Genome-wide association study (GWAS) of seed germination-related traits in rice and identification of candidate genes

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Abstract

Background

Genome-wide association study (GWAS) has become an accepted and powerful method for understanding the associations between phenotypes and genotypes. In agricultural production, uniform and rapid germination is an important prerequisite in crop production. Here, a rice (*Oryza sativa* L.) GWAS with 33,934 SNPs (MAF > 0.05) for eight germination traits including germination percentage (GP), shoot (SL) and root length (RL), root (RFW) and shoot fresh weight (SFW), root (RDW) and shoot (SDW) dry weight, and number of days to germinate (NDG) was performed to define genomic regions influencing seed germination.

Results

Loci (43) with 70 significant germination-associated markers were detected across all rice chromosomes. Some of novel candidate associated genes were: LOC_Os01g26210 (OsWAK6) co-located with qGR-1 that is seed vigor QTL, LOC_Os07g23944 (GH31) with an α -glucosidases /starch lyase activity; id7000519 marker that corresponds to a gene cluster containing glutathione S-transferase and glucan endo-1,3- β -glucosidase co-located with qAG3 germination-related marker, LOC_Os06g47640 (calmodulin-related calcium sensor protein 29) involved in the inhibition of ABA during seed germination, and id4006430 marker that corresponds to a gene cluster containing three GH17 hydrolytic enzyme that are co-located with qHD4 and qGI1 markers.

Conclusion

The germination process is an initial and important step in the production of agricultural products, especially for rice, which is a crop plant that is grown in flooded lands. Here, the genetic diversity of rice genotypes was put under scrutiny for germination. Our GWAS results identified several likely candidate genes for germination traits that will greatly contribute to our understanding of the genetic complexity underlying the corresponding traits. The associated genes with the germination traits can be generally classified as hydrolytic enzymes and regulatory proteins that can directly or indirectly influence germination.

Background

Successful germination, with direct association to crop yield, is an important step in seedlings establishment (Almansouri et al. 2001). Cereals seeds have a similar structure that include seed coat, bran, starchy endosperm and embryo (Linkies et al. 2010). Germination in cereals initiates with the activation of hydrolytic enzymes present in aleurone following imbibition and uptake of water. The enzymes, mainly amylases and glucanases, digest the starch present in endosperm to release glucosyl residues that then can be transported through scutellum to embryo. The embryo uses glucose as energy source to germinate (Joshi 2018). The germination is regulated by abscisic acid (ABA) and gibberellins (GAs) with contradictory effect on the process: ABA has inhibitory- (Nambara et al. 2010), while GAs have stimulatory- roles in hydrolytic enzyme secretion (Mrva et al. 2006; Guzmán-Ortiz et al. 2019). Other factors such as cGMP, calcium, calmodulin, and protein kinases regulate the aleurone response to hormones during germination (Ritchie et al. 2000). In addition to the starch-degrading enzymes, other enzymes that break down cell wall components, including enzymes such as arabinoxylan hydrolases, xylopyranosidase, α -galactosidase and endoxylanase have also

been shown to be increased during germination (Guzmán-Ortiz et al. 2019; Ha et al. 2018; Andriotis et al. 2016; González-Calle et al. 2015; Gomez et al. 2009; Grant et al. 2003; Leubner-Metzger, 2003; Caspers et al. 2001). Catalase has been suggested to be involved in the promotion of dormant seed germination (Ayşe et al. 2012). Lipids have been shown to be an important source of energy in the early stages of germination, and lipase activity increases during germination (Kawiński et al. 2021; Vijayakumar and Gowda 2012). Phytases, also found in aleurone, scutellum, and embryo (Guzmán-Ortiz et al. 2019), improve the bioavailability of inorganic phosphate to help in seedlings growth with their temporal expression (Sung et al. 2005). Other enzymes that are activated in the germination process are protein degradation enzymes. Hydrolysis of proteins stored in seeds helps to release free amino acids, and these free amino acids help the biosynthesis of proteins in the endosperm and embryo (Joshi 2018). In this research, the main goal was to perform a GWAS to identify the genomic regions affecting the germination in an international rice population in order to identify candidate genes associated with germination traits.

Results

Phenotypic variation (PV) in rice accessions

Most of the traits were almost normally distributed, except for RDW, SDW and RFW showing skewness (Supplementary Fig. 1). We considered the emergence of the radical tip as the beginning of germination. Most of the cultivars reached this stage after 4 d, but the range was 2–7 d (Table 1), meaning how many seeds are germinated. The broad-sense heritability (h_b^2) was between 0.465 for RDW to 0.746 for SL (Table 1), indicating that most of traits are relatively under the control of environment with a lot of genes being involved in defining the germination in rice.

Table 1
Descriptive statistics of germination traits in a collection of rice pupulation. Standard deviation, minimum and maximum values for germination traits in the *Oryza sativa* collection are provided.

	NDG	RDW (mg)	SDW (mg)	GP	SL (cm)	RL (cm)	RFW (mg)	SFW (mg)
Average	4	0.0040	0.0273	0.910	6.800	5.210	0.0210	0.212
Minimum	2	0.0007	0.0040	0.230	13.800	1.030	0.0005	0.010
Maximum	7	0.0414	0.2730	0.950	1.530	12.000	0.3033	0.440
H_b^2	-	0.4650	0.5340	-	0.746	0.632	0.6150	0.497
SD	1.507	0.0050	0.0380	0.116	2.055	2.345	0.0440	0.075

Population stratification, LD levels and GWAS

The kinship matrix summarized the distribution of the pairwise relative relationship coefficients among the accessions in the association panel based on SNPs information (Fig. 1a). The results showed relatively higher genetic relatedness within populations. All twelve rice chromosomes showed a significant and very slow LD decay; as were dropped to 0.2 in around ~ 400 kb (nearly 2 cM) and to 0.1 in around 1.2 Mbp (more than 5 cM) (Fig. 1b). Our data were consistent with an earlier result obtained in rice (Mather et al. 2007). Scree plot from GAPIT showed the selection of PCs, representative of population structure, for association study in three main groups (Fig. 1c). The first three PCs explained 9.4%, 7.1%, and 0.9% of the variation (Fig. 1d).

Marker-trait association (MTA) analysis

In order to identify genetic loci responsible for variation in germination traits in rice accessions, GWAS was carried out on a high-quality SNP array. The Manhattan and Q-Q plots for germination traits are shown in Fig. 2 and supplementary Fig. 2 respectively. Markers with $-\log_{10}(P) > 3$ were considered as significant markers for NDG, RDW, SL, and SFW. For SDW, RL, and RFW, markers with $-\log_{10}(P) > 4$ were considered as significant. Markers with $-\log_{10}(P) > 10$ were considered as significant markers for GP. The MLM model identified a total of 70 significant marker-trait associations (Table 2). For NDG, five genomic regions (a total of 11 SNP markers) were identified on chromosomes 1, 2, 6, 8, 12, demonstrating 5.0-6.23% of the PV. For RDW, three genomic regions tagged by five SNP markers were identified on chromosomes 4, 7 and 8 with 6.65–8.03% of the PV. For SDW, four genomic regions tagged to seven SNP markers on chromosomes 2, 3, 7 and 8 were identified. The PV for SDW was between 7.4–18.7%. For GP, five genomic regions tagged by 15 SNP markers were identified that explained 22.38-58.0% of the PV. These regions were on chromosomes 1, 2, 4, 7, and 12. For SL, five significant SNP markers identified in 2 genomic regions (chromosomes 7 and 12), which explained between 6.62–7.38% of the PV. For RL, five SNPs tagged in 2 genomic regions (chromosomes 8 and 12) were identified that explained 7.46–9.11% of the PV. For RFW, 12 SNPs in 4 genomic regions located on chromosomes 1, 2, 6 and 8 were identified with a PV of 7.52–18.66%. For SFW, eight SNPs were found on chromosomes 1 and 6 with a PV of 5.46–7.45% (Table 2). The physical locations of the markers and the genes are shown in Fig. 3.

Table 2
SNPs-association with NDG, RDW, SDW, GP, SL, RL, RFW, and SFW.

Trait	Marker	Chr.	R ²	-log ₁₀ P-value	Position (MBP)	No. of significant SNPs in QTL regions	Associated genes to SNP marker
NDG	id12001382	12	5.46	3.79	3407246 ± 50	1	LOC_Os12g07110 (acyl-CoA synthetase)
	id8001343	8	5.46	3.24	4081390 ± 50	3	LOC_Os08g07270 (O-glucosyltransferase 2), LOC_Os08g07290 (HEAT protein), LOC_Os08g07300 (haloacid dehalogenase 3)
	id1009883	1	6.23	3.16	14857164 ± 50	2	LOC_Os01g26210 (OsWAK6), LOC_Os01g26160 (Exportin 1)
	id6010296	6	5.40	3.14	19454974 ± 50	1	LOC_Os06g33320 (G-protein)
	id2016360	2	5.00	3.14	35519431 ± 50	4	LOC_Os02g58020 (ABC transporter), LOC_Os02g57940 (OsFBX74), LOC_Os02g57960 (Leucine Rich protein), LOC_Os02g58080 (SUT4)
RDW	id8007379	8	8.03	4.21	27025397 ± 50	1	LOC_Os08g42720 (solute carrier family 35)
	id4010508	4	6.56	3.64	31004419 ± 50	1	LOC_Os04g52250 (Protease inhibitor/seed storage/ ltp family)
	id8006888	8	6.52	3.56	24882466 ± 50	1	LOC_Os08g39370 (citrate transporter)
	wd7001684	7	6.86	3.43	12533004 ± 50	1	LOC_Os07g22350 (glucose-6-phosphate 1-dehydrogenase)
	id8007277	8	6.65	3.41	26539291 ± 50	1	LOC_Os08g42010 (nodulin)
SDW	id3017683	3	18.70	8	35509733 ± 50	1	LOC_Os03g62860 (glucan endo-1,3-beta-glucosidase)
	id2014670	2	8.38	4.17	32979137 ± 50	3	LOC_Os02g53820 (endoglucanase), LOC_Os02g54010 (lipase 3), LOC_Os02g54030 (polygalacturonase)

Trait	Marker	Chr.	R ²	-log ₁₀ P-value	Position (MBP)	No. of significant SNPs in QTL regions	Associated genes to SNP marker
	id7005737	7	9.27	4.13	28557782 ± 50	1	LOC_Os07g47820 (acyl-CoA dehydrogenase 10)
	ud8000043	8	7.70	4.12	580911 ± 50	1	LOC_Os08g01940 (non-lysosomal glucosylceramidase)
	id8000288	8	7.40	4	706101 ± 50	1	LOC_Os08g02094 (lipase/acyl hydrolase)
GP	wd1001610	1	58.00	21.23	21440232 ± 50	1	LOC_Os01g38229 (peptidyl-prolyl isomerase)
	ud7000240	7	40.90	16.5	3358568 ± 50	1	LOC_Os07g06860 (gibberellin receptor GID1L2)
	id7003209	7	36.35	15.45	20393040 ± 50	3	LOC_Os07g34190 (Stilbene and chalcone synthases), LOC_Os07g34140 (chalcone synthase), LOC_Os07g34110 (DUF6)
	id4006430	4	33.1	14.26	20380040 ± 50	3	LOC_Os04g33590 (hydrolase, alpha/beta fold family protein), LOC_Os04g33640 (glycosyl hydrolases family 17), LOC_Os04g33720 (glycosyl hydrolases)
	id1007796	1	25.36	11.43	10825444 ± 50	2	LOC_Os01g19170 (polygalacturonase), LOC_Os01g19220 (beta-D-xylosidase)
	wd1000531	1	25.36	11.42	10713826 ± 50	1	LOC_Os01g18950 (peroxidase)
	id12006484	12	25.24	11.19	19320321 ± 50	1	LOC_Os12g32250 (WRKY96)
	id7000519	7	24.88	10.62	3649192 ± 50	2	LOC_Os07g07320 (glutathione S-transferase), LOC_Os07g07340 (glucan endo-1,3-beta-glucosidase)
	id2001900	2	22.38	10.27	3404588 ± 50	1	LOC_Os02g06840 (galactosyltransferase)

Trait	Marker	Chr.	R ²	$-\log_{10}P\text{-value}$	Position (MBP)	No. of significant SNPs in QTL regions	Associated genes to SNP marker
SL	id12001349	12	7.88	4.29	3280034 $\pm$ 50	2	LOC_Os12g06800 (serine esterase), LOC_Os12g06810 (WD domain 48)
	id12001382	12	6.97	3.88	3407246 $\pm$ 50	1	LOC_Os12g07110 (acyl-CoA synthetase)
	id7001911	7	8.86	3.76	10811204 $\pm$ 50	2	LOC_Os07g18240 (lectin-like receptor kinase), LOC_Os07g18120 (aldehyde oxidase)
	id7002230	7	6.62	3.73	13595959 $\pm$ 50	1	LOC_Os07g23944 (GH31)
	wd7001684	7	7.38	3.58	12533004 $\pm$ 50	1	LOC_Os07g22350 (glucose-6-phosphate 1-dehydrogenase)
RL	id12001349	12	7.46	4.24	3280034 $\pm$ 50	2	LOC_Os12g06800 (serine esterase), LOC_Os12g06810 (WD domain 48)
	id8007314	8	9.11	4	26837234 $\pm$ 50	3	LOC_Os08g42550 (AP2 protein), LOC_Os08g42410 (transketolase), LOC_Os08g42450 (Annexin A7)
RFW	id1011623	1	18.66	8.25	19509449 $\pm$ 50	2	LOC_Os01g35230 (L-aminocyclopropane-L-carboxylate oxidase), LOC_Os01g35160 (DUF26)
	id1023217	1	12.29	6.16	36606107 $\pm$ 50	2	LOC_Os01g63160 (MYB family transcription factor), LOC_Os01g63180 (laccase-6)
	id6012421	6	9.83	4.98	23912891 $\pm$ 50	1	LOC_Os06g40170 (phospholipase D)
	id6015037	6	8.74	4.65	26809019 $\pm$ 50	2	LOC_Os06g44370 (WD domain), LOC_Os06g44460 (D-3-phosphoglycerate dehydrogenase)

Trait	Marker	Chr.	R ²	-log ₁₀ P-value	Position (MBP)	No. of significant SNPs in QTL regions	Associated genes to SNP marker
	id8007573	8	8.36	4.46	27592676 ± 50	3	LOC_Os08g43700 (Auxin-responsive SAUR), LOC_Os08g43570 (beta-galactosidase), LOC_Os08g43560 (Ascorbate Peroxidase)
	id8004844	8	7.52	4.08	18343102 ± 50	1	LOC_Os08g29770 (endoglucanase)
	id2006290	2	10.31	4.07	15705734 ± 50	1	LOC_Os02g26814 (UDP-N-acetylglucosamine transferase subunit ALG13)
SFW	id1013001	1	7.45	3.73	22806870 ± 50	1	LOC_Os01g40430 (WRKY27)
	id1026302	1	5.90	3.32	41101759 ± 50	2	LOC_Os01g71000 (protein kinase APK1B), LOC_Os01g71010 (lipase)
	wd6000411	6	6.15	3.08	8871246 ± 50	1	LOC_Os06g15620 (GASR7)
	id6016397	6	5.46	3.03	28799533 ± 50	3	LOC_Os06g47620 (peptidase), LOC_Os06g47700 (serine/threonine-protein kinase BRI1-like 2), LOC_Os06g47640 (Calmodulin-related calcium sensor protein 29)
	id6005326	6	5.46	3.03	8186912 ± 50	1	LOC_Os06g14630 (GDSL-like lipase/acyl hydrolase)

The results showed that the marker id12001382 that corresponds to the acyl-CoA synthetase gene was a common marker between SL and NDG. The marker wd7001684 corresponds to glucose-6-phosphate 1-dehydrogenase gene was a common candidate gene for SL and RDW and the marker id12001349 that corresponds to serine esterase and LWD domain 48 was a common candidate gene for SL and RL (Table 2).

Discussion

Unlike other grains, rice grain has the ability to germinate under anaerobic and hypoxic conditions (Miro and Ismail 2013), but in early stages of seedling growth, it is extremely sensitive to anaerobic conditions (Facon 2000; Ismail et al. 2009 and 2013). This study presents a comprehensive genetic dissection of germination traits in a rice population resulting in the identification of a total of 70-related QTLs.

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Associated genes with NDG

In the germination process of each seed, the number of days required for germination is an important issue. Because seeds that do not germinate within a certain period of time may rot or be eaten by macro and micro-organisms. GWAS results showed 11 candidate genes, acyl-CoA synthetase, *O*-glucosyltransferase 2, HEAT protein, haloacid dehalogenase 3, *OsWAK6*, Exportin 1, G-protein, ABC transporter, *OsFBX74*, leucine rich protein and sucrose transporter *SUT4* for NDG (Table 2).

Degradation of fatty acids begins with the activation of acyl-CoAs by the enzyme acyl-CoA synthetase (ACS; Hayashi et al. 2002) that follows with β -oxidation cycle to decompose acetate units one after the other (Hayashi et al. 2002). In our study, *ACS* was a common candidate gene between NDG and SL. Earlier, its relevance to germination was established (Fulda et al. 2004; Zhang et al. 2020). *O*-glucosyltransferase 2 previously was shown to be involved in Arabidopsis seed germination (Zhang et al. 2016). HEAT protein among many other functions (Al-Whaibi 2011), have been shown to be involved in the germination of wheat, maize, Arabidopsis, and tomatoes (Helm et al. 1989; Su and Li, 2008; Abreu et al. 2016; Ma et al. 2019). Haloacid dehalogenase 3 has hydrolase activity and its overexpression in rice resulted in enhanced phosphatase activity (Pandey et al. 2017). Phosphorus deficiency has been shown to reduce seed size and number (Silva et al. 2014). On the other hand, larger seeds have the ability to germinate in deep soil layers that gives the plant the ability to be more drought resistant (Yi et al. 2019). A study showed that seeds with more phosphate have faster germination and more seedling growth (Ros et al. 1997). Therefore, this gene is likely to affect germination by interfering with the amount of phosphate in the seed during germination.

Other associated gene was *OsWAK6*, a receptor-like protein kinase to sense both intra- and extra-cellular signals (de Oliveira et al. 2014). Although no relationship has been established between this protein and germination, due to their involvement in hormone signaling (Li et al. 2017) similar functions are being speculated here. Wang et al. (2010) demonstrated qGR-1, a rice seed vigor QTL, to be located in this chromosomal region. G-protein, the other associated gene to NDG, was shown to be involved in the germination process in crops (Ullah et al. 2002; Pandey et al. 2006; Botto et al. 2009; Ma et al. 2017). Gibberellins, the germination intrinsic factor, is being stimulated by G-protein family of enzymes (Assmann 2005).

Other associated gene to NDG was an ABC transporter. ABC transporters are essential for plant growth and development and play an important role in processes such as germination, organ formation and organ growth. They are involved in germination by interfering with various cellular processes (Kanai et al. 2010; Basu et al. 2019). For example, they can be negatively involved in the germination process by transferring abscisic acid (Ko et al. 2014). Study by Fedi et al. (2017) showed that mutation in an ABC transporter speeded up the germination. Other candidate gene was *OsFBX74* that is part of a large family of proteins in plants, with various roles (Wang et al. 2016; Baute et al. 2017) including germination (Majee et al. 2018). It has been shown that overexpression of *OsFBX74* in rice delays germination and increases grain size (Chen et al. 2013). Leucine rich protein, the other associated gene with NDG, are abscisic acid responsive (Finkelstein et al. 2002). The loss-of-function of this gene showed ABA insensitivity and improved seed germination (Osakabe et al. 2005). Sucrose transporter *SUT4* was also showed to be associated to NDG as its involvement in germination was reported previously (Siao et al. 2011; Li et al. 2012). Overexpression of this gene has been shown to increase susceptibility to ABA during seed incubation (Wang et al. 2020).

Associated genes with GP

GWAS results showed nine associated markers with 15 genes including peptidyl-prolyl isomerase, gibberellin receptor GID1L2, chalcone synthase, DUF6, hydrolase, alpha/beta fold family protein, glycosyl hydrolases family 17, glycosyl hydrolases, Polygalacturonase, beta-D-xylosidase, peroxidase, WRKY96, glutathione S-transferase, glucan endo-1,3-beta-glucosidase, galactosyltransferase with GP.

Peptidyl prolyl isomerase was shown to negatively regulate ABA signaling, negative regulator of seed germination, in *Arabidopsis* (Jung et al. 2020). Gibberellin receptor GID1L2 plays an important role in germination via signal perception of gibberellins (Voegelé et al. 2011; Munteanu et al. 2014). Chalcone synthase is a key enzyme involved in the flavonoid biosynthesis pathway in higher plants (Yonekura-Sakakibara et al. 2019). This gene can control the germination of plant seeds (Debeaujon et al. 2000). It has also been shown that this gene could affect seed dormancy by regulating the expression of ABA signaling-related genes (Gao et al. 2018). Therefore, this gene seems to play a role in initiating seed germination.

DUF6 is part of the plant metabolite transporter family. DUF6 was introduced for germinating ability for cold (Borjas et al. 2016) and salt tolerance (Zeng et al. 2020). The other associated marker to PG was id4006430 on chromosome 4 that corresponds to a gene cluster containing three hydrolytic enzyme (GH17) encoding genes (Mindrebo et al. 2016). Glycosyl hydrolysing enzymes are the prerequisite for seed germination (Minic and Jouanin 2006; Ferreira et al. 2009). The marker is co-localised with qHD4 and qGI1 markers, which have been introduced as a germination-related gene (Wang et al. 2010; Fujino et al. 2015). The next associated gene with PG was a polygalacturonase with demonstrated function in seed germination in rice and *Arabidopsis* (Chen et al. 2019). The role of peroxidase, the other associated gene with PG, with germination process has already been determined (Lakra et al. 2019; Mousavizadeh et al. 2013; Bellani et al. 2002). Another associated gene was WRKY96, a transcription factor family with demonstrated role in seed germination and plant development (Han et al. 2020). The marker id7000519 on chromosome 7 corresponds to a gene cluster containing glutathione S-transferase and glucan endo-1,3- β -glucosidase. This marker is co-localised with the qAG3 marker, a germination-related marker (Baltazar et al. 2019). Over expression of glutathione S-transferase in rice (Takesawa et al. 2002) and *Arabidopsis* (Kumar and Trivedi, 2018) showed enhancement of germination rate that led to early bolting (Kumar et al. 2015).

Associated genes with RDW

Root and seedling dry matter can be defined as seed reserve mobilization efficiency. The result of GWAS showed five associated genes including solute carrier family 35, Protease inhibitor/seed storage/LTP family, citrate transporter, glucose-6-phosphate 1-dehydrogenase and nodulin with RDW. Solute carrier protein is responsible for solute transfer across biological membranes, including inorganic ions, amino acids, lipids, sugars (Bai et al. 2017). The role of these proteins in germination has not yet been established. However, due to the metabolism that occurs during germination, many carriers are likely to be activated in this process. During germination, seed proteins are broken down by proteases to produce nitrogen for the biosynthesis of other substances (Martinez et al. 2019). It has been shown that protease inhibitor activities such as protease inhibitor/seed storage/LTP family decreases during the germination process (Pagnussat et al. 2015). Other associated gene was a nodulin with possible function in germination in *Arabidopsis* (Denancé et al. 2014).

Associated genes with SDW

GWAS found seven associated genes with SDW, interestingly all of which were hydrolytic enzymes for carbohydrates and lipids. Glucan endo-1,3- β -glucosidase, a cell wall degradative enzyme, has been shown to be involved in germination (Leubner-Metzger, 2003). Endoglucanase, an enzyme that randomly hydrolyse β -1,4-glucan

Loading [MathJax]/jax/output/CommonHTML/fonts/TeX/fontdata.js associated gene. The wall needs to be loosened up during

germination and endoglucanase can efficiently contribute in this process. Polygalacturonase with role in germination (Hu et al. 2017; Chen et al. 2019) was the other associated gene with SDW. Lipase 3 showed association with SDW in GWAS. During seed germination, stored lipids are expected to be broken down and to be used as an energy resource for root and seedling production (Penfield et al. 2005; Graham 2008). Acyl-CoA dehydrogenase 10 (ACADs), key enzymes in fatty acid and amino acid catabolism (Shen et al. 2009), was found to be associated with SDW. The activity of this enzyme in cotyledon tissue has been determined during germination (Masterson et al. 2000). Other associated gene with SDW was non-lysosomal glucosylceramidase that catalyses glucosylceramide to glucose and ceramide (Boot et al. 2007). It has been shown this hydrolytic enzyme increase its activity during germination (Guzmán-Ortiz et al. 2019).

Associated genes with SL

Our GWAS results showed five associated genes with SL. First candidate marker was id12001349 that corresponds to two candidate genes, serine esterase and WD domain 48 that are common genes with root length. Lectin-like receptor kinase, negative regulator of ABA during germination (Deng et al. 2009; Chen et al. 2013; Sun et al. 2013), was the other associated gene with SL. In another GWAS in maize, its role as a chilling tolerant gene was established (Hu et al. 2017). Aldehyde oxidase, an enzyme that catalyses the final step of ABA biosynthesis (Seo et al. 2000), was the other associated gene with SL. An α -glucosidases (GH31) with starch hydrolysis activity (Rozeboom et al. 2013) was also found in our GWAS. A glucose-6-phosphate 1-dehydrogenase was the other associated gene with SL. Its involvement in *Arabidopsis* germination has been previously reported (Yang^b et al. 2019).

Associated genes with RL

Root length is an important factor in cereals germination (Hammer et al. 2009). It contributes to the germination via nutrient and water uptake and helps plant to become stabile in soil (Lilley and Kirkegaard 2007). Our results showed five associated genes for RL: a serine esterase, WD domain 48, AP2 protein, transketolase, and annexin A7. Serine esterase has been shown to be involved in *Arabidopsis* germination (Huang et al. 2015). WD domain 48 was reported to be involved in *Arabidopsis* germination inhibition (Chuang et al. 2015). AP2 transcription factor, among many other developmental functions (Sekhwal et al. 2014), have been shown in germination regulation (Yamagishi et al. 2009). Transketolase, a restriction enzyme in the pentose phosphate pathway, was showed to be associated with RL here and by Jiang et al. (2017) as qGRR11 QTL. Transketolase and ribose phosphate isomerase increase immediately after the onset of germination (Rajjou et al. 2006). Last candidate gene corresponded to root length is Annexin A7 was the other physically associated gene with RL. Annexins are expressed during germination and in the early stages of seedling growth (Cantero et al. 2006; Yan et al. 2016; He et al. 2019).

Associated genes with RFW

GWAS results showed 12 physically associated genes to RFW. The first one was 1-aminocyclopropane-1-carboxylate oxidase, an enzyme involved in ethylene biosynthesis. Ethylene, as a hormone, involves in many cellular processes such as germination, seed dormancy, and seedling formation (Corbineau et al. 2014; Wilson et al. 2014). DUF26, the other associated gene with RFW, is a cysteine-containing protein with varieties of functions in various cellular processes (Vaattovaara et al. 2019). Knocking down of this gene in rice has been shown to result in seed germination in an NaCl-containing medium (Zhang et al. 2009). MYB family of transcription factors (TFs) with roles in hormonal signal transduction (Zhang et al. 2018) was the other associated gene with RFW. In *Arabidopsis* it has been shown that MYBs are involved in the germination process in response to ABA as a positive regulator (Roy et al. 2019). In sheepgrass, MYB2 increases during germination under

drought stress (Zhao et al. 2019). Recently, rice qCAD-2-2 QTL (MYB factor) was reported to be involved in anaerobic germination tolerance (Yang^a et al. 2019). Other RFW-associated gene was laccase-6. This gene is involved in lignin degradation and detoxification of lignin-derived products. The seed coat of many species contains hydrophobic lignins and husk lignin decomposition may be required for germination. Phospholipase D (PLD) was physically associated with RFW. The enzyme breaks phosphodiester bonds between glycerophospholipids and thus phosphatidic acid is released as an energy source for germination (McDermott 2004; Parthibane et al. 2012). A rice GWAS showed its involvement in germination (Li et al. 2007).

D-3-phosphoglycerate dehydrogenase, a plant gene responsible for serine biosynthesis (Okamura and Hirai, 2017), showed a physical association with RFW. The gene has been proposed to be related to carbohydrate metabolism during germination in *Morus notabilis* and *Nelumbo nucifera* (Zaynab et al. 2018). Auxin-responsive SAUR, a negative auxin synthesis and transport regulator (Kant and Rothstein 2009) and positive gibberellic acid regulator (Ren and Gray 2015), showed to be associated with RFW. Loss-of-function of auxin synthesis in *Arabidopsis* has been shown to delay seed germination (Hussain et al. 2020). Other associated gene with RFW was β -galactosidase. As reported earlier, corresponding enzyme activity will increase during germination in rice (*Oryza sativa* L.) (Chantarangsee et al. 2007) to possibly change cell wall structure for better expansion upon cellular divisions (Ramos and Malcata, 2011). Ascorbate peroxidase, a reactive oxygen species scavenging enzyme (Ozyigit et al. 2016) showed to be associated with RFW. In tobacco, overexpression of the corresponding gene has been shown to improve germination under drought stress (Faize et al. 2015). Our results showed the association of endoglucanase to RFW. During germination, the activity of degrading enzymes such as endoglucanases occurs to soften the micropylar region so that radicle emergence becomes possible (Leubner-Metzger and Meins 2000). Other candidate gene was UDP-N-acetylglucosamine transferase subunit ALG13 with synthetic function of lipid-bound oligosaccharides (Sonkar et al. 2017). It has been shown that the activity of this enzyme increases during the germination in *Arabidopsis* (Tatematsu et al. 2008).

Associated genes with SFW

GWAS results showed eight candidate genes for SFW. WRKY27 is gibberellins-responsive transcription factor (Gu et al. 2019). Here and for the first time we have reported its possible association with SFW and seed germination as gibberellins are intrinsic factors involved in the germination process (Stejskalová et al. 2015). Protein kinase APK1B, the other SFW-associated gene, is required for ABA signaling during germination (Fujii et al. 2007; He et al. 2011). This QTL is co-located with qNGR1 QTL for seed germination under low temperature for rice by Jiang et al. (2017). GASR7, a GA-induced protein (Zhang et al. 2015), was the other SFW associated gene. Peptidases hydrolyse peptides during seed germination (Martinez et al. 2019). In our study a peptidase gene showed to be associated with the marker responsible to SFW. Other candidate gene was a serine/threonine-protein kinase BRI1-like 2. This protein is involved in hormone-mediated signaling pathway and auxin-activated signaling pathway. Auxin is positively related to the germination process (Hussain et al. 2020). This QTL is similar to S1_1910174 QTL for SDW under salinity condition reported by Luo et al. (2020). Other candidate gene was calmodulin-related calcium sensor protein. Calcium plays a key role in the plant's response to environmental stimuli such as biotic and abiotic stresses as well as plant hormones (Pandey et al. 2004). Calmodulin signals are involved in the inhibition of ABA during seed germination and seedling growth (Zhou et al. 2018) and ABA promotes seed dormancy and inhibits seed germination and post-germination growth (Zhou et al. 2018). It seems this gene has negative relationship with germination. GDSL-like lipase/acylhydrolase that shows lipase activity was the other gene to be associated with SFW. Lipase activity and its involvement in the germination process has already been established (Graham 2008).

Conclusion

The germination process is an initial and important step in the production of agricultural products, especially for rice, which is a crop plant that is grown in flooded lands. Here, the genetic diversity of rice genotypes was put under scrutiny for germination. Our GWAS results identified several likely candidate genes for germination traits that will greatly contribute to our understanding of the genetic complexity underlying the corresponding traits. The associated genes with the germination traits can be generally classified as hydrolytic enzymes and regulatory proteins that can directly or indirectly influence germination.

Methods

Rice material

A collection of 283 accessions of rice (*O. sativa* L.) genotypes from 82 countries (Supplementary file S1) were sown in plots of $2 \times 2 \text{ m}^2$ with 25 cm within rows spacing in Sari Agricultural and Natural Resources University (Northern Iran). Superphosphate triple (180 kg/ha at plowing), urea (100 kg/ha at seedling stage), and potash (80 kg/ha at plowing stage) were given to plants. Grains of panicles from each plant in three biological replicates were collected at full maturity. Accessions were TEJ (Temperate Japonica), IND (Indica), AUS (aus), ARO (Aroaatic), TRJ (Tropical Japonica), and ADMIX subpopulations. Rice seeds may be dormant for up to 80 days after harvest, depending on the variety and wild and traditional rice cultivars have higher degrees of dormancy than modern ones. In order to break dormancy in the seeds, grains were stored for 90 days at 4 °C and then germination rate was measured. Rice 44.1 K SNPs array was downloaded from the Gramene portal (<http://gramene.org>) to use genotyping by sequencing data (Zhao et al. 2011). We used 220 rice accessions in this study.

Evaluation of seed germination

A total of 20 healthy grains of each accession by three biological replicates were surface-sterilized with 10 % sodium hypochlorite solution for 1 min and washed three times with sterile dH_2O . The seeds were placed in a petri dish with two sheets of wet filter paper. Seeds were germinated under normal conditions at $25 \pm 1^\circ\text{C}$ for 14 d with a 12 h light/12 h dark photoperiod. The plates were checked daily to determine number of days to germinate and coleoptile appearance (NDG). The estimated germination percentage ($\text{GP} = \text{seeds germinated}/\text{total seeds} \times 100$) is a measure of seed viability (Abdel-Haleem and El-Shaieny, 2015). At day 14th, five germinated grains were randomly selected from each replicate and root (RL) and shoot (SL) length, fresh weight of shoots (SFW) and roots (RFW) were measured. The shoots (SDW) and roots (RDW) were placed at 60 °C for 24 h and the corresponding dry weights were measured. In total, all traits were measured in three biological replications. Statistical distribution of phenotypes as well as correlation between the traits were plotted at R package of programs (<https://cran.r-project.org/>). Broad-sense heritability (H_b^2) was estimated by R rptR package (<https://cran.r-project.org/web/packages/rpart/index.html>) using phenotypic data.

Population structure, estimation of LD decay in rice genome and GWAS

Analysis of population structure among all rice accessions was performed by principal components analysis (PCA) in TASSEL v5 (Bradbury et al. 2007). The PCA analysis and plots were generated using the genomic association and prediction integrated tool (GAPIT) (Lipka et al. 2012). The kinship matrix was obtained at TASSEL v5 and heatmap of the kinship matrix was produced at GAPIT (Zhang et al. 2010).

Genome-wide linkage disequilibrium (LD) analyses were performed in the association panel in order to evaluate the resolution of LD. Pairwise calculations of LD between SNPs were made in TASSEL using r^2 with a sliding window of 50 markers. Graphs depicting the decay of LD with physical distance between SNPs were visualized using ggplot2 in R (Wickham, 2016).

Association analyses were performed using 33,934 SNPs with a minor allele frequency (MAF) above 5% and obtained phenotypic data using MLM models in TASSEL. Quantile-quantile (Q-Q) plots (observed versus expected $-\log_{10}P\text{-values}$) were generated by R software using qqman package with the results obtained from Tassel to determine the model that fits the data best (Chen et al. 2016). The Manhattan plots were drawn with the results obtained from tassell in R software using qqman package (Turner, 2014).

Bioinformatics and gene identification

In order to identify genes underlying the QTLs of seed germination-related traits that overlapped with the genomic regions (i.e., their associated SNPs), genes deposited on the Rice Annotation Project database (<http://rice.plantbiology.msu.edu/>) and also IRGSP-1.0 (<https://rapdb.dna.affrc.go.jp/>) were assessed. All hypothetical genes were ignored in order to put protein coding sequences and transposable elements into prospect for further analyses. We searched for genes in an interval extending 50 kb in either direction from SNP markers (i.e., a total of 100 kb since LD decay is very low in rice; Mather et al. 2007) in order to account for map order uncertainty and LD. To ascertain whether candidate genes underlying our QTLs were previously cloned and functionally annotated, a thorough literature search was made for all genes.

Abbreviations

GWAS: genome-wide association study; LD: linkage disequilibrium; MLM: mixed linear model; PV: Phenotypic variation; SNP: single nucleotide polymorphism; QTL: quantitative trait loci; GP: germination percentage; SL: shoot length; RL: root length; SFW: shoot fresh weight; RFW: root fresh weight; RDW: root dry weight; SDW: shoot dry weight; NDG: number of days to germinate.

Declarations

Ethical Approval and Consent to participate

Not applicable as no animal experiments were involved in the entire project.

Consent for publication

All the authors have given their consent to publish the manuscript.

Availability of supporting data

All relevant data have been provided as Tables, Figures with in the text and in the following supplementary data.

Competing interests

The authors declare that they have no competing interests.

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Authors' Contributions

Rahele Panahabadi performed experiments, carried out the GWAS analysis and prepared the draft of manuscript. Asadollah AhmadiKhah co-supervised the research, provided all samples used in the project and carried out phenotypic data analysis. Naser Farokhi co-supervised the research carried out phenotypic data analysis. All authors read and approved the final manuscript.

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Figures

Figure 1

Kinship, LD decay of the whole population, principal components (PC). a) Heatmap of kinship matrix of 220 rice accessions using SNPs data. Red indicates the highest correlation between pairs of individuals and yellow indicates the lowest correlation. A hierarchical clustering tree based on the pairwise kinship values for all accessions is displayed along the top and left axes. b) population structure of rice germplasm collection as reflected by PCs. First two PCs explain 8.5% and 7.4% of the variations, respectively. The third PC explains 2.6% of the variation; c) LD decay of genome-wide association study (GWAS) population, each dot represents LD (r^2) between a pair of SNPs, the regression used to describe the relationship between

the pairwise distance and r^2 . Significant LD decay was observed in population; d) Scree plot from GAPIT showing the selection of PCs for association study and results showed three main groups.

Figure 2

Manhattan plots of germination data from GWAS using the MLM model. The $-\log_{10}(p)$ values from the GWAS were plotted according to genomic position on each of the 12 rice chromosome in left side of each Manhattan plot. a) NDG, b) RDW, c) SDW, d) GP, e) SL, f) RL, g) RFW, h) SFW.

Figure 3

Schematic representation of the 12 rice linkage groups analyzed for genome-wide association scan for germination traits. On the right side of each chromosome the marker position is shown, and on the left, the marker Id is presented. Yellow dots are the associated SNPs resulted from GWAS for NDG, red dots for RDW, green dots for SDW, blue dots for GP, orange dots for SL, pink dots for RL, purple dot for RFW, dark blue dot for SFW. Figure is drawn manually and scaled to reality.

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