, direct seeding, in which seeds are sown directly in rice fields without going through the most labor-intensive part of the rice cultivation process, i.e., seedling production and transplanting, has been recommended. However, prevalent elite rice varieties are known to be unsuitable for direct seeding due to their poor seed germination ability under low-temperature conditions. In this study, we show for the first time that *GF14h* gene from the Portuguese variety Arroz da Terra improves seed germination at low temperatures (LTG). In addition, a novel cross-bred line was generated by introducing the *GF14h*-containing genomic segment from Arroz da Terra into Hitomebore, a widely cultivated variety in northern Japan. This line is expected to be used as a pre-breeding material to enhance LTG. This study will provide a genetic basis for LTG and contribute to basic and applied research progress.

Introduction

Low-temperature seed germination (LTG) is a pivotal agronomic trait in rice (*Oryza sativa*). As rice originated from tropical and subtropical regions, it is highly susceptible to low-temperature conditions compared to other cereal crops such as wheat (*Triticum*

71 *aestivum*) and barley (*Hordeum vulgare*) [1]. Nevertheless, rice is produced in temperate
72 and high-altitude regions, where it frequently experiences temperatures below 20°C. In
73 Japan, rice is abundantly cultivated in relatively cold areas such as Tohoku and Hokkaido.
74 In recent years, there has been an increasing demand to shift from conventional
75 transplantation-based rice cultivation to direct seeding to reduce labor and costs. However,
76 direct seeding raises the risk of exposure to low temperatures during seed germination
77 [2]. Therefore, to expand the use of direct seeding, it is crucial to breed rice cultivars with
78 enhanced LTG.

79 LTG is a quantitative trait regulated by complex molecular mechanisms. Linkage
80 mapping and genome-wide association studies (GWAS) have identified over 30 LTG-
81 related quantitative trait loci (QTLs) or genomic regions associated with this trait, located
82 on all 12 rice chromosomes [3-20]. However, only a few genes involved in LTG have
83 been described, such as *qLTG3-1* [3] and *STRESS-ASSOCIATED PROTEIN16*
84 (*OsSAP16*) [15]. The *qLTG3-1* gene, encoding a protein of unknown function, has a
85 substantial influence on LTG [3]. During seed germination, *qLTG3-1* expression is
86 strongly induced in embryos, which leads to the loosening of the tissues covering the
87 embryo by promoting vacuolation [3]. *OsSAP16* encodes a stress-associated protein with
88 two AN1-C2H2 zinc finger domains [15]. OsSAP16 presumably acts as a regulator of
89 LTG.

90 14-3-3 proteins are regulatory proteins that are widely conserved in eukaryotes.
91 These proteins bind to phosphorylated serine and tyrosine residues in their target proteins
92 that participate in signal transduction and the regulation of gene expression [21, 22], thus
93 altering their enzymatic activity, subcellular localization, stability, or protein–protein
94 interactions [23-25]. The rice genome encodes eight 14-3-3 proteins, named GF14a–h for

GENERAL REGULATORY FACTOR14 [26]. *GF14h* is involved in rice seed germination under optimal temperature conditions [27, 28]. In addition, GF14h contributes to phytohormone signaling, including abscisic acid and gibberellin signaling [27, 28]. However, it remains unclear whether *GF14h* promotes seed germination under low-temperature conditions [28].

QTL pyramiding has been proposed as a breeding concept [29] for bringing together several QTLs (or genes) related to agronomically important traits in the genetic background of locally adapted elite cultivars. In practice, it is essential to generate pre-breeding materials for QTL pyramiding, i.e., near-isogenic lines (NILs) that harbor one or a few genomic segments introgressed from the donor parent into the genome of the recipient parent through a combination of continuous backcrossing and selfing via marker-assisted selection [30]. In this study, we determined that *GF14h* is responsible for an LTG-related QTL in Portuguese rice variety Arroz da Terra. We generated a NIL in the background of rice cultivar Hitomebore, which is adapted for growth in northern Japan, by replacing its *GF14h* genomic fragment with that from Arroz da Terra and tested its LTG performance.

Results

Evaluation of QTLs associated with low-temperature germination using the Portuguese rice variety Arroz da Terra

We investigated seed germination characteristics of a Portuguese rice variety Arroz da Terra and a Japanese varieties Iwatekko and Hitomebore. Under low-temperature

118 conditions (15ºC), Arroz da Terra exhibited significantly higher germination rates
119 compared to Iwatekko and Hitomebore during days 7−15, particularly showing a 30−40%
120 increase in germination rate 10−11 days after imbibition (Fig 1A and 1B). Similarly, at
121 normal temperature conditions (25ºC), Arroz da Terra showed superior germination rates
122 2−4 days after imbibition, especially with a 30% difference observed 3 days after
123 imbibition (Fig 1A and 1C). These findings indicate that Arroz da Terra exhibits more
124 vigorous germination under normal and low-temperature conditions than the Japanese
125 cultivars Iwatekko and Hitomebore. To identify the genes responsible for this difference,
126 we searched for QTLs involved in the high LTG of Arroz da Terra. We previously
127 generated a set of 200 RILs at the F_7 generation derived from a cross between Arroz da
128 Terra and Iwatekko (S1 Fig) [31]. We phenotyped all RILs for LTG at 13ºC and selected
129 the 20 RILs with the highest LTG and the 20 RILs with the lowest LTG. We assembled
130 two pools of seedlings with low or high LTG and extracted their genomic DNA for whole-
131 genome sequencing on the Illumina platform (S1 Fig) [31]. We mapped the resulting
132 sequencing reads to the Nipponbare rice reference genome (IRGSP-1.0) and performed
133 QTL-seq analysis using our new high-performance pipeline [32]. Based on the ΔSNP
134 index, we identified three QTLs related to LTG on chromosome 3 (*qLTG3-1* and *qLTG3-*
135 *2*) and chromosome 11 (*qLTG11*) (Fig 1D), which is consistent with the results of a
136 previous study [31].

137 The *qLTG3-1* region contained the gene Os03g0103300, which was reported to be
138 involved in LTG in a study using rice cultivar Italica Livorno, which has high LTG, and
139 Hayamasari, which has low LTG [3]. An examination of its coding sequences in Arroz da
140 Terra, as well as Iwatekko and Hitomebore, revealed that they were identical to those

141 found in Italica Livorno and Hayamasari, respectively (S2 Fig). While Italica Livorno
142 harbored a functional haplotype for this gene, Hayamasari carried a loss-of-function
143 haplotype due to a 71-bp deletion (S2 Fig) [3]. Therefore, we propose that the causal gene
144 for the QTL *LTG3-1* is Os03g0103300.

145 To evaluate the contribution of the two other QTLs to LTG, we generated NILs
146 harboring a segment from the Arroz da Terra genome for each QTL (approximately 5 Mb)
147 in the Hitomebore background (Fig 1E; S3A and S4A Figs). We detected no clear effect
148 of *qLTG3-2* on LTG, as *qLTG3-2*-NIL and Hitomebore showed similar seed germination
149 rates at 15ºC (S4B Fig). By contrast, *qLTG11*-NIL showed a significantly higher rate of
150 germination than Hitomebore 7−12 days after imbibition at 15ºC, particularly after 8 and
151 9 days, showing a difference of more than 40% (Fig 1E and 1F), indicating that *qLTG11*
152 enhances LTG. *qLTG11*-NIL seeds also germinated more rapidly than Hitomebore seeds
153 under normal conditions (25ºC), although with a smaller difference between the two
154 genotypes than at low temperature (S5 Fig). We therefore focused our analysis on *qLTG11*.
155

156 Identification of *GF14h* as the candidate gene for *qLTG11*

157 To delineate the *qLTG11* region, we carried out map-based cloning using a segregating
158 population derived from a cross between BC_2F_3 line *qLTG11*-NIL and Japanese elite
159 cultivar Hitomebore (S3A Fig). For mapping, we conducted germination tests at 15°C.
160 We narrowed down the genomic region containing the QTL to a 52-kb segment (from
161 23.512 bp to 23.564 Mb) on chromosome 11 based on the Nipponbare reference genome
162 (IRGSP-1.0) (Fig 2A). This interval contains two annotated genes based on the
163 Nipponbare genome sequence (Fig 2B). We compared the genomic sequence of
164 Hitomebore and Arroz da Terra across the candidate region using *de novo* genome

165 assembly obtained from Nanopore long reads. The cultivars Hitomebore and Nipponbare
166 had an identical genomic sequence over the entire candidate region (S6A Fig). By contrast,
167 the genome sequence from Arroz da Terra was substantially different from that of
168 Nipponbare, with the equivalent candidate region spanning approximately 94 kb (Fig 2B
169 and S6B Fig).

170 As the causal gene behind the variation in LTG is likely expressed in seeds, we
171 performed transcriptome deep sequencing (RNA-seq) during seed germination in
172 Hitomebore and *qLTG11*-NIL (S1 Table). Within the candidate region, the gene
173 Os11g0609600, corresponding to the *14-3-3* gene *GF14h*, was expressed in both
174 Hitomebore and *qLTG11*-NIL, whereas Os11g0609500 (*Jacalin-like lectin domain*
175 *containing protein*) was not expressed in seeds (S7 Fig), thus suggesting that *GF14h* is a
176 strong candidate gene for LTG. The *GF14h* gene structure and haplotypes in Arroz da
177 Terra and Hitomebore are shown in Fig 2C. We detected a 4-bp deletion in the *GF14h*
178 coding region in Hitomebore, causing a frameshift mutation predicted to introduce a
179 premature stop codon (Fig 2C and S8 Fig). These results suggest that Hitomebore carries
180 a loss-of-function allele of *GF14h*. To assess the role of *GF14h* in LTG, we examined the
181 expression pattern of the putative functional *GF14h* ($GF14h^{Arroz}$) allele during seed
182 germination at low temperature (15°C) using *qLTG11*-NIL. RT-qPCR analysis of *GF14h*
183 expression levels showed that they were comparable in the embryo and endosperm at 1
184 and 3 days after the onset of seed imbibition (Fig 2D). At the beginning of germination,
185 when a white coleoptile was visible (5 and 7 days after seed imbibition), *GF14h*
186 expression levels rose in the endosperm, but not in the embryo (Fig 2D). By nine days of
187 imbibition, when most seeds had germinated, *GF14h* expression in the endosperm
188 returned to basal levels (Fig 2D). These results support the notion that *GF14h* plays a role

189 in seed germination at low temperature.

190

191 *GF14h* plays a vital role in LTG

192 To investigate the contribution of GF14h to LTG, we knocked out the functional *GF14h*
193 copy present in *qLTG11*-NIL by clustered regularly interspersed short palindromic repeat
194 (CRISPR)/CRISPR-associated nuclease 9 (Cas9)-mediated gene editing and evaluated
195 LTG. Specifically, we introduced two single guide RNA (sgRNA) constructs targeting the
196 exons of *GF14h* individually into *qLTG11*-NIL by Agrobacterium-mediated
197 transformation. We chose to knock out *GF14h* in the *qLTG11*-NIL background rather than
198 Arroz da Terra to evaluate the specific contribution of *GF14h* to LTG without the
199 influence of *qLTG3-1*, which would be present in the Arroz da Terra background. To
200 accurately evaluate the phenotypes of the edited plants, we selected heterozygous plants
201 in the T_0 generation and isolated homozygous mutant lines and their unedited
202 homozygous siblings in the T_1 generation. We obtained four knockout lines (*gf14h-1*,
203 *gf14h-2*, *gf14h-3*, and *gf14h-4*) and their wild-type sibling (WT^{Arroz}) (S9 Fig). We detected
204 significant drops in the germination percentage in all four knockout lines compared to
205 WT^{Arroz} (Fig 3A and 3B). We also found that the knockout lines tended to have lower
206 germination rates than WT^{Arroz} under normal temperature conditions (25°C) (S10 Fig),
207 which is consistent with the previous report [27]. Furthermore, we generated transgenic
208 lines in the Hitomebore background overexpressing the functional *GF14h* allele from
209 Arroz da Terra under the control of the CaMV 35S promoter. In these overexpression
210 lines, *GF14h* expression increased approximately 1,000-fold compared to the wild-type
211 sibling ($WT^{Hitomebore}$) (S11 Fig). Importantly, the overexpression lines showed higher LTG
212 than $WT^{Hitomebore}$ when tested at 15ºC (Fig 3C and 3D). Taken together, these data indicate

213 that *GF14h* is a key gene involved in LTG.

214

215 Loss-of-function alleles *GF14h* and *qLTG3-1* increased in frequency 216 during rice breeding in Japan

217 We reconstructed the *GF14h* haplotype network using genotype data obtained from 218 whole-genome resequencing of 492 *O. sativa* accessions from various collections, 219 including the World Rice Core Collection [33], the Rice Core Collection of Japanese 220 Landraces [34], and a set of Japanese landraces and modern varieties [35], in addition to 221 11 wild rice (*O. rufipogon*) accessions [36] (S2 Table). We distinguished 10 haplotypes 222 for the *GF14h* coding region based on 11 polymorphic sites comprising one frameshift 223 mutation caused by a 4-bp deletion, four nonsynonymous single nucleotide 224 polymorphisms (SNPs), and six synonymous SNPs (S3 Table). The conversion of the 225 functional allele Hap2 to its nonfunctional allele Hap1 required only a single step: a 4-bp 226 deletion (S12 Fig).

227 We analyzed the haplotype frequencies of *GF14h* and *qLTG3-1* in Japanese 228 landraces and cultivars, which we grouped according to their time of release. More than 229 half of all Japanese landraces carried functional alleles of both *GF14h* and *LTG3-1* (Fig 230 4A). The next most common allele combination among the Japanese landraces was a 231 nonfunctional *GF14h* allele with a functional *LTG3-1* allele (Fig 4A). The percentage of 232 lines with loss-of-function alleles at both *GF14h* and *LTG3-1* has increased since the 233 beginning of crossbreeding in Japan in the early 20th century, with more than 80% of 234 varieties released after 2001 carrying loss-of-function alleles for both genes (Fig 4A and 235 4B). Looking at each gene separately in landraces, only a few lines carried a loss-of-

236 function allele for *LTG3-1*, whereas roughly half of all lines already harbored a loss-of-
237 function allele for *GF14h* (Fig 4B). Modern breeding thus appears to have increased the
238 proportion of loss-of-function alleles for these two genes, with a substantial increase in
239 *LTG3-1*, reaching almost 90% among lines bred after 2001 (Fig 4B).
240

241 **The Arroz da Terra *GF14h* allele could be valuable for rice breeding**

242 To assess how useful the above findings might be to practical breeding programs, we
243 developed new breeding materials. QTL pyramiding, a strategy for introducing multiple
244 QTLs for desired traits into a single genetic background, is a key strategy employed in
245 current breeding. An essential step in QTL pyramiding is the generation of NILs
246 containing the desired QTLs. Therefore, we developed a NIL, termed NIL-$GF14h^{Arroz}$,
247 using the elite cultivar Hitomebore as the genetic background into which we introgressed
248 a 172-kb region from the Arroz da Terra genome containing *GF14h* (S3 Fig). This NIL
249 showed a higher seed germination rate under low-temperature conditions compared to
250 Hitomebore (Fig 5A and 5B). In addition, although no significant difference was observed,
251 it is likely that NIL-$GF14h^{Arroz}$ tends to be more susceptible to pre-harvest sprouting than
252 Hitomebore (S13 Fig). Importantly, we observed no substantial differences in five
253 agronomic traits (culm length, panicle length, panicle number, grain number, and grain
254 weight) between NIL-$GF14h^{Arroz}$ and Hitomebore (Fig 5C–5G). Brown rice yield was
255 slightly lower in NIL-$GF14h^{Arroz}$ compared to Hitomebore, but a sufficient yield was
256 guaranteed (Fig 5H and 5I). These results suggest that NIL-$GF14h^{Arroz}$ could be a valuable
257 parental line for breeding via QTL pyramiding.
258

Discussion

Here, we demonstrated that the functional *GF14h* allele present in Portuguese rice variety Arroz da Terra plays a pivotal role in supporting seed germinability under low-temperature conditions. Although the regulation of seed germination by *GF14h* was recently documented, its activity under low-temperature conditions remained unclear [27, 28]. While many genomic regions associated with LTG have been detected through QTL mapping and GWAS, only a few studies have identified the causal genes [3-20]. Indeed, LTG is a quantitative trait involving the cumulative effects of multiple genes and their epistatic relationships, making it difficult to assess the specific effect of a single genomic region on LTG. To eliminate the influence of other chromosomal regions on LTG, we first generated *qLTG11*-NIL containing only one of three QTLs detected in the Arroz da Terra background for genetic analysis. The analysis of *qLTG11*-NIL revealed that *GF14h* participates in LTG. Significantly, the NIL harboring the functional *GF14h* allele from Arroz da Terra in the Hitomebore background provides valuable pre-breeding materials for QTL pyramiding. These findings provide a genetic understanding of low-temperature germinability as well as new resources for rice breeding.

Influence of *GF14h* on low-temperature germination

In this study, we identified *GF14h* as being implicated in LTG. In a previous study, a genetic complementation assay with a functional *GF14h* allele introduced into the rice cultivar Nipponbare background increased the germination rate at 30°C, but only to a limited extent at 15°C [28]. This result is not consistent with our finding that introducing functional *GF14h* into Hitomebore resulted in a significant improvement in germination

282 at low temperature. Perhaps this discrepancy is due to differences in the rice varieties
283 used in the germination assays. Notably, the haplotypes of *qLTG3-1*, a major QTL behind
284 LTG [3], are different between Nipponbare and Hitomebore: whereas Nipponbare, which
285 was released in 1961, harbors the functional allele of *qLTG3-1*, Hitomebore carries a loss-
286 of-function allele with a deletion of 71 bp (S2 Fig) [37]. Moreover, the cultivars
287 Koshihikari and Hayamasari, which carry the same loss-of-function *qLTG3-1* allele as
288 the Hitomebore variety, were reported to exhibit lower germination rates at low
289 temperatures than Nipponbare [37]. LTG tests using chromosome segment substitution
290 lines derived from a cross between Koshihikari and Nipponbare indicated that *qLTG3-1*
291 contributes to the difference in LTG between the two varieties [37]. Based on these
292 observations, it is likely that Nipponbare has a better LTG ability than Hitomebore, which
293 may have masked the effect of functional *GF14h* on LTG in the previous study [28]. Our
294 study confirmed the involvement of *GF14h* in LTG through map-based cloning and
295 analysis of knockout and overexpression lines. It is also worth mentioning that our
296 experiments were performed in the *qLTG11*-NIL background, which allowed us to isolate
297 the contribution of GF14h to LTG without any influence from *qLTG3-1* or other genes in
298 the Arroz da Terra background. In summary, we provided multiple lines of evidence that
299 *GF14h* contributes to LTG.

300 The expression pattern of functional *GF14h* during seed germination was
301 previously unclear. While *GF14h* has been shown to be expressed in the aleurone layer
302 surrounding the embryo [28], it is also highly expressed in the endosperm [27]. In the
303 current study, we showed that *GF14h* was expressed throughout the seeds, but with a
304 transient induction in expression in the endosperm at roughly the time of initiation of seed
305 germination. GF14h was reported to regulate seed germination by interacting with the

306 abscisic acid and gibberellin signaling pathways at optimal temperatures [27, 28]. We
307 therefore suggest that GF14h controls LTG by interacting with various phytohormone
308 signaling pathways.
309

310 Low-temperature germinability in rice was lost due to selection in 311 modern Japanese breeding

312 In this study, we performed haplotype network analysis of *GF14h* using many Japanese
313 rice varieties. We identified ten distinct haplotypes based on 11 polymorphic sites in the
314 *GF14h* coding region. Of these, Hap4, encompassing the aus, indica, tropical japonica,
315 temperate japonica, and *O. rufipogon* accessions, was defined at the center of the
316 haplotype network. Furthermore, we determined that a 4-bp deletion converted the
317 functional haplotype Hap2, which was derived from Hap4, into the nonfunctional
318 haplotype Hap1. This finding is consistent with the relationship between Hap6 and Hap1
319 (which we defined as Hap2 and Hap1, respectively) observed by [27]. These results
320 suggest that the nonfunctional allele represented by Hap1 was introduced into temperate
321 *japonica* varieties from tropical *japonica* varieties carrying Hap2 and then spread to
322 Japanese cultivars.

323 We studied the haplotype frequencies of *GF14h* and *qLTG3-1* in various Japanese
324 landraces and cultivars, considering their time of release from breeding programs into the
325 field. More than half of the Japanese landraces analyzed carried both functional *GF14h*
326 and *qLTG3-1* alleles. However, the frequency of varieties carrying loss-of-function alleles
327 for both *GF14h* and *qLTG3-1* has increased since crossbreeding began in the early 20th
328 century. This trend has continued to the present, perhaps as a result of artificial selection

329 to improve resistance to pre-harvest sprouting, with more than 80% of all varieties bred
330 since 2001 carrying these loss-of-function alleles.

331 Our study provides a historical perspective on allelic shifts in Japanese rice breeding
332 while highlighting the influence of modern breeding on genetic diversity. Further research
333 is needed to elucidate the potential effects of higher frequencies of loss-of-function alleles
334 on the overall phenotypic characteristics and ecological adaptability of Japanese rice
335 varieties. In addition, as mentioned in previous reports [27, 28], we believe that the
336 reintroduction of functional alleles should be considered in order to develop cultivars
337 suitable for labor-saving cultivation techniques such as direct seeding.

338

339 Application to direct seeding for rice cultivation

340 Cultivation stability under direct seeding conditions is important for managing rice
341 production costs and reducing labor. However, since rice is sensitive to low temperatures,
342 improving seed germination and seedling establishment at low temperatures is a desirable
343 breeding trait in high-latitude rice production areas such as Japan. In the current study,
344 we developed a potentially useful NIL (NIL-*GF14h*^{Arroz}) by introducing the functional
345 *GF14h* allele into the Hitomebore background. Overexpressing this functional *GF14h*
346 allele was previously shown to improve anaerobic germination and tolerance to seedling
347 establishment under anaerobic conditions in laboratory experiments [27]. However,
348 whether NIL-*GF14h*^{Arroz} exhibits strong seedling vigor at low temperatures in rice fields
349 remains to be determined. We previously identified a QTL associated with seedling vigor,
350 *qPHS3-2* (QTL for plant height of seedling 3-2) [38]. *qPHS3-2* most likely corresponds
351 to the gibberellin biosynthesis gene *GA20 oxidase1* (*OsGA20ox1*), a paralog of *Semi*
352 *Dwarf1* (*sd-1*, corresponding to *OsGA20ox2*) [38]. Therefore, the pyramiding of *qPHS3-*

353 *2* in NIL-*GF14h*^{Arroz} by marker-assisted selection represents a promising approach for
354 further improving seedling vigor in NIL-*GF14h*^{Arroz}. On the other hand, enhancing LTG
355 may conversely increase the risk of pre-harvest sprouting. Should the level of pre-harvest
356 sprouting in NIL-*GF14h*^{Arroz} pose practical issues, the pyramiding of QTLs for pre-
357 harvest sprouting resistance, such as *Seed Dormancy 4* [39], may offer a solution.

358 In recent years, "early-winter direct seeding" has been experimentally tested as a
359 new system of direct seeding for rice production in Japan [40]. In this system, seeds are
360 directly sown in the early winter of the previous year instead of the spring. The sown
361 seeds thus overwinter in snow-covered soil and germinate the following spring. The
362 major advantage of this approach is that it can significantly decrease the amount of labor
363 required by farmers during the busy spring season. However, it is challenging to
364 overwinter the seeds of modern rice varieties in the soil and achieve good seedling
365 establishment [40-42]. We expect that reintroducing beneficial alleles like *GF14h*^{Arroz} that
366 were lost during modern breeding into future rice varieties will enable the implementation
367 of new cultivation practices and increase productivity.

368

369 # Materials and Methods

370 ## Plant materials

371 Rice was cultivated in a paddy field at Iwate Agricultural Research Center (39°35'N,
372 141°11'E). A recombinant inbred line (RIL) population of 200 F_7 lines was generated
373 from a cross between Japanese variety Iwatekko and the high-LTG variety Arroz da Terra
374 (S1 Fig) [31]. To develop NILs, rice cultivar Hitomebore was used as the recipient parent
375 to generate NILs harboring the target genomic region from Arroz da Terra (S3A Fig),

376 thereby establishing *qLTG3-2*-NIL, *qLTG11*-NIL, and NIL-*GF14h*^{Arroz} (Fig 1E ; S3B and
377 S4A Figs).
378

379 **Evaluation of germination rate**

380 Seeds for each line were harvested 45 days after heading, air-dried at 30°C for two days,
381 and stored at 4°C until use. The seeds were air-dried at 50°C for seven days in the dark
382 to break dormancy. For germination tests, 50 seeds per replicate were incubated in a Petri
383 dish filled with distilled water in the dark at 15°C (low-temperature conditions) or 25°C
384 (optimal temperature conditions). For QTL-seq analysis, germination tests were
385 conducted at 13°C [31]. The germination rate was calculated as the total number of
386 germinated seeds at each time point divided by the number of seeds tested. Seeds were
387 considered to have germinated when the white coleoptile was visible.
388 To evaluate resistance to pre-harvest sprouting, panicles were harvested from
389 NIL-*GF14h*^{Arroz} and Hitomebore 30 days after heading. The panicles were incubated in
390 dark, wet conditions (by covering them with filter paper moistened with water) at 28°C
391 for 10 days, and seed germination was scored.
392

393 **QTL-seq analysis**

394 LTG data for the 200 RILs derived from a cross between Iwatekko and Arroz da Terra
395 (S1 Fig) were analyzed [31]. The top 20 RILs showing high-LTG and the bottom 20 RILs
396 showing low-LTG phenotypes were selected to assemble the two bulk samples with
397 contrasting LTG phenotypes. All seedlings with high or low LTG were pooled, and DNA
398 was extracted from each bulk as previously described [31]. The genomic DNA of the two

399 bulks was used to generate DNA-seq libraries and sequenced on a GAIIx sequencer
400 (Illumina, CA, USA). QTL-seq was performed to identify QTLs related to LTG [31, 32].
401

402 **Map-based cloning of *qLTG11***

403 To narrow down the *qLTG11* region, genotyping was performed using a cross population
404 of *qLTG11*-NIL (BC_2F_3) backcrossed to Hitomebore. The germination rate under low-
405 temperature conditions (15°C) was measured to characterize LTG activity. High-
406 resolution fine mapping with ten markers (markers B−K) between 23.466 Mb and 23.609
407 Mb on chromosome 11 identified six informative recombinants in the target region.
408 Primers used for mapping are listed in S4 Table.
409

410 ***De novo* assembly of the Hitomebore and Arroz da Terra genomes**

411 To reconstruct the *qLTG11* regions in Hitomebore and Arroz da Terra, *de novo* assembly
412 was performed for each genome using Nanopore long reads and Illumina short reads
413 according to a published method [43]. To extract high-molecular-weight genomic DNA
414 from leaf tissue for Nanopore sequencing, a NucleoBond high-molecular-weight DNA
415 kit (MACHEREY-NAGEL, Germany) was used. Following DNA extraction, low-
416 molecular-weight DNA was eliminated using a Short Read Eliminator Kit XL
417 (Circulomics, MD, USA). Library preparation was then performed using a Ligation
418 Sequencing Kit (SQK-LSK-109; Oxford Nanopore Technologies [ONT], United
419 Kingdom) according to the manufacturer's instructions, and sequencing was performed
420 using MinION (ONT, UK) for Arroz da Terra. For Hitomebore, Nanopore long reads
421 sequenced by [43] were used. Base-calling of the Nanopore long reads was performed

422 using Guppy 4.4.2 (ONT, UK). Sequences derived from the lambda phage genome were
423 removed from the raw reads with NanoLyse v1.1.0 [44]. The first 50 bp of each read were
424 then removed, as were reads with an average read quality score below 7 and reads shorter
425 than 3,000 bases, using NanoFilt v2.7.1 [44]. The clean Nanopore long reads were
426 assembled using NECAT v0.0.1 [45], setting the genome size to 380 Mb. To improve the
427 accuracy of assembly, Racon v1.4.20 [46] was used twice for error correction using
428 Nanopore reads, and Medaka v1.4.1 (https://github.com/nanoporetech/medaka) was
429 subsequently used to correct mis-assembly. Two rounds of consensus correction were
430 then performed using bwa-mem v0.7.17 [47] and HyPo v1.0.3 [48] with the Illumina
431 short reads. Redundant contigs were removed using purge-haplotigs v1.1.1 [49], resulting
432 in a *de novo* assembly of 374.8 Mb comprising 82 contigs for Hitomebore and 376.3 Mb
433 consisting of 82 contigs for Arroz da Terra. The resulting genome sequences have been
434 deposited in Zenodo (https://doi.org/10.5281/zenodo.10460309).
435

436 **Plant transformation**

437 To generate *GF14h* knockout mutants, two single guide RNAs (sgRNAs) targeting exon
438 4 or exon 5 of *GF14h* were designed using the web-based service CRISPRdirect
439 (crispr.dbcls.jp) [50] and cloned individually into the pZH::OsU6gRNA::MMCas9 vector
440 [51]. The resulting vectors were introduced into Agrobacterium (*Agrobacterium*
441 *tumefaciens*) strain EHA105 for transformation into *qLTG11*-NIL plants [52]. The target
442 sites in the positive transformants were sequenced by Sanger sequencing to detect
443 mutations. To obtain overexpression constructs, the full-length coding sequence of
444 functional *GF14h* was amplified from total RNA extracted from *qLTG11*-NIL and cloned
445 into the plant binary vector pCAMBIA1300 under the control of the cauliflower mosaic

446 virus (CaMV) 35S promoter. The overexpression plasmid was introduced into
447 Agrobacterium strain EHA105 for transformation of rice variety Hitomebore [52]. All
448 primers used are listed in S4 Table.
449

450 **Expression analysis**

451 Total RNA was extracted from germinating seeds using an RNA-suisui S kit (Rizo). Total
452 RNA was treated with RNase-free DNase I (Nippon Gene). The resulting samples were
453 reverse transcribed into first-strand cDNA using a PrimeScript RT Reagent Kit (Takara
454 Bio). Quantitative PCR (qPCR) was conducted using a QuantStudio 3 system (Thermo
455 Fisher Scientific) with Luna Universal qPCR Master Mix (New England Biolabs). The
456 cycling parameters were 1 min at 95°C, followed by 40 cycles of amplification (95°C for
457 15 sec and 60°C for 30 sec). The *Actin* gene (Os03g0718100) served as an internal control,
458 and the Delta CT method was used to calculate the relative expression levels. The primer
459 sets are listed in S4 Table.
460

461 **RNA-seq**

462 Total RNA was extracted from Hitomebore and *qLTG11*-NIL seeds at 0, 1, 2, and 3 days
463 after the onset of seed hydration under low (15°C) or optimum (25°C) temperature
464 conditions using an RNA-suisui S kit (Rizo, Ibaraki, Japan). Sequencing libraries were
465 prepared using an NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New
466 England Biolabs Japan, Tokyo, Japan) following the manufacturer's protocol. The
467 libraries were sequenced in paired-end mode using an Illumina HiSeq X instrument
468 (Illumina, CA, USA). The raw reads have been deposited in the DNA Databank of Japan

469 (BioProject accession No. PRJDB17450; S5 Table). For quality control, reads shorter
470 than 50 bases and those with an average read quality below 20 were discarded using
471 Trimmomatic v0.36 [53], and poly(A) sequences were trimmed using PRINSEQ++ v1.2
472 [54]. The resulting clean reads were aligned to the *de novo* assembled Hitomebore and
473 Arroz da Terra genomes with HISAT2 v2.1 [55]. BAM files were sorted and indexed with
474 SAMtools v1.10 [56], and aligned reads were assembled into transcripts with StringTie
475 [57] by combining bam files for each variety. In a similar manner, the expression data
476 were generated using the Nipponbare reference genome downloaded from IRGSP-1.0
477 (https://rapdb.dna.affrc.go.jp/download/irgsp1.html).
478

479 **Haplotype network analysis**

480 Sequencing datasets were obtained for 503 rice accessions. Of these, 379 were FASTQ
481 files downloaded from the DNA Data Bank of Japan Sequence Read Archive (DRA) [33-
482 35, 58] and 124 were sequenced in this study (S2 Table). Details about DNA extraction,
483 whole-genome sequencing techniques, and construction of the genotype datasets in VCF
484 format are provided in a previous report [35]. This study specifically focused on the
485 coding region of *GF14h*. Genotype information related to the coding region of *GF14h*
486 was extracted from the VCF dataset. In addition, the *k*-mer analysis program
487 (https://github.com/taitoh1970/kmer) [59] was used with Illumina short reads to detect
488 the 4-bp deletion with high sensitivity. Genotype information for the presence of the 4-
489 bp deletion was added to the VCF file, and 81 samples with heterozygous genotypes were
490 discarded. A haplotype network was then constructed using the median-joining network
491 algorithm [60] implemented in Popart v1.7 [61].
492

Evaluation of agronomic traits and yield performance of NIL-*GF14h*^{Arroz}

The grain yields of Hitomebore and NIL-*GF14h*^{Arroz} were investigated in experimental paddy fields in 2023. Field experiments were conducted at the Iwate Agricultural Research Center (39°35'N, 141°11'E) in Kitakami, Iwate, Japan. A fertilization regime of $N:P_2O_5:K_2O$ = 6:6:6 g m^{-2} was applied as a basal dressing, and $N:K_2O$ = 2:2 g m^{-2} was applied as a top dressing. Seeds were sown in a seedling nursery box on 21 April, and seedlings were transplanted to the paddy field on 18 May. To evaluate agronomic traits, the seedlings were transplanted at a rate of one plant per hill, with a planting density of 22.2 hills m^{-2}. Culm length, panicle length, panicle number, grain number per plant, and grain weight per plant were measured at maturity. To evaluate yield performance, seedlings were transplanted with three plants per hill at a planting density of 16.7 hills m^{-2}. The 0.9 × 5.0 m experimental plots in the paddy fields were arranged in a randomized complete block design with three replicates. At maturity, 50 hills were harvested from each plot to measure brown rice yield. The hulls were removed using a rice huller (Model 25MC, Ohya Tanzo Factory Co., Ltd., Japan), and the hulled grains were screened with a grain sorter (1.9-mm sieve size). Brown rice yields were adjusted to 15% moisture content and converted to weight per hectare.

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Competing interests

None declared.

Author contributions

Conceptualization: Akira Abe, Hiroki Takagi, Ryohei Terauchi

Data curation: Yusaku Sugimura, Akira Abe

Formal analysis: Yusaku Sugimura, Yu Sugihara, Akira Abe

Funding acquisition: Akira Abe, Hiroki Takagi, Ryohei Terauchi

Investigation: Yusaku Sugimura, Akira Abe, Kaori Oikawa, Yu Sugihara, Hiroe Utsushi, Eiko Kanzaki, Kazue Ito, Yumiko Ogasawara, Tomoaki Fujioka, Hiroki Takagi, Motoki Shimizu, Hiroyuki Shimono

Supervision: Akira Abe, Ryohei Terauchi

Validation: Yusaku Sugimura, Akira Abe

Visualization: Yusaku Sugimura, Yu Sugihara, Akira Abe

Writing – original draft: Yusaku Sugimura, Akira Abe

Writing – review & editing: Yusaku Sugimura, Akira Abe, Ryohei Terauchi

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Figure legends

Fig 1. Effects of quantitative trait loci (QTLs) on low-temperature seed germinability.

(A) Representative photographs showing the germination of seeds from the Iwatekko, Hitomebore, and Arroz da Terra varieties 11 days (15°C) or 3 days (25°C) after the onset of seed imbibition. Scale bar, 1 cm. **(B–C)** Germination time courses of Iwatekko, Hitomebore, and Arroz da Terra at 15°C (B) or 25 °C (C). Values are means ± standard deviation (SD) from biologically independent samples (n = 8). Dunnett's test shows significant differences in germination for Hitomebore (upper) and Iwatekko (lower) compared with Arroz da Terra at each time point ($^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$). **(D)** Map positions of QTLs for low-temperature germination, as determined by QTL-seq. The Δ (SNP-index) values (red lines) were plotted for chromosomes 3 and 11, with statistical confidence intervals under the null hypothesis of no QTL (green, $P < 0.05$; orange, $P < 0.01$). **(E)** Diagram showing the genotype of *qLTG11*-NIL. *qLTG11*-NIL harbors the Arroz da Terra allele at *qLTG11* on chromosome 11. Light blue indicates genomic fragments from Hitomebore; red indicates genomic fragments from Arroz da Terra; dark blue indicates heterozygous regions. **(F)** Germination time courses of Hitomebore and *qLTG11*-NIL at 15°C. Values are means ± SD from biologically independent samples (n = 10). Two-tailed t-test was used between *qLTG11*-NIL and Hitomebore for each time point ($^{*}P < 0.05$ and $^{***}P < 0.001$).

801 **Fig 2. Positional cloning of *qLTG11*.**

802 **(A)** Fine mapping of *qLTG11* to a 52-kb region between markers E and J. The
803 chromosomal positions are based on the Nipponbare reference genome (Os-Nipponbare-
804 Reference-IRGSP-1.0). Germination percentage was determined at 9 days of incubation
805 at 15°C. Red and blue rectangles indicate chromosomal segments homozygous for Arroz
806 da Terra or Hitomebore, respectively. Different lowercase letters indicate significant
807 differences (n = 3 biologically independent samples, $P < 0.001$, Tukey's HSD test). **(B)**
808 Genomic structure of the candidate genomic region in Arroz da Terra and Hitomebore.
809 Os11g0609600 (shown in red), encoding GF14h, is expressed in germinating seeds. **(C)**
810 Diagram of the *GF14h* gene structure and sequence polymorphisms between Arroz da
811 Terra and Hitomebore. The chromosomal positions are based on the Nipponbare reference
812 genome. The coding region of *GF14h* in Hitomebore is identical to that in Nipponbare.
813 The 4-bp deletion in Hitomebore causes a frameshift and the introduction of a premature
814 stop codon. **(D)** Relative *GF14h* expression levels in germinating seeds of *qLTG11*-NIL.
815 This expression analysis was conducted by RT-qPCR. In the boxplots, the box edges
816 represent the upper and lower quantiles, the horizontal line in the middle of the box
817 represents the median value, whiskers represent the lowest quantile to the top quantile,
818 and the black squares show the mean. Five biological replicates were measured
819 independently. Different lowercase letters indicate significant differences based on
820 Tukey's HSD test ($P < 0.05$). *OsActin1* (Os03g0718100) was used for normalization.

821

822 **Fig 3. Effect of *GF14h* mutation and overexpression on low-temperature**
823 **germination.**

824 **(A)** Representative photographs showing seed germination in wild-type harboring Arroz-

825 type *GF14h* (WT^{Arroz}) and CRISPR/Cas9 knockout lines (*gf14-1*) at 8 days after the onset
826 of seed imbibition. Scale bar, 1 cm. **(B)** Seed germination rate of WT^{Arroz} and its
827 CRISPR/Cas9 knockout lines at 7 days of seed imbibition at 15°C. The two target
828 constructs (S9 Fig) were introduced into the *qLTG11*-NIL line. Data are means ± standard
829 error (SE, n = 3). Different lowercase letters indicate significant differences based on
830 Tukey's HSD test ($P < 0.01$). **(C)** Representative photographs showing seed germination
831 of wild-type ($WT^{Hitomebore}$) and $OsGF14h^{Arroz}$ overexpression lines ($OsGF14h^{Arroz}$-Ox #2)
832 at 7 days of seed imbibition at 15ºC. Scale bar, 1 cm. **(D)** Seed germination rate of
833 $WT^{Hitomebore}$ and $GF14h^{Arroz}$ overexpression lines in the Hitomebore background at 7 days
834 of seed imbibition at 15°C. Data are means ± SE (n = 3). Different lowercase letters
835 indicate significant differences based on Tukey's HSD test ($P < 0.05$).
836
837 **Fig 4. Gradual selection of loss-of-function alleles in *GF14h* and *qLTG3-1* during**
838 **rice breeding in Japan.**
839 A total of 350 Japanese varieties were examined, including the World Rice Core
840 Collection [33], the Rice Core Collection of Japanese Landraces [34], and the collection
841 of Japanese core cultivars [35]. The allele type at each gene was determined to be
842 functional or nonfunctional by the k-mer method using Illumina short reads for each
843 variety. **(A)** Proportion of allele type combinations at *GF14h* and *qLTG3-1* sorted by
844 breeding year. **(B)** Proportion of nonfunctional allele types at *GF14h* and *qLTG3-1* sorted
845 by breeding year.
846
847 **Fig 5. Phenotypic analysis of a near-isogenic line homozygous for Arroz-type *GF14h***
848 **in the Hitomebore genetic background (NIL-$GF14h^{Arroz}$).**

849 **(A)** Gross morphology of Hitomebore and NIL-*GF14h*^{Arroz} at 87 days after transplanting
850 (top row), and seed germination at 9 days after the onset of seed imbibition at 15°C
851 (bottom row). Scale bars, 20 cm (top), 1 cm (bottom). **(B)** Low-temperature germination
852 ability of NIL-*GF14h*^{Arroz} at 8 days after the onset of seed imbibition at 15°C. Values are
853 means ± SE of biologically independent samples (n = 3). Asterisks indicate significant
854 differences, as determined by two-tailed t-test. **(C–G)** Agronomic traits in Hitomebore
855 and NIL-*GF14h*^{Arroz}: culm length (**C**), panicle length (**D**), panicle number (**E**), grain
856 number per plant (**F**), and grain weight per plant (**G**). Values are means ± SE of
857 biologically independent plants (n = 40). **(H)** Side view of Hitomebore and NIL-
858 *GF14h*^{Arroz} growing in the field under typical rice-growing conditions at the maturity
859 stage. **(I)** Brown rice yield per unit area. Values are means ± SE of biologically
860 independent plots (n = 3). P-values calculated by t-tests are listed throughout the figure.
861

862 # Supporting information

863 **S1 Fig. Frequency distribution of germination rates in the RIL population and**
864 **germination rates of selected RILs for QTL-seq analysis.**
865 **(A)** Frequency distribution of germination rates at 13°C after eight days from seed
866 imbibition in 200 F7 RILs derived from a cross between Iwatekko and Arroz da Terra
867 [31]. **(B)** Selection of RILs with low cold germination rates. The 62 RILs with the lower
868 germination rates from the first test (A) were tested for the second, and then the 20 RILs
869 with the lowest germination rates were selected as a bulk sample for QTL-seq analysis.
870 The bar graph shows the mean values of the two tests. **(C)** Selection of RILs with high
871 cold germination rates. The 37 RILs with the higher germination rates from the first test

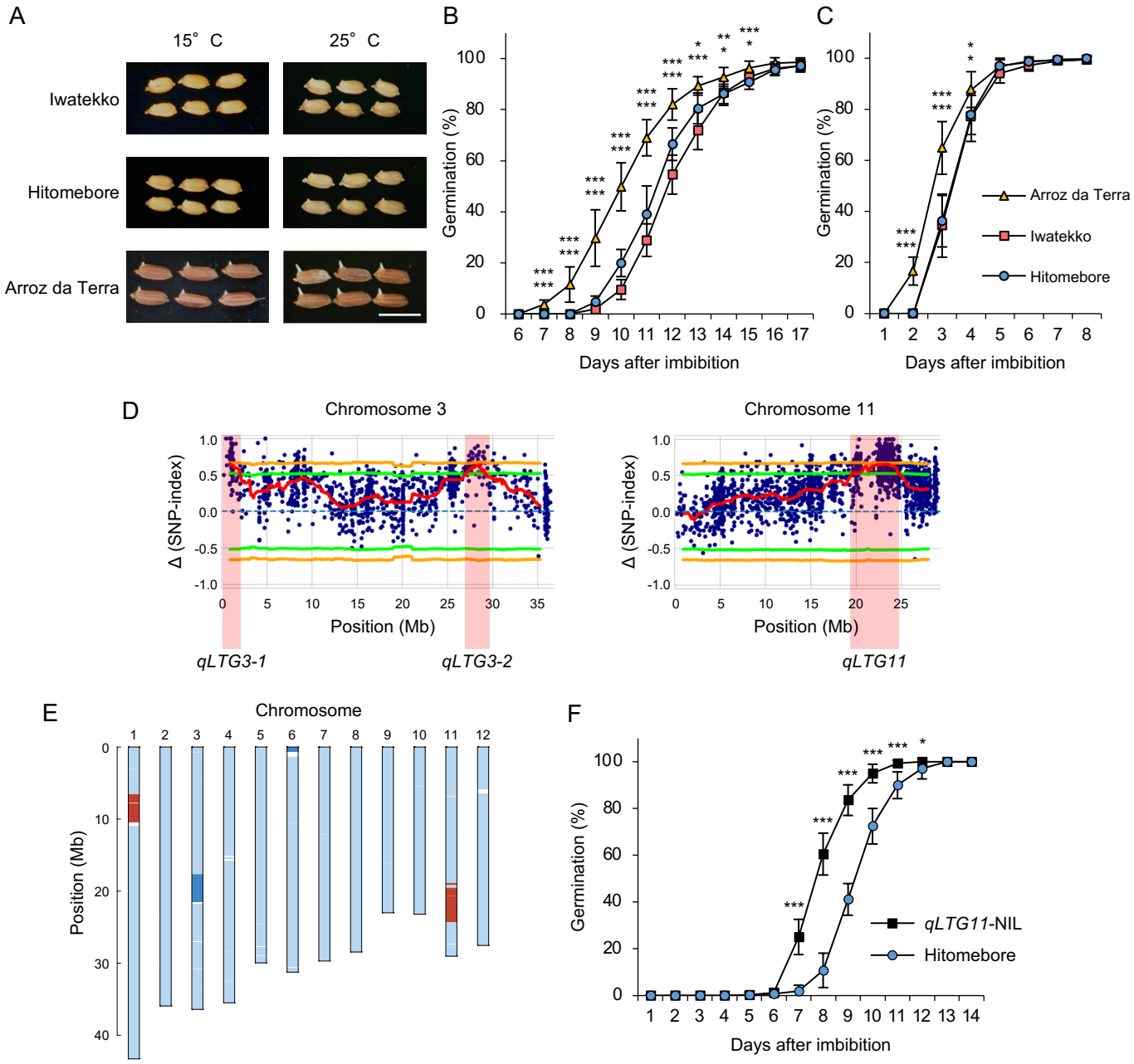
872 (A) were tested for the second, and then the 20 RILs with the highest germination rates
873 were selected for a bulk sample for QTL-seq analysis. The bar graph shows the average
874 values of the two tests.
875
876 **S2 Fig. Multiple DNA sequence alignment of *qLTG3-1* variants.**
877 Arroz da Terra and Italica Livorno harbor a functional *qLTG3-1* variant. Nipponbare
878 carries another functional *qLTG3-1* variant due to the nonsynonymous substitution (*).
879 Iwatekko, Hitomebore, and Hayamasari contain a loss-of-function variant for *qLTG3-1*
880 due to a 71-bp deletion.
881
882 **S3 Fig. Generation of a near-isogenic line with high LTG in the Hitomebore**
883 **background.**
884 **(A)** Strategy for the development of *qLTG3-2*-NIL, *qLTG11*-NIL, and NIL-$GF14h^{Arroz}$.
885 Molecular markers were used for foreground and background selection. **(B)** Diagram
886 showing the genotype of NIL-$GF14h^{Arroz}$. NIL-$GF14h^{Arroz}$ contains a 172-kb region on
887 chromosome 11 harboring the Arroz da Terra allele of *GF14h*. Light blue bars indicate
888 genomic fragments from Hitomebore; red bars indicate genomic fragments from Arroz
889 da Terra.
890
891 **S4 Fig. Summary of *qLTG3-2*.**
892 **(A)** Diagram showing the genotype of *qLTG3-2*-NIL containing the Arroz da Terra allele
893 at *qLTG3-2* on chromosome 3. Light blue bars indicate genomic fragments from
894 Hitomebore; red bars indicate genomic fragments from Arroz da Terra; dark blue bars
895 indicate heterozygous regions. **(B)** Germination time courses for seeds of Hitomebore,

896 the *qLTG3-2*-NIL, and Arroz da Terra at 15°C. Values are means ± SD of biologically
897 independent samples (Hitomebore and NIL n = 10, Arroz da Terra n = 5).
898
899 **S5 Fig. Seed germination of *qLTG11*-NIL under optimal temperature conditions.**
900 Germination time courses of seeds from Hitomebore and *qLTG11*-NIL at 25°C. Values
901 are means ± SD of biologically independent samples (n = 3). Two-tailed t-test was used
902 between *qLTG11*-NIL and Hitomebore for each time point (*P < 0.05 and **P < 0.01).
903
904 **S6 Fig. Comparison of the *qLTG11* genomic region in Hitomebore, Arroz da Terra,**
905 **and Nipponbare.**
906 Dot blot analyses of the genomic sequence in the *qLTG11* candidate region between **(A)**
907 Hitomebore and Nipponbare and **(B)** Hitomebore and Arroz da Terra, using D-GENIES
908 [62]. Based on the Nipponbare genome (IRGSP-1.0), the genomic region containing the
909 causative gene is located at 23.512–23.564 Mb (approximately 52 kb) on chromosome
910 11. The genome sequence of Hitomebore is identical to that of Nipponbare. The candidate
911 region corresponds to a fragment of approximately 94 kb in the Arroz da Terra genome.
912
913 **S7 Fig. Expression levels of the two annotated genes in the candidate genomic region**
914 **of *qLTG11* based on RNA-seq data.**
915 Total RNA was extracted from Hitomebore and *qLTG11*-NIL seeds at 0, 1, 2, and 3 days
916 after the onset of seed imbibition under 15 or 25°C temperature conditions, followed by
917 RNA-seq. The sequence reads were mapped to the Nipponbare genome (IRGSP-1.0), and
918 expression data were obtained. **(A–B)** The expression levels of Os11g0609600 (*GF14h*)
919 during seed germination under 15°C (A) and 25°C (B) are shown. Data are presented as

920 means ± SE. n = 3 biologically independent samples. **(C–D)** The expression levels of
921 Os11g0609500 (*Jacalin-like lectin domain containing protein*) during seed germination
922 under 15°C (C) and 25°C (D) are shown. Data are presented as means ± SE. n = 3
923 biologically independent samples.
924
925 **S8 Fig. Multiple DNA sequence alignment of *GF14h* variants.**
926 Arroz da Terra carries a functional *GF14h* variant. Hitomebore and Nipponbare harbors
927 a loss-of-function variant of *GF14h* due to a 4-bp deletion (black line).
928
929 **S9 Fig. CRISPR/Cas9-mediated genome editing of *GF14h*.**
930 Top, diagram showing the *GF14h* locus, with the locations of the two sgRNA target sites
931 marked by inverted red triangles. Bottom, sequencing results of putative *gf14h* mutants.
932 The sgRNA target sites are underlined, and the PAMs are highlighted. The mutation sites
933 in $GF14h^{Arroz}$ for the four mutants (*gf14h-1*, *gf14h-2*, *gf14h-3,* and *gf14h-4*) are indicated.
934
935 **S10 Fig. Effect of *GF14h* mutation on optimal-temperature germination.**
936 **(A)** Representative photographs showing seed germination in wild-type harboring Arroz-
937 type *GF14h* (WT^{Arroz}) and CRISPR/Cas9 knockout lines (*gf14-1*) at 3 days after the onset
938 of seed imbibition. Scale bar, 1 cm. **(B)** Seed germination rate of WT^{Arroz} and its
939 CRISPR/Cas9 knockout lines at 2 days of seed imbibition at 25°C. The two target
940 constructs (S9 Fig) were introduced into the *qLTG11*-NIL line. Data are means ± standard
941 error (WT^{Arroz}, *gf14h-1*, *gf14h-2* and *gf14h-4*, n = 3; *gf14h-3*, n = 2). Different lowercase
942 letters indicate significant differences based on Tukey's HSD test ($P < 0.05$).
943

944 **S11 Fig. Relative *GF14h* expression levels in germinating seeds of *GF14h*^{Arroz}**
945 **overexpression lines and the parental line.**
946 The *GF14h*^{Arroz} overexpression construct was introduced into Hitomebore. *OsActin1*
947 (Os03g0718100) was used for normalization. Values are means ± SE (n = 3 or 4).
948 Different lowercase letters indicate significant differences based on Tukey's HSD test (P
949 < 0.001).
950
951 **S12 Fig. Haplotype network of *GF14h*.**
952 The *GF14h* genomic sequences obtained from 411 *O. sativa* varieties and 11 *O. rufipogon*
953 accessions were used for analysis (S1 Table). The haplotype network was reconstructed
954 by the median joining network algorithm [60] implemented in Popart v1.7 [61]. The
955 haplotype Hap1 evolved from Hap2 by acquiring the 4-bp sequence, resulting in a
956 nonfunctional *GF14h* gene. The Hitomebore cultivar contains Hap1 (nonfunctional), and
957 the Arroz da Terra cultivar contains Hap9 (functional).
958
959 **S13 Fig. Pre-harvest sprouting of NIL-*GF14h*^{Arroz}.**
960 Germination time courses of seeds from Hitomebore (blue circles) and the NIL-
961 *GF14h*^{Arroz} (pink triangles) under wet conditions at 28°C. Seeds were harvested from
962 tagged panicles 30 days after heading. Values are means ± SE of biologically independent
963 samples (n = 3). The P-values calculated from t-tests at each time point are shown in the
964 figure.
965
966 **S14 Fig. Development of a functional marker based on the 4-bp deletion in *GF14h*.**
967 **(A)** Diagram of the sequence around the 4-bp InDel of *GF14h*. In the Hitomebore (loss-

968 of-function) allele, the 4-bp deletion creates a SmlI restriction site. **(B)** Genotyping of the
969 4-bp deletion in *GF14h*. A genomic fragment containing the 4-bp InDel of *GF14h* was
970 amplified by PCR and digested with SmlI. The products were separated on a 3% (w/v)
971 agarose gel and stained with Midori Green. The PCR product from $GF14h^{Arroz}$
972 (approximately 500 bp) was not cleaved, whereas the PCR product from $GF14h^{Hitomebore}$
973 was cleaved, producing two fragments of approximately 250 bp each. Both bands were
974 detected in heterozygous plants.
975
976 **S1 Table. Expression profile (TPM) during seed germination by RNA-seq.**
977
978 **S2 Table. List of rice varieties used in this study and their sequence read archive**
979 **(SRA) IDs.**
980
981 **S3 Table. Haplotypes of the *GF14h* gene analyzed in S12 Fig.**
982
983 **S4 Table. Primers used in this study.**
984
985 **S5 Table. List of RNA-seq samples used in this study and their sequence read archive**
986 **(SRA) IDs.**
987



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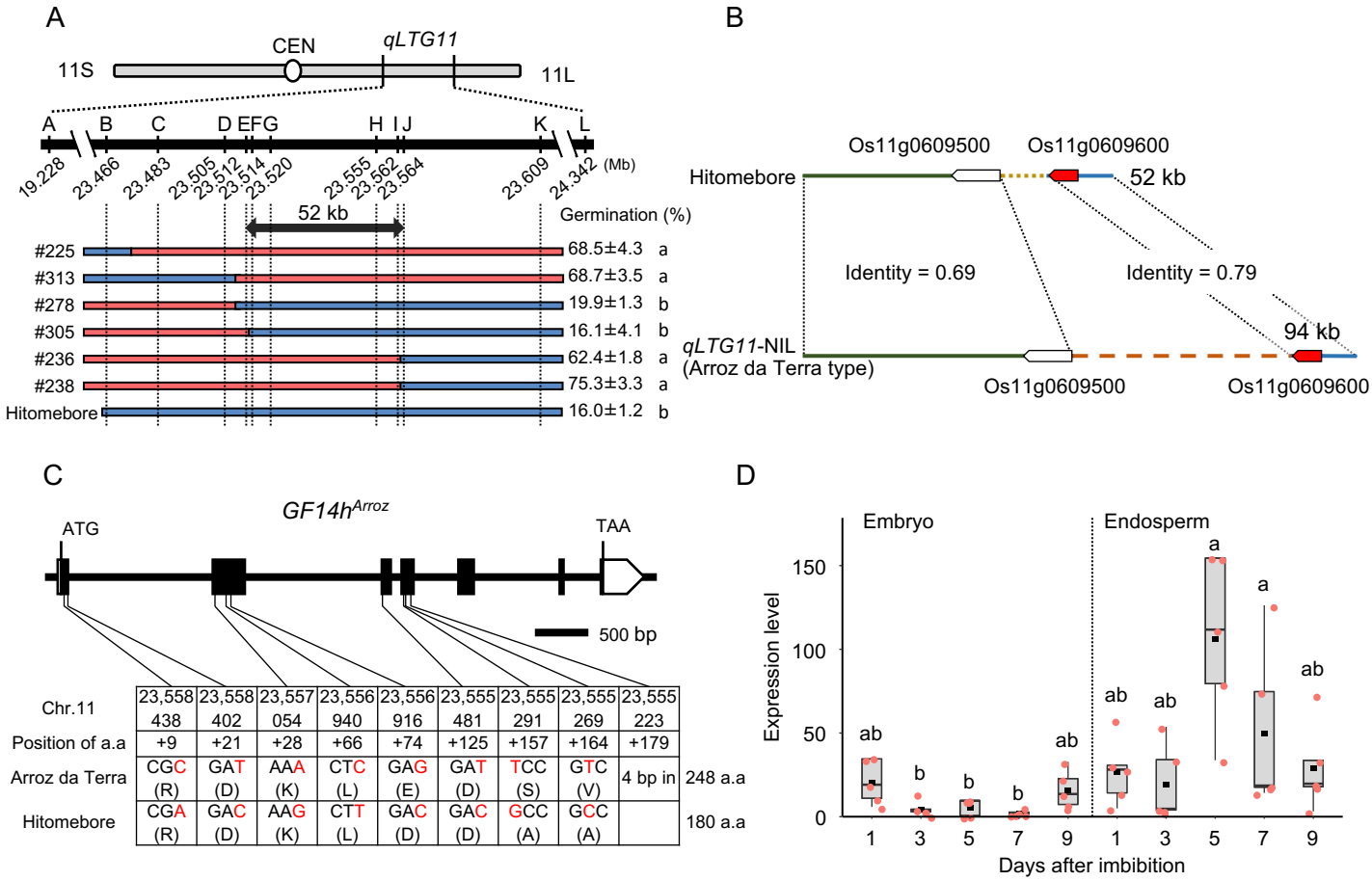

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(A) Fine mapping of *qLTG11* to a 52-kb region between markers E and J. The chromosomal positions are based on the Nipponbare reference genome (Os-Nipponbare-Reference-IRGSP-1.0). Germination percentage was determined at 9 days of incubation at 15° C. Red and blue rectangles indicate chromosomal segments homozygous for Arroz da Terra or Hitomebore, respectively. Different lowercase letters indicate significant differences (n = 3 biologically independent samples, $P < 0.001$, Tukey's HSD test). **(B)** Genomic structure of the candidate genomic region in Arroz da Terra and Hitomebore. Os11g0609600 (shown in red), encoding GF14h, is expressed in germinating seeds. **(C)** Diagram of the *GF14h* gene structure and sequence polymorphisms between Arroz da Terra and Hitomebore. The chromosomal positions are based on the Nipponbare reference genome. The coding region of *GF14h* in Hitomebore is identical to that in Nipponbare. The 4-bp deletion in Hitomebore causes a frameshift and the introduction of a premature stop codon. **(D)** Relative *GF14h* expression levels in germinating seeds of *qLTG11*-NIL. This expression analysis was conducted by RT-qPCR. In the boxplots, the box edges represent the upper and lower quantiles, the horizontal line in the middle of the box represents the median value, whiskers represent the lowest quantile to the top quantile, and the black squares show the mean. Five biological replicates were measured independently. Different lowercase letters indicate significant differences based on Tukey's HSD test ($P < 0.05$). *OsActin1* (Os03g0718100) was used for normalization.

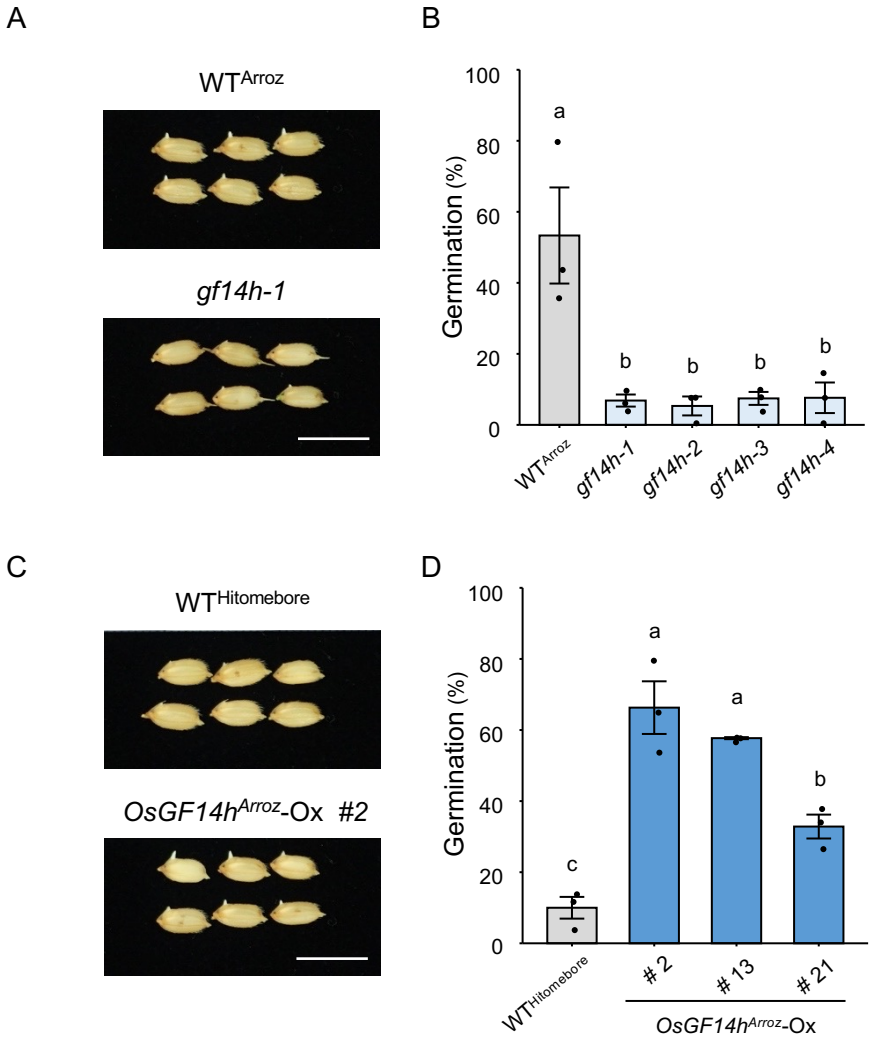
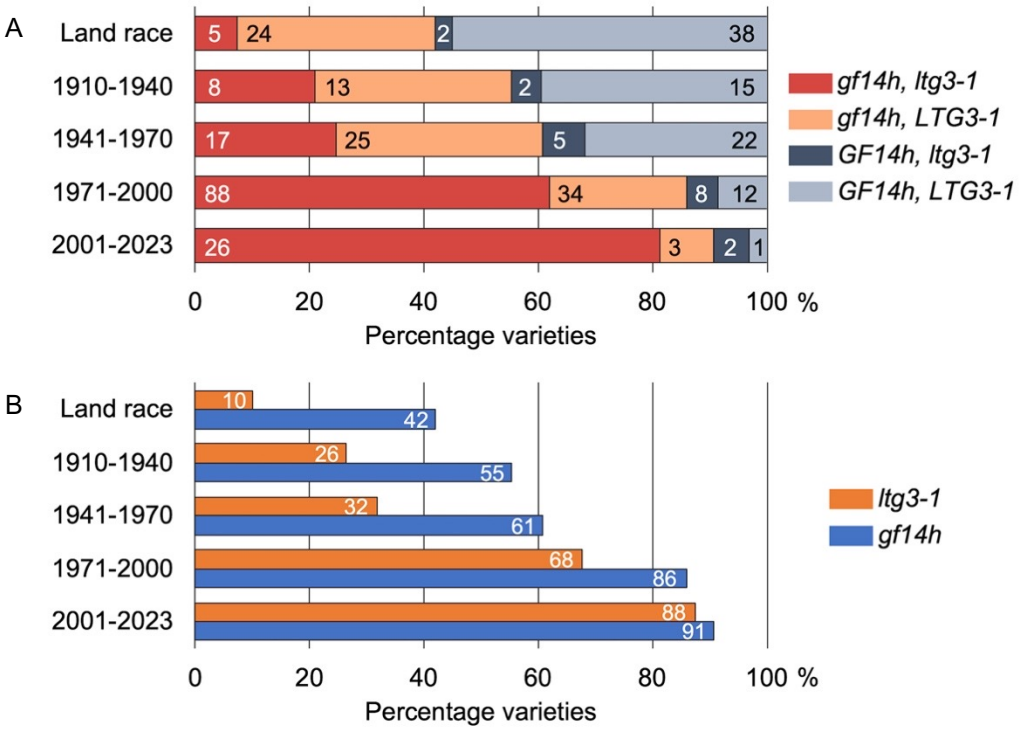


Fig 3. Effect of *GF14h* mutation and overexpression on low-temperature germination. **(A)** Representative photographs showing seed germination in wild-type harboring Arroz-type *GF14h* (WT^{Arroz}) and CRISPR/Cas9 knockout lines (*gf14-1*) at 8 days after the onset of seed imbibition. Scale bar, 1 cm. **(B)** Seed germination rate of WT^{Arroz} and its CRISPR/Cas9 knockout lines at 7 days of seed imbibition at 15° C. The two target constructs (S9 Fig) were introduced into the *qLTG11*-NIL line. Data are means ± standard error (SE, n = 3). Different lowercase letters indicate significant differences based on Tukey's HSD test ($P < 0.01$). **(C)** Representative photographs showing seed germination of wild-type ($WT^{Hitomebore}$) and $OsGF14h^{Arroz}$ overexpression lines ($OsGF14h^{Arroz}$-Ox #2) at 7 days of seed imbibition at 15ºC. Scale bar, 1 cm. **(D)** Seed germination rate of $WT^{Hitomebore}$ and $GF14h^{Arroz}$ overexpression lines in the Hitomebore background at 7 days of seed imbibition at 15° C. Data are means ± SE (n = 3). Different lowercase letters indicate significant differences based on Tukey's HSD test ($P < 0.05$).



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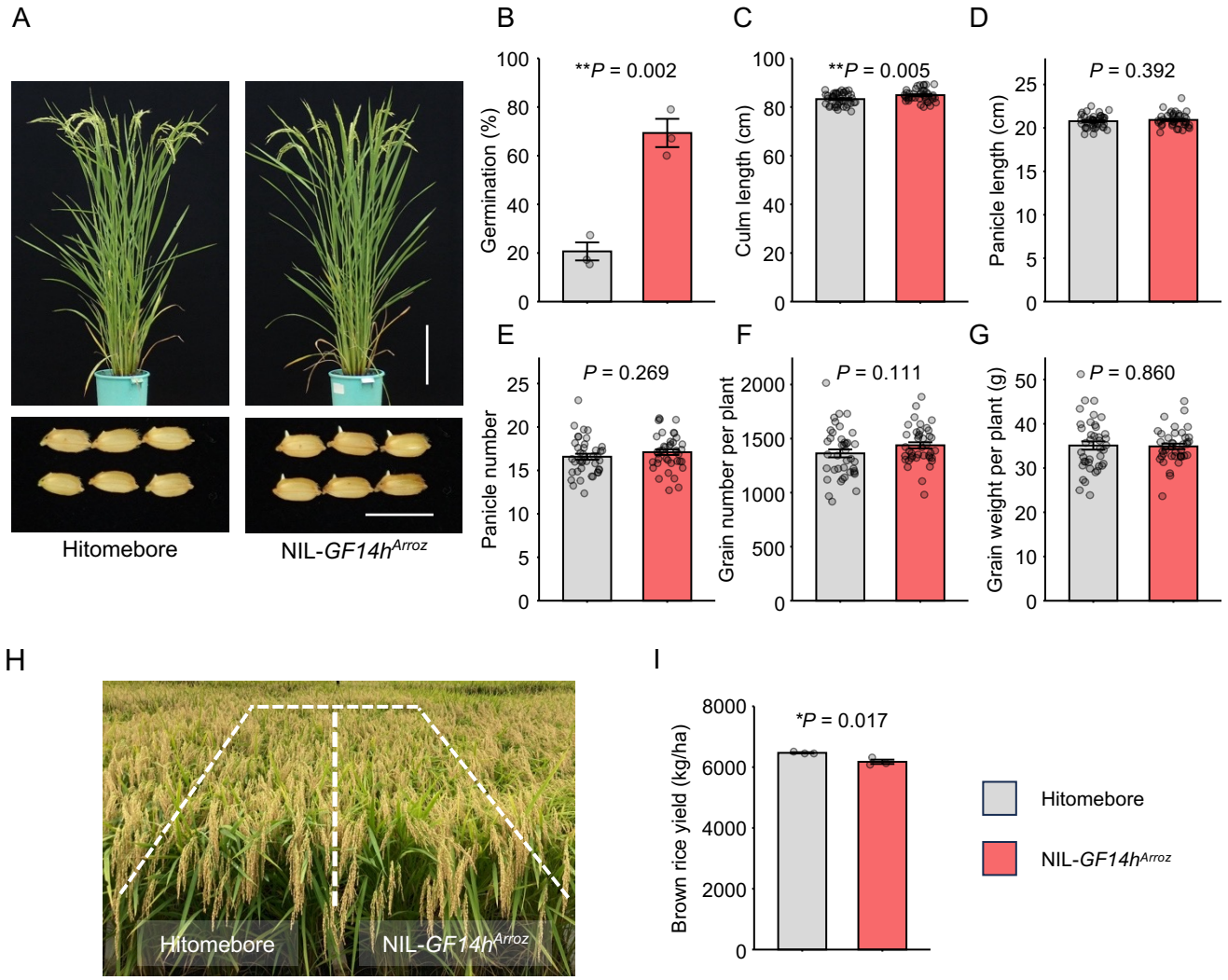
A total of 350 Japanese varieties were examined, including the World Rice Core Collection [33], the Rice Core Collection of Japanese Landraces [34], and the collection of Japanese core cultivars [35]. The allele type at each gene was determined to be functional or nonfunctional by the k-mer method using Illumina short reads for each variety. **(A)** Proportion of allele type combinations at *GF14h* and *qLTG3-1* sorted by breeding year. **(B)** Proportion of nonfunctional allele types at *GF14h* and *qLTG3-1* sorted by breeding year.



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